The Effects of Honey on the Inflammatory Response of Cells With Respect to Wound Healing

The University of Waikato

Te Whare Wānanga o Waikato

A thesis
submitted in partial fulfilment
of the requirement for the Degree of

Doctor of Philosophy
in Biomedicine

at

The University of Waikato

by

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ABSTRACT

The primary aim of this thesis was to test the effect of two types of honey, a manuka honey and an Otago pasture honey, on a range of key cell types involved in the inflammatory response of wound healing. A range of *in vitro* assays were used to test the effect of honey on various cells with a view to the implications of the results for wound healing, focusing on the mechanisms by which honey has been observed to have beneficial effects on both scar formation and inflammation.

To investigate whether honey could stimulate bovine T cells to proliferate *in vitro*, both MTT and BrDU assays of proliferation and flow cytometry analysis were used. It was found that low concentrations of honey stimulated resting T cells to proliferate and express the IL-2 receptor in a dose-dependent manner with progressive dilution. This suggests that honey contains lymphomitogenic factors. Manuka honey was stimulatory at higher dilutions than pasture honey. Low concentrations of honey induced cell division profiles similar to those obtained with Con A-stimulated cells. The stimulatory activity of honey was found to be in a high molecular weight fraction. Sugars alone had no effect on T cell proliferation, as demonstrated by use of artificial honey (a syrup of sugars as in honey).

The ability for honey to induce messenger RNA expression for key cytokines involved in wound healing was investigated. Conventional reverse transcriptase-PCR was used to detect the production of mRNA for honey at 0.25% concentrations for various times (0–24 h). The more sensitive molecular technique, quantitative real-time RT-PCR was then used to quantify the abundance of cytokine mRNA transcripts expressed in bovine blood exposed to 0.25% manuka honey as compared with Con A or control cultures. Transcriptional activity of ten genes, IL-1, IL-5, IL-12, IL-18, IFN-γ, HSP70, HSP90, i-NOS, TNF-α, and TGF-β were studied at the mRNA level during a 0–24 h exposure of whole blood to honey. To test for any modulatory effects of honey on gene expression in an inflammatory model, whole blood was exposed to honey at the same time as LPS and the mRNA expression for the genes was measured. The results show that honey up-regulates a wide range of mediators, including TNF-α, IL-1β, and TGF-β, and this supports the hypothesis that honey induces cytokine release. Honey gave a transient and moderate induction of cytokine mRNA compared with a massive and prolonged

induction by the mitogens, Con A and LPS. The inclusion of honey with LPS led to a reduced expression of mRNA for key inflammatory mediators, including iNOS and TGF-β, compared with LPS alone. This supports the hypothesis that honey modulates inflammation.

To investigate whether honey could induce THP-1 monocytes to release TNF-α, bioassays using WEHI cells were carried out to measure TNF-α production after the monocytes were exposed to honey. Honey was found to stimulate release of TNF-α by the monocytes when at a range of concentrations between 0.000025–0.1%, with no differences between the levels produced at the various concentrations of honey. At concentrations of honey from 0.25–1% the TNF-α production decreased as the concentration of honey increased. This may indicate that an anti-inflammatory action overrides the stimulatory effect at concentrations of honey greater than 0.25%. Sugar content had no effect upon TNF-α release, as demonstrated by the artificial honey control. There were no differences between honey types (manuka honey and pasture honey) in induction of TNF-α release. Time-course analysis confirmed that a 4–6 h incubation period of cells with 0.25% honey gave maximal TNF-α production. A 2 h minimum exposure period of cells to honey was critical for TNF-α production. Incubation of LPS-stimulated monocytes with honey had no effect on their subsequent TNF-α production. A good correlation was found between the TNF measurements detected by ELISA and the WEHI Bioassay.

To test whether honey could modulate LPS-stimulated NO production by THP-1 monocytes and bovine peripheral blood mononuclear cells, Greiss assays were performed. Both manuka honey and pasture honey at 0.5% and 1% concentrations suppressed LPS-induced nitrite release in a dose-wise manner, indicating modulation of nitric oxide production. Manuka honey had a more potent modulating effect on LPS-driven nitrite production than pasture honey, and maintained activity at 0.25%. Sugars alone had no effect. High molecular weight dialysis fractions of either honey contained the activity, but some of the activity was lost by fractionation. An ether extract of manuka honey led to the greatest modulation of nitrite production by LPS-stimulated monocytes.

To investigate whether honey has an effect on phagocytosis, whole blood was incubated with honey and the ability of neutrophils to take up fluorescent-labelled bacteria was measured using the Phagotest® assay. The artificial honey control provided clear evidence that low concentrations of honey (optimal at 0.25%) induce phagocytosis by neutrophils due to the supply of sugars. Manuka honey had an additional opsonizing effect on bacteria, which enhances the phagocytic response beyond that seen with sugars alone.

The effects of honey on tight junction (TJ) resistance were assessed for MDCK cell monolayers subjected to an EGTA challenge. It was found that manuka honey and pasture honey have protective effects on TJ following the challenge, and enhance post-challenge recovery of transepithelial resistance. Manuka honey had greater modulatory activity on TJ with increased concentration from 0.1–1%, and 1% concentrations of both honeys gave the greatest protective effects. Manuka honey appeared to have greater protective effects than pasture honey. Application of manuka honey (at 1% concentrations) to both the apical and basolateral sides of the MDCK cell monolayer significantly enhanced TJ tightness beyond the control. Dialysis of the honey confirmed that the high molecular weight fraction contained the active component. Diffusate fractions from either honey type had no effect on TJ. Artificial honey had no effect.

The effect of various honey concentrations on the proliferation of the 3T3-L1 fibroblast cell-line *in vitro* was investigated using MTT proliferation assays. Both manuka honey and pasture honey (0.25%) increased fibroblast proliferation. Artificial honey had no effect on fibroblast proliferation, indicating sugars have no role in mitogenic activity. This suggests that honey contains factors which directly promote cell division in fibroblasts.

An *in vitro* model was used to test whether honey-induced factors produced by peripheral blood mononuclear cells (PBMC) could activate fibroblast proliferation. These assays were performed to examine whether honey could have an indirect stimulatory effect on fibroblasts. Incubating fibroblasts with supernatants derived from honey-stimulated PBMCs (at 0.025% concentrations of honey) led to significant fibroblast proliferation. Low concentrations of honey (less than 0.1%) do not directly stimulate fibroblast proliferation, therefore factors produced by honey-stimulated

PBMCs must promote fibroblast proliferation. A high molecular weight fraction of manuka honey obtained from dialysis contained the active component. The diffusate obtained by dialysis (containing sugars) had no activity.

To investigate whether honey could modulate the response of fibroblasts to an inflammatory agent, fibroblasts were exposed to honey for various times prior to and at the same time as IL-1β. Honey did not augment fibroblast proliferation when added at the same time as IL-1β. Prior incubation of fibroblasts with honey (0.25–1%) for 2 h before IL-1β-stimulation decreased the cell response to IL-1β, and this anti-inflammatory active component was of a high molecular weight.

It is proposed, on the basis of this *in vitro* study, that honey provides a neatly controlled therapy for optimising tissue repair, with potential for use in inflammatory disorders. It is the central thesis of this study that the stimulatory agent in honey induces cytokine production necessary for healing to occur, but that the oxidant species produced by these cells are effectively regulated by a second agent, thereby creating a feedback-regulated delivery system. The results presented in the current study suggest that honey can both stimulate and modulate cell activity, and show that honey interferes with a large number of regulatory steps in the inflammatory pathway, *e.g.* cell division, transcription, tight junction integrity, production of oxidant species. While the stimulatory activity was observed at lower concentrations, the modulatory effects required higher concentrations of honey. The dual ability for honey to stimulate moderate cellular activation in the absence of an immune stimulus, yet not augment mitogenic stimulation, and in some cases to modulate cell response to a mitogen, indicates that it will promote healing without setting up harmful inflammation.

If further experiments confirm this to be the case, honey will have potential for therapeutic application. The work also identifies some new areas of research, which if completed would further enhance the understanding of the role honey plays in tissue healing.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my profound appreciation to Professor Peter Molan, whose passionate quest to understand the complexities of honey continues to be an inspiration. You made me believe in myself at times when I had lost faith. Thank you so much for your support.

I wish to extend a huge thank you to Dr Ray Cursons, who helped me to pull it all together at the end when I needed it most.

I wish to acknowledge the many people who have provided advice and feedback during my study: Barry O'Brien, Kerry Allen, Rae Towers and Lance McLeay at Waikato University; Harold Henderson and Ray Littler for advice with the statistics (Waikato University), Megan Callaghan, Robin McDonald, Rob McLaren and Craig Smith at AgResearch, Ruakura.

I would like to acknowledge my financial support from AGMARDT and the National Honey Board (USA), without which this research would not have been possible. Also to those who have employed me and helped me to expand my horizons; Drs Alison Campbell, Liz laFranchie, and Ryan Martinus (Waikato University). Thankyou to the Waikato Museum for enabling me to complete the revision process during the first few months of my new job!

Thanks to those who supported me and *tried* to keep me sane over the last 5 years, I'll see you in the padded cell! Most of all thank you to my family; to my boys who have grown up with a distracted, over-committed mother - I hope this hasn't put you off doing a doctorate, and to Steve who has been my rock and some-time advisor. But most of all to my Mum, to whom I dedicate this thesis, for her overwhelming love and support-you know I couldn't have done this without you! I hope I make you proud.

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ABBREVIATIONS

ANOVA	analysis of variance
BrDU	bromodeoxyuridine
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-amino-ethyl ether)
FCS	foetal calf serum
g	gravitational force
HEPES	N-2-(hydroxyethyl) piperazine-N'-2-ethanesulfonic acid
HIMF	hypoxia-induced mitogenic factor
HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon-γ
IL-1β	interleukin-1β
LPS	lipopolysaccharide
MDCK	Madin Darby Canine Kidney
Milli Q	Milli Q water
MTP	microtitre plate
MTT	3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide
n	number of samples
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	nuclear transcription factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
Nox	nitrite plus nitrate
NSAIDs	Nonsteroidal Antiinflammatory Drugs
OD	optical density units
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
pen-strep	penicillin-streptomycin
PMN	polymorphonuclear cells
rpm	revolutions per minute

RT	room temperature
SAS	Statistical Analysis System
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TER	transepithelial resistance
TGF-β	tumour growth factor-beta
TJ	tight junctions
TMB	tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
TNF-α	tumor necrosis factor-alpha

PREAMBLE

Chapter 1 begins with a brief introduction and concludes with a literature review comprising:

1) A review of what was known about the tissue-healing properties of honey prior to the current study.
2) A review of established knowledge of the inflammatory process, with an emphasis on wound healing.
3) A review of the current understanding of the pathogenesis of some key inflammatory diseases, with a focus on fibrosis.
4) A review of the existing treatments for keloid scars, with an emphasis on the need for new therapies.
5) Primary aims of the current study.

Chapter 2 describes the effect of honey on T cell cultures as measured by proliferation assays (BrDU and MTT), along with flow cytometry analysis of cellular activation markers. Chapter 3 describes the effect of honey on the kinetics of cytokine mRNA expression for several genes involved in wound healing, in whole blood cultures. The molecular techniques, conventional reverse transcriptase-PCR and quantitative real-time reverse transcriptase-PCR, were used to examine both the direct effect of honey on mRNA amplicons and the effect of adding honey at the same time as lipopolysaccharide on mRNA amplicons. Based on the results obtained in Chapter 3, in Chapter 4 a WEHI bioassay was used to further examine the effect of honey on TNF-α production by peripheral blood mononuclear cells (PBMCs) and a THP-1 monocyte cell line.

In Chapter 5 the effect of honey on nitric oxide production by PBMCs and THP-1 monocytes was measured using the Griess reagent. Based on the observation that honey down regulated the mRNA expression for iNOS in Chapter 3, the effect on nitroc oxide production of exposing THP-1 monocytes to honey at the same time as LPS, was further examined. In Chapter 6, the effect of honey on bead uptake by neutrophils was examined using both flow cytometry analysis and haemocytometry.

After establishing that honey has effects on key cells functioning in the inflammatory pathway, the effect of honey on blood-tissue barriers was examined. In Chapter 7, measurement of transepithelial resistance across an epithelial cell monolayer was used to measure the effect of honey on tight junction integrity during an inflammatory challenge. Chapter 8 describes the effect of honey on fibroblast cultures as measured by MTT proliferation assays. Fibroblasts represent one of the key cell types functioning in the later inflammatory stage of wound repair.

Finally, in Chapter 9, a discussion of the relevance and importance of the findings in the present study is presented.

PREFACE

All honey concentrations are expressed as % (v/v) unless otherwise stated (taking 1 ml honey as 1.37 g honey). Where the concentration of honey is expressed as the degree of dilution of the original honey, the dilution is by volume. All concentrations of honey are given as final concentrations unless otherwise stated.

Chapter One: General Introduction

"Whether it's got reliable evidence is the key - not whether it's got a conventional or alternative label" (Rogers, 2003)

The study in this thesis investigates the effect of honey on key cells involved in the inflammatory phase of tissue repair. Therefore this review will briefly examine the avenues of work which led to the acceptance in modern times of honey as a therapeutic agent, as well as the mechanisms by which honey was known to promote wound healing prior to commencement of this study. A section on the process of tissue repair will indicate steps where therapeutic agents may intervene. Finally a discussion of the aims of this study will be presented.

1.1 DEFINING THE FIRST STEPS TO INVESTIGATE WHETHER HONEY HAS SCIENTIFIC VALIDITY AS A WOUND HEALING THERAPY

The aim of current therapeutic discovery research is to identify drugs which are highly specific for a single target, do not produce side-effects, and are active *in vivo*. Generally major drug development programs screen thousands of compounds in order to identify an agent with some therapeutic activity. The exploration of honey as a therapeutic agent is being conducted in reverse. Having been a successful therapy for several millennia, the scientific research to understand how honey acts physiologically to promote wound healing is only now being done. Commonly, therapies which optimise tissue healing have effects upon receptors, enzymes or ion channels (Nicholson, 2000). Therefore, the immune system is a relevant place to begin a search for the mechanisms by which honey has these effects, as the inflammatory response to injury or infection is the start of the healing/repair process. Further, aspects of the immune system are easily modelled in vitro by culturing specific cell types and challenging them with inflammatory conditions.

The key objective of this thesis was to investigate the effect of honey on the immune system. The first step was to identify the cells upon which honey has a significant effect. Then, if honey provided encouraging results in these cell-based assays, more specific analyses would be performed to demonstrate how honey produced the biological response. While the study undertaken here was not the first of its kind to examine the effect of honey on immune cell function, previous research had been limited. The two published studies conducted prior to this thesis identified the potential for honey to stimulate lymphocyte cell division and to increase phagocytic activity of neutrophils (Abuharfeil *et al.*, 1999), and also the potential for honey to increase cytokine production by monocytes and to reduce the production of oxygen radicals by these cells (Tonks *et al.*, 2001). These studies only tested a few concentrations of honey (0.1, 0.25% Jordanian wild flower honey and 1% New Zealand manuka and Otago pasture honey). With this in mind, it was logical to commence research in this thesis by repeating the assays described in those studies, and extending the range of honey concentrations.

1.2 CURRENT KNOWLEDGE ABOUT HONEY AS A TISSUE - HEALING AGENT

Molan (2001) has comprehensively reviewed all that is currently known about the tissue - healing properties of honey. Honey has been used as a folk medicine since ancient times (Adcock, 1962; Ransome, 1937). It appears in historical records as a pharmaceutical used to successfully treat a myriad of ailments from wounds to intestinal disorders (reviewed by Molan, 1992a). With the advent of bacterial resistance to antibiotics resulting from their widespread use in the 20th century, honey has been rediscovered as a medicine. The proven ability of honey to promote wound healing by removing bacteria, debriding, and by stimulating re-epithelialisation, growth of fibroblasts and new blood capillaries, has been reviewed by Molan (2001). Though there was limited scientific evidence to explain the mechanisms by which honey optimises wound healing prior to the present study, a wealth of anecdotal reports document the ability of honey to remove infection in cases where skin was broken or unbroken (Molan, 1998; Postmes *et al.*, 1997; Subrahmanyam,

1993; Harris, 1994; Adesunkanmi and Oyelami, 1994; Suguna *et al.*, 1993; Postmes *et al.*, 1993; Ndayisaba *et al.*, 1993; Efem, 1993; Gupta *et al.*, 1992; Ankra-Badu, 1992; Subrahmanyam, 1991; Zumla and Lulat, 1989; Farouk *et al.*, 1988; Efem, 1988; Dumronglert, 1983; Bergman *et al.*, 1983; Bose, 1982; Armon, 1980; Cavanagh *et al.*, 1970; Bulman, 1955). While much of the evidence for honey as a wound healing agent is based on topical application of honey to the skin (Cooper *et al.*, 2001; Vardi *et al.*, 1998; Subrahmanyam, 1998; Wood *et al.*, 1997; Harris, 1994; Subrahmanyam, 1994; Subrahmanyam, 1993; Phuapradit and Saropola, 1992; Subrahmanyam, 1991; Efem, 1988; Bergman *et al.*, 1983) there is evidence that honey has a protective effect on the gut mucosa (Ghosh and Playford, 2003; Bilsel *et al.*, 2002; Gharzouli *et al.*, 2002; Ali *et al.*, 1997; Haffejee and Moosa, 1985; Salem, 1981). The ability for honey to have direct activity on bacteria has been well documented (Kingsley, 2001; Moore *et al.*, 2001; Dunford *et al.*, 2000; Thomas *et al.*, 1998; Vardi *et al.*, 1998; Wood *et al.*, 1997; Ndayisaba *et al.*, 1993; Efem, 1988), and honey is allowed to be sold as a medicine in Australia based on this scientific evidence. Clinical observations that honey clears infection beneath unbroken skin is thought to be due to the existence of at least one of the following factors in honey: antibacterial activity, immunostimulatory activity or anti-inflammatory activity (Ahmed *et al.*, 2003; Biswal *et al.*, 2003; Gheldof *et al.*, 2003; Misirlioglu *et al.*, 2003; Schramm *et al.*, 2003; Swellam *et al.*, 2003; Lusby *et al.*, 2002; Mahgoub *et al.*, 2002; Topham, 2002; Al-Waili, 2001; Ali *et al.*, 1997; Duddukuri *et al.*, 1997). The present study sought to identify which of these factors was most likely to be responsible for observed effects on tissue healing in the absence of clinical infection.

1.3 THERAPEUTIC BENEFITS OF HONEY

1.3.1 Honey as an antibacterial agent

Infection of wounds result from the presence of micro-organisms, and honey has been used successfully to clear bacteria from a variety of infected wounds (reviewed by Molan, 2001). The most compelling evidence for the ability of honey to act as an antibacterial

agent is the clearance of antibiotic-resistant bacterial strains (Dunford *et al.*, 2000; Vardi *et al.*, 1998). A brief review of the known mechanisms by which honey has antibacterial activity is given here. Antibacterial activity of honey has been attributed to a number of factors: the osmotic effect of the sugars, acidity, and the presence of hydrogen peroxide along with other unidentified substances probably originating from the floral source (Wahdan, 1998). The "Unique Manuka Factor" (UMF) is an example of a floral-derived compound with antibacterial activity which is only found in manuka honey (Molan and Russell, 1988). The rating of the level of UMF has been adopted as a marketing tool for manuka honey to be sold as a medicine in many countries. Though, despite many years of research, UMF has not been isolated to identify its chemical structure (Weston, 2000). Evidence that antibacterial activity of generic honey types is conveyed by more than the osmotic effect is demonstrated by studies where honey gave complete bacterial inhibition, and sugars alone had no effect (Cooper and Molan, 1999).

1.3.1.1 Osmotic effects

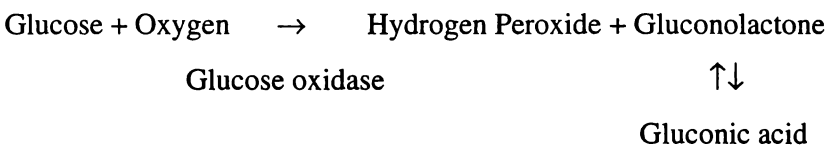
One of the ways honey is known to inhibit microbial growth is due to its high sugar content. Honey is hypertonic, a saturated or supersaturated solution of sugars, fructose and glucose being the key components (Rüegg and Blanc, 1981). Honey only has a 15-21% water content (w/v), and the low water activity deprives bacteria of the water needed for cell function, resulting in bacterial inhibition (Molan, 1992a; Chirife *et al.*, 1982. Honey may also draw water from bacterial cells (Majno, 1975). This has been demonstrated using artificial honey, made using the same proportions of sugars found in natural honey, and measuring bacterial inhibition (Postmes *et al.*, 1993).

Dilution of honey results in corresponding reductions of osmotic effect. The lowest concentration of sugar that inhibits *Staphlococcus aureus* growth has a water activity of 0.86 (equivalent to a 29% v/v honey solution). Yet, much lower concentrations (2-4%) can completely inhibit *S. aureus*, indicating the antibacterial activity obtained by honey can not be attributed solely to osmolarity effects (Cooper *et al.*, 1999; Chirife *et al.*,

1982). This has important implications for potential use of honey as a topical treatment for infected wounds, due to the dilution effect which will occur with lymph or plasma (Molan, 2001). It will also be diluted by gastric fluid when taken orally (Molan, 2001).

1.3.1.2 Hydrogen peroxide

A key component of honey responsible for antibacterial activity is hydrogen peroxide (Weston, 2000; Molan, 1992a; 1992b). The hydrogen peroxide content of honey was first identified in 1962, and in 1963 its presence was attributed to the activity of the enzyme glucose oxidase in honey (White *et al.*,1963; Adcock, 1962). At that time, the antibacterial activity of honey was attributed to hydrogen peroxide for the first time, and it was observed that either light or catalase could remove its activity.



This reaction occurs when bees secrete the glucose oxidase enzyme from their hypopharyngeal glands into the nectar during the formation of honey (White, 1975a;1975b). White *et al.* (1963) demonstrated that hydrogen peroxide is only produced after honey has been diluted, as glucose oxidase is almost inactive in full strength honey. This has important implications for its use as a topical treatment, again due to the dilution effects of lymph, plasma or stomach acids when orally administered. Further, it is important that hydrogen peroxide is only present at low concentrations as it has a cytotoxicity exceeding its bacterial potency at concentrations beyond 100 μM (Burdon, 1995; Lineaweaver *et al.*, 1985).

1.3.1.3 Acidity

Due to the presence of gluconolactone/gluconic acid produced enzymically during the ripening of nectar into honey, honey has a characteristically low pH, typically 3.2-4.5 (White, 1975b; Stinson *et al.*, 1960). Though this means that honey has a lower pH than the minimum tolerated values for survival by some key wound-infecting bacteria species (*e.g. Pseudomonas aeruginosa*, pH 4.4), no correlation has been found between antibacterial activity and pH of honey (Molan, 1992a; Thimann, 1963). Again, dilution of honey applied to wounds will change the pH and this will affect the extent to which pH will negatively affect bacterial species.

1.3.1.4 Non-peroxide antibacterial components

Flavonoids, including chrysin, pinobanksin and pinocembrine (Marcucci *et al.*, 2001), along with flavonone and pinocembrine (Bogdanov, 1984), have been isolated in small amounts from honey. While these substances have proven antibacterial activity (Rivera-Vargas *et al.*, 1993), they could not have significant antibacterial effects at the concentrations present in honey (Weston, 2000). The antibacterial activity of the phenolic acids, caffeic acid and ferulic acid, has also been identified (Cizmarik and Matel, 1970) and these have been isolated from honey (Wahdan, 1998), but these occur at concentrations well below those necessary for potent effects (Weston, 2000). The presence of UMF in manuka honey has already been described (Section 1.3.1).

1.3.1.5 Variation between honey types

The demonstrated variation in antibacterial activity between honeys obtained from different floral origins and geographical sources confirms that different honey types will have different therapeutic properties (Allen *et al.*, 1991; Farouk *et al.*, 1988; Molan, 1992b). The knowledge that honey type could be matched to therapeutic useage was understood by the ancient physicians: Aristotle (384-322 BC) recommended pale honey

be used as a salve for sore eyes and wounds, and Discorides (c.50 AD) promoted pale honey from Attica as the best cure for ulceration (Molan, 1999). While manuka honey is likely to be rich in glycated proteins and antioxidants, it has a low hydrogen peroxide content compared with a pasture honey type, which has the reverse profile (Molan, 1992b). The hydrogen peroxide content of individual honey types is likely to be related to the catalase content of that honey, and this is derived from the plant of origin (Dustmann, 1971; Schepartz and Subers, 1966). The ascorbic acid content of host plasma, along with the presence of iron has also been documented to affect the rate at which hydrogen peroxide breaks down via chemical reactions (Savini *et al.*, 1999). This highlights the importance of interactions between the host and the honey type in any observed variation of potency. Darker honeys have been found to have a higher antioxidant content (Molan, 2001), which is a reflection of the high content of plant phenols (P. C. Molan; personal communication). While the white pasture honey type obtained from the Otago plains, is largely devoid of tree pollens due to the lack of tree cover, and therefore, antioxidant content is low, manuka honey has a high antioxidant content.

1.4 MECHANISMS BY WHICH HONEY PROMOTES TISSUE REPAIR

1.4.1 The existing evidence for honey as a cell growth stimulant

Of direct relevance to the aims of this study, there are a number of anecdotal reports which suggest that honey promotes cellular anabolism (Lusby *et al.*, 2002; Wood *et al.*, 1997; Ndayisaba *et al.*, 1993; Somerfield, 1991; Efem, 1988). Though the mechanisms by which honey promotes cell growth have been speculated, they have not yet been identified (Molan, 1993). Acidification of wounds by lowering the pH has been determined experimentally to increase the rate of epithelialisation (Kaufman *et al.*, 1985), and this may contribute to the observed stimulatory effects of honey on tissue repair. Of relevance to this study, 0.1% manuka honey concentrations stimulated epithelial cell division in a respiratory epithelial cell line model and increasing the concentration beyond this had no effect (D. Adams, Child Health Research Institute, Adelaide; personal

communication). Honey has been demonstrated to: promote the development of new connective tissue around regenerating blood vessels (Subrahmanyam, 1998; Efem, 1993; Farouk *et al.*, 1988; Efem, 1988), stimulate epithelial cover (Misirlioglu *et al.*, 2003; Topham, 2002; Subrahmanyam, 1998; Efem, 1993; Efem, 1988), stimulate collagen synthesis (Kumar *et al.*, 1993; Suguna *et al.*, 1992) and stimulate development of new blood vessels to increase tissue oxygenation (Kumar *et al.*, 1993; Gupta *et al.*, 1992). Further, the osmotic outflow of lymph which occurs when honey is applied topically to wounds may increase nutrient supply to the tissues (Lusby *et al.*, 2002). It would appear that honey has a superior ability to promote re-epithelialisation compared with sugar (Bose, 1982). In addition to its sugar content, honey has a direct nutrient effect on cell metabolism (Molan, 1999). Hydrogen peroxide has been demonstrated to have a direct stimulatory effect on fibroblast proliferation when added to cell cultures *in vitro* at low concentrations (Burdon, 1995; Chung *et al.*, 1993; Schmidt *et al.*, 1992; Rao and Berk, 1992; Murrell *et al.*, 1990). The role of hydrogen peroxide as an immune stimulant is discussed in Section 1.4.3.

1.4.2 Cleansing and debriding action of honey on wounds

Honey has been documented to provide cleansing action on wounds due to flushing which occurs as lymph is drawn out by the osmotic action of sugars (Topham, 2002; Molan, 1999). In addition to removing any potential contaminants from the wound bed, the osmotic activity lifts dead tissue eliminating the need for surgical debridement (McInerney, 1990; Efem, 1988; Farouk *et al.*, 1988; Wadi *et al.*, 1987). Debridement of the wound bed is critical because a contaminated wound cannot heal (McInerney, 1990) due to the propensity for dead tissue to support the growth of bacteria (Cavanagh *et al.*, 1970). The importance of maintaining a moist wound-healing environment for optimal tissue repair has been reported (Thomas *et al.*, 1998). This is because protein-digesting enzymes function more efficiently in moist environments (Molan, 2001; Bradley *et al.*, 1999; Archer *et al.*, 1990). Fibroblast proliferation is optimised by moist conditions, and such an environment is also necessary for keratinocyte migration across the wound bed to

restore epithelial cover, so that a scar is less likely to form (Misirlioglu *et al.*, 2003; Niessen *et al.*, 1999; Kirsner and Eaglstein, 1993). Evidence from Topham (2002), suggests that an additional contribution of honey to scar-free healing is its ability to enable wound-healing proteoglycans to function without excessive production of collagens. Furthermore, it was demonstrated that the ability of hyaluronic acid to be continually generated from glucose due to the presence of a glucose supply reduced the formation of fibre-forming collagens (Topham, 2002). These observations help to explain why honey is associated with scar-free healing (Molan, 1993; Topham, 2002; Tejero-Trujeque, 2001).

The ability of hydrogen peroxide to mediate the observed chemical and enzymic debriding processes imparted by honey on wounds has been postulated by Molan (2001). Metalloproteases are known to be activated by oxidants (van Wart and Birkedal-Hansen, 1990), along with deactivation of inhibitors of tissue serine proteases (Flohé *et al.*, 1985). Fibroblast collagenase, normally present in tissues in the latent form, can be activated by oxidants, enabling breakdown of collagen so that tissue remodelling can proceed (Weiss *et al.*, 1985). The ability of honey to reduce post-operative peritoneal adhesions has been speculated to be due to inhibition of peritoneal plasminogen activator (Aysan *et al.*, 2002). They postulated this may have been achieved through antioxidant activity (Section 1.4.4.1), or because of the physical properties of honey including its hygroscopicity, hypertonicity and low pH (Sections 1.3.1.1, 1.3.1.3) which inhibits mechanical contact between the cecal and ileal surfaces (Aysan *et al.*, 2002) so that adhesion could not occur.

1.4.3 Evidence that honey is an immune stimulant

In addition to the proven antibacterial activity of honey, and the clinical observations that it stimulates re-epithelialisation and minimises scar formation, there is limited evidence that honey may act on immune cells in the first phase of inflammation. Studies conducted by Abuharfeil *et al.* (1999), have shown that honey stimulates B- and T-lymphocytes , and activates neutrophils. A study by Al-Waili (2003) showed that oral ingestion of honey

increased both the number of circulating monocytes and lymphocytes in blood. Investigations by Blair (2000) and Tonks *et al.* (2003), have demonstrated that honey stimulates monocytes *in vitro* to release tumour necrosis factor α (TNF-α) and interleukins 1 and 6 (IL-1 and IL-6). Further, Simuth *et al.* (2004) have reported that the presence of royal jelly proteins in honey stimulated macrophages to release TNF-α. These cytokines play a crucial role in activation of the immune response (Nathan, 2002). There are a number of mechanisms which have been hypothesised to account for immune stimulation by honey. The direct supply of glucose to macrophages has been demonstrated to enhance their respiratory burst (Ryan and Majno, 1977). Incubation of T cell lymphocytes with low concentrations of hydrogen peroxide has been reported to stimulate cell proliferation *in vitro* (Reth, 2002) and stimulate fibroblast mitosis *in vitro* (Chung *et al.*, 1993). There are a number of current theories about how hydrogen peroxide stimulates cell proliferation, and the possibility that the hydrogen peroxide content of honey may induce these changes. Direct exposure of various cells to low concentrations of hydrogen peroxide *in vitro* has been demonstrated to have various effects: it regulates signal transduction pathways, activates transcription factors and controls the expression of genes governing growth, differentiation and immune function in various cell types (Reth, 2002; Mahadev *et al.*, 2001; Nappi and Vass, 1997; Burdon, 1995).

Hydrogen peroxide potentiates the phosphorylation of endogenous cellular proteins which are involved in regulation of cell activity. Studies by Golpalakrishna *et al.* (1986) and Kass *et al.* (1989) have suggested that hydrogen peroxide may temporarily alter the kinase-phosphatase balance within cells. Hydrogen peroxide modulates the redox status of the cell as a result of changes in cellular levels of oxidised glutathione (GSH) and its reduced form (GSSG) (Burdon, 1995). High GSH levels are required for IL-2-dependent proliferation, and for cytotoxic T-lymphocyte activity. Proliferating fibroblasts *in vitro* release low levels of superoxide (and its dismutation product, hydrogen peroxide), from the action of NADPH oxidase present in the cell membranes (Meister, 1988). The cytokines, IL-1 and TNF-α, stimulate further superoxide release in the presence of low

levels of hydrogen peroxide (Burdon, 1995). The observations made in these studies suggest that cells use hydrogen peroxide to stimulate their growth and division, and that hydrogen peroxide functions as an intercellular messenger. Hydrogen peroxide has been shown to inactivate the inhibitor of matrix metalloproteinases (MMPs), thereby activating the MMPs. MMPs cleave TNF from monocytes and macrophages, which enhance the neutrophil response to the injured tissue site and ensure the early inflammation phase proceeds at the required rate (Nathan, 2002).

The insulin-like effects of hydrogen peroxide may mediate growth stimulation of cells, and these effects include activation of glucose transport and increased incorporation of lipids into glycogen and lipids, along with activation of the receptor tyrosine kinase through increased IGF-II binding (Mahadev *et al.*, 2001; Kadota *et al.*, 1986). Hydrogen peroxide is known to have a direct effect on the insulin receptor (Koshio *et al.*, 1988). Low concentrations of hydrogen peroxide have also been reported to directly stimulate angiogenesis (Tur *et al.*, 1995).

Glycosylated proteins have been demonstrated to have a direct stimulatory effect on endothelial cells (Brownlee, 1995; Vlassara *et al.*, 1988; Takata *et al.*, 1988) and it is probable that honey would contain these as protein stored in the presence of high levels of monosaccharides are likely to react. The proportion of proteins varies according to honey type, and the possibility that glycated proteins contained in honey may have immunogenic effects has been suggested by Tonks *et al.* (2001). The terms "glycated" and "glycosylated" are here acknowledged to refer to different protein products. Glycated proteins are created by the spontaneous reaction of sugars with proteins leading to the production of advanced glycation end products (AGEs), occurring without enzyme activity (Yim *et al.*, 2001). Glycosylated proteins are created through the enzymatically driven N-or O-linked transfer of sugars to proteins. It is highly likely that honey would contain AGEs due to the proteins being in contact with sugars during storage. It is possible that glycosylated proteins would be found in honey due to the potential for the

non specific transfer of sugars onto proteins by the invertase enzyme. The AGEs will stimulate endothelial cells (Price *et al.*, 2004).

1.4.4 Honey as an anti-inflammatory agent

Though the process by which honey acts as an anti-inflammatory has yet to be demonstrated, the effect has been observed clinically in numerous reports where honey applied to wounds reduces the localised swelling, heat and pain associated with inflammation (Molan, 1999). Anecdotal evidence from using manuka honey incorporated in a gel for topical use has reported suppression of itching and erythema associated with mosquito bites and other inflammatory skin reactions due to local anaphylactic reactions (P. C. Molan; personal communication). These symptoms are produced by the increased liberation of prostaglandins (especially PGE2), which trigger the hypothalmus to increase heat production, resulting in development of fever or localised hot areas (Dascombe, 1985). This m\ay indicate that honey acts to shortcircuit prostaglandin synthesis. Of particular note, honey has been observed to reduce inflammation when applied to unbroken skin, indicating that the anti-inflammatory activity may be able to diffuse through the skin (Molan, 1992a). Indeed, hydrogen peroxide has been demonstrated to diffuse through cell membranes and enter the cell nucleus (Reth, 2002) and therefore it is plausible that this may occur when honey is applied topically (Molan, 1992a). Microscopic examination confirmed that application of honey to inflamed tissues significantly reduced the leukocyte count (Postmes *et al.*, 1997). In studies by Postmes *et al.* (1997); Kumar *et al.* (1993); Oryan *et al.* (1998); Gupta *et al.* (1992) and Church (1954), reduction of inflammation in wounds where honey was applied in the absence of infection was taken as evidence that honey has a direct anti-inflammatory effect. As infection was not present, the observed anti-inflammatory effects could not have be attributed to any removal of inflammation promoting bacteria. Honey can be used as a topical treatment for localised inflammation, and has been used successfully to alleviate pain and damage to burned tissues (Subrahmanyam, 1993;1991).

1.4.4.1 The possibility that the antioxidant content of honey may account for observed anti-inflammatory effects

It is not known what part of the observed anti-inflammatory effect may be due to the presence of antioxidants. The variation in antioxidant content according to honey type has been discussed in Section 1.3.1.5. Direct application of antioxidants to burns has been demonstrated to reduce inflammation (Burlando, 1978; Subrahmanyam, 1991). There is a burgeoning body of evidence which demonstrates that antioxidants have potent effects on immune cell function (Bourdon *et al.*, 1999; Grimble, 1994; Ames *et al.*, 1993) and much of the evidence for bioactive agents to have immunosuppressive effects on inflammation is based on their antioxidant content (Basova *et al.*, 2002; Meydani and Erickson, 2001; Alliangana, 1996; Rao *et al.*, 2003). Antioxidants are known to alleviate tissue damage by harmful oxidant species through stabilising ions via covalent bonding of unpaired electrons (Grimble, 1994). The observation that topical administration of honey reduced post-operative peritoneal adhesions has been speculated to be due to its antioxidant content, particularly its content of caffeic acid, benzoic acid, phenolic acid, and flavonoid glycones (Aysan *et al.*, 2002). The likely mechanism of observed anti-inflammatory action is prevention of the feed-back amplification of inflammation that is due to hydrogen peroxide. Clinical studies have confirmed that hydrogen peroxide is directly inflammatory to skin and this was why it ceased to be used as an antiseptic on tissues (Saïssy *et al.*, 1995; Halliwell and Cross, 1994; Salahudeen *et al.*, 1991; Lineaweaver *et al.*, 1985). Antioxidants have been demonstrated to block the stimulatory effects of hydrogen peroxide and prevent the oxidation of regulatory proteins which occurs when free radicals form from it (Grimble, 1994).

1.5 HOW DOES THE IMMUNE SYSTEM FUNCTION TO PROMOTE NORMAL WOUND HEALING?

Inflammation is the first crucial phase of tissue repair. It arises through the actions of immune cells and their production of cytokines, growth factors and oxidant species (Enoch and Harding, 2003; Nathan, 2002). When the interplay between these

inflammatory cells and their mediator products deviates from the normal sequence, due to some breakdown in regulation, clinical disease may develop (Enoch and Harding, 2003; Nathan, 2002). The pathogenesis of some key inflammatory diseases is outlined in Section 1.6. The process of tissue repair conforms to a series of overlapping but well defined phases of inflammation, proliferation and remodelling (Nathan, 2002) as shown in Figure 1.1. Where bleeding has occurred in the tissues, a hemostatic phase in which the fibrin clot is formed, must precede the arrival of the phagocytes (not shown in Figure 1.1).

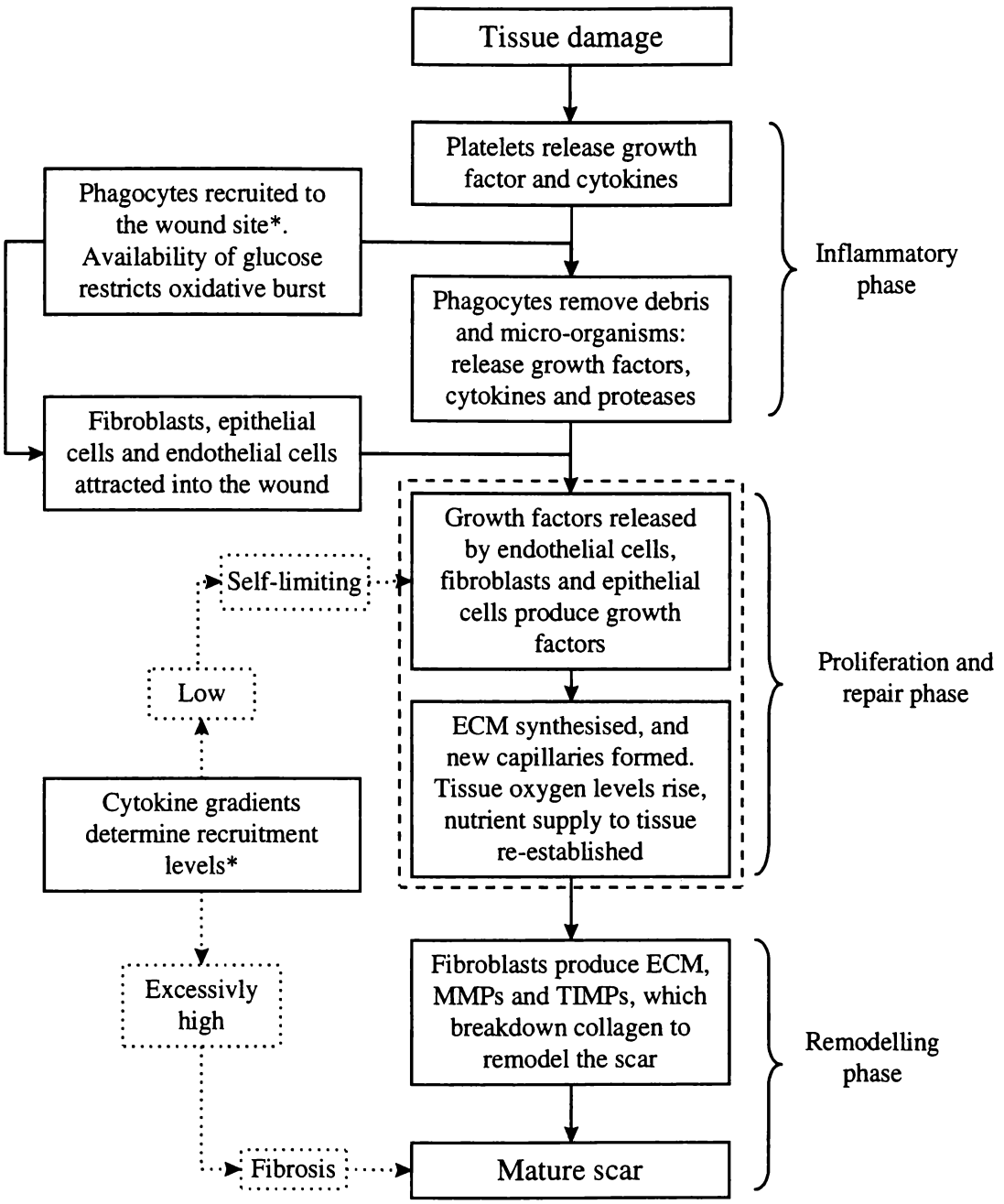


Figure 1.1: Concept map illustrating the overlapping phases of the normal wound repair process. In the normal individual, wound healing occurs according to a predictable sequence of phases, in which the production of cytokines, growth factors and oxidant species is tightly regulated. Points at which honey may be hypothesised to have some activity are indicated by *. (This is a modification of a figure presented by Enoch and Harding, 2003). Definitions: ECM extracellular matrix, MMPs matrix metalloproteinases, TIMPs tissue inhibitors of metalloproteinases.

In order to recognise the steps in the tissue repair process at which honey has been hypothesised to intervene, a brief outline of the normal wound healing process is given. However, it should be noted that other studies have demonstrated that honey has effects on cells during the final remodelling phase of tissue repair (Section 1.4.1).

1.5.1 Events during the inflammatory phase

The following description of inflammatory cell recruitment mechanisms is a comprehensive review of the literature. When tissue damage occurs, soluble mediators (histamine from mast cells, kinins from precursor molecules undergoing proteolysis) directly cause vascular changes (Nathan, 2002). These include the classic hallmarks of inflammation characterised by Celsus some two thousand years ago; tumour, rubor, calor and dolor (Benjamini *et al.*, 1996). In simple terms, Celsus was describing local changes in vascular permeability and accompanying changes in blood flow. These changes are necessary in order for immune cells (leukocytes) to be recruited to the damaged tissues, so that phagocytes can destroy foreign invaders and clean up the tissues, and for the repair process to be initiated (Nathan, 2002; Enoch and Harding, 2003).

The first phase of repair is dominated by inflammation of the wound and mobilisation of the cells in to the injured tissues enabling synthesis of granulation tissue to occur. This phase is characterised by infiltration of the polymorphonuclear leukocytes in to damaged tissues (Enoch and Harding, 2003; Nathan, 2002). Neutrophils and monocytes migrate to damaged tissues in response to chemotactic factors produced by the coagulation cascade (Kirsner *et al.*, 1993). Recent studies by Marsland *et al.*, (2005) demonstrate that the recruitment of leukocyte subtypes is tightly regulated by chemotactic cytokines, termed chemokines. Chemokines are produced and released by a variety of cell types during the initial phase of the inflammatory response. To date, there have been 40 chemokine proteins identified (Simon and Green, 2005). The chemokines bind to specific seven transmembrane spanning G protein-linked receptors on target cells, and this binding action causes reconfiguration of adhesion proteins, such as β integrins, on the surface of

the responding cells. Chemokines stimulate chemotaxis and extravasation of leukocytes and can only act on cells carrying the specific chemokine receptor. For example, members of the CXC family of chemokines signal through the chemokine receptors CXCR1-5 (Christopherson and Hromas, 2001). In general chemokines from the CC (β-chemokine) family chemoattract monocytes, eosinophils, basophils, and T cells (Sauty *et al.*, 1999). In wounded tissue, both macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), and Rantes are primary chemokines attracting macrophages to the injury site, and the time of expression differs for each molecule (Jackman *et al.*, 2000). MCP-1 also acts on a subset of T cells and on mast cells bearing the CCR3 receptor (Baggiolini, 2001). Conversely, the chemokine Interleukin 8 (IL-8), a member of the CXC family (α-chemokine), results in inflammatory reactions dominated by neutrophils (Marshall *et al.*, 2003), and is synthesised by the endothelium. It is now well recognised that chemokines such as CCL19 and CCL21do more than simply guide migration of immune cells, but through induction of dendritic cell maturation, prime T cell responses (Kaiser *et al.*, 2005; Marsland *et al.*, 2005; Yopp *et al.*, 2005). The group of CXC chemokines lacking a NH_2-terminal ELR sequence are induced through interferons (IFN) as a lymphocyte chemoattractant, and include IP-10, Mig, and I-TAC (Sauty *et al.*, 1999). In addition to the timing of chemokine release, tight regulation of leukocyte migration is effected by their interplay with cytokines and growth factors (Werner and Grose, 2003). There is evidence for the existence of immunological synapses and that when conjugates form between T cells and antigen-presenting cells (APC), chemokine receptors are only sensitive to chemokines released by the APC (Trautmann, 2005). APC comprise a variety of cells with a common function of processing antigens and re-expressing fragments of the antigen on their surface for T cell recognition, including dendritic cells, macrophages and B-cells. Therefore, the existence of immunological synapses explains why T cells are not distracted by irrelevant, exogenous chemokines. It has been understood for some time that while chemokines provide "start" signals for T cells to stimulate directional movement, TCR-induced calcium flux immobilises the T cell (Trautmann, 2005). Recently, it has been proposed that the immunological synapses formed between T cells and APC stabilise the T cell-APC

conjugate and act as a costimulatory signal (Trautmann, 2005). Thus, chemokines play a key role in both direct and indirect recruitment of leukocytes.

A mixture of chemokine receptors, integrins and selectins expressed by leukocytes and endothelial cells are involved in the recruitment of leukocytes from the blood into the tissues. Changes in vascular permeability lower the shear stress so that leukocyte movement through the circulatory system is slowed and the immune cells have greater contact with postcapillary venules (Nathan, 2002). As the avidity of the integrins expressed on the leukocyte membranes toward adhesion molecules on the endothelium increases, leukocytes become immobilised (Trautmann, 2005). It is the exposed endothelium present in these venules that enables leukocytes to interact with endothelium, the primary step in "directing" the immune cells through to the injured tissues (Benjamini *et al.*, 1996). The general paradigm governing the adhesion events by which immune cells undergo diapedesis (the passage of blood cells through the blood vessel walls after the cells have changed shape) are considered in more detail in Section 7.1. Briefly, these events include; capture of the leukocytes via interactions with activated endothelium (selectins and their carbohydrate ligands), initiating rolling of the leukocytes along the endothelium so that integrin molecules on the immune cells bind with counter-receptors on the endothelial cells (creating firm adhesion) (Enoch and Harding, 2003).

Following the reconfiguration of adhesion proteins, such as B integrins, in response to chemokine binding to surface receptors, leukocytes can then bind to endothelial cells lining blood vessel walls (Trautmann, 2005). Integrins are cell-adhesion receptors which enable cell-cell interactions and cell-extracellular matrix communication. The integrins are composed of two subunits, one belonging to the α family, and the other belonging to the β family (Hynes, 2002). Integrins are one of four types of cell-surface adhesion molecules (CAMs), and perform a distinct function from the other three types of CAMs (cadherins, Immunoglobulin superfamily, and selectins). Integrins need to be activated before they will bind their ligand (*e.g.* to VCAM1, MAdCAM1, ICAMs expressed on tissues), and clustering of integrins on the cell membrane is necessary for a full biological

response (Van Buul and Hordijk, 2004; Worthylake and Burridge, 2001). Re-circulating leukocytes also bear adhesion-molecule receptors for the mucin family (GlyCAM-1 and CD34) along with the immunoglobulin family (ICAM-1-3, VCAM-1, and MAdCAM-1) (Murray *et al.*, 2003). Within seconds of an inflammatory signal being released by endothelial cells, P-selectin is expressed on the cell surface, and the leukocyte binds to the endothelium via a ligand called the sialyl Lewis-x antigen. After the rolling leukocytes slow down through binding with P-selectin (tethering), tight adhesion of the leukocyte is effected through activated integrins. The integrin then binds to leukocyte integrin ligands, ICAM-1 and ICAM-2, expressed by endothelium (Garbacki *et al.*, 2005). Leukocytes do not migrate through epithelium easily without selectin binding, and therefore, the combination of integrin binding to integrin ligands, and ligands to endothelial E and P-selectin are both necessary. The differential expression of these homing receptors provides tight regulation of leukocytes moving into different tissues.

Once the flattened leukocytes are firmly adhered, diapedesis is facilitated by products of the immunoglobulin gene family, enabling the cells to squeeze through the endothelial sheet and move toward chemotactic gradients in the target tissues (Benjamini *et al.*, 1996). Aggregation and shape change of leukocytes undergoing diapedesis are guided by various adhesion molecules including CD11b/ CD18 (Mac-1), CD11a/ CD18 (LFA-1) and P-Selectin. Migration through the endothelial wall is guided by various adhesion molecules including, β_2 integrins, ICAM-1, VCAM-1 and PECAM-1 (Simon and Green, 2005; Murray *et al.*, 2003). Chemokines are particularly important in this part of the inflammatory response, as they guide leukocytes to the injured or infected site and trigger neutrophil phagocytosis. During acute inflammation macrophages derived from monocytes destroy microbes, debride wound tissue and release soluble growth factors which initiate the second, proliferative, phase of tissue repair. The epithelial tissues surrounding the injury site thicken as a result of mitosis and hypertrophy. Ehrlich (1996) has stated that optimum thresholds exist for normal wound healing to occur according to both time of onset, and duration, of the inflammatory response. Wounds which have late onset of inflammation are accompanied by late wound closure and frequently are

susceptible to the development of infection. Tissues in which inflammation persists beyond the normal time period (such as chronic wounds), or where hypersensitivity occurs (increased inflammation intensity) will also be subject to problems associated with abnormal healing.

1.5.2 The proliferation and repair phase

The development of new blood vessels along the wound margins constitute an integral function of the repair phase (Risau, 1997; Slavkin, 2000; Bennett and Schultz, 1993b). It is the formation of a blood clot immediately following injury that enables migration of cells in to the wound site (Risau, 1997; Slavkin, 2000). Fibrin provides a matrix for migration of fibroblasts, keratinocytes and monocytes. Where vasodilation occurs in tissue surrounding a burn site, blood flow is restricted in the damaged tissue preventing normal cellular migration. As a result of restricted cell movement, thrombus formation will frequently occur in arterioles which in turn may lead to local ischemia and tissue necrosis (Ehrlich, 1996). Where ischemia and necrosis of the tissues has occurred, it can be expected that the wound will take significantly longer for closure to be achieved. The appropriate synthesis of growth factors by platelets, such as fibroblast growth factor (FGF), TGF-α, TGF-β, and in particular platelet-derived growth factor (PDGF), is necessary for migration of fibroblasts (Eswarakumar *et al.*, 2005; Bonner, 2004; Falanga *et al.*, 1988). Where growth factor availability is not sufficient for establishment of normal mitogenic and chemotactic gradients for fibroblasts, it may be expected that delayed development of the collagen matrix will occur (Presta *et al.*, 2005; Ciano *et al.*, 1986). For these reasons, delayed wound closure is typically correlated with abnormal scar formation.

It is during the proliferative phase of tissue repair that induction of angiogenesis occurs and granulation tissue is formed. Collagen and proteoglycan production by the granulation tissue leads to an increase in the mechanical strength of the wound. Where the wound healing process occurs under optimum conditions, granulation tissue usually

appears around 3 days after injury has occurred (Niessen *et al.*, 1998). Granulation tissue contains new capillaries, fibroblasts, macrophages and mast cells. The number of mononuclear leukocytes present in the damaged tissue continue to increase until they are the dominant cell type by the fifth day (Enoch and Harding, 2003). Fibroblasts are recruited to the wound site in response to local cytokine production, and divide readily so that a very high density of fibroblasts is obtained rapidly (Cook *et al.*, 2000; Mendez *et al.*, 1998). Where the inflammatory phase has been prolonged, increased cytokine activity may occur and this has been found to correlate with increased keloid formation (Deitch *et al.*, 1983). Fibroblast cells continue to increase in number during the proliferative phase, and are responsible for the extracellular synthesis of collagen, proteoglycans and fibronectin. By the fifth day, granulation tissue fills the entire wound and collagen is abundant (Cook *et al.*, 2000).

Kirsner *et al.* (1993) describes the migration of epidermal cells as the advancement of an epidermal tongue in which the basal cells express selective cell surface markers (such as CD44) which are not normally expressed by non-squamous cells. These surface markers enable basal cells to move independently of the basement membrane due to loss of binding between the cell types. Early studies by Bullough and Mitrani (1978) suggested that epithelial cells migrate according to contact inhibition. When loss of contact with other cells occurs, individual epithelial cells become motile, and cease migration when contact is re-established.

During angiogenesis, it is thought that factors such as low oxygen tension, lactic acid and biogenic amines may stimulate growth regulator production (specifically TGF-β) and collagen synthesis (Kirsner and Eaglestein, 1992). Excessive fibrosis, and therefore deposition of collagen, may result in abnormal scar development where angiogenesis has occurred under hypoxic conditions. The study by Gessin *et al.* (1988) demonstrated the need for vitamin C to be present as a co-factor with detectable oxygen levels for normal collagen cross-linking to occur. Where the co-factor was lacking (such as in patients with scurvy) or the wound conditions were anoxic, poor wound healing was obtained.

1.5.3 Remodelling phase

The third (remodelling) phase of tissue repair occurs as the open wound is re-epithelialised and closure of the wound occurs. During re-epithelialisation, the granulation tissue matures to form scar tissue. In the normal healing process, fibroblast density is reduced via apoptosis as the oxygen levels rise due to neovascularisation (Phillips *et al.*, 1998). Interferons produced by T cells inhibit fibroblastic collagen synthesis and retard fibroblast proliferation (Kalvakolanu and Borden, 1996). Fibroblast translocation of collagen fibrils results in increased packing, giving wound contracture. Phenotypic changes to fibroblasts at the margins of the wound to create myofibroblasts (containing contractile proteins) enable participation of the cells in wound contracture (Ciano *et al.*, 1986). By pulling the surrounding skin in to the scar, contracture increases the breaking strength of the healed wound (up to a maximum of 80% of the original tensile strength of the uninjured tissue). There is a change in the type of collagen present in the wound during the remodelling phase, so that type III collagen is lysed by collagenases produced by a number of cells (epithelial cells, fibroblasts, macrophages, and leukocytes) and be replaced by type I collagen (Enoch and Harding, 2003). Thinning of the epithelium occurs around the healed site and the tissue acquires an attachment to the underlying dermis as it matures. The change of collagen type in the mature healed wound leads to a change in orientation of the scar tissue over the following 6 to 12 months (Kanzler *et al.*, 1986). Therefore, adequate collagen breakdown by tissue serine proteases and proteolytic enzymes is a crucial factor in determining the final appearance of a scar.

1.6 THE PATHOGENESIS OF SOME KEY INFLAMMATORY DISEASES

Inflammation is a double-edged sword, as the inflammatory process which fights off pathogens and enables tissue repair may induce pathological diseases in some inflammatory situations (Enoch and Harding, 2003; Coussens and Werb, 2002; Benoist

and Mathis, 2002). Honey may provide the therapeutic answer to the immune dichotomy (Molan, 1993).

1.6.1 The compromised immune system prevents normal healing

When patients have an abnormal leukocyte count, either through primary disorders caused by illness (leukemia, AIDS) or through secondary disorders caused by medical treatment (chemotherapy) this will have dire consequences for production of the soluble mediators which orchestrate inflammation (Dow *et al.*, 1999)(Figure 1.2 illustrates this). Where leukocytes are present at abnormally low densities, cytokine production will be limited and therefore, suboptimal recruitment of endothelial and epithelial cells to the injury site will result (Dow *et al.*, 1999). Phagocytes present at low densities would be unable to clear contaminants from the injured tissues, and if bacteria were present they could thrive unchecked in the necrotic tissue (Thomson, 2000; Dow *et al.*, 1999; Bucalo *et al.*, 1993; Facklam and Washington, 1991). Moreover, if epithelial cells are not signalled to close the wound in the final phase of tissue repair, normal healing cannot be achieved (Benjamini *et al.*, 1996), and the condition of the wound will deteriorate further (Dow *et al.*, 1999). Fibroblasts taken from the edges of chronic venous leg ulcers have been found to be less proliferative than normal fibroblasts and it was concluded from their altered morphology that these cells had become senescent (Stanley *et al.*, 1997;

Campisi *et al.*, 1996). If chronic wounds persist for long periods of time it becomes increasingly difficult to stimulate healing in the wound environment due to breakdown of protease inhibitors such as α2-macroglobulin and α1-proteinase inhibitor (Grinnell and Zhu, 1996; Rao *et al.*, 1975). When proteases are no longer regulated, the glycoprotein fibronectin, which plays an essential role in keratinocyte migration, is constantly degraded (Grinnell and Zhu, 1996; Wysocki *et al.*, 1993). Therefore, the development of therapeutic agents which can stimulate healing in immune-compromised patients, and cause minimal side-effects is of immense value. Based on the observations by Tonks *et al.* (2001) that honey induced cytokine release by resting monocytes, it has been hypothesised that honey may boost the immune system and this would be beneficial to

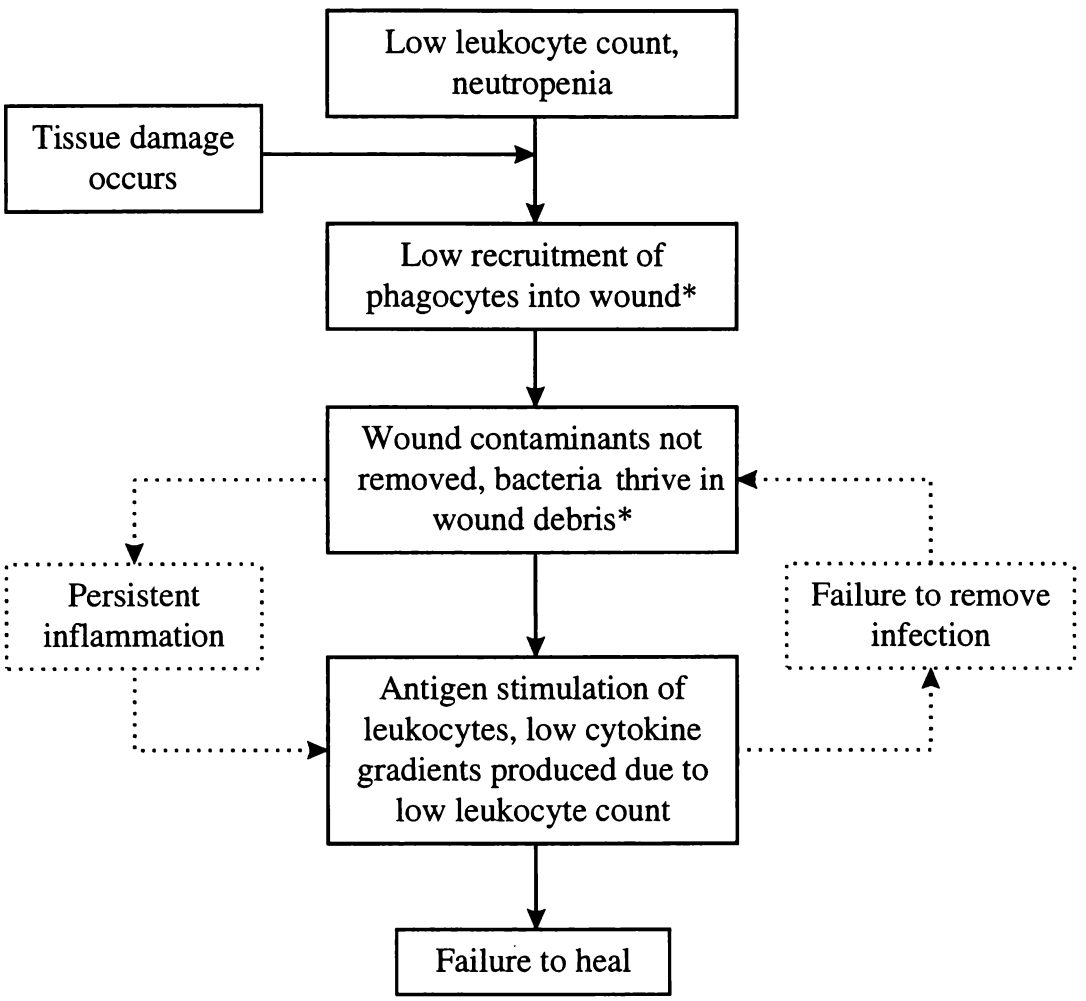


Figure 1.2: Concept diagram illustrating the development of chronic infection in an immune compromised patient. Low leukocyte counts following chemotherapy treatment create suboptimal gradients of intercellular messages, and the immune system is slow to respond to infection. Points at which honey is hypothesised to have some activity are indicated by *.

immune-compromised individuals. Once cytokines have been produced, bystander activation leads to the stimulation of neighbouring cells (Figure 1.1).

1.6.2 Safety issues associated with use of honey as a therapeutic agent

Where antiseptics are used to clean the wound, their cytotoxic nature damages the healing tissues, thus prolonging the healing process. Molan (1998) has noted that no adverse side-effects from using honey as a wound dressing have been reported in clinical and histological observations of humans and experimentally for animals. None were reported either in the many papers on the use of honey in opthalmology (Molan, 2001). In a survey conducted by Kiistala *et al.* (1995) allergies to honey were found to be rare. However, Helbling *et al.* (1992) proposed that severe allergies can occur due to the presence of pollen and bee proteins in honey. Molan and Allen (1996) demonstrated that the potential risk of wound botulism could be avoided by gamma-irradiation of honey to remove spores of *Clostridium botulinum* with no associated loss of antibacterial activity. With regard to the possible use of honey as an anti-inflammatory, it is ideal that honey produces virtually no side-effects. In the reports reviewed by Molan (1999) only 5 cases out of 470 cases treated with honey failed to successfully heal. Only one case in this study exhibited side-effects from the honey, in this case painful reactivity to honey as a topical treatment.

1.6.3 Fibrosis as an example of excessive inflammation

Clinically, keloid scarring is characterised by the presence of excessive dermal collagen beyond the confines of the original wound, so that the scar tissue is raised (Figure 1.3). Keloid scarring provides a key example of an inflammatory condition which results from the unregulated proliferation of fibroblasts during the proliferative phase of tissue repair (Figure 1.4). Histologically, keloids consist of the haphazard deposition of collagen fibres within the dermis, surrounded by a mucinous extracellular matrix with few macrophages and an abundance of eosinophils, mast cells, plasma cells, and lymphocytes (Urioste *et al.*, 1999).

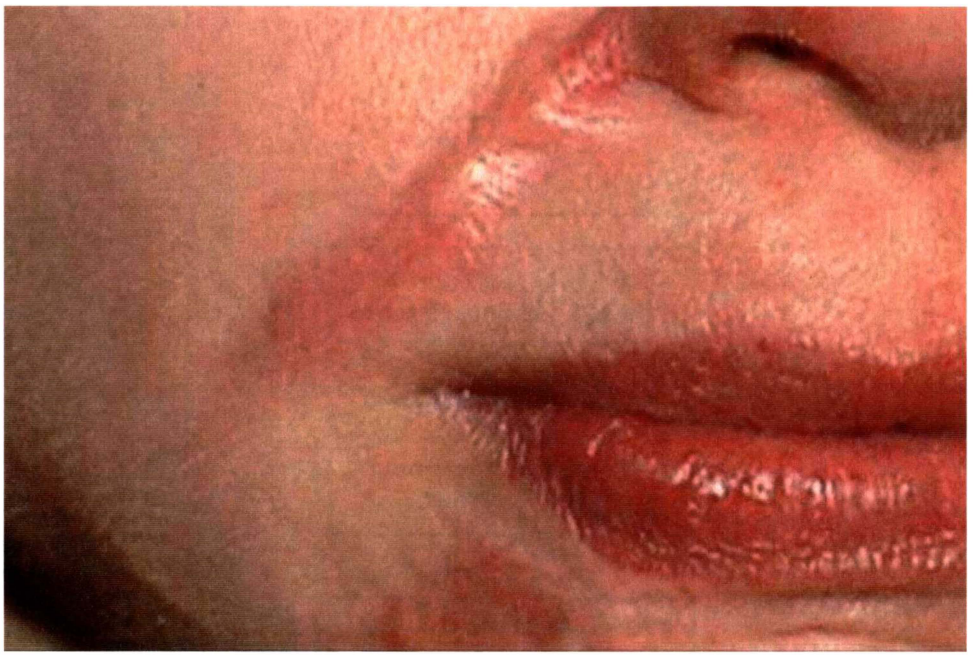


Figure 1.3: Characteristic appearance of a keloid scar. Note how excess collagen deposition has resulted in the characteristically raised and inflamed scar tissue.

The existence of a keloid scar is the result of persistent inflammation during the proliferation phase of wound repair, so that cytokines were produced at excessive levels leading to fibrosis (Urioste *et al.*, 1999) as summarised in Figure 1.4. Keloids are frequently pruitic, may form strictures and cause much pain for sufferers. Emotional suffering as a consequence of cosmetic disfigurement is common to those who exhibit keloids. Once developed, keloid scars tend to persist over time as compared to hypertrophic scars, which frequently undergo some degree of regression. There are many factors which can affect wound healing: the patient's health, nutritional status and intervention in the healing process can affect the outcome and affect the incidence of excessive scarring. Keloids only occur in humans, and it appears that ethnicity predisposes some individuals to keloid development (Ketchum, 1977; Alhady and Sivanantharajah, 1969). Though keloids have long been recognised as areas of abnormal healed tissue in records dating back as far as the *Smith Papyrus* around 1,700 BC, the exact mechanism by which they are formed and the best course of treatment for and indeed prevention of them continues to be elusive (Urioste *et al.*, 1999).

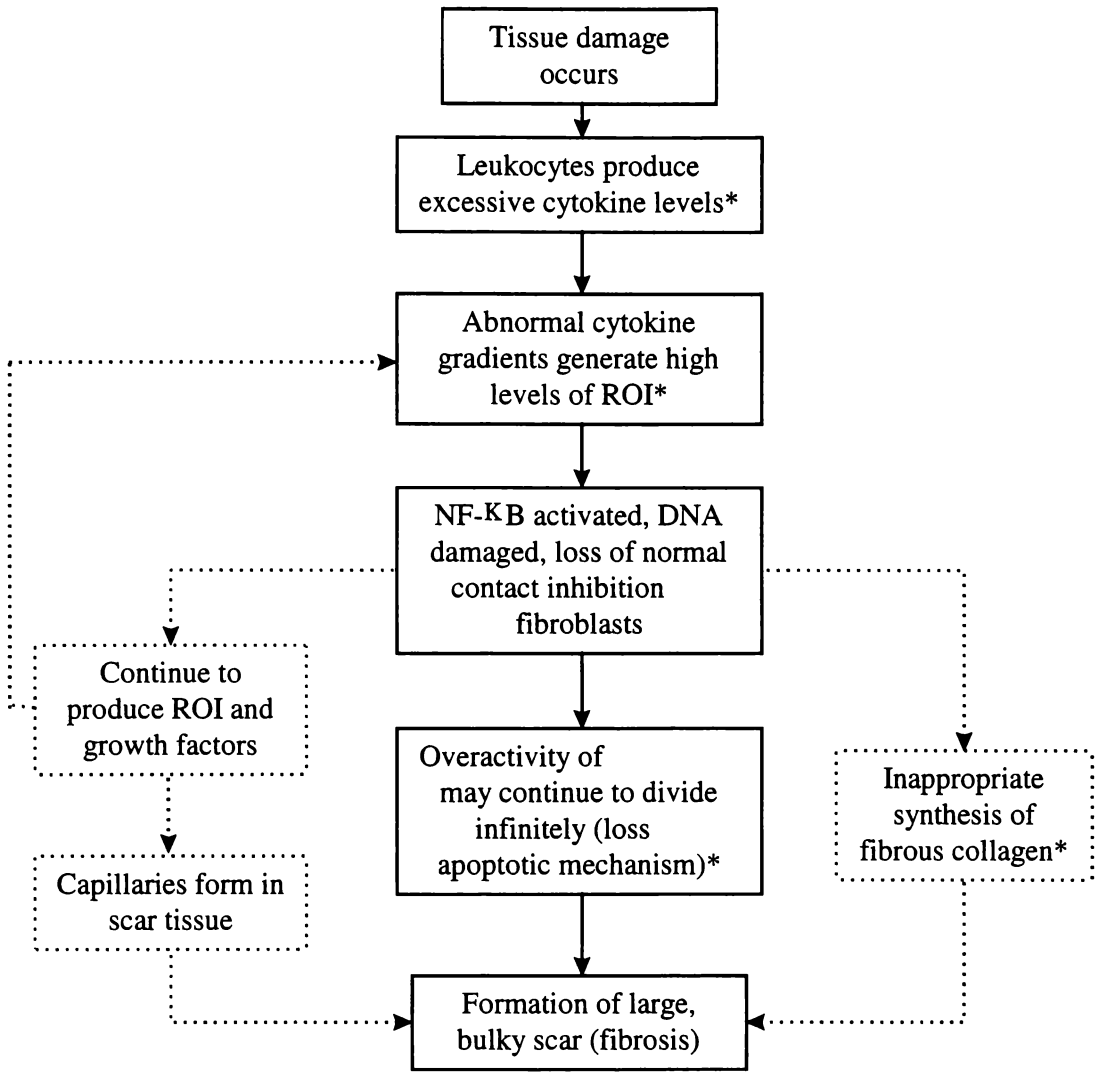


Figure 1.4: Concept diagram illustrating one mechanism by which a keloid scar may develop as a result of excessive inflammation (fibrosis). There are a number of theories about the causal mechanisms for keloid development, but ultimately they occur when the normal mechanisms governing fibroblast proliferation are lost. One theory is that the excessive production of cytokines and oxidant species by endothelial cells drives fibrosis. Possible points at which honey may intervene are indicated by *. Definitions: ROI reactive oxygen intermediates, NF-κB nuclear transcription factor κB.

Though the actual cause of keloid development remains to be elicited, the best accepted theory is that keloids occur when normal control over cell proliferation has been lost. In patients exhibiting keloid development, collagen synthesis occurs at a rate 20 times greater than for patients with normal skin (Cohen *et al.*, 1971). Oxidant species have been reported to alter the DNA of cells and may lead to development of abberant cell types such as occur in keloid scars and cancerous lesions (Coussens and Werb, 2002; Finkel and Holbrook, 2000; Li and Karin, 1999; Ames *et al.*, 1993). Changes in DNA may lead to the loss of tight regulation over inflammatory mediator production (Enoch and Harding, 2003), and the ability for cells involved in the wound repair process to function according to normal parameters (Coussens and Werb, 2002; Finkel and Holbrook, 2000; Li and Karin, 1999; Ames *et al.*, 1993). Antioxidants have been demonstrated to prevent damage to DNA caused by oxidant species (Grimble, 1994).

A long-held theory for keloid development is that abnormal molecular defects produce abnormal signals from cytokines and growth factors. Cytokines and growth factors transmit signals in to the cytoplasm that start a cascade of growth signal transduction reactions. The sensitivity of keloid-derived fibroblasts to growth factors may also be significant, as some growth factors induce collagen accumulation (Slavkin, 2000; Overall *et al.*, 1989; Ignotz and Massague, 1986). There may be a defect in genes contained in keloid-derived fibroblasts that prevents switching off of collagen synthesis. The inability of aberrant genes (oncogenes) to regulate cell division has been identified as a main factor in tumour development (Weinberg, 1989). Mutations in the genes involved in the cell cycle could explain observed increases in proliferation and yet decreased apoptosis as described for keloid-derived fibroblasts (Yoshimoto *et al.*, 1999).

Cohen *et al.* (1971) demonstrated that a disproportional increase in collagen anabolism over catabolism contributed to the uncontrollable collagen synthesis by keloids. The collagen contains dense masses of fibroblasts within hyalinized bands of eosinophilic nodules (Kischer, 1992). The failure of tissue serine protease and proteolytic enzymes to breakdown collagen leads to persistence of collagen in the scar, which appears shiny and

bulky (Cook *et al.*, 2000; Urioste *et al.*, 1999; Overall *et al.*, 1989). Hydrogen peroxide has been demonstrated to activate serine tissue proteases and proteolytic enzymes, and therefore it is hypothesised that honey may act here (Section 1.4.1). Excessive endothelial cell proliferation, and subsequent perpetuation of both cytokines and oxidant species, results in the development of numerous microvessels encircling the fibroblast-containing nodules. Direct application of antioxidants has been shown to reduce excessive cytokine production and mediate production of oxidant species (Grimble, 1994), and it is hypothesised that the antioxidant content of honey means it might have activity here (Section 1.4.1.1). The observation that keloid-derived fibroblasts require very low oxygen levels to proliferate (Kischer *et al.*, 1982), raises the possibility that stimulation of angiogenesis by hydrogen peroxide (Burdon, 1995; Tur *et al.*, 1995), with the accompanying rise in oxygen levels, may inhibit the activity of keloid-derived fibroblasts.

In an earlier investigation, Diegelmann *et al.* (1977) reported collagenase inhibitors (specifically, α_1-antitrypsin and α_2-macroglobulin) were present at much higher levels in the keloid. The prevention of collagen breakdown by collagenase is further enhanced by the occurrence of chondroitin-sulphate at significantly higher levels in the keloid scar (Linares *et al.*, 1993). Chondroitin-sulphate functions to strengthen collagen fibres to withstand degradation by collagenase. In normal skin, proteoglycans are responsible for mediating fibrosis, and these have been found to occur at subnormal levels in keloids, therefore the potential for hydrogen peroxide to stimulate protease activity suggests that it may overcome problems with collagen remodelling.

Other growth factors which occur at elevated levels in keloid scar tissues include; IL-1, insulin-like growth factor-1, platelet-derived growth factor, and tumour necrosis factor (TNF) (Ghahany *et al.*, 1995; 1993; Lynch *et al.*, 1989). The observation that honey can alter cytokine expression (Tonks *et al.*, 2001) leads to the hypothesis that it may manipulate at this step in keloid development.

1.6.3.1 Existing treatments for keloid scars

The existing treatment methods available for prevention and alleviation of keloid scarring are briefly reviewed as they illustrate the need which exists for development of a new therapy. Furthermore, a comparison of honey to existing treatments highlights the potential benefits of its use.

Existing treatment methods for keloid scars are limited and are used with varying success, but many of the more successful methods have undesirable side-effects (Berman and Bieley, 1995; Lawrence, 1991; Sherris *et al.*, 1995; Boyadjiev *et al.*, 1995; Darzi *et al.*, 1992; Tang, 1992; Kiil, 1977). Honey has been reported to be free of side-effects (Section 1.6.2). Despite poor success, surgical excision has been one of the long-standing treatment methods for keloids (Lawrence, 1991; Rockwell *et al.*, 1989). Pressure therapy is another keloid treatment method with a long history and a better success rate than excision, with recurrence rates of 15-40% (Rockwell *et al.*, 1989; Linares *et al.*, 1993; Ward, 1995). Steroidal treatments have been widely used for keloid scars (Berman *et al.*, 1995; Lawrence, 1991; Sherris *et al.*, 1995; Boyadjiev *et al.*, 1995; Darzi *et al.*, 1992; Tang, 1992; Kiil, 1977). Steroids bring some initial relief from the painful aspects of keloids, itching and burning, and limited flattening and softening of the scar but have been associated with various undesirable side-effects (Murray, 1993). Gastrointestinal side-effects are a common consequence of steroid use, and may range from mild bowel upsets to ulceration (Nemeth, 1993). Impaired wound healing often results from steroid use (Hunt *et al.*, 1969). Skin hypopigmentation, depigmentation, telangiectasia, and necrosis may result (Boyadjiev *et al.*, 1995; Friedman *et al.*, 1988). Though many of these side-effects are reversible when the steroid source is removed, complications have been recorded where the steroid has entered the surrounding dermis tissue (Murray, 1993). The long term use of steroids is not desirable due to development of tolerance in the patient and the need for increased dose (Murray, 1993). Radiation therapy continues to give moderate success, with a mean recurrence rate of 44% of cases (Berman *et al.*, 1995). When combined with surgical excision, mean recurrence rates have been reduced to 24%

cases (Berman *et al.*, 1995). The other major category of anti-inflammatory drugs are the NSAIDS (non-steroidal anti-inflammatory drugs). The mode of action of the NSAIDS in tissues is mainly mediated by inhibition of cyclooxygenase activity (Goldstein, 1988). Cyclooxygenase is the enzyme responsible for the biosynthesis of prostaglandins, released from damaged tissues. NSAIDS include the common drugs, aspirin and ibuprofen (administered for rheumatoid athritis), diclofenac (Voltaren), and sulidac. The NSAIDS vary widely in their effects on inflammation. Gabriel *et al.* (1991), identified the high incidence with which gastric ulceration accompanies NSAID use.

1.7 AIMS OF THIS STUDY

The general aim of the current study was to test the effect of honey on a variety of cells active at different steps in the inflammatory process, pertinent to wound healing. As inflammation is a broad and complex process, and it is not possible to investigate all of the cellular interactions involved in a time-limited doctoral study, assays were restricted to a small selection of key experiments. The objectives of each experiment are addressed in detail at the beginning of each section.

Prior to this study, few, limited studies had been published which elucidated the mechanism by which honey has activity on cells involve in the inflammatory response, *in vitro*. Therefore, a starting point for this thesis was to repeat these limited studies (Tonks *et al.*, 2001; Abuharfeil *et al.*, 1999), extending the range of honeys tested and assays employed. One of the key objectives of this thesis was to test whether honey has any effect on cells active in the early inflammatory phase, and specifically whether honey has activity on those cells involved in cytokine production, and therefore cellular recruitment (peripheral blood mononuclear cells, focusing on T cells and monocytes). Following a series of investigations to determine whether honey has additional activity on T cell proliferation beyond that reported by Abuharfeil *et al.* (1999), receptor staining would be used to examine how honey was having this stimulatory effect. Measurement of mRNA expression would be used to investigate whether honey had an effect on gene expression

for cytokines involved in wound healing. Further, measurement of TNF protein and NO would be used to examine whether honey has any effect on production of functional protein beyond that which had been reported by Tonks *et al.* (2001). Inclusion of known mitogens would be used to provide positive controls against which to compare the magnitude of any effects of honey, with a view to assessing whether honey could exacerbate inflammation. The inclusion of cells incubated with known mitogens and honey would provide a model on which to test for any modulatory effects of honey, and therefore, to examine whether honey has any anti-inflammatory effects at this level.

To investigate whether honey might have any effect on the movement of cells through the tissues, the effect of honey on tight junction integrity would be examined. This would indicate whether honey might affect the permeability of tissues to leukocytes. The ability for honey to modify the uptake of bacteria by neutrophils would be evaluated using a commercially available phagocytosis assay, with implications for debridement of wounds. To test whether honey plays a role in wound closure, assays would be conducted to measure whether honey has activity on fibroblast proliferation. With specific relevance to fibrosis, fibroblasts would be cultured with honey and IL-1β, to examine whether honey would have any anti-inflammatory effect on the response of fibroblasts to a mitogen known to drive fibrotic scar formation.

Anecdotal reports suggest that honey has both immunostimulatory and anti-inflammatory activity, although the mechanism by which this occurs has not yet been established. The present study aimed to characterise any activity into fractions of different molecular weights, and where activity was found, to discuss the likelihood of the active agent in light of published reports. Therefore, a final objective of this thesis was to present a comprehensive discussion about the mechanisms by which honey has activity *in vitro* and the feasibility of these observations being realised *in vivo*. Thus, the objectives of this thesis were to screen a broad range of cells for the effect of honey at various steps of the inflammatory process, and consider the implications of these observations for wound healing.

Chapter Two: Effects Of Honey On The Proliferation Of T Cells

SUMMARY

To investigate whether honey could stimulate bovine T cells to proliferate *in vitro*, both MTT and BrDU assays of proliferation and flow cytometry analysis were used. It was found that low concentrations of honey stimulated resting T cells to proliferate and express the IL-2 receptor in a dose-dependent manner with progressive dilution. This suggests that honey contains lymphomitogenic factors. Manuka honey was stimulatory at higher dilutions than pasture honey. Low concentrations of honey induced cell division profiles similar to those obtained with Con A-stimulated cells. The stimulatory activity of honey was found to be in a high molecular weight fraction. Sugars alone had no effect on T cell proliferation, as demonstrated by use of artificial honey (a syrup of sugars as in honey).

2.1 INTRODUCTION

Prior to this study several clinical trials had been conducted which demonstrated the potential for honey to optimise wound healing. Honey has been observed to promote healing of a range of wounds in humans (Molan, 1999; Molan, 1998; Efem, 1988) and in experimental animal wound studies (Oryan and Zaker, 1998; Postmes *et al.*, 1997; Kumar *et al.*, 1993; Gupta *et al.*, 1992; Bergman *et al.*, 1983, Burlando, 1978). Of particular relevance to this chapter it was noted that topical application of honey to a variety of wounds led to increased cell recruitment in the wound bed (Subrahmanyam, 1999; Efem, 1988; Subrahmanyam, 1998) and specifically of lymphocytes (Oryan and Zaker, 1998), a key cell type involved in the early stages of repair. Lymphocytes constitute a key group of immune cells involved in the inflammation pathway. A study by Abuharfeil *et al.* (1999) demonstrated that low concentrations of honey (0.1%) induced proliferation of lymphocytes *in vitro*. As topical application of honey on wounds is effective despite the likelihood that the concentration of active component(s) diffusing into the tissues is low, this suggested that honey may have activity at concentrations below those previously tested. Further, studies on experimental wounds in animals reported that orally-administered honey was more effective in treating surface wounds than was topically-applied honey (Suguna *et al.*, 1993; Suguna *et al.*, 1992; El-Banby *et al.*, 1989; Kandil *et al.*, 1987) so that the effective dose of active component would be lower at the wound site. Therefore, the current study aimed to investigate the possibility that honey would have activity on lymphocytes at lower concentrations than had previously been tested.

Due to the availability of bovine blood and cell lines from various origins, along with the constraints of ethical approval and resources for human studies, assays were restricted to the use of these cells. While the activity of cytokines on specific cells is known to vary slightly between human and bovine systems (Collins *et al.*, 1999), it is assumed that the ability for honey to activate the inflammatory pathway would be applicable to both. The use of non-human cell-based inflammation models for human application studies is accepted as a viable model in several key wound healing studies (Goncalves *et al.*, 2001; Greenwel *et al.*, 2000; Klyubin *et al.*, 1996 and Granstein *et*

al., 1990). These *in vitro* assays in no way include or contain the complex interactions of multiple defense mechanisms that are active in the human body or indeed *in vivo* in a cow.

Lymphocytes are comprised of two sub-populations, the T cells (including T-helper cells) active in cell-mediated immunity, and the B cells which produce antibodies after antigenic stimulation (during infection). T cells are the focus of this chapter as they play a pivotal role in the inflammation pathway, lysing infected cells and producing cytokines which cause subsequent cell recruitment in the inflammation cascade. For this reason, the following background has been restricted to a brief overview of T cell biology.

T cells play a central role in host defence and mucosal immunity, and are continuously moving through the blood, tissue and lymph (Cunningham, 2002). T cells are mediators of immunity and their activation is controlled through interactions with APCs. Macrophages play an important role in antigen presentation along with dendritic cells. Within the thymus T cells are differentiated from lymphoid cells, and are released as mature T cells into the circulation. T cell differentiation in the thymus is accompanied by changes in surface markers, with DNA rearrangements generating a diversity of T cell receptors (TCRs). Within the thymus there are three selection steps for the development of mature T cells. Initially there is positive selection for T cells which express both CD4 and CD8 accessory molecules, followed by further selection for cells expressing the mature TCR CD4+CD8+ (double positive). The final selection stage is the deletion of T cells recognising self antigen, so that the T cells released into the circulation will exhibit self-tolerance. Positive selection is unique to T cells which are selected for a moderate affinity for self major histocompatability complex MHC-peptide complexes. MHC are self marker proteins that either link to antigens so they can be presented to CD8 cytotoxic T cells (class I), or are found on APC and display antigen to CD4 T cells (class II). For the life of each T cell, they will only recognise those antigens for which they are specific, and combined in a complex with MHC. T cells will enter apotosis if they cannot express their TCR, are not positively selected or are removed by negative selection. Those T cells which are removed by negative

selection and are not controlled have implications for the development of autoimmune diseases. The positively selected mature T cells then lose either their CD4 molecules or their CD8 molecules and become differentiated as T cell subsets.

The presence of a complex of transmembrane polypeptides (such as CD3, CD4 and CD8) on the cell surface influences antigen binding. The CD3 marker indicates T cells as compared with B cells (CD3 negative). Antigen-specific T accessory cell interactions occur through the TCR/CD3-MHC/peptide complex (Dewar *et al.*, 2005). Individuals possess millions of different T cells, each with a unique TcR for antigen recognition. CD4+ T cells exist in two subsets, Th1 and Th2, producing distinct cytokine profiles, with distinct cellular targets. Th0 precursor cells make a diverse range of cytokines, and are differentiated after activation in the presence of IL-12 and IL-18 (released by dendritic cells) into Th1cells, or in the presence of IL-4 (released by B cells) into Th2 cells (Vos *et al.*, 2000; Murphy, 1998; Sallusto *et al.*, 1998; Abbas *et al.*, 1996). Th1 cells produce IFN-γ, TNF-α, TNF-γ, and IL-2, and regulate classical delayed hypersensitivity reactions around macrophage activation and T cell-mediated immunity. The Th2 subset on the other hand produce IL-4, IL-5, IL-6, IL-10 and regulate immediate hypersensitivity reactions and participate in B-cell antibody-mediated immunity (Sauty *et al.*, 1999; Murphy, 1998; Sallusto *et al.*, 1998; Abbas *et al.*, 1996). CD8+ T cells respond to viral infections, tumours and also participate in some bacterial and protozoan infections. Termed cytotoxic T cells, CD8+ cells kill host cells with surface expression of foreign antigens (*e.g.* harbouring a virus). The recognition of a peptide/MHC complex on the surface of an APC by CD8+ T cells is so highly sensitive that antigens can be detected at very low densities (Gonzalez *et al.*, 2005).

On leaving the thymus, T cells recirculate between blood and lymph nodes or mucosal lymphatic tissues. It is in the secondary lymphoid organs that proliferation occurs after activation by antigen presented on APC. While naïve lymphocytes appear to navigate all tissues without bias, memory cells return to tissues in which they first encountered antigenic stimulation. Protein antigens are ingested and processed into antigenic fragments by activated macrophages or other host APCs (dendritic cells, Langerhan's cells). The antigenic fragments are complexed with MHC and presented on the surface of the APC to receptors for antigen on helper T cells (CD4+). B cells can also process

and present antigen to helper T cells. Naïve T cells encounter dendritic cells carrying antigen in the lymph nodes which stimulates the T cells to proliferate and to express activation molecules. Until naïve T cells have been stimulated by antigen, they have no effector functions, and their sole activity is to recirculate through the peripheral lymph organs. In the case of inflammatory skin diseases, the expression of the cutaneous lymphocyte-associated antigen (CLA) marker on these previously naïve T cells leaving the lymph node means that they can home to the site of antigen origin (Cunningham, 2002). T cells require two signals for full activation to be achieved, the first being antigen-specific and delivered through the TCR. A co-stimulatory signal is transmitted through the receptor-ligand interaction occurring between surface molecules on APCs, B cells and T cells. For example, it is the binding of the CD40 costimulatory molecule on the T cells to the CD40L ligand on the dendritic cells which enables antigen presentation to be initiated. If adhesion molecule expression can be blocked then it is possible to stop T cell proliferation (Dewar *et al.*, 2005). Conversely, overexpression of T cell integrins is linked to the development of autoimmunity (Yung *et al.*, 2003).

The APCs then produce more cytokines (*e.g.* IL-2) which act to enhance T cell proliferation. Indeed, cell-to-cell interactions are critical for T cell proliferation to occur (Dewar *et al.*, 2005). The costimulatory signal provided by the APC is received by the T cells via CD28/CTLA-4, and this drives the cell to enter the cell cycle and to proliferate. T cells themselves clump together in order to initiate proliferation through the interaction of LFA-1 and ICAM-1. The interaction of LFA-1 and ICAM-1 is therefore of key significance for many cell-cell mediated activities including; T cell proliferation, the binding of T cells to endothelium and part of the T cell immunological synapse with dendritic cells.

The antigen signal must be sustained for long enough for the T cells to fully commit to mounting an immune response (Gonzalez *et al.*, 2005), and must be withdrawn before the T cells become committed to apoptosis. The concept of an immunological synapse was introduced in Section 1.5.1. At the immunological synapse, a central cluster of T cell receptors are surrounded by a ring of adhesion molecules, and this is where full activation of the T cell by the antigen occurs (Trauttman, 2005).

Molecules expressed on the endothelium recruit T cells into the tissues. In Section 1.5.1, the need for T cell-specific chemokines and cytokines to direct movement of the cells toward infected and injured tissues was outlined. As discussed in Section 1.5.1, T cells can only receive chemokines and cytokines for which they have the corresponding receptors.

There are a number of pathways by which T cells can be activated. Antigen-independent interactions are achieved via costimulatory molecules, adhesion molecules and cytokines (Dewar *et al.*, 2005). Natural Killer cells exposed to microbial factors (*e.g.* LPS) produce IFN-γ and IL-18, in turn stimulating the release of IL-12 from macrophages and dendritic cells. The APCs themselves will be induced to produce IL-12 by LPS. The IL-12 is received by the IL-12Rα,β receptor on Th1 T cells (Luzzati *et al.*, 1997). Changes in Stat-4 induce the Th-1 cell to produce IFN-γ, a self perpetuating stimulation then occurs as Th1 cytokines further Th1 responses and indeed downregulate Th2 responses. Stat are a family of cytokine receptor signalling molecules. Th-1 cells receive RANTES via the CCR1 chemokine receptor, Macrophage Inflammatory Protein-1 (MIP-1α,β) via the CCR5 receptor and IP-10 via the CXCR3 receptor. TGF-β released by activated Th-0 cells may co-stimulate Th-1 cells along with IL-12. Th-2 cells may be co-stimulated by TGF-β and IL-4. Exogenous IL-4 (*e.g.* released by activated Th0 cells in response to dust mite allergens) is received by the IL-4R receptor on the Th2 cell, and corresponding changes in Stat-6 stimulate the Th2 cells to release IL-4, creating a self perpetuating stimulation cycle. The chemokines Eotaxin (binds to CCR3) and Macrophage derived Chemokine (MDC) which is received by the CCR4 receptor further stimulate Th2 cells. The Th2 cytokine profile promote the allergy pathway, with IL-2 and IFN-γ promoting IgG2, IL-4 promoting IgG1 and IgE, and IL-5 promoting IgA. Once the Th cell response has been polarised and full activation has occurred the antigen can be presented directly or indirectly to activate different effector cells, macrophages, neutrophils and CD8+ T cells for Th1 responses, and eosinophils, mast cells and B cells for the Th2 response (Fallon, 2000; Hammond *et al.*, 1999; Louis *et al.*, 1998; Murphy, 1998; Sallusto *et al.*, 1998; Zelenika *et al.*, 1998; Carter and Swain, 1997; Groux *et al.*, 1997; Abbas *et al.*, 1996;

Mosmann and Sad, 1996). Other key cells known to activate T cells include mast cells, which can promote T cell activation by means of soluble factors, and enhance T cell proliferation and activation (Nakae *et al.*, 2005).

Once T cells have encountered antigen in the lymph nodes and become fully activated, they home to the site of antigen origin. In proximity to the post capillary venule, the T cell encounters E-selectin on the endothelial cell surface, and becomes tethered (Cunningham, 2002). The leukocyte homing process to target tissues was outlined in Section 1.5.1. After becoming tethered to the endothelial cell surface, T cell integrins (*e.g.* LFA1 or VLA4) interact with adhesion molecules released by the endothelium (*e.g.* ICAM 1 and VCAM 1), and undergo extravasation to the inflammation site (Cunningham, 2002). While the α chemokines are primarily responsible for activation of T cells, the β chemokines assist chemotaxis of CD4 and CD8 T cells and also monocytes (Cunningham, 2002). As mentioned in Section 1.5.1, the efficiency of T cell activation depends on a complex interplay between receptor-mediator-antigen kinetics and epitope density on the APC. Further evidence that there are a number of signals which determine recruitment of leukocytes, comes from the recent discovery that a fat-like compound, Sphingosine 1-phosphare (S1P), binds to a receptor on T cells and assists in the migration of T cells to inflamed or injured tissues (Goetzl and Rosen, 2005). When excess S1P is produced, T cells infiltrate healthy tissues, leading to the development of autoimmune diseases. All of the immunoregulatory lipid molecules have effects on cells through G protein-coupled receptors. The lipid molecules have diverse effects from proliferation and survival through to migration and signalling. However, unlike S1P, which has a generalised effect on T cells (and many other cell types), many of the other lipid mediators have effects only on specific subsets of T cells.

The prime function of T cells is to communicate with other cells via soluble cytokine mediators and to recognise and kill cells with foreign antigen expressed on their surface. During inflammation the ability of T cells to proliferate plays a key role in the production of cytokines at adequate levels for subsequent immune cell recruitment to the site of tissue injury. When a specific T cell encounters a pathogen it recognises, it becomes activated. It must then amplify in order to deal with the infection. The

amplification step (T cell proliferation) is dependent on the T cell growth factor, Interleukin-2 (IL-2), which is produced by the Th1 subset of CD4+ T cells. T cells must first increase expression of receptors for IL-2 before they can respond to this chemical signal. Once they can receive IL-2 they proliferate, subsequently boosting the lymphocyte population (Los *et al.*, 1995). It is noted that the terms T cell activation and T cell proliferation are not necessarily interchangeable.

However, proliferation of the local T cell population is not sufficient for inflammation to proceed at the optimal rate. Homing of circulating lymphocytes to sites of tissue injury is the other key process by which recruitment occurs. The process of homing is discussed in detail in Chapter 1, and is therefore mentioned briefly here.

It is evident that there is vast potential for therapeutic agents to intervene at any step in T cell function. T cell activation *in vivo* can be distinguished from that induced *in vitro* by diverse agents such as antigens, plant mitogens, and cytokines (Peacock *et al.*, 1990; Kern *et al.*, 1986; Weiss *et al.*, 1986; Meuer *et al.*, 1984). Antibody stimulation may also be used to activate T cells *in vitro* but may have *in vivo* implications. The two parameters which were chosen for investigation in this study were the effect of honey on T cell proliferation and the effect of honey on expression of the interleukin-2 receptor (IL-2 receptor) on the T cell membrane. These aspects were chosen because they are easily measured and the techniques used have been well documented in the published literature.

Testing the ability of substances (in this case honey) to induce T cell proliferation is easily achieved in an *in vitro* system. This type of experimental work has been a starting point for many investigations aiming to screen various substances for potential immunogenic effects (Colic *et al.*, 2002; Wilasrusmee *et al.*, 2002; Wong *et al.*, 1996; Yun *et al.*, 1996). Moreover, lymphocytes can be readily used in an *in vitro* system to model inflammation and test the effect of therapeutic agents on inflammation. An inflammatory response is easily induced in T cell populations by the mitogenic substance Concanavalin A (Con A). Con A is termed a mitogen because it stimulates T cells to divide (Kirjavainen *et al.*, 1999). The rationale for using a mitogen to stimulate T cell proliferation in these assays was to simulate the inflammatory situation where T

cell activation, and subsequent proliferation, occurs along the same pathway. Many published studies have used lectins (*e.g.* Con A) successfully to create a proliferative response by lymphocytes in an *in vitro* culture system (Miller *et al.*, 1991; Young *et al.*, 1994; Abuharfeil *et al.*, 1999). The binding of Con A to surface glycoproteins initiates a series of early activation events including the phosphorylation of intracellular proteins, breakdown of phospholipids and a rise in intracellular calcium levels (McCole *et al.*, 1998). These changes induce nuclear effects and have impacts upon transcription of genes in the nucleus. It is these changes that induce the expression of IL-2 receptor and the production of IL-2. To investigate whether honey had any stimulatory activity on T cell proliferation it was planned to compare the cell response to honey to that induced by Con A.

While it is essential for lymphocytes to undergo proliferation in order to drive the inflammatory process, excessive proliferation is harmful to the patient. The importance of obtaining cytokine production at levels deemed to be within the normal range to create normal wound healing has been discussed at length in Chapter 1. It should be emphasised here that cytokine levels falling either side of the normal range may lead to impaired wound healing. Low levels of white blood cell activity and, specifically, subnormal recruitment of lymphocytes and monocytes will result in inadequate production of cytokines needed to stimulate the healing process. Conversely, excessive T cell proliferation and subsequent production of cytokines at very high levels can contribute to development of various diseases. Where the inflammatory process continues for longer than deemed normal, persistent inflammation may result. The role of persistent inflammation in keloid development (and auto-immune diseases such as arthritis) have been discussed in Chapter 1. However, autoimmune diseases have the added complication of populations of aberrant T cells which have escaped positive selection in the thymus and are able to respond to antigen on host cells. Effectively this means that during autoimmune disease lymphocytes attack the host's own immune system, a battle which can only be alleviated by therapeutic intervention or death, and produce inappropriate levels of cytokines as a side-effect. Thus in the search for a therapeutic agent which enhances T cell activity it is important to remember that promotion of excessive mitosis would be a dangerous feature. Taking Con A-stimulated

cultures to represent a model of chronic activation, the potency of honey could then be compared.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

RPMI complete medium

All PBMC cultures used RPMI complete medium. A 200 ml volume consisted of 1 X RPMI 1640 (GibcoBRL, Grand Island, NY, USA, Cat #11875-093), supplemented with L-glutamine (2 mmol/L); 1% Hepes for buffering in air at 37°C (20 mmol/L, BDH Laboratory Supplies, Poole, England); sodium bicarbonate (0.396 g, Scientific Supplies Ltd, East Tamaki, Auckland); 10% foetal calf serum, NZ origin (GibcoBRL, Cat # 10 091-148); 10 µl of 2-Mercaptoethanol (0.5 µM, Sigma Chemical Co, St Louis, MO, USA); and 1% Penicillin-Streptomycin (containing 5,000 units/ml penicillin G sodium and 5000 µg/ml streptomycin sulfate in 0.85% saline, GibcoBRL, Cat # 15140-122). The final volume was achieved by adding RPMI 1640. The pH was adjusted with 1N NaOH or 1N HCl to 7.3 and sterilised by passing small amounts through a sterile 0.2 µm Sartorius Minisart filter (Sartorius, Göttingen, Germany). The final pH was approximately 7.5.

Ammonium chloride lysis buffer (NH_4CL)

A lysis buffer was made by first stirring 2 g of Tris (0.017 mol/L, AppliChem, Ottoweg, Damstadt) and 7.5 g of NH_4Cl (0.14 mol/L, APS Ajax Finechem, Auburn, Australia) in to 900 ml of Milli Q water. The solution was adjusted to pH 7.2 using concentrated HCl, and diluted to a final volume of 1 litre. The buffer was sterilised by passing it through a sterile 0.2 µm Sartorius Minisart filter, and stored at 4 °C.

Phosphate-buffered saline (PBS)

NaCl 8 g (0.138 M)

KCl 0.2 g (0.0027 M)

KH_2PO_4 (anhydrous) 0.2 g (0.0014 M)

Na_2HPO_4 (anhydrous) 1.15 g (0.0032 M)

All of the reagents were purchased from BDH Chemicals Ltd., L. R. The phosphate-buffered saline was made up to 1 litre by adding sterile Milli Q water. The pH approximated 7.2 and the phosphate-buffered saline was passed through a sterile 0.2 µm Sartorius Minisart filter. The phosphate-buffered saline was stored at 4 °C.

Concanavalin A (Con A)

In a cytotoxicity cabinet, 5 mg of Con A (Sigma C5275) was weighed in a sterile boat and added to 10 ml of sterile RPMI complete medium giving a final concentration of 500 µg/ml. Aliquots of this solution were stored at -20°C.

All glassware and metal spatulas were rendered sterile by autoclaving, and plasticware was certified endotoxin free and purchased new. All reagents and RPMI complete medium was brought to room temperature prior to use (unless otherwise stated).

2.2.2 Honey solutions

Both M109 manuka honey (a batch of manuka honey with non-peroxide activity equivalent to 18% w/v phenol as determined by Allen *et al.*, 1991) and a pasture honey from the Otago region were tested. These were selected from the samples supplied to the University of Waikato Honey Research Unit by various beekeepers for research projects. Honey solutions were prepared by diluting raw honey (1.37 g = 1 ml) with RPMI complete medium (1 ml) to achieve a 50% v/v concentration. The diluted honey was warmed in the incubator (37°C), and gently stirred to ensure thorough mixing. Further dilution of the 50% honey solutions was performed to obtain the stated final concentrations (as presented in all figures throughout the thesis). Addition of an equal volume of RPMI medium to the 50% honey solution gave a 25% solution (*i.e.* a 4-fold dilution) and ten-fold dilution steps of this with either cell suspension or medium gave honey concentrations of 2.5%, 0.25%, 0.025% *e.t.c.* Allowance was made for the subsequent dilution by other components of the incubation mixture when calculating the final concentrations of honey. This meant that an additional dilution step was required to compensate for the ratio of honey to other reactants in the assay. For example, a serial dilution of 7.5% was required to allow for a 1:3 dilution of honey solutions. As honey was diluted by volume the resultant solution was expressed as '%

v/v" and has no units being x% honey. The solution was then passed through a 0.25 μm filter (Sartorius), and maintained at room temperature, for use within an hour of preparation. The honey used in the assays described in this thesis was always subsampled from the same batch of honey. Consistency was maintained in preparation technique.

Artificial honey

Artificial honey was made according to a recipe described by White (1975a), formulated from the average composition of at least 400 samples of major commercial honey types in the USA. The following were added (% by weight) to prepare the sugar syrup: water 17.2%, fructose 38.4%, glucose 30.3%, sucrose 1.3%, maltose 8.6%, maltodextrin 1.4%. The syrup was stored at 4°C and solutions were prepared for use as described for natural honey. To make a 50% solution of artificial honey, 1.43 g (taken to be 1 ml) of this sugar syrup was diluted with 1 ml of RPMI complete medium.

2.2.2.1 Preparation of honey fractions

Manuka honey and Otago pasture honey were fractionated according to four techniques; dialysis, ether extraction (manuka honey only), reverse phase chromatography and ultrafiltration (as described by Pingoud *et al.*, 2002).

Dialysis

Dialysis was performed to separate the high molecular weight (>10 kDa) and low molecular weight (<10 kDa) components of the honey. A 25% (v/v) honey solution was made by warming 13.70 g (= 10 ml) of manuka honey with 30 ml of Milli Q water in the incubator. It was necessary to dilute the honey solution as a pilot test had shown that using concentrated honey would prevent air from escaping from the tubing. Honey (20 ml) was dialysed in 29 mm diameter 10kDa dialysis tubing (Spectra/Por®) against 10 x volume Milli Q water at 4°C, in the dark. The water was changed three times over 48 h and the yellow diffusate was collected and concentrated by freeze-drying (VirTis, NY, USA). The diffusate was poured into three round bottomed flasks and shell-frozen by

rotating each flask in a slush bath comprised of ethanol and dry ice. Once the flask was coated in a fine layer of ice and all of the solution had frozen, the flasks were transported to the freeze drier on dry ice. The flasks were attached to the freeze-drier and left overnight. Once all of the cool spots and condensation had disappeared, freeze-drying was complete. At this point the freeze-dried dialysate was reconstituted by adding enough Milli Q water to make the volume back up to the original 20 ml volume of the honey solution. This again represented a 25% (v/v) honey concentration. Osmotic measurement confirmed that the osmolarity of the high molecular weight fraction did not exceed that of medium alone. The dialysed honey was frozen at -20°C until required.

Ether extraction

This procedure was kindly performed by Melanie Snow in the Chemistry Department of the University of Waikato, using a liquid/ liquid extraction technique as described by Tan *et al.* (1988).

Reverse phase chromatography

Using reverse phase chromatography, the hydrophilic and hydrophobic fractions of honey were separated. A 25% (v/v) honey solution was prepared as for dialysis. The SepPac cartridge (Waters Corporation, Massachusetts) was prepared by injecting 5 ml of ethanol through with a syringe. This was discarded and followed by injection of 10 ml of water through the cartridge. After the washing step, 15 ml of honey solution was injected through the cartridge, followed by air to flush through the honey solution which was collected in a sterile bottle then 15 ml of ethanol was injected through the cartridge, followed by air and was collected in another sterile bottle. In a final washing step, 10 ml of water was injected through the cartridge and discarded, along with 5 ml of ethanol which was also stored at -20°C until needed. The 15 ml of honey solution collected, which would have contained the hydrophilic components, was at the correct concentration for use in the assay. However, the ethanol-eluted hydrophobic components and washings were rotary evaporated to remove the ethanol. To perform rotary evaporation, the hydrophobic fraction was placed in a round flask, attached to a Büchi Rotavapor R rotary evaporator run at 37°C under vacuum. Evaporation was complete when a viscous yellow solution was all that remained in the flask. The

hydrophobic fraction was reconstituted by adding enough Milli Q water to make the volume up to the original volume (15 ml) of the honey solution.

Ultrafiltration

Ultrafiltration of honey was performed to separate components of the honey by molecular weight. It was achieved by passing a pre-filtered honey solution through a 10 kDa cut-off membrane (Amicon/ Millipore) using nitrogen gas pressure. A 25% (v/v) honey solution was prepared as for dialysis. It was pre-filtered through a series of membranes (100 μm, 50 μm, Millipore Corporation) to remove the extraneous matter (bee tissues, pollen *etc*) as this will clog the membrane pores. The resulting solution was then passed through a glass membrane overlying an 8 μm microporous membrane (both from Millipore Corporation). A 10 ml volume was loaded into the ultrafiltration chamber, and the ultrafilter left to run in the dark, until all of the solution had moved through the membrane. The filtrate was collected and stored at -20°C. The retentate components were restored to their original concentration in the honey solution by adding 10 ml of Milli Q water and stored at -20°C.

2.2.2.2 Dilution of the honey solutions to achieve final concentrations for testing

Final honey concentrations were achieved by conducting serial dilutions of the honey solutions with RPMI complete medium. All honey solutions were protected from light by wrapping in aluminium foil, to prevent denaturation of the enzymes. Artificial honey was used to provide a control for the osmotic effects of the sugars. Solutions of honey were prepared immediately prior to use.

2.2.2.3 Osmolarity of the honey solutions

The osmolarity of the honey solutions prepared for addition to T cells was measured to establish whether: (a) at the concentrations tested, osmolarity caused cell damage; (b) osmolarity varied between honey types which might account for any differences in

activity. Immediately after preparation of a range of honey solutions diluted in RPMI complete medium, the osmolarity of the solutions was determined using a freezing point osmometer (3D3 Osmometer). A plot of osmolarity versus honey concentration was constructed and a curve fitted to enable extrapolation to higher honey concentrations (see Figure 2.1).

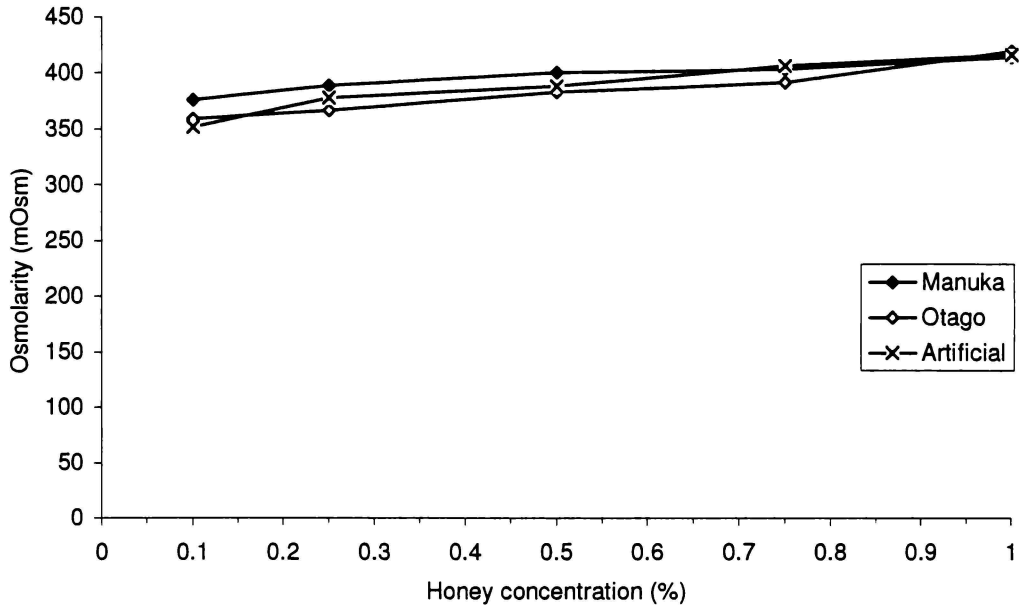
It can be seen that there were no significant differences in osmolarity between honey types at the same concentration. The osmolarity of normal blood is approximately 370 mOsm (Marieb, 2001).

2.2.3 T cell extraction procedure

2.2.3.1 Blood collection

Blood was collected from the severed jugular vein of a heifer during slaughter at the AgResearch Ruakura abbatoir. This blood was collected as a by-product of the routine processing of the meat industry and therefore did not require ethics approval. Collection of the blood directly in to sterile 50 ml Nunc tubes containing sodium heparin solution (1.25 ml of 5000 IU heparin in 5 ml of sodium chloride) minimised contamination. Directly prior to blood collection the tubes were coated with the heparin, and carefully inverted several times upon addition of the blood to prevent clotting. Immediately following blood collection, the T cell isolation procedure was undertaken.

A).



B).

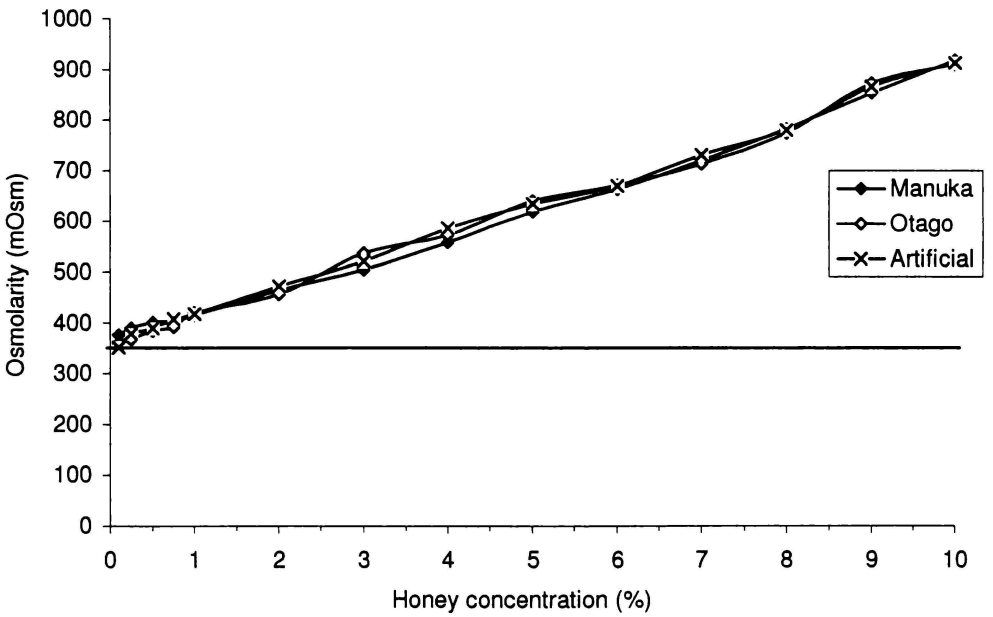


Figure 2.1: Plots of osmolarity versus concentration of manuka, Otago pasture and artificial honeys. A). concentrations in the range between 0–1% and B). concentrations in the range between 0–10%. Honey dilutions were made in RPMI complete medium and the osmolarity measured using a freezing point osmometer.

According to standard tissue culture techniques, aseptic conditions were maintained at all times and all steps were performed in a laminar flow workstation (Westinghouse Pty Ltd).

2.2.3.2 Isolation of Peripheral Blood Mononuclear Cells

Lymphocytes were isolated from whole blood according to a method described by Carlson and Kaneko (1973) with some modifications. Whole blood was first lysed by mixing a 5 ml portion of the heparinised blood with 10 ml of NH_4Cl lysis buffer and spun on a rotor (Chiltern Scientific) for 15 min at room temperature. The lysed blood was centrifuged for 10 min at 1 879 x *g* (Sorval H-400), and the supernatant removed. The pellet was resuspended in 5 ml of phosphate-buffered saline, and vortexed (Cenco Instrumenten, Breda, Netherlands) for 30 sec to ensure the pellet was broken up. A 5 ml portion of the resuspended leukocyte pellet was carefully layered over 3 ml Ficoll-Paque (Pharmacia Biotech) according to the manufacturer's instructions, and centrifuged at 900 x *g* (35 min) at room temperature with no brake. The interface layer (containing monocytes and lymphocytes) between plasma and Ficoll Paque was washed in 10 ml phosphate-buffered saline (450 x *g*, 10 min). The supernatant was discarded and the pellet was resuspended in a further 5 ml of lysis buffer solution and incubated for 10 min (37°C, 5 % CO_2, 95% air). An equal volume of phosphate-buffered saline was then added and the tube centrifuged for 10 min (at 450 x *g*). The supernatant was again discarded and the pellet resuspended in 5 ml of phosphate-buffered saline before centrifuging for 8 min (at 250 x *g*). This step was repeated, to ensure that any platelets had been removed. The cells were then suspended in RPMI complete medium and a cell count performed by haemocytometer according to standard methods. Lymphocytes were not differentiated from monocytes. The cell suspension was then diluted as required using RPMI complete medium. This procedure typically produced cell fractions with >99 % viability as determined by trypan blue staining. The average harvest for bovine lymphocytes within a PBMC fraction prepared in this manner is 88% (12% monocytes).

2.2.4 The lymphocyte proliferation assay

After the cells were obtained from the isolation procedure described in section 2.2.3.2 they were then referred to as a PBMC cell fraction because the actual proportion of T cells was not established.

2.2.4.1 Assay to measure the stimulatory effect of honey on lymphocyte proliferation

The standard method for testing the effect of honey on T cell proliferation was to place 100 µl of PBMC suspension (obtained as described in Section 2.2.3.2) in a 96-well flat bottom microtitre plate (Maxisorp, Nunc) at a density of 2.5 x 10^6 cells/ml. In addition to the cell suspension, wells received 50 µl of honey solutions (diluted in RPMI complete medium as described in Section 2.2.2.2). Controls received either 50 µl of RPMI complete medium or 50 µl of RPMI complete medium containing Con A (at a final concentration in wells of 5 µg/ml). The final volume was 150 µl. Cells were incubated in a humidified atmosphere at 37°C (5% CO_2, 95% air) for periods of time as stated in each experimental outline (72 h was standard). Where this standard assay method was modified for specific experiments this has been stated in the experimental outline.

As the PBMC fractions contained monocytes, and these cells are activated by LPS (Beutler and Cerami, 1988), it was necessary to determine whether endotoxin contamination had occurred. To verify that stimulatory effects were not due to endotoxin contamination, contamination cultures were set up with and without polymyxin B (PMB; Pfizer, Karlsruhe, Germany) at 10 µg/ml. PBMC in the contamination cultures were exposed to polymyxin B for 30 min at 37°C (5% CO_2, 95% air), prior to adding treatments, to inactivate LPS. No LPS contamination was detected.

For the proliferation assays analysed using flow cytometry, the cells were added in 1 ml aliquots to 24-well flat bottom microtitre plates (Nunc), maintaining the same cell density (2.5 x 10^6 cells/ml). This modification was made to facilitate the staining of

cells. Honey solutions, RPMI complete medium or RPMI complete medium containing Con A (final concentration of 5 μg/ml) were added to the cell suspensions in 50 μl aliquots. Cells were incubated at 37°C (5% CO_2, 95% air) for periods of time as stated in each experimental outline (72 h was standard). The pH of the honey solutions was measured prior to adding cells (Radiometer Analytical MeterLab, PHM210), and at the concentrations tested, were not found to differ from RPMI complete medium alone.

2.2.5 Stimulation of lymphocytes with Con A to activate T cells

Con A is a lectin that binds to glycoproteins on the lymphocyte cell surface and causes activation of T cells, stimulating the production of cytokines by T cells (such as IL-2) which in turn activate other T cells to proliferate (global T cell stimulation). As Abuharfeil *et al.* (1999) had used 5 μg/ml successfully to stimulate proliferation of blood-derived lymphocytes for measurement by MTT assay, this protocol was developed in this study as it was directly applicable to the experimental conditions. An early pilot study conducted prior to the investigations confirmed that 5 μg/ ml of Con A produced good levels of T cell proliferation which enabled clear treatment effects to be measured when compared with resting cells (data not shown).

2.2.6 Measurement of lymphocyte proliferation and activation

Measurement of lymphocyte proliferation was used to assess the ability of honey to activate resting PBMC, via BrDU (bromodeoxyuridine) ELISA and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The MTT assay is one of the most commonly used assays for measuring cell proliferation because it is easy to use and has been adapted to a microtitre plate-format (Liu *et al.*, 1997). MTT is taken into the cells by endocytosis and metabolically reduced into blue insoluble crystals by endosomal and lysosomal vesicle enzymes and mitochondrial enzymes (Liu *et al.*, 1997). The blue crystals are subsequently dissolved in added dimethylformamide and the absorbance of the solution measured spectrophotometrically. Prior to use of these assays it was necessary to establish a standard curve to ensure that the production of the blue formazan product was linear with respect to the number of cells in the PBMC culture. Once a linear relationship was established it was possible to estimate the

number of viable cells in a sample. Increased MTT response of a PBMC culture with honey or a mitogen compared to a control culture indicated that proliferation of these cells was increased.

BrDU ELISA works along the same principles as MTT to indicate cell division status of a cell population. BrDU analysis provided an alternative measure of lymphocyte proliferation. As no published studies had used the BrDU ELISA method to evaluate the effects of honey on lymphocyte proliferation before, it was necessary to adapt the methods used from other studies performed with bovine lymphocytes. A study by Fisher *et al.* (1999), using BrDU measurement of lymphocyte proliferation to detect stress-induced diseases in cows was selected for this purpose. The BrDU ELISA works by incorporation of a labelled bromodeoxyuridine in to the DNA of actively dividing cells. In the final step of the BrDU assay, an antibody with a peroxidase enzyme linked becomes bound to the BrDU label. It is the binding of the substrate-antibody complex which enables development of a blue colour, quantified spectrophotometrically. The absorbance obtained is proportional to the number of cell divisions and a standard curve enables this information to be interpreted. As with the MTT assay method, the starting density of cells, concentration of mitogen used and the amount of time the assay is run for are crucial to the sensitivity of the BrDU ELISA. BrDU analysis provides a "snapshot" of proliferation so that the results of a BrDU ELISA will reflect how much of the labelled base was incorporated into cellular DNA during a 24 hour period. Thus, to maximise the sensitivity of the assay it was necessary to pinpoint the time at which the lymphocytes in the experimental set up underwent high levels of mitotic activity, and then to add the label during this time.

In order to confirm the results obtained by the BrDU ELISA, flow cytometry was used to provide another technique to track cell division. Flow cytometry is an invaluable tool for imaging lymphocytes and enables the investigator to confirm that transformation has occurred in these cells due to changes in the size and granularity of the cells (Parish, 1999). These changes are indicated by linear forward scatter and log side scatter respectively. Flow cytometry was used in this study to quantify the number of cell divisions undertaken by the T cells and to quantify the proportion of cells in each of these generations. This was achieved by incubating lymphocytes with CFSE dye at the

same time as the test substances. CFSE (5-carboxyfluorescein diacetate succinimidyl ester) is a membrane-permanent fluorescent dye which is used to track cell division as the fluorescence intensity halves with each cell division. Thus, flow cytometry was used to reveal the proportion of cells which fell into various cell divisions based on their fluorescent intensity. Detection of the CD3+ T cell receptor using a fluorescent antibody specific to this enabled the proportion of cells activated via this receptor to be quantified. Flow cytometry was also used to identify the expression of the IL-2 receptor on T cells. This was achieved by staining T cells with FITC (fluorescein isothiocyanate)-labelled antibodies against the IL-2 receptor.

2.2.6.1 Trypan blue exclusion

Cells with an intact membrane exclude trypan blue. Staining of the cells with trypan blue distinguishes between those newly committed to apoptosis and those in the later stages of cell death as the membrane remains intact in the first stages (Slater, 2001). The combination of trypan blue exclusion and proliferation assays together provides a useful tool for assessing any cytotoxic effects of honey. Thus, trypan blue exclusion was used to establish the osmotic tolerance range of honey prior to commencing assays. Trypan blue dye exclusion assays were performed according to standard methods (Gorman *et al.*, 1997). In brief, after exposure of lymphocytes to treatment solutions in 24-well plates, an equal volume of trypan blue solution (0.4% trypan blue in phosphate-buffered saline) was added to each well containing 100 µl of cell suspension (2.5 x 10^6 cells/ml) mixed on a plate shaker and incubated for 1 min (37°C, 5% CO_2, 95% air). Cell viability was determined using a haemocytometer and bright field microscopy (Olympus, 40x) according to the method described by Gorman *et al.* (1997).

2.2.6.2 MTT assay

The 96-well plate MTT assay was a modification of the method of Burres and Clement (1989). The MTT assay performed in this study was purchased as a kit (Roche Molecular Biochemicals, Cat. No. 1 465 007). After 2–3 days incubation (37°C, 5% CO_2, 95% air) of the cell suspension with various treatments in 96-well flat bottom

microtitre plates, the MTT assay was performed. To a well containing 150 µl of cell suspension, 15 µl of MTT solution was added. The original method was adapted due to honey interfering with the MTT assay directly. It was suggested that spontaneous conversion of the MTT salts with honey is likely to be a result of the glucose oxidase enzyme in the honey interfering with the assay, giving false positives (M. Berridge, Malaghan Institute, Wellington: personal communication). The enzyme glucose oxidase becomes more active as it is diluted out, and this would explain why the control wells with honey in RPMI complete medium without cells had higher absorbance values for 0.25% manuka honey than 10% manuka honey. The absence of any colour development in the control wells containing artificial honey supported this. Microscopic analysis of the wells ruled out the presence of a micro-contaminant.

False positives were avoided by washing the microtitre plates with 100 µl phosphate-buffered saline (room temperature, 320 x *g*, 15 min) three times to pellet the cells in the bottom of the wells, flicking out and resuspending the cells in 100 µl RPMI complete medium prior to addition of the MTT reagent. The plate was then returned to the tissue culture incubator for 2 h (37°C, 5% CO_2, 95% air) to enable the tetrazolium salt to be enzymatically reduced by viable cells in the culture. MTT solubiliser (10% sodium dodecyl sulfate (SDS), 45% dimethylformamide, adjusted to pH 4.5 with acetic acid) was added (0.1 ml/ well) and the plate returned to the incubator overnight (37°C, 5% CO_2, 95% air), to dissolve the blue formazan precipitate. The absorbances of the solutions in the wells were then read at 570 nm wavelength (Bio-Tek instruments). Control wells with RPMI complete medium or honey solutions and MTT alone (no cells) provided the blank, and the absorbance of the blanks were subtracted from all samples.

2.2.6.3 BrDU ELISA

The BrDU colormetric cell proliferation ELISA (kit from Boehringer Manheim Cat. No. 1 647 229) was performed according to the manufacturer's instructions. Cell suspensions were incubated with honey solutions (and control solutions) at 37°C (5% CO_2, 95% air) for 2–3 days prior to addition of a BrDU label (Section 2.2.4). The study

by Fisher *et al.* (1999) had indicated that adding the BrDU label 72 h after setting up the assay gave a good capture of mitotic activity when the starting density of lymphocytes was 2.5 x 10^6 cells per ml. An 18 h incubation time for the cells with the BrDU label was chosen because pilot assays performed in the current study had established that this enabled sensitive detection of the amount of proliferation which occurred for cells exposed to RPMI complete medium alone as compared with cells stimulated with Con A. After this time, 15 µl of BrDU label was added to each well and the plates were incubated (37°C, 5% CO_2, 95% air) for a further 24 h. After centrifugation (10 min at 438 x *g*, 18°C) the supernatant was removed and plates dried prior to adding 200 µl of FixDenat to each of the wells. The plates were incubated at room temperature for 30 min, and 100 µl of anti-BrDU-POD working solution added to each well. The plates were then incubated for 120 min at 37°C (5% CO_2, 95% air). Though the manufacturer recommended a 45 min incubation time at room temperature, an increased incubation time of 120 min at 37°C (5% CO_2, 95% air) was found to increase the sensitivity of the assay as more binding occurred. The plates were then washed three times with 300 µl of washing buffer provided in the kit, and 100 µl of the substrate solution (tetramethylbenzidine and peroxide) was added to each well. The plates were incubated for a further 10 min at room temperature, and 25 µl of 1 M sulphuric acid added to each of the wells to stop the reaction. After 1 min on a plate shaker the absorbance was determined at 490 nm with a reference wavelength of 690 nm (BioRad Model 550). Control wells with RPMI complete medium or honey solutions and BrDU alone (no cells) provided the blank, and the absorbance of the blanks were subtracted from all samples.

2.2.6.4 Flow cytometry analysis

Flow cytometry was used to confirm the BrDU assay results and to investigate some key aspects of T cell response to honey: induction of cell blasting, the number of cell division events, identification of the subset of T cell-responders, and induction of the IL-2 receptor on the cell membrane. The profile obtained for cells incubated with and without honey could thus be compared, and the effects of honey on lymphocytes could be determined at a cellular level.

2.2.6.4.1 Staining methods

To enable cells to be analysed using flow cytometry, they were stained for various cell surface markers. The murine anti-CD antibodies are primarily bovine specific.

Tracking T cell divisions using CFSE-staining

CFSE (5-and 6-carboxyfluorescein succinimidyl ester diacetate) stain (Molecular Probes, Eugene, USA) forms conjugates with proteins within the cell which are retained through mitosis and one half of the label is inherited by each daughter cell after division. CFSE staining enables independent examination of individual cells within a T cell sample. To stain for CFSE analysis, CFSE (10 mM in DMSO) was diluted 1:4 000 in phosphate-buffered saline to give a 4 µmol/l working solution and an equal volume was added to the cells (2 x 10^7 cells/ml in phosphate-buffered saline) to achieve a final CFSE concentration of 2 µmol/l. The tube contents were mixed several times during incubation at room temperature for 10 min. An equal volume of ice-cold foetal calf serum was added to the tube and the contents mixed gently. The tube was centrifuged for 20 min at 250 x *g*. The supernatant was decanted, the tube was then filled with RPMI complete medium and the cells resuspended gently, then centrifuged for 10 min (250 x *g*). This washing step was repeated three times. The cell density of the resuspended cells was then adjusted with RPMI complete medium to achieve a final concentration of 2.5 x 10^6 cells/ ml and 1 ml of suspension was transferred into each well of a 24-well microtitre plate (Nunc). Then 50 µl of either honey solutions, Con A or RPMI complete medium was added to each well and the cells were incubated for 72 h (37°C, 5% CO_2, 95% air), to enable activation to occur. If the cells were to be double-stained (using cell surface markers for lymphocyte subsets), the addition of these fluorescent-labelled antibodies was done after this incubation period.

Identification of the actively dividing T cell subset

As lymphocytes consist of several subset types (Section 2.1) it was decided to investigate which type were undergoing cell division in response to honey, and this was achieved by staining cells for surface antigens. Figure 2.2 shows that T cell sub-populations may also be discriminated from each other according to the presence of thiols attached to the surface antigens, though these were not identified in the current

study. Figure 2.2 shows that T cell populations all express CD3 markers and staining for this marker gives assurance that T cells are being analysed. So, for example, if T cells from a PBMC fraction were double-stained for CFSE and CD3, the proportion of dividing T cells could be accurately tracked.

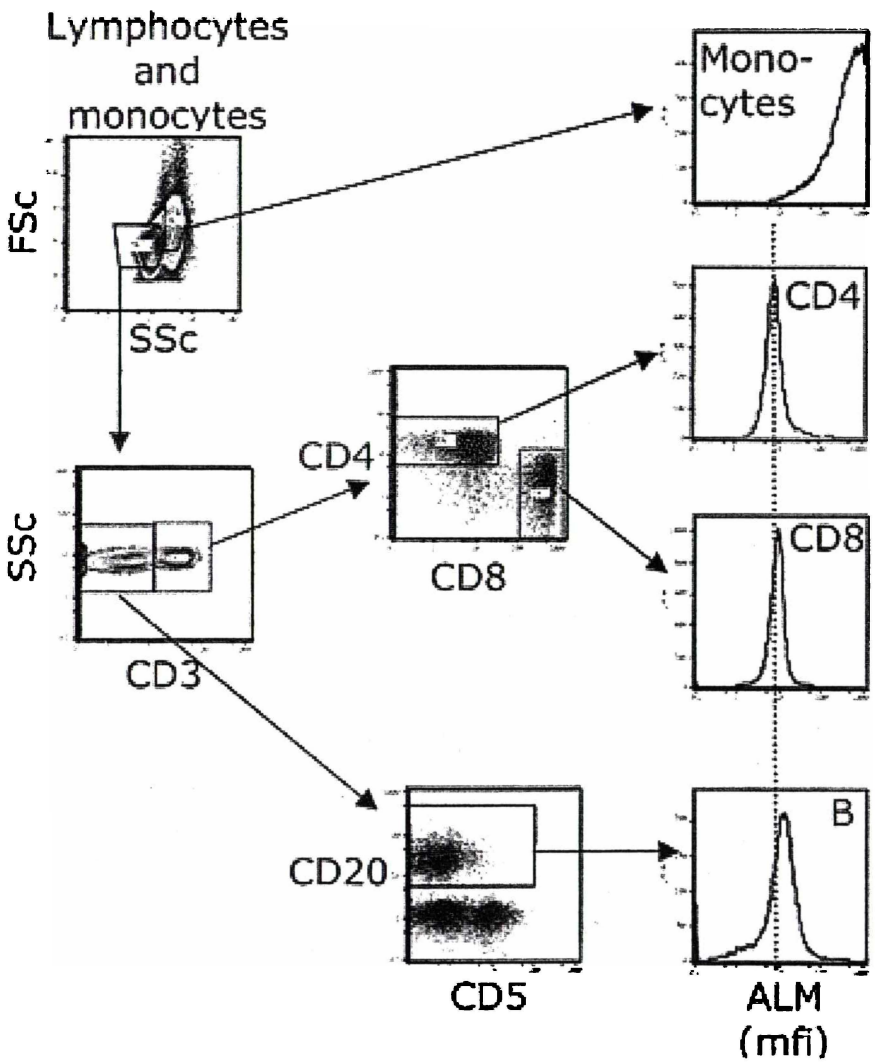


Figure 2.2: Lymphocyte subsets in freshly isolated PBMCs. PBMCs were stained for surface markers to detect monocyte and lymphocyte subsets and gated according to their position in a two-parameter dot plot. PBMCs were also stained by Sahaf *et al.* (2002) for the presence of surface thiols on cell surface markers which differ among cell subsets (detected by binding of the fluorescent molecule, ALM (mfi), Alexa-maleimide) (figure taken from Sahaf *et al.* 2002).

With reference to Figure 2.2, the T-helper (T_H) cells display a specific cell surface antigen (CD4) on their membrane. T cytotoxic cells can express either the CD4 or the CD8 epitope (Meuer *et al.*, 1982). T-suppressor cells display the CD8 antigen on their surfaces. The T-helper to T-cytotoxic /suppressor cell ratio, also called the CD4+:CD8+ T cell ratio is often used as a measure of immune status. According to Rapaport *et al.* (1986) an increase in the CD4+:CD8+ T cell ratio indicates an increased immune function with greater responsiveness of lymphocytes to mitogens.

The expression of the IL-2 receptor on the surface of T cells is a marker of activation (Jan, 1994; Los *et al.*, 1995; Cross and Gill, 1999; Carswell and Papoutsakis, 2000; Kunitomi *et al.*, 2000). Cells were stained with antibodies binding to the IL-2 receptor, using a two-step procedure, with the second antibody being fluorescent and detected by the flow cytometer. Antibodies to bovine CD4 markers and bovine CD8 markers were included to track the expression of the IL-2 receptor by these T cell subsets.

Cells were stained with a fluorescence-labelled antibody for the various surface markers according to a two stage process, adding first the primary antibody (*e.g.* anti-CD3) and then the secondary antibody which binds the first and gives the fluorescence. PBMC (10^6 cells/tube) were placed in 5 ml tubes (Becton-Dickson) after passing through 100 μm gauze to remove clumps. An equal volume of phosphate-buffered saline containing 1% foetal calf serum was added and the tubes centrifuged (322 x *g*, 5 min, 4°C). The supernatants were decanted and the cells were resuspended in the drop of supernatant remaining. Then, 50 μl aliquots of primary antibody: murine anti-CD3 (MM1A, diluted 1: 400 in phosphate-buffered saline: Veterinary Medical Research and Development, USA) murine anti-CD4 (CC8 diluted 1: 500 in phosphate-buffered saline: gift from N. Parlane, AgResearch, Wallaceville), murine anti-CD8 (CC63 diluted 1: 500 in phosphate-buffered saline: gift from N. Parlane, AgResearch, Wallaceville) or murine anti-IL-2R (CACT116A diluted 1: 400 in phosphate-buffered saline: Veterinary Medical Research and Development, USA), were added to tubes. Where cells were double-stained for both the IL-2 receptor and either the CD4 or CD8 surface markers, this enabled identification of the T cell subset expressing the IL-2 receptor.

Duplicate tubes containing cells stained with same and GC270 IgG2a (Silenus) were prepared in the same manner as those stained for specific surface markers, to provide a control for non-specific binding by the secondary antibody, and to provide baseline data for background. When cells were stained for the IL-2 receptor only, the non-specific primary antibody murine IgG1 (Ci4 diluted 1: 400 in phosphate-buffered saline: Silenus) was used.

The tube contents were mixed and incubated in the dark at 4°C for 20 min. After incubation, 500 µl of 1% phosphate-buffered saline was added and the cells were centrifuged for 6 min (322 x *g*, 4°C) the supernatant removed and resuspended in the remaining supernatant. The washing step was repeated three times to remove unbound antibody. Following the final wash, the secondary antibody was added. If cells were stained with CFSE, the secondary antibody was PE-labelled rat anti-murine IgG_1 (Jackson ImmunoResearch Laboratories Inc., PA) used at a dilution of 1:1 000 in phosphate-buffered saline. For single-stained cells (*i.e.* no CFSE staining) goat anti-murine-IgG with a FITC label (diluted 1:50 in phosphate-buffered saline: BD PharMingen, San Diego, CA) was used as an alternative. This was because the fluorescent dye needed to be attached to bound antibody needed to be clearly distinguished from the CFSE signal when double staining had been performed. Once the antibody was prepared, 50 µl was added to each tube and these were incubated in the dark for 30 min at 4°C. After incubation, 500 µl of phosphate-buffered saline containing 1% foetal calf serum was added to each tube and the tubes were centrifuged at 322 x *g* (6 min, 4°C) and the supernatant removed. The cells were resuspended in the remaining supernatant. The washing step was repeated three times, and the cells were finally resuspended in 500 µl of phosphate-buffered saline (containing 1% foetal calf serum). The cells were taken to the flow cytometer for analysis.

2.2.6.5 T cell identification and marker analysis

All flow cytometry analysis was performed according to a sequence of gating steps (which are summarised in Figure 2.3).

Identification of the lymphocyte population

The first step in flow cytometry analysis was to distinguish lymphocytes from the other cell types within the PBMC suspension. Cell size and granularity were determined by forward scatter (FSC) and side scatter (SSC) creating a dot plot. The essence of flow cytometry is the analysis of the effects produced by the passage of a particle of interest through a beam of light, termed an event. In this case the particle is a cell and the beam of light was from a blue-green excitation light (488 nm argon-ion laser). Light was directed at the cells and the deflection of this, or the absorption of light, was measured. Clear demarcation in size and granularity exists between polymorphonuclear cells (including neutrophils) and PBMCs, with no overlap in the analysis regions between these two cell populations (data not shown). In all of these experiments, a minimum of 10 000 cells were counted in list mode for each event. On the dot plots obtained cells were separated into distinct populations of lymphocytes, monocytes and granulocytes (including neutrophils), according to their FSC and SSC. A gate was drawn around the lymphocyte population (Figure 2.4) by drawing either a rectangle, elipse or free-form encirclement around the population of interest and only cells within this gate were included in further analysis.

Identification of actively dividing T cells in a lymphocyte population

Once the captured data had excluded all of the debris, monocytes and granulocytes, a second dot plot was created by using FSC and SSC characteristics of the lymphocytes alone. Blasting (large), and therefore actively dividing cells, were selected for analysis by gating around the discrete population of cells with greater forward scatter than the rest of the population (Figure 2.5). Using the negative controls (cells in RPMI complete medium) and positive controls (Con A stimulated cells) the position of the gate was set so that all samples were compared with the controls, and positive activation could be identified. The position of the gate was subjective, capturing only the cells of interest. There was a larger population of blasting cells when lymphocytes were incubated with Con A than when incubated with RPMI complete medium alone (Figure 2.5).

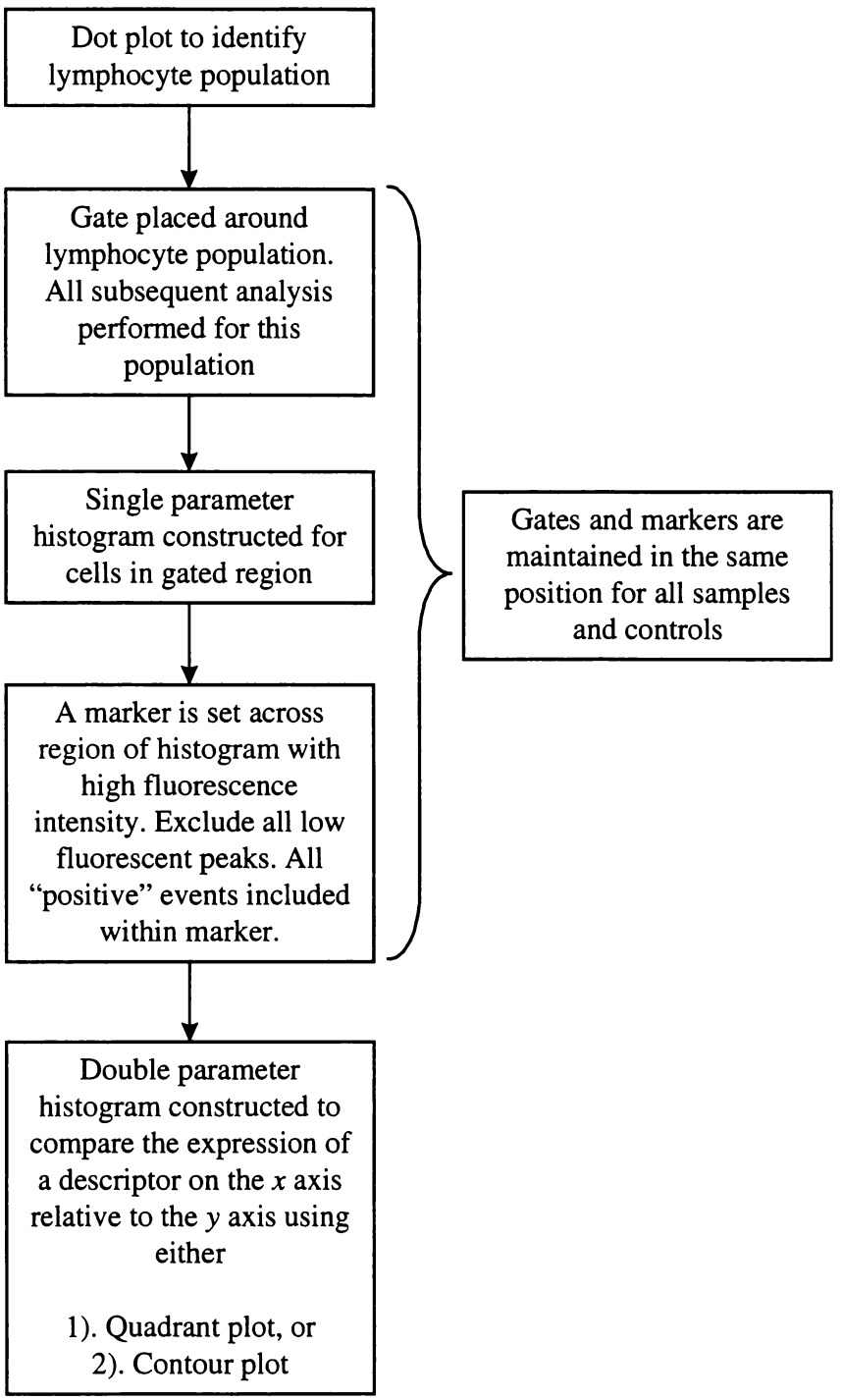


Figure 2.3: Sequence of gating steps to identify cell types in flow cytometry

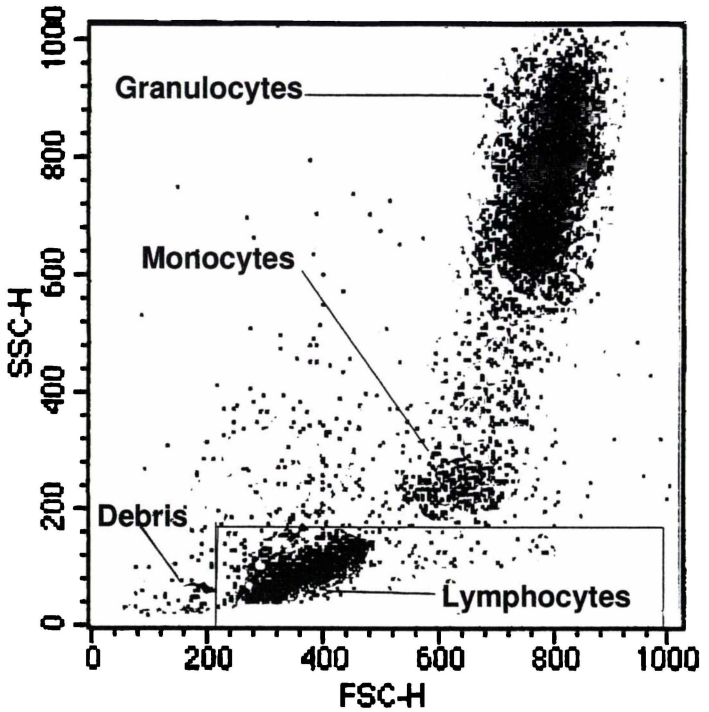


Figure 2.4: Dot plot showing different populations of leukocytes according to their specific characteristics (source: Anon (a), 2003). The lymphocyte population has been gated by drawing a rectangle around the cells exhibiting characteristic FSC and SSC characteristics.

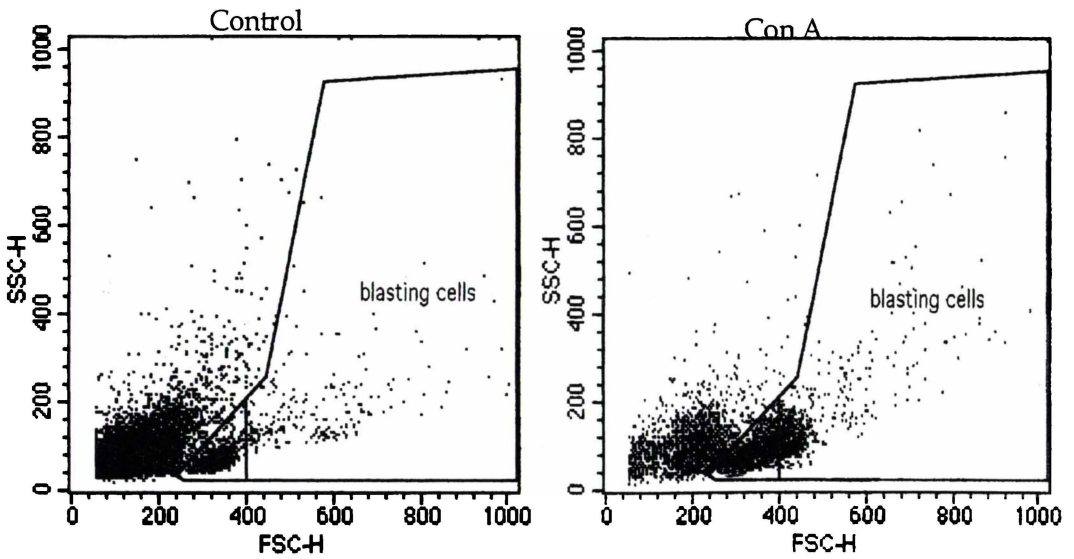


Figure 2.5: Dot plots showing characteristic differences between the blasting profiles of T cells stimulated with negative and positive controls. T cells ($2.5x10^6$ cells/ml) were incubated with RPMI complete medium with or without Con A (5µg/ml) for 72 h prior to flow cytometry analysis. A gate (R1) was placed around the large, blasting cells and the % of cells within this region compared between treatments. All subsequent analysis was carried out on cells within the gated region, R1. When T cells are activated they increase in size (known as blasting). This can be assessed by comparing the FSC of cells. The effects of incubating T cells with: A, RPMI complete medium (negative control) and B, Con A (positive control), indicate that the mitogen is a potent activator of lymphoblasting.

The percentage of blasting cells was determined for each treatment and compared to non-dividing smaller cells, according to a histogram plot of cell count (number of events) versus FSC (forward scatter) (Figure 2.6). All subsequent analysis was carried out on cells within the region R1 of Figure 2.6.

Figure 2.6 shows that a single-parameter histogram was created which included all of the cells in the gated region, R1 (from Figure 2.5). The single-parameter histogram (shown in Figure 2.6) has a logarithmic x-axis representing forward scatter and the y-axis representing the number of cells counted in each channel along the x-axis. In Figure 2.6, the gate (marker) was set based on the leading edge of the negative control (cells in RPMI complete medium). All events to the left of the marker were considered to be negative. The number of positive events were then expressed as a percentage of total events (cells) in the histogram. The median and mean for each histogram were compared between the negative and positive (Con A) controls, along with the percentage of cells in the marker region. Ideally, the marker was positioned so that less than 1% of the negative control background fell in the marker region. A geometric mean was calculated by the CELLQuest programme. Multiple differing sub-populations within a sample were seen as multiple peaks on a histogram.

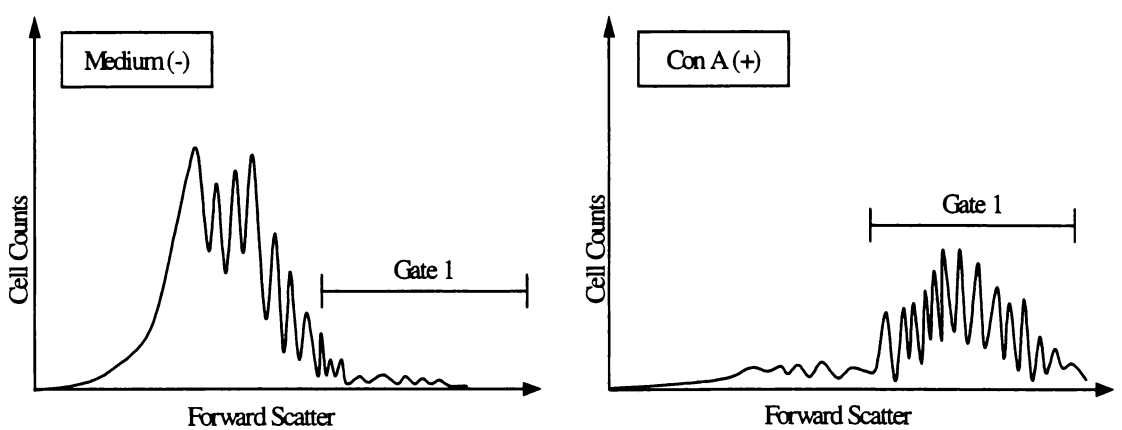


Figure 2.6: Cell counts of the positive and negative controls in single-parameter FSC histogram plots as shown in Figure 2.4 Single-parameter histograms were constructed from the cells contained within R1 (from Fig. 2.6). The gate (Gate 1) was placed across the population with highest forward scatter, and these cells were taken to be activated. The position of the gate was maintained for all samples so that they could be compared to each other and to the controls. These are representative examples.

Analysis of various cell surface markers

A two-parameter dot plot was constructed for the cells captured in the gate 1 region of the single-parameter histogram (as shown in Figure 2.5). The two-parameter dot plot enables measurement of an event with a particular combination of signal intensity for two detectors (*e.g.* FSC and SSC). In order to identify T cells, they were stained for the CD3 surface marker and their SSC characteristics examined. The percentage of cells in each quadrant of the two-parameter dot plot could be calculated as compared with all cells represented in the plot (Figure 2.6). The quadrants are described according to location in the plot so that LL (lower left) represents cells negative for both descriptors, UR (upper right) represents cells dual positive for both descriptors, UL (upper left) represents cells positive for the *y*-axis descriptor and negative for the *x*-axis descriptor, and LR (lower right) represents cells positive for the *x*-axis descriptor and negative for the *y*-axis descriptor. The use of double-parameter dot plots enabled the percentage of events in each quadrant to be compared among samples. Where T cell had been stained for only one surface marker, they were gated according to positive staining for the *x*-axis descriptor in relation to their SSC as the *y*-axis descriptor. For example, T cells were gated based on positive staining for the CD3 marker and low SSC characteristics (Figure 2.7). The proportion of cells expressing any combination of the surface markers could be analysed this way, and the position of double-stained cells in the plot (*e.g.* cells stained for both CD3 and CFSE) could be used to discriminate populations based on two parameters. For example, cells positive for both CD3 markers and negative for CFSE would be dividing T cells.

By double-staining cells for the CD3 marker and CFSE, this enabled the division of T cells to be tracked using fluorescence channels 1 (FL1) and 2 (FL2). The fluorescence channels were used to measure the amount of fluorescent light emitted from each cell and this enabled contour plots to be constructed. A contour plot introduces event frequency as a third dimension so that the number of events with a particular combination of descriptors can be shown. In a dot plot overlapping events obscure one another, however in a contour plot a particular combination of colour intensities (or shading intensities) are joined between adjacent points with equal density. Contour lines sharing the same colour shading represent areas with cells of the same fluorescence intensity within. The proximity of contour lines to each other indicate the

relative number of cells present in regions of the plot, so that contour lines located close together represent a concentrated population of cells. Such representations allow the visual discrimination of differing components within the sample, as they will appear as disjoint islands of points. By convention, CFSE intensity was represented on the *x* axis (measured using the FL1 channel). Fluorescence height is proportional to the maximal signal level from cells in the channel (hence FL1-H uses the FL1 channel). The *y* axis represented the maximal signal level from the other descriptor (*i.e.* CD3 expression) as FL2-H values (shown in the example provided in Figure 2.7). To set the FL2 threshold, cells stained with IgG_1 non-specific antibodies and PE were used to check for any non-specific binding by the secondary antibody and to provide baseline data for background.

After the computer had created the contour plot, a box was placed around the most intense cell population (as indicated by an area of closely spaced contour lines) and equal-sized regions were gated to the left until all cells were included (Figure 2.7). Based on the knowledge that CFSE fluorescence intensity halves with each mitotic division, the proportion of cells in each region (and therefore, generation) as indicated by the relative colours of the contour lines could be compared between treatments by drawing boxes around each. The mean fluorescence of the cells expressing CFSE was then obtained as arbitrary fluorescence units (flU) on a logarithmic scale.

A contour plot was used to identify the double-stained cells expressing the CD3 marker and containing CFSE. Using the markers CD3 and CFSE as an example, the FL1 channel was used to measure the intensity of CFSE-stained cells (represented on the *x* axis). The FL2 channel was used to measure the intensity of the PE-labelled cells (represented on the *y* axis). Cells positive for CD3 and CFSE are located at the far end of the *x* axis. Division of the CD3 cells is indicated by a shift in the position of clusters toward the *y* axis (with loss of the CFSE dye and therefore, fluorescence intensity). Therefore, reference to the relative position of cells along the *y* axis with respect to the *x* axis identified the $CD3^+$ population (Figure 2.7). The use of relative positioning of cells in the contour plot could be used to define T cell sub-populations according to any two of the cell markers (IL-2 receptor, CD3, CD4, CD8 or CFSE) in combination.

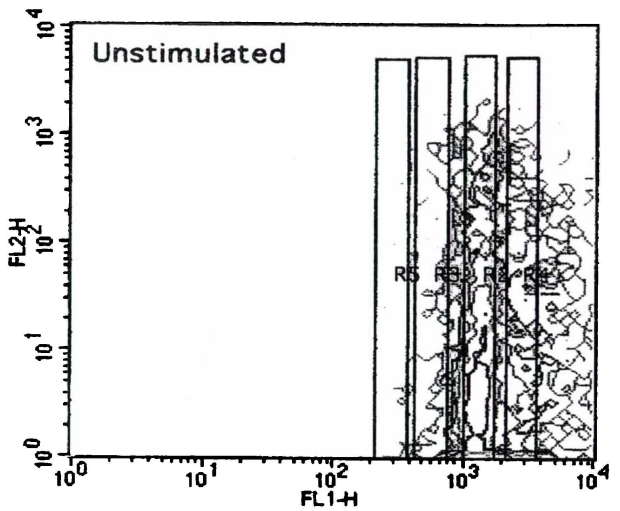


Figure 2.7: Use of progressive gating of a contour plot to determine the proportion of cells in each generation. T cells were double stained with PE-labelled anti-CD3 and CFSE, and a contour plot constructed to identify the populations according to their fluorescent intensity. Contour lines located close together indicate concentrated cell populations, and contour lines sharing the same colour represent cells sharing the same relative fluorescence intensity. Based on the knowledge that CFSE stained cells lose intensity with each mitotic division, the regions, R, were first set by bracketing the intense population (far right of *X* axis), and then adding equal sized boxes to the left. By using the boxes as gates to separate out populations according to their fluorescence, the proportion of T cells in each generation could be calculated by the CELLQuest programme, and histograms constructed.

The quantitative data derived from the contour plots could be converted into histograms which showed visually the number of times each cell had divided with each generation of daughter cells appearing as a distinct peak on the histogram (see Figure 2.8). Data were then expressed as the percentage of cells which had divided among total T cells counted.

2.2.7 Preliminary experiment to find the osmotic tolerance of PBMC

To establish an osmolarity range for PBMC and enable selection of a honey concentration which would not cause cell damage, PBMC were incubated for 24 h with

various concentrations (0.1–10%) of different honeys (artificial, manuka, Otago pasture) or RPMI complete medium alone according to the assay method described in Section 2.2.4.1. Cells were cultured for 24 h at 37°C (5% CO_2, 95% air) and viability was determined using trypan blue exclusion (as described in Section 2.2.6.1).

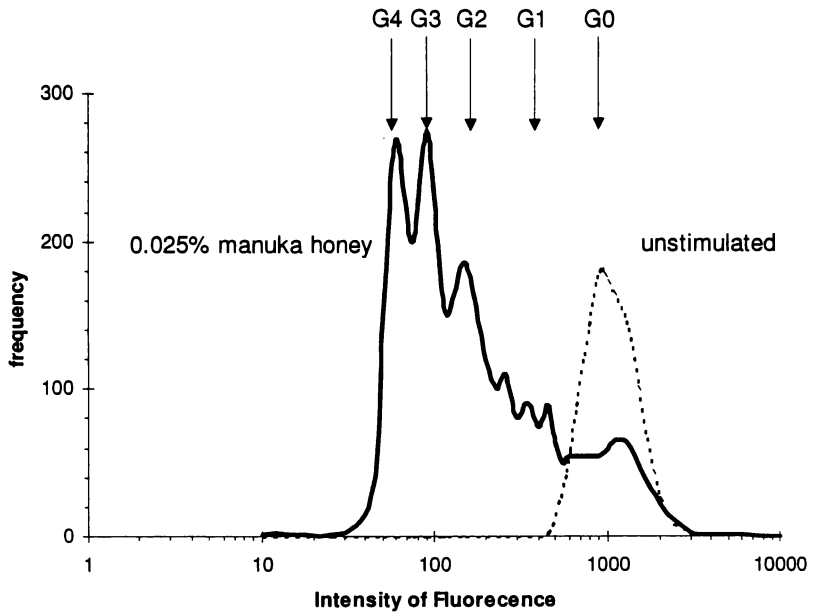


Figure 2.8: A CFSE histogram plot showing the fluorescence intensity, after a 72 h incubation period, of unstimulated lymphocytes and stimulated lymphocytes with 0.025% manuka honey. This plot demonstrates that most of the cells incubated in RPMI complete medium alone (unstimulated) remain in G0, compared to those stimulated with honey which have divided through 1-4 generations (G1-G4). CFSE fluorescence intensity halves with each mitotic division. Frequency refers to the number of cells. Two separate experiments were performed. The data presented is a representative example of a single experiment.

The effect of incubating PBMC with a range of concentrations (0.1–10%) of different honeys on cell viability are shown in Figure 2.9.

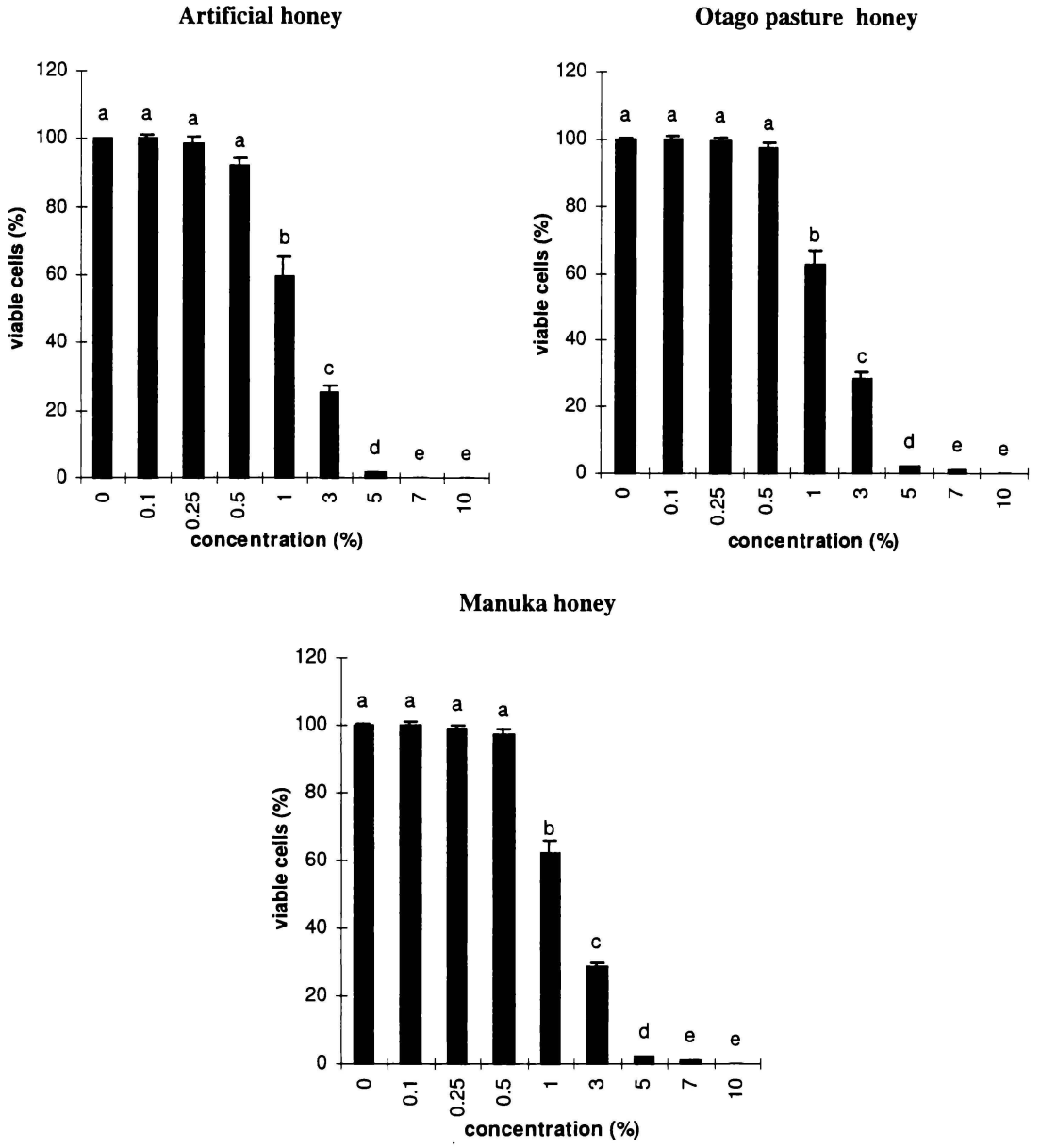


Figure 2.9: The effect of honey concentration on PBMC viability. PBMC were incubated for 24 h with various concentrations of artificial honey, manuka honey, Otago pasture honey or RPMI complete medium alone. Cell viability was assessed using trypan blue exclusion. Data are presented as the mean of nine measurements ± SEM (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within a graph are significantly different ($P<0.05$). Means did not differ significantly between the graphs for corresponding concentrations of honey.

The results in Figure 2.9 show that honey of all types significantly reduced the viability of PBMC at 1% concentrations but had no significant effect on viability at concentrations of 0.5% and less.

2.2.8 Statistical analysis

Unless otherwise stated, all tests were performed in triplicate wells and repeated at least twice in time, using blood from two different animals. Each datapoint represents the mean ± SEM of OD measurements. The results from trypan blue experiments were not derived from OD measurements. As the overall level of BrDU incorporation varies each time, data were presented as "% of control". Therefore, to compare treatment data to the controls, data was normalised by taking the mean for triplicate treatment wells as a proportion of the mean values obtained for triplicate control wells. SEM for the control wells were calculated on raw data and multiplied by the same proportion as used to convert the control to 100%. For multiple group comparisons, the data were subjected to one way analysis of variance (ANOVA) to determine overall difference between the group means. The between group effect was the amount of proliferation (or the proportion of cells expressing a fluorescent marker). The levels of the repeated measures were at least n=6 for proliferation assays, and at least n=4 for flow cytometry analyses. Significant effects were further analysed using Tukey's honestly significant difference (HSD) for pairwise differences for within-group comparisons. Systat software version 7.0 (SPSS Inc.) was used for all analyses.

2.3 EXPERIMENTS

There were two aspects to be examined for the effect of honey on PBMC proliferation. The first was whether honey could stimulate cell division in resting PBMC cultures. The second was whether honey could modify the response of PBMC to Con A.

2.3.1 The effect of honey on resting PBMC

2.3.1.1 The effect of various honeys at concentrations ranging from 0.1–1% on proliferation of resting PBMC

In order to determine whether various honeys had stimulatory activity on resting PBMC, bovine cells were incubated with a range of honey concentrations. Proliferation was assessed by uptake of BrDU after 72 h incubation (Sections 2.2.4.1 and 2.2.6.3).

The effect of incubating PBMC with (0.1–1%) concentrations of honey or RPMI complete medium alone for 72 h on PBMC proliferation are shown in Figure 2.10.

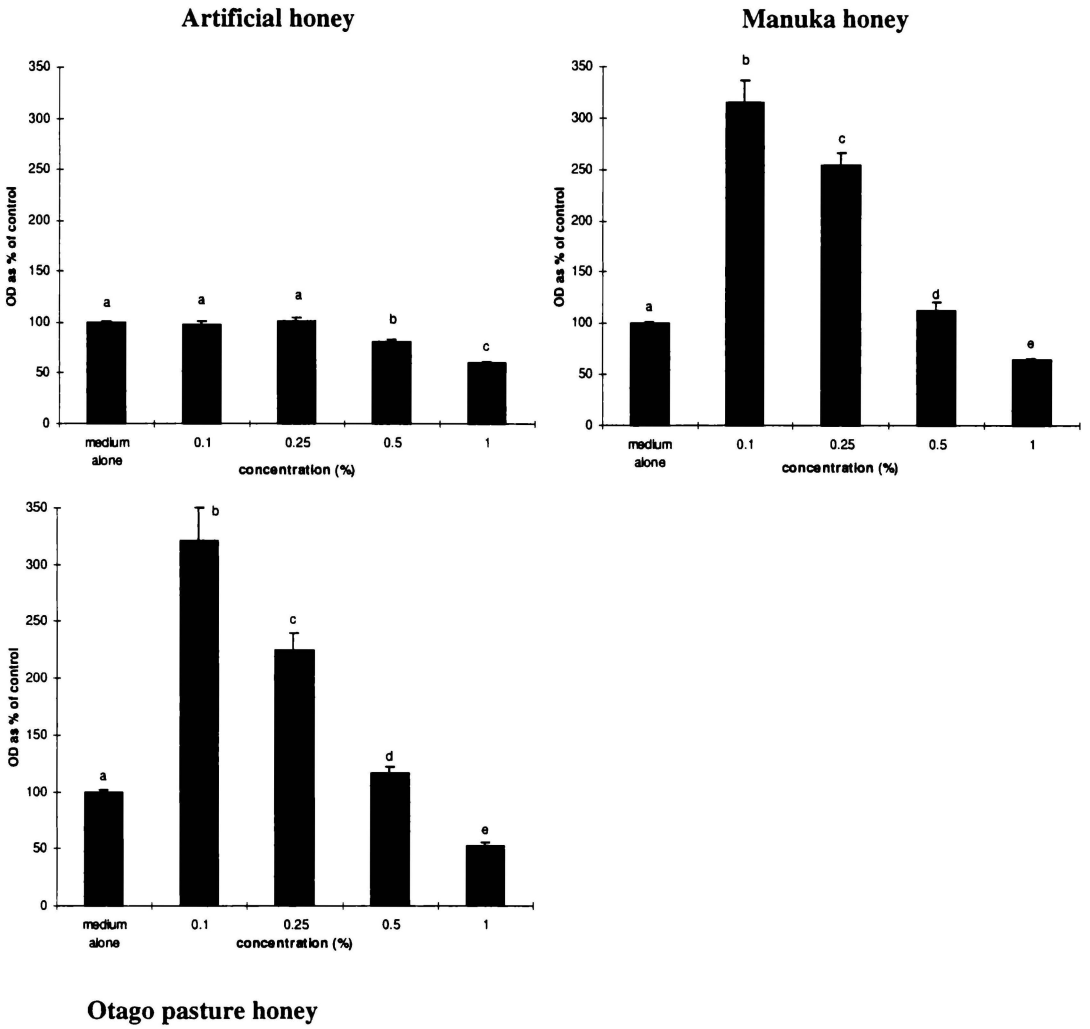


Figure 2.10: Effect of honey concentration on PBMC proliferation. PBMC were incubated with honeys (artificial, manuka or Otago pasture) or RPMI complete medium alone. Cells were cultured for 72 h and PBMC proliferation over the final 18 h of culture was determined via incorporation of a BrDU label. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Differences between the means are indicated by superscript letters, where means not sharing common letters within a graph are significantly different ($P<0.05$). Treatments were conducted in triplicate wells and these means are derived from three independent experiments (n = 9).

The results in Figure 2.10 show that BrDU incorporation by cells varied according to concentration for each honey type. Artificial honey did not significantly increase BrDU incorporation by cells beyond the control at any concentration. The decrease in cell proliferation compared with that of cells with RPMI complete medium alone, recorded

for PBMC incubated with 1% concentrations of all honey types can be attributed to the osmotic effects of sugar on the cells, as trypan blue staining had demonstrated this effect of sugars on cell viability (Figure 2.9). There was decreased viability for the cells exposed to 0.5% artificial honey but this was not significantly less than the control.

The observation that BrDU incorporation by lymphocytes increased beyond the control as the dose of honey decreased (0.5–0.1%) indicated that natural honey had a significantly greater effect on proliferation at lower concentrations (Figure 2.10). Tests for significance in the amount of proliferation induced between honeys at these lower concentrations confirmed that the natural honeys were statistically different from artificial honey ($P<0.05$) but only different from each other at the 0.25% concentration with manuka honey increasing proliferation more than Otago pasture honey did at this concentration ($P<0.05$).

2.3.1.2 The effect of various honeys at final concentrations of 0.0000025–0.025% on proliferation of resting lymphocytes

Based on the initial observation that 0.1% natural honeys induced the greatest cell response, the objective was to determine at what concentration the stimulatory effect would be lost. Therefore, the range of honey concentrations tested was extended by serial dilution of prediluted 25% honey solutions to obtain final concentrations in culture of 0.0000025–0.025%. PBMC were added to a 96-well microtitre plate according to the assay method described in Section 2.2.4.1, along with honey solutions (manuka, Otago pasture or artificial) or RPMI complete medium alone. Serial dilutions of the initial 25% honey solution enabled final concentrations to be obtained, taking into account the dilution effects of all well contents. The cells were incubated for 72 h (37°C, 5% CO_2, 95% air). Proliferation was determined using incorporation of a BrDU label (as described in Section 2.2.6.3).

The effect of incubating PBMC with honey at very low concentrations are shown in Figure 2.11.

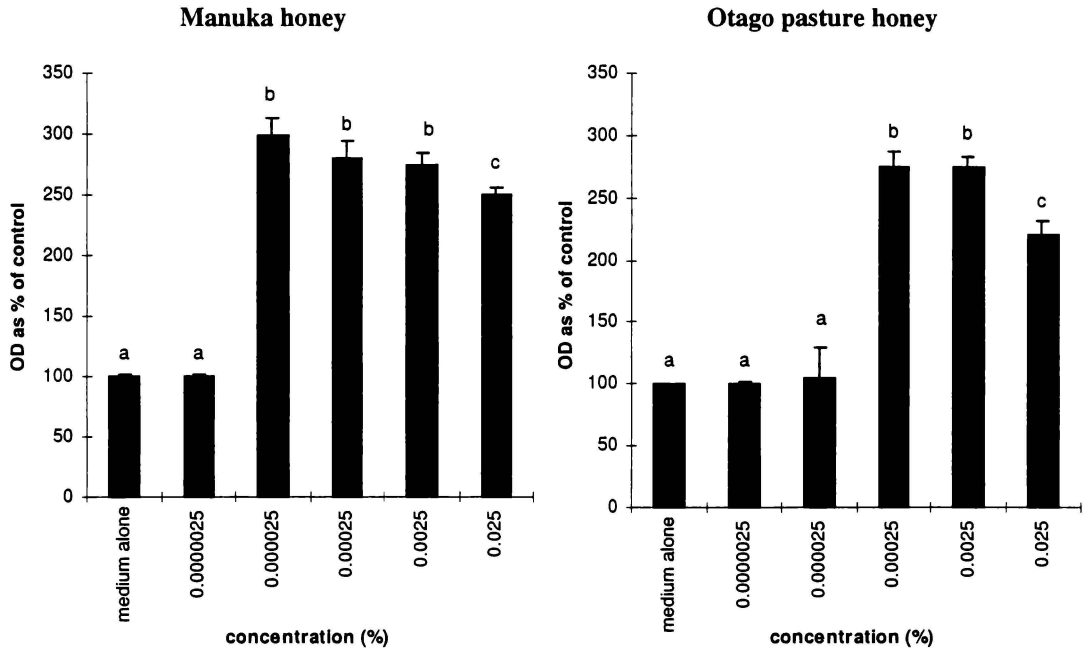


Figure 2.11: Effect of honey concentration on PBMC proliferation. PBMC were incubated with either manuka honey or Otago pasture honey at final concentrations of 0.0000025–0.025% or RPMI complete medium alone. Cells were cultured for 72 h (37°C, 5% CO_2, 95% air), and PBMC proliferation over the final 18 h of culture was determined via incorporation of a BrDU label. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from four independent experiments (n = 12).

The results in Figure 2.11 show that manuka honey at a final concentration of 0.000025% increased BrDU incorporation by PBMC compared with control cells incubated in RPMI complete medium only, but had no effect above the control at a lower concentration of 0.0000025%. Manuka honey had a greater stimulatory effect on PBMC proliferation when the concentration was reduced tenfold from 0.025–0.0025% (Figure 2.11). Otago pasture honey increased BrDU incorporation of cells beyond that obtained with the control at a concentration of 0.00025%, but not at the lower concentration of 0.000025% as seen with manuka honey. Artificial honey had no effect on PBMC proliferation at all concentrations compared with the control (data not shown). The stimulatory effect on PBMC proliferation of both natural honey types increased as honey was serially diluted below a concentration of 0.025%.

In view of the results obtained in Figure 2.11, the assays were repeated but this time using MTT assay to assess the effect of honey concentrations on PBMC proliferation

when measured through different mechanisms. The MTT assays gave the same results as the BrDU assays (data not shown) and therefore the MTT assay data was not presented as it did not differ significantly. However, the MTT assay method development was retained in this thesis as a useful reference for future experimental design. These two types of assay provided measurement of cell proliferation using different biochemical processes: the BrDU assay shows increase in DNA synthesised and the MTT assay shows increase in cytoplasmic metabolic activity.

2.3.1.3 The effect of various honey fractions on proliferation of resting lymphocytes

In order to characterise the stimulatory agent in honey, manuka honey was fractionated as described in Section 2.2.2.1. Bovine PBMC were incubated for 72 h with honey fractions according to the method described in Section 2.2.4.1. The fractions were: dialysis retentate, dialysis diffusate, high and low molecular weight fractions from ultrafiltration, or an ether extract at final concentrations of 0.000025–0.1%. Dialysis fractions of Otago pasture honey were also tested for stimulatory effects at the same concentrations as equivalent to natural honey. Proliferation was determined using incorporation of a BrDU label (as described in Section 2.2.6.3).

The ether extract of manuka honey had no effect on PBMC proliferation beyond that obtained with RPMI complete medium alone at any of the concentrations tested (0.000025–0.1%) (data not shown). Further, both the low and high molecular weight fractions of ultrafiltered honey (using a 10 kDa separation) had no activity beyond that obtained for the control (data not shown).

The effect of exposing PBMC to dialysis retentate fractions at concentrations equivalent to 0.000025–0.1%[1] natural honey are shown in Figure 2.12.

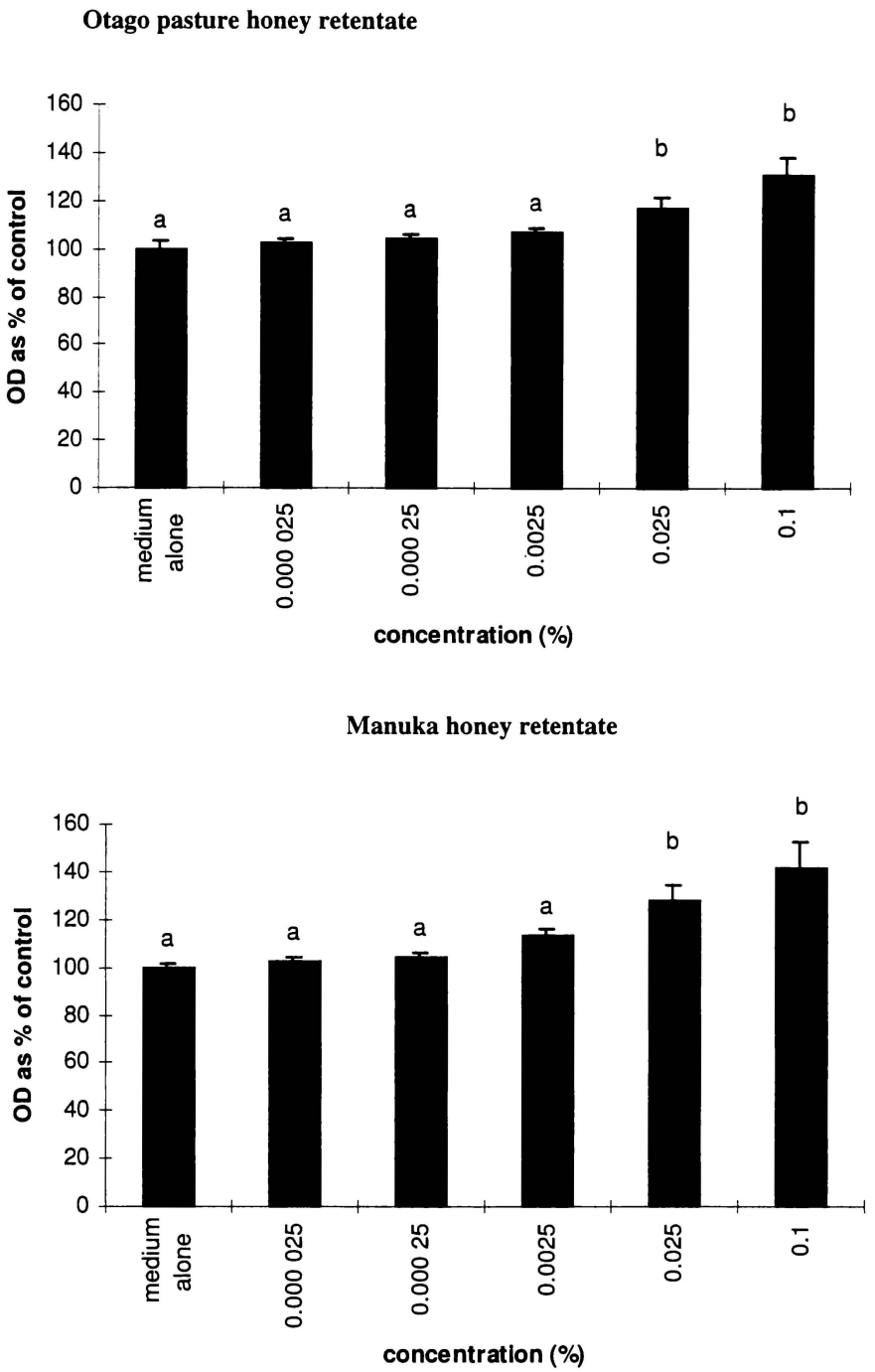


Figure 2.12: The effect of dialysis retentate fractions at concentrations equivalent to 0.000025–0.1% natural honey on PBMC proliferation. PBMC were incubated with high molecular weight fractions of honeys (Otago pasture or manuka) obtained by dialysis, or RPMI complete medium alone. Cells were cultured for 72 h (37°C, 5% CO_2, 95% air), and PBMC proliferation over the final 18 h of culture was determined via incorporation of a BrDU label. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n = 6).

The results in Figure 2.12 show that dialysis retentate fractions at concentrations equivalent to 0.025–0.1% manuka honey induced significant proliferation beyond that obtained for the control. At concentrations equivalent to 0.000025–0.025% manuka honey, retentate fractions had no activity on lymphocytes beyond that obtained with RPMI complete medium alone. Retentate fractions of Otago pasture honey had significant stimulatory activity on lymphocytes at concentrations equivalent to 0.025–0.1% honey. At concentrations where proliferation was induced there were no significant differences in the levels of BrDU incorporation obtained between the honeys. These results indicate that the active component in honey is present in the high molecular weight fraction (>10 kDa in size) and that it exerts its effects in manuka honey diluted 0.025–0.1%.

As it was observed that increased concentrations of retentate (of either honey) produced an upwards trend for proliferation levels (Figure 2.12) further assays were conducted to identify activity at higher concentrations. Bovine PBMC were incubated with retentate at concentrations equivalent to 0.1–0.5% manuka honey or RPMI complete medium alone as described. Proliferation was determined using incorporation of a BrDU label (as described in Section 2.2.6.3). The effect of exposing PBMC to higher doses of manuka honey retenate are shown in Figure 2.13.

The results in Figure 2.13 show that retentate fractions at concentrations equivalent to 0.1–0.25% manuka honey induced significant PBMC proliferation compared with the control. There was a downwards trend in the amount of cell proliferation as the retentate concentration increased. There was no activity beyond that obtained for the control when PBMC were exposed to retentate fractions equivalent to 0.5% manuka honey.

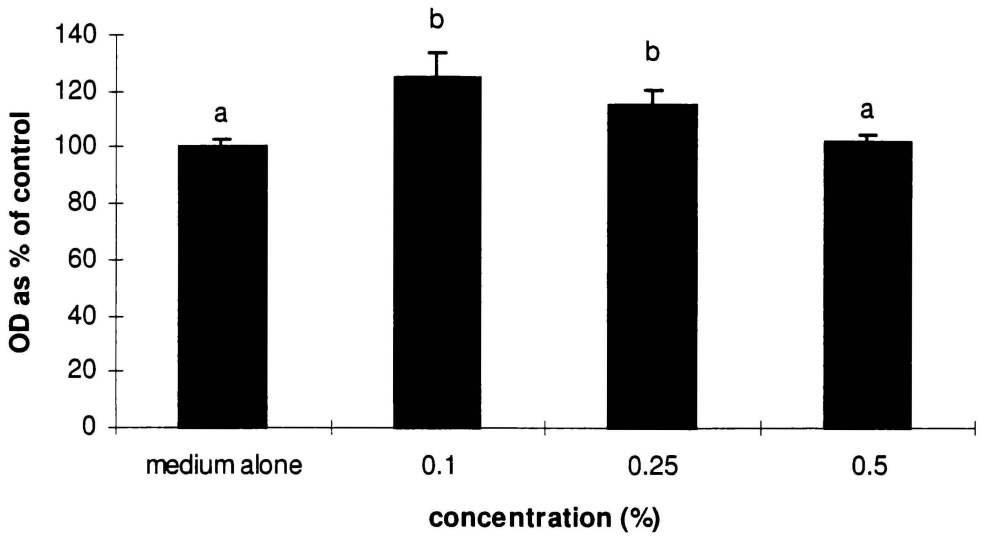


Figure 2.13: The effect of dialysis retentate fractions at concentrations equivalent to 0.1– 0.5% manuka honey on PBMC proliferation. Bovine PBMC were incubated with high molecular weight fractions, obtained by dialysis, at concentrations equivalent to 0.1–0.5% manuka honey or RPMI complete medium alone. Cells were cultured for 72 h (37°C, 5% CO_2, 95% air), and PBMC proliferation over the final 18 h of culture was determined via incorporation of a BrDU label. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Differences between the means are indicated by superscript letters where means not sharing letters are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n = 6).

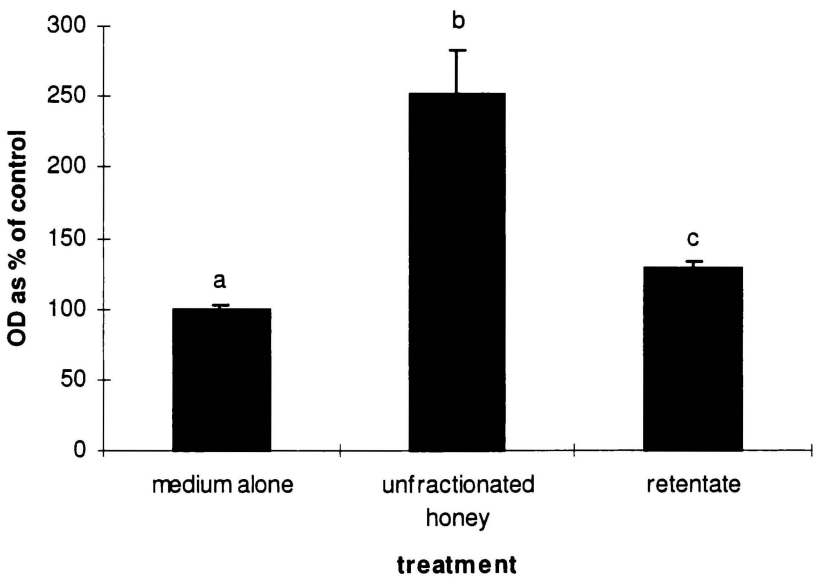


Figure 2.14: The effect of dialysis retentate fractions at a concentration equivalent to 0.1% manuka honey on Bovine PBMC proliferation compared with unfractionated manuka honey (0.1%). PBMC were incubated with 0.1% concentrations of either unfractionated manuka honey or dialysis retentate fractions at a concentration equivalent to 0.1% manuka honey, or RPMI complete medium alone. Cells were cultured for 72 h (37°C, 5% CO_2, 95% air), and PBMC proliferation over the final 18 h of culture was determined via incorporation of a BrDU label. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Differences between the means are indicated by superscript letters where means not sharing letters are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n = 6).

In view of stimulation being less than was seen in Figure 2.10 and Figure 2.11 with unfractionated honey, further assays were conducted to compare the magnitude of the stimulatory effect induced by the retentate fraction and unfractionated manuka honey. Bovine PBMC were cultured with either a retentate fraction (at a concentration equivalent to 0.1% manuka honey) or unfractionated manuka honey (0.1%) or RPMI complete medium alone. Proliferation was determined using incorporation of a BrDU label (as described in Section 2.2.6.3). A 0.1% concentration was chosen as it had been shown to produce optimal PBMC stimulation for both honey treatments.

The results in Figure 2.14 show that most of the stimulatory activity is lost by dialysing manuka honey, as indicated by the reduction in proliferation which occurred when PBMC were exposed to retentate fractions compared with that with the unfractionated manuka honey. Both honey solutions induced significant PBMC proliferation beyond levels obtained for the control.

2.3.1.4 The effect of exposing PBMC to honey for various times on proliferation of resting lymphocytes

Further assays were conducted to establish the minimum time necessary for PBMC to be exposed to 0.1% manuka honey for significant effects on PBMC proliferation to be obtained. Bovine PBMC were incubated with honey as described previously, except that the assay method was modified so that addition of the cell suspensions to the plate was staggered in time, at 72 h, 48 h, 24 h, 12 h, 8 h, 6 h, 4 h, 2 h, and the last series of wells were loaded with cells 30 min prior to conducting a MTT assay (as described in Section 2.2.6.2). Cell suspensions were loaded into 96-well plates immediately following harvest, and maintained in RPMI complete medium alone until transfer to the assay plate and addition of the honey solution occurred. Honey solutions were made fresh for each time period. Trypan blue staining had shown that PBMC were still viable when maintained in these conditions for 72 h (data not shown). All cells were incubated at 37°C (5% CO_2, 95% air). The effect of incubating PBMC with 0.1% manuka honey for various lengths of time on PBMC proliferation are shown in Figure 2.15.

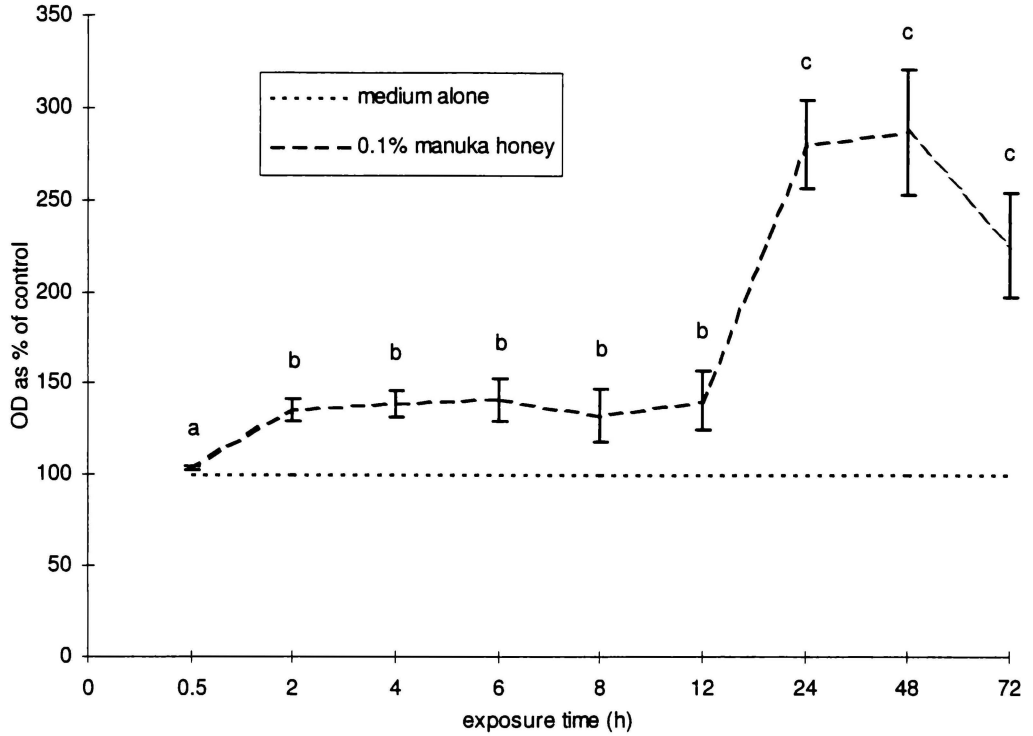


Figure 2.15: The effect on proliferation of PBMC of exposing the resting cells to 0.1% manuka honey for various times. Bovine PBMC were incubated with 0.1% manuka honey for various times (0.5–72 h) prior to MTT assay. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (RPMI complete medium only). The absorbance value obtained for each timepoint represents the amount of MTT which, in the 2 h period of incubation, has been metabolically converted as a proportion of the absorbance values obtained for cells incubated in RPMI complete medium alone for the same period. Data represent the mean ± SEM of OD readings for each treatment, expressed as a proportion of the control (cells in RPMI complete medium only). Significant differences in proliferation between times of exposure to honey are indicated by superscript letters where means not sharing letters are significantly different ($P<0.05$). Significant differences between means for honey and RPMI complete medium alone at each time-point are indicated $P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n = 6).

The results in Figure 2.15 show that a minimum period of exposure of 2 h is necessary for 0.1% manuka honey to have any stimulatory effects on PBMC proliferation. While a 2–12 h exposure time for PBMC to honey significantly increased proliferation beyond that obtained for RPMI complete medium alone, an exposure time of 24 h or more had the greatest effect on proliferation. There were no significant changes in proliferation when cells were incubated with honey beyond 24 h. There was some suggestion from the plotted data that proliferation levels had begun to decline after 72 h of exposure.

2.3.1.5 Assessment of the effect of honey on PBMC proliferation using CFSE staining

Flow cytometry was used to provide an independent measure of activation of proliferation of T cells, to further verify the results obtained from the BrDU and MTT assays. By staining with CD3 antibodies it would be possible to determine the extent of T cell proliferation. According to the method described in Section 2.2.6.4, bovine PBMC were stained with CFSE prior to incubation with honey (0.0025%). Cells were also incubated with Con A as a positive control. After 72 h the cells were removed and stained for CD3 expression. Analysis was undertaken using flow cytometry (as described in Section 2.2.6.4).

With regard to the contour plots in Figure 2.16, relative colour shading indicates the relative fluorescence intensity of cells in the plot, so that contour lines sharing the same colour represent cells with the same fluorescence intensity. The proximity of the contour lines to each other indicates the concentration of cells present in plot regions, so that dense populations have lines close to each other. The effect on the cell division profile of CD3-labelled cells exposed to honey as compared with Con A or RPMI complete medium alone is shown in Figure 2.16.

Cells cultured for 72 h in the absence of Con A (control, lower left panel in Figure 2.16) have very little cell division, as shown by the majority of the cells remaining at resting stage fluorescence (approximately 2 x 10^3 FL1). Those cells which have divided following incubation with honey (top panels) have also stained negative for CD3 (B cells), indicating the honey acts as a T cell stimulus. The quantitative results for the cell division profiles for T cells exposed to honey, Con A or RPMI complete medium alone (as shown in Figure 2.16) are presented in Figure 2.17.

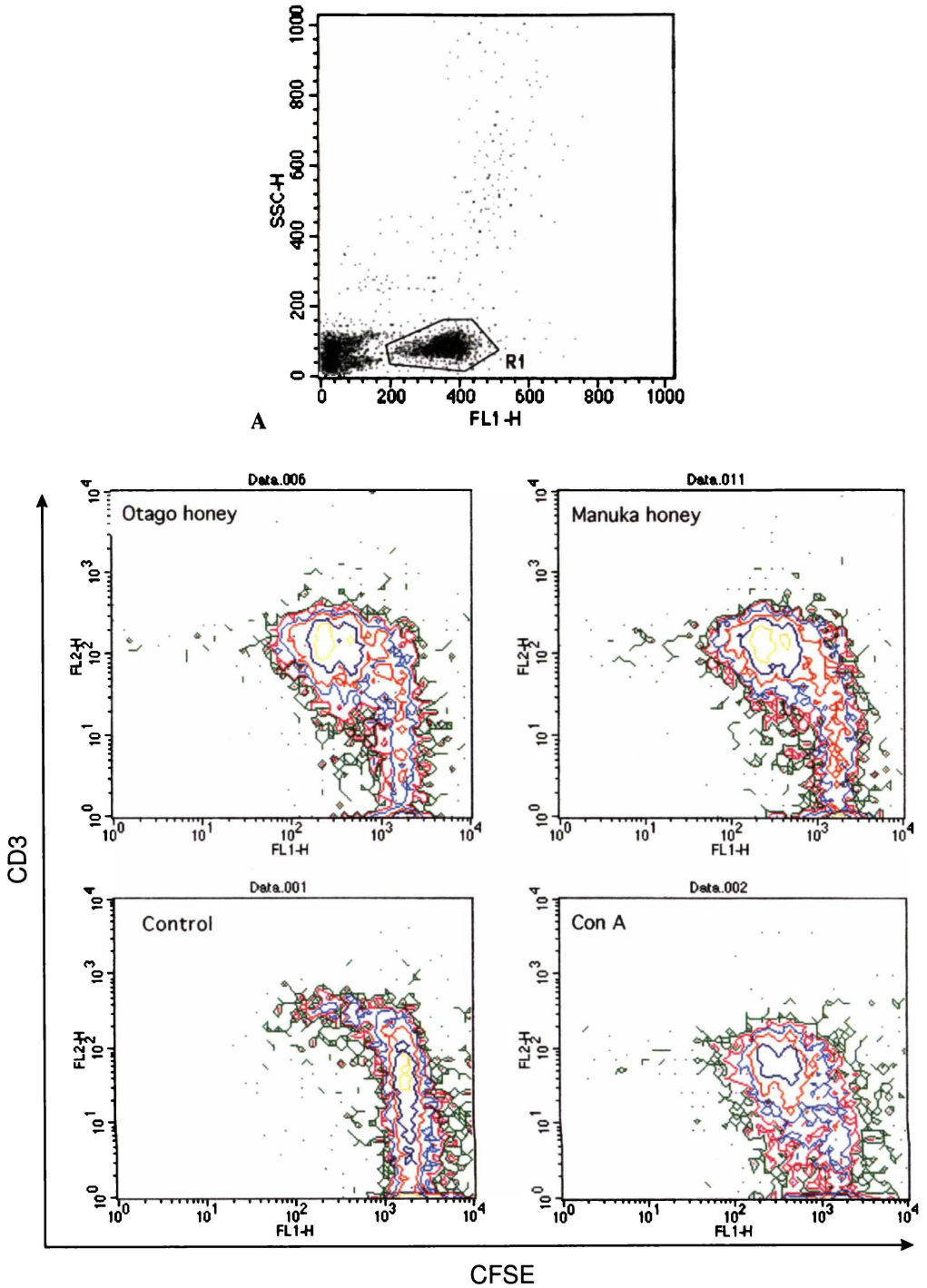


Figure 2.16: A. T cells were identified by gating on CD3+SSC (low) characteristics. B. The effect of honey on T cell division as indicated by progressive loss of CFSE stain by CD3 cells. Bovine PBMC (2.5 x 10^6 cells/ml) were costained with primary antibody anti-CD3 and secondary antibody anti-IgG_1 PE, and CFSE. PBMC were exposed to either: RPMI complete medium, Con A (5 µg/ml) or honey (manuka or Otago pasture at final concentrations of 0.0025%), for 72 h prior to flow cytometry analysis. Cells positive for both CD3 and CFSE are located at the far end of the *x* axis, and division of the CD3 cells (with loss of CFSE stain) resulted in a shift of the population toward the *y* axis. Cells negative for the CD3 marker were located in the lower half of the plot. Two separate experiments were performed. The data presented is a representative example of a single experiment.

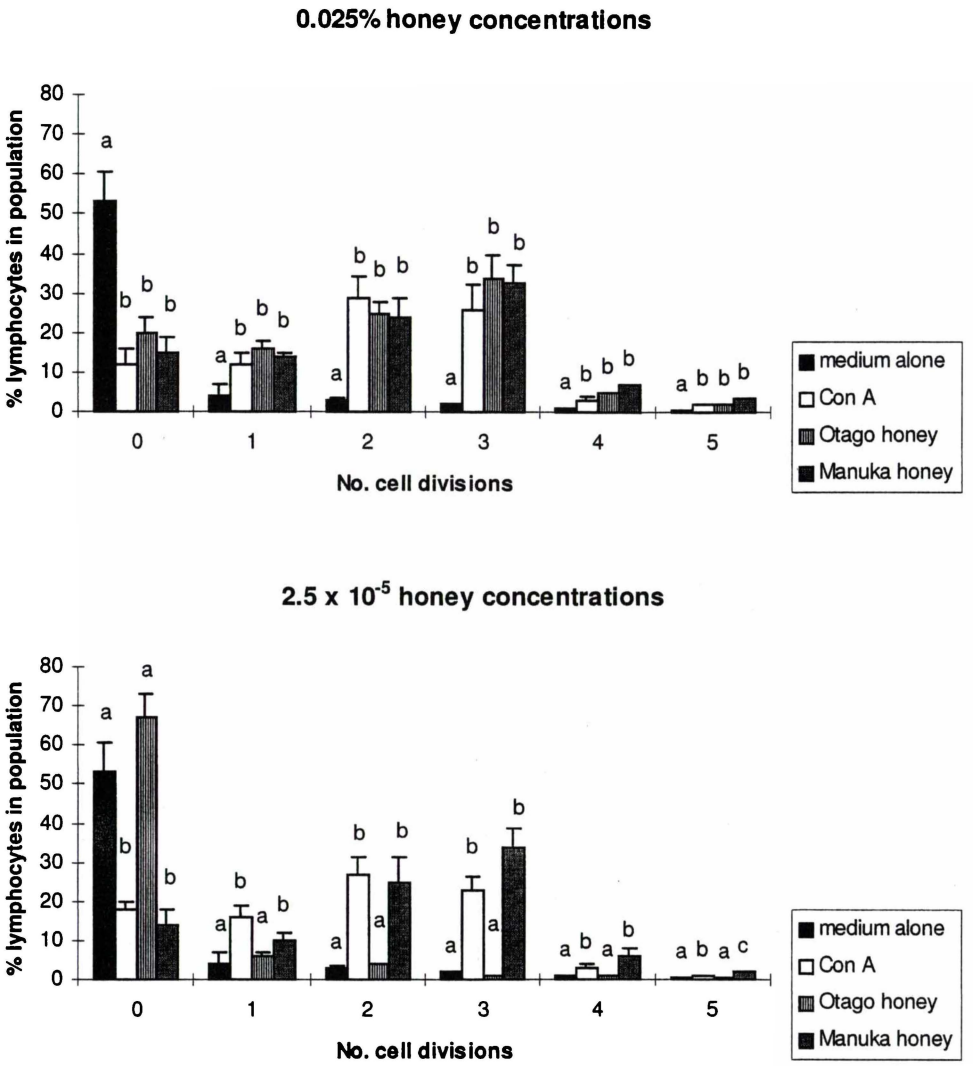


Figure 2.17: The proportion of lymphocytes in each daughter T cell generation according to progressive loss of CFSE fluorescence. Lymphocytes (2.5 x 10^6 cells/ ml) were incubated (37°C, 5% CO_2, 95% air), with either honeys (Otago pasture or manuka at final concentrations 0.000025-0.025%), Con A (5 µg/ ml), or RPMI complete medium alone for 72 h. Differences between the means for the proportion of cells within each generation are indicated by superscript letters, where means not sharing letters within a division are significantly different ($P<0.05$). Data in triplicate are shown as the mean ± SEM of a single experiment (n = 3).

The results in Figure 2.17 show that five rounds of cell division were identified for the analysis. When T cells were incubated with 0.025% concentrations of either honey, the proportion of cells undergoing a fifth round of mitosis was similar to those

incubated with Con A and significantly higher than for the control group. It is possible that the cells in the treatment groups had continued to divide beyond five rounds but that loss of fluorescent intensity may have prevented resolution from background levels at this point. Further, the proportion of cells in the generations 1-5 (G1-5), were significantly higher for cells incubated with Con A or honey (0.025%) than for RPMI complete medium alone indicating that honey, like Con A, induced T cell division. There was a higher proportion of undivided cells (G0) for cells exposed to RPMI complete medium alone than to Con A or honeys. Otago pasture honey had no activity on T cell proliferation beyond that obtained for RPMI complete medium alone at the lowest concentration, 0.000025%. The stimulatory effect of manuka honey at a 0.000025% concentration was equal to that of Con A indicating that it has significant activity at very low levels as shown earlier with the results obtained from MTT and BrDU analysis.

2.3.1.6 Activation of resting lymphocytes by honey as determined by expression of the Interleukin-2 receptor

Recently activated T cells express the IL-2 receptor on their cell surface (Pereira *et al.*, 1999). CFSE stained bovine PBMC that had been cultured with various concentrations of honey (Section 2.2.4) were stained for expression of the IL-2 receptor. Con A was included as a positive control (Section 2.2.4.1). Non CFSE stained cells were also cultured and stained for expression of CD4 or CD8 and IL-2 receptor expression.

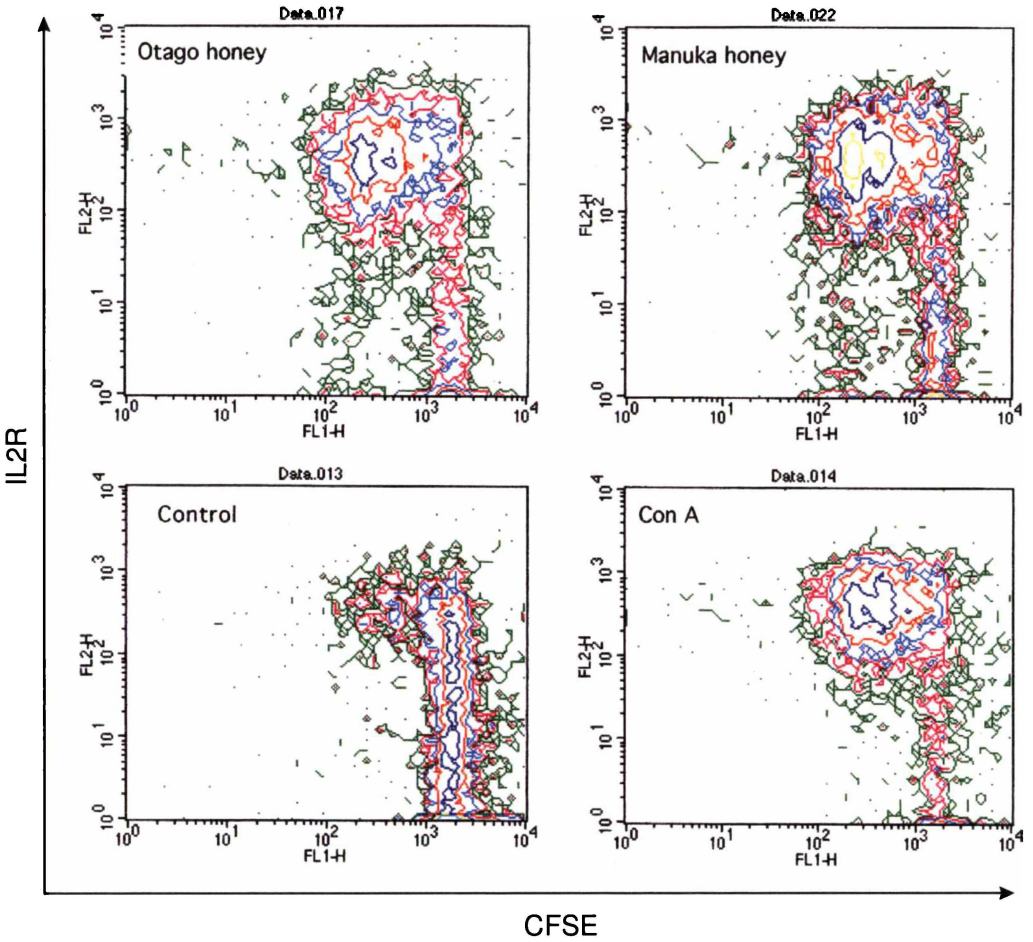


Figure 2.18: The effect of honey on the expression of the IL-2 receptor on lymphocytes. PBMC (2.5 x 10^6 cells/ml) were costained with anti-IL-2 monoclonal antibodies and CFSE. PBMC were exposed to either: RPMI complete medium, Con A (5 µg/ml) or honey at a 0.0025% concentration (manuka or Otago pasture) for 72 h prior to flow cytometry analysis. Cells positive for both the IL-2 receptor and CFSE are located in the top right of the plot (within the 10^2–10^3 flU height range as measured by the FL2 channel and shown on the *y* axis). With progressive loss of the CFSE stain (indicating cell divison) the population expressing the IL-2 receptor shifts toward the *y* axis. Cells negative for IL-2 receptor expression are located in the lower half of the plot. Two separate experiments were performed. The data presented is a representative example of a single experiment.

As was previously described for Section 2.3.1.5, relative colour shading in the contour plots presented in Figure 2.18 indicates the relative fluorescence intensity of cells in the plot, so that contour lines sharing the same colour represent cells with the same fluorescence intensity. The proximity of the contour lines to each other indicates the concentration of cells present in plot regions, so that dense populations have lines close to each other. The effect on the expression of the IL-2 receptor by T cells exposed to honey as compared with Con A or RPMI complete medium alone are shown in Figure 2.18.

The contour profile exhibited by the cells incubated with honey at a 0.0025% concentration, indicates that honey increased their expression of the IL-2 receptor and underwent several rounds of cell division. Consequently, by G3 or G4, the expression of the IL-2 receptor was lost. The results in Figure 2.18 did not identify which T cell subsets were expressing the IL-2 receptor.

The objective of costaining T cells for the IL-2 receptor and various CD markers was to quantify the response of T cell subsets to honey as compared with Con A or RPMI complete medium alone. The effect on the expression of the IL-2 receptor by the CD8+ T cell subset after exposure to honey, Con A or RPMI complete medium alone is shown in Figure 2.19.

The results in Figure 2.19 show visually the differences in the proportion of CD8+ T cells expressing the IL-2 receptor (in gated regions) when T cells had been exposed to honey as compared with Con A or RPMI complete medium alone. While the results presented in Figure 2.19 give percentages for CD8+ T cells expressing the IL-2 receptor in each plot, these percentages are obtained from one sample only, and therefore the means for the values obtained for two independent experiments are given in Figure 2.20.

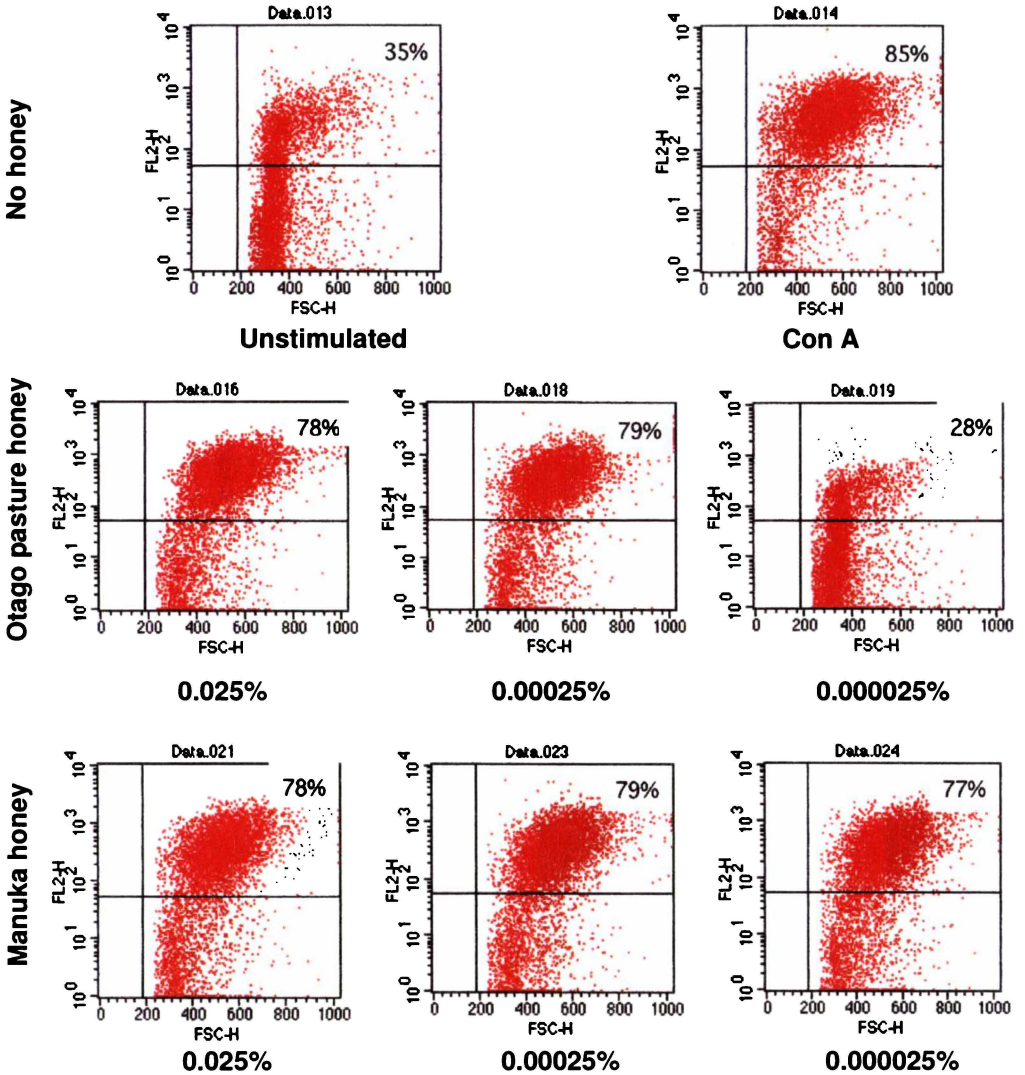


Figure 2.19: The effect on the expression of the IL-2 receptor by CD8+ T cells exposed to honey or Con A. After a 72 h exposure to honey (manuka or Otago pasture diluted to final concentrations of 0.000025-0.025%), Con A (5 µg/ml) or RPMI complete medium alone, PBMC (2.5 x 10^6 cells/ml) were double stained with an anti-IL-2 receptor antibody and anti-CD8. FL2 fluorescence is IL-2R, and FSC-H is CD8 fluorescence. CD8+ T cells were initially gated using the Con A and control cultures as a reference. The presence of cells in the upper right quadrant of a plot indicated they are positive for both descriptors. The proportion of cells present in the upper right quadrant was compared among treatments (honey or Con A) with the control. Two separate experiments were performed. The data presented is a representative example of a single experiment. The percentage values shown in the gated areas were assigned by CELLQuest and represent the values obtained for one sample.

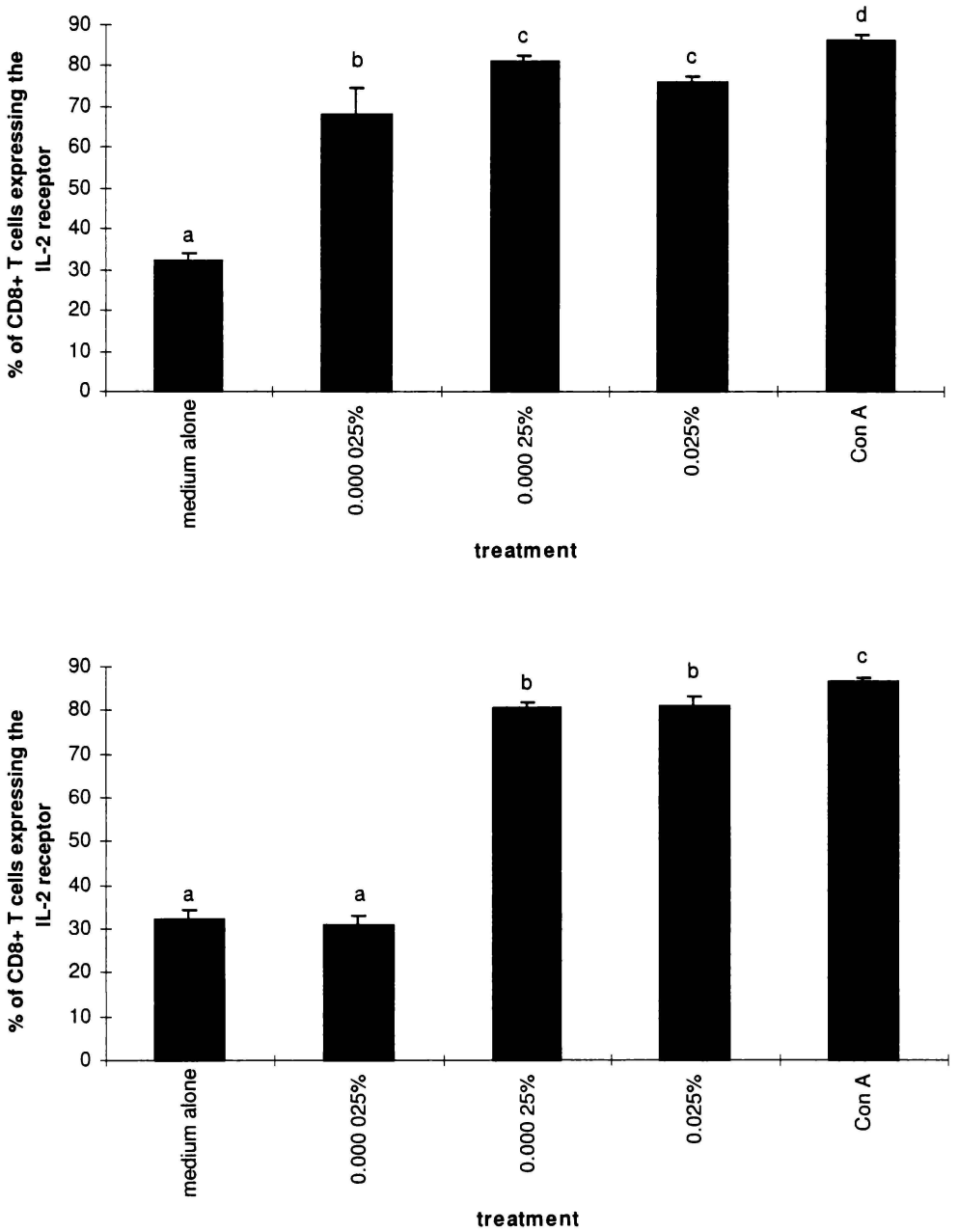


Figure 2.20: IL-2 receptor expression on bovine CD8+ T cells cultured with honey. After a 72 h exposure to honey (manuka or Otago pasture diluted to final concentrations 0.000025–0.025%) Con A (5 μg/ml) or RPMI complete medium alone, PBMC (2.5 x 10^6 cells/ml) were costained with an anti-IL-2 receptor antibody and anti-CD8. CD8+ T cells were initially gated using the Con A and control cultures as a reference. Data represent the mean ± SEM proportion of gated CD8+ T cells (as shown in Figure 2.19) expressing the IL-2 receptor for each treatment. Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$). Treatments were conducted in duplicate and these means are derived from two independent experiments (n = 4).

The results presented in Figure 2.19 and Figure 2.20 show that the proportion of CD8+ T cells expressing the IL-2 receptor was greater when T cells had been incubated with honey (at final concentrations of 0.00025% and 0.025%) or Con A compared to cells incubated with RPMI complete medium alone. At both of the higher honey concentrations, more than 70% of CD8+ T cells expressed the IL-2 receptor compared with more than 80% in the Con A-stimulated group. The proportion of CD8+ T cells expressing the IL-2 receptor did not differ among the two natural honey types at these higher concentrations. However, at 0.000025% concentrations, only manuka honey increased the proportion of cells expressing the IL-2 receptor beyond the levels obtained for RPMI complete medium, but to a lesser effect than with the higher concentrations. These results indicate that low concentrations of honey induce T cells to express a key receptor needed for proliferation to occur. Manuka honey is more potent than Otago pasture honey in inducing IL-2 receptor expression. The staining for expression of the CD4 surface protein failed to work on the two occasions tested and was therefore not continued.

In addition to performing proliferation assays and measuring cell activation status using flow cytometry, cells were checked visually for stimulation by assessing the amount of clumping (proliferating cells) using an inverted light microscope. PBMC cultures were established as described in Section 2.2.4.1, and the cell cultures exposed to manuka honey alone at 0.05% and 0.5% were selected for photographs as they provided evidence of the activating effects on T cells of honey at both a low concentration, and a concentration at the limit of the osmotic tolerance range. These results are shown in Figure 2.21.

The visual results presented in Figure 2.21 show that very low concentrations of manuka honey induce T cells to clump in the same way as a mitogen. At the higher concentration (0.5%) of manuka honey, no clumping was evident and the cell cultures looked the same as those exposed to RPMI complete medium alone. Further, there is no evidence of osmotic stress in the cell cultures exposed to 0.5% manuka honey. This evidence suggests that the stimulatory component in manuka honey has been suppressed at the higher concentration, and that it is not likely to be due to osmotic stress alone.

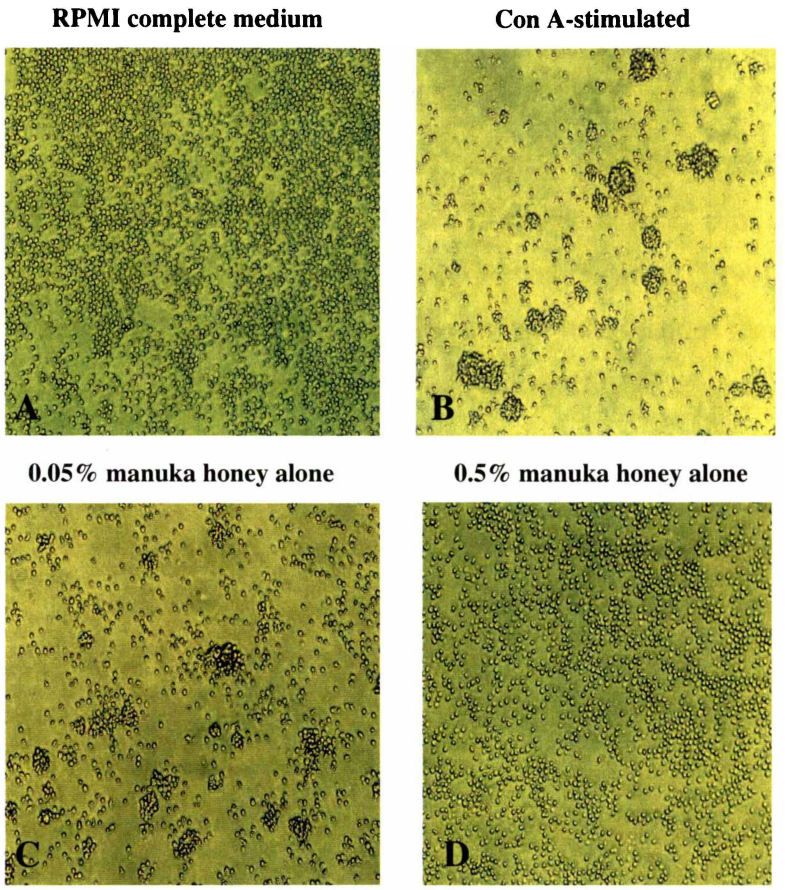


Figure 2.21: The visual appearance of bovine PBMC cultures exposed to manuka honey at various concentrations as compared with Con A or RPMI complete medium alone. PBMC were incubated with manuka honey (at C: 0.05% and D: 0.5% concentrations) B: Con A (5 μg/ml) or A: RPMI complete medium alone for 72 h. For each treatment, a representative PBMC culture was photographed after 24 h incubation (Olympus 300x). These results are representative of three independent assays.

2.4 DISCUSSION

This study aimed to investigate whether honey had any effect on T cell proliferation. The results of the current study provide evidence that low concentrations of honey stimulate T cells to proliferate *in vitro*, but not to the same extent as a classical mitogen.

Cell proliferation encompasses DNA synthesis, mitosis and an increase in cell number. The ability of low concentrations of honey to stimulate T cell proliferation *in vitro* are in agreement with observations reported by Abuharfeil *et al.* (1999), although activity in the present study has been identified for lower concentrations than reported elsewhere. Physiologically, the concentration of unidentified immunostimulatory component of honey reaching the circulation is likely to be in the micromolar concentration range. Based on the composition of an average honey type as given by White (1975), only 2.4% of the total honey content can be attributed to that other than sugars, water and minerals. Taking into account the water content (75%) of an average North American adult female (62 kg) being 45 litres, consumption of 10 g of honey would deliver a maximum potential dose of 240 mg of the unidentified agent.

Whether honey is ingested orally or applied topically, the active ingredient will be subject to various unknown factors: dilution with wound fluid or digestive juices, pH of the interface between honey and wound or honey and gut wall, variable oxygen levels, and the presence of enzymes and ions in the body. This means that the effective concentration reaching the "target" tissues will be significantly reduced from the original dose. However, the most significant point to be taken from these results is that T cell proliferation is enhanced by hundred-fold dilutions of honey. If the immunostimulatory components of honey are absorbed from the gut and become distributed in the total body fluid, then an approximately 0.01% concentration could be expected in the circulation after a teaspoonful of honey has been administered orally. A study by Al-Waili (2003) demonstrated that oral ingestion of small quantities of honey led to significant changes in the haematological indices of blood. This means that honey could be expected to have systemic as well as local immunostimulatory

effects. If there is no immune response occurring then honey will stimulate one even if only trace quantities of honey are there. It is not currently known how biotransformation may affect the active ingredient(s) following consumption.

Abuharfeil *et al.* (1999) reported that B cell proliferation increased as honey concentrations were diluted from 0.25–0.1%. A study on the effects of oral ingestion of honey on haematological indices in blood showed that it elevated both the populations of circulating monocytes and lymphocytes (Al-Waili, 2003). This data supports the observations made in the present study.

Inclusion of the artificial honey control was designed to examine the effect of the sugars alone on T cell function. The observations made in this study reveal that sugars alone have no effect on T cell proliferation apart from the negative effect of the osmotic pressures they exert on cells when osmolarity exceeds 315 mOsm (0.5% concentration). In view of the increased stimulatory effect as honey is further diluted, it is significant that sugars alone were shown to have no effect on T cell activity. Testing of dialysis retentate fractions of honey (with sugars removed) established that the osmotic effect cannot be responsible for the suppression of stimulation seen with higher concentrations.

Further, the demonstrated effects on T cell proliferation by dialysis retentate fractions confirm that activation cannot be due to supply of glucose to the cells alone. Dialysis fractions had a reduced effect on T cell activation suggesting some of the activity had been lost during fractionation. That activating effects on T cells are greatest at higher honey dilutions means that they are unlikely to be due to a direct nutrient effect on cells. These results are in agreement with the other studies which have shown that honey stimulates wound healing whereas sugars alone do not (Bose, 1982; Burlando, 1978; Hutton, 1966). The sugar component of honey has many significant biological effects, however these are not relevant to T cells when the availability of glucose in the culture medium is not lacking. It is possible that honey could give increased metabolic activity to cells without an increase in the number of cells, to give the increased activity seen in the MTT assay. However, use of BrDU assays and flow cytometry imaging (through CFSE staining) has measured the direct effect of honey

on cell division. Differences have been observed in the osmotic tolerance of cells by authors. While Tonks *et al.* (2001) reported that 1% final honey concentrations stimulated activity in the MM6 monocytic cell line with no osmotic damage, this was not the case in the current study. Cell types will exhibit differences in their osmotic tolerance according to many factors, including their biological origin.

There are a number of plausible mechanisms by which honey may activate T cell proliferation and these can be divided into three broad groups based on the likely component in honey responsible; proteins, non-protein components of high molecular weight, and hydrogen peroxide.

A number of studies have shown that proteins can activate T cells, and in particular proteins with sugars attached (Brownlee, 1995; Vlassara, 1995; Vlassara *et al.*, 1995; Takata *et al.*, 1988; Vlassara *et al.*, 1985). Honey is known to contain proteins (National Honey Board USA, 2003; Khalil *et al.*, 2001; Molan, 1992b; White, 1975a; 1963), and it is likely that proteins in honey would be glycated (Tonks *et al.*, 2003), due to the propensity for the enzyme invertase (which has glucose transferase activity) to transfer glucose on to other groups (*e.g.* proteins) (White, 1963). Further, long-term exposure of the proteins in honey to monosaccharides increases the likelihood that sugars will be added onto the proteins via the Maillard reaction (White, 1963). It is possible that different honey types have different protein compositions.

Studies have established that glycated proteins bind to cellular receptors in a similar manner to lectins (*e.g.* Con A) (Jensen *et al.*, 2003; MacCarthy *et al.*, 1994; Takata *et al.*, 1988). However, glycated proteins activate T cells through the TcR receptor on the cell membranes (Sitkovsky *et al.*, 1984). Full strength honey contains a low protein content (Molan, 1992b; White, 1963). That an average honey has an average total protein content of 168.6 mg/100g honey (National Honey Board USA, 2003), and dilution to the very low levels tested in the current study would reduce these further, raises doubts about the feasibility of such low concentrations of protein having significant stimulatory effects on T cells. Con A loses its effect on T cell activation when diluted to 0.01 µg/ml (Beineke *et al.*, 2004). Using the figures reported by White (1975a) for the free amino acid content in an average honey, at a

0.25% concentration the free amino acid content would be around 1.12 μg/ml. This does not take into account the content of any polypeptides. Taking into consideration the content of active proteins (such as those derived from royal jelly as reported by Simuth *et al.*, 2004) and enzymes, the possible contribution of proteins to the observed stimulatory effects cannot be discounted.

There is extensive evidence indicating that hydrogen peroxide is a cellular activator (Reth, 2002; Lee *et al.*, 1999; Li and Karin, 1999; Los *et al.*, 1995; Schreck *et al.*, 1991, Flohé *et al.*, 1985), specifically for T cells (Reth, 2002; Los *et al.*, 1995). This study has shown that honey similarly upregulates expression of the IL-2 receptor and induces T cell proliferation. Direct addition of micromolar concentrations of hydrogen peroxide to T cells for short periods of time upregulates IL-2 production, and in turn, cell proliferation (Los *et al.*, 1995). The results of the current study were in agreement with Los *et al.* (1995), who observed that IL-2 receptor expression was induced by exposing Esb-L T lymphoma cells to micromolar concentrations of hydrogen peroxide for short periods of time. In addition, it has been shown that honey will induce IL-2 production by a murine cell line (de Jong, 2003). Further, it has been demonstrated by Pani *et al.* (2000) and Alliangana (1996) that altering the balance between exogenously added hydrogen peroxide and antioxidants to a cell culture had a profound effect on whether cells proliferated or not.

Extrapolating from the values for hydrogen peroxide production by honey given by Bang *et al.* (2003), the likely concentration of hydrogen peroxide accumulating in cell cultures incubated with 0.25% honey for 30 min would be approximately 0.05 mM. This is well below the minimum concentrations of hydrogen peroxide (0.2 mM) reported to induce oxidative stress in fibroblasts (Wang *et al.*, 2001). Yet this level is well above the minimum levels of oxidant species (0.2–0.1 nmol) observed to stimulate fibroblast proliferation (Murrell *et al.*, 1990). A further point to consider is that dialysis retentate fractions contained the active, stimulatory component and this supports the possibility of hydrogen peroxide being responsible, since glucose oxidase would be in the retentate. The glucose in the medium would provide the substrate for glucose oxidase to produce hydrogen peroxide. With hindsight, it would have been

useful to run the retentate fraction through a separation column to enable purified fractions of this to be tested.

While there is an abundance of evidence for the activating effects of hydrogen peroxide on T cells, there are several factors which complicate the likelihood of its involvement in the results presented here. The first is the observation that progressive dilution of honey below a 30% concentration (120-fold more concentrated solution than the 0.25% concentrations found to have activity in the current study) leads to significant reduction in the ability of the glucose oxidase enzyme to produce hydrogen peroxide (Bang *et al.*, 2003). The second is that manuka honey has a more potent stimulatory effect than Otago pasture honey yet has a lower hydrogen peroxide content (Molan, 1992b).

Hydrogen peroxide has been demonstrated to stimulate cell proliferation when co-incubated with cells for short periods of time (8 h optimal), and stimulation effects were reduced with increased time (Los *et al.*, 1995). However, in the current study it was observed that while a 2 h incubation period was sufficient to activate some proliferation, maximal effects were obtained after 24 h. It should be noted however, that the investigations performed by Los *et al.* (1995) delivered hydrogen peroxide as a bolus to the cells, whereas hydrogen peroxide would have been delivered continuously through glucose oxidase activity in the current study. Comparison of the amount of time for different honey types to release maximal levels of hydrogen peroxide has illustrated that there were large differences according to honey composition (Bang *et al.* 2003).

While some of the results suggest hydrogen peroxide is the active stimulant, practically it is unlikely as manuka honey has a lower hydrogen peroxide content and yet a greater stimulatory effect. Manuka honey has a reported hydrogen peroxide content which is negligible compared with Otago pasture honey (Molan 1992b; 1998).

Of particular relevance to the investigations reported here, hydrogen peroxide-driven stimulation is enhanced when cells are maintained in the presence of the reducing agent 2-mercaptoethanol (2-ME)(Los *et al.*, 1995). Many cell culture mediums

include 2-ME, and it was included in all PBMC cultures presented in this thesis. The presence of 2-ME may provide some explanation for the greater magnitude of proliferation levels obtained for PBMCs incubated with honey in the current study as compared with those reported by Abuharfeil *et al.* (1999) who did not use 2-mercaptoethanol in their medium. The proliferation levels obtained in the current study were up to 2-fold greater for PBMC incubated with the same honey concentrations (0.1%) than reported for the lymphocytes in the Abuharfeil *et al.* (1999) study. Further evidence for the ability of a reducing environment to maximise lymphocyte proliferation comes from a study by Carswell and Papaoutsakis. (2000), which demonstrated that low oxygen tension promotes T cell proliferation. Free radical scavengers themselves have been reported to activate NF-κB expression, strengthening the importance of maintaining a reducing environment to regulate normal cell proliferation (Murley *et al.*, 2001). Within the cell, the enzyme-SH groups are readily oxidised by hydrogen peroxide (Ahn and Thiele, 2003).

That honey does not stimulate the same levels of proliferation as a classical mitogen is of itself an important observation. The implications of this for the healing wound are important, because it is vital for inflammation to proceed at an appropriate level for infection to be cleared and for repair to be achieved, and to avoid the problems associated with excessive inflammation (*e.g.* fibrosis or erosion of tissue) or inadequate inflammation (failure to heal).

It was noted that there was often reproducibility problems between assays. This problem has also been experienced by de Jong (2003) when conducting proliferation assays with T cells obtained from bovine blood and is likely to reflect the physiological differences between individual animals. As independent assays contained blood from different animals the possibility that an animal exhibited an attenuated response to some *in vivo* event could not be excluded as a source of variation in cell activity. A recent study by Baechler *et al.* (2004) has demonstrated the extreme sensitivity of blood cells to *ex vivo* handling. Of relevance to the current study, Baechler *et al.* (2004) reported that subtle changes which occur in the regulation of mRNA expression during handling of blood *ex vivo* have profound implications for activation status of the cells and therefore the outcome of various

ELISA measurements. Measurement of pH in various honey solutions and medium confirmed that there was no significant variation in pH between treatment cultures.

The significance to the T cell proliferation response of having accessory cells present to variable degrees has not been investigated. Certainly it has been shown by Yancy *et al.* (2001) that the presence of various primary cell populations *in vitro* assays has a profound effect on the activation response. The effect of honey on these other cell populations and subsequent effects of chemical messages produced by these cells on T cells during the assay have not been evaluated. The use of a non-purified T cell fraction creates scope for variation between experiments based on the undetermined interactions with other cells in the culture. It is noted that the protocol used in the time series experiments may have resulted in the transfer of T cells and monocytes in different proportions, and therefore it cannot be determined whether the data in Figure 2.15 is representative of a mixed population or for T cells only.

In summary, this study has shown that low concentrations of natural honey induce lymphocyte proliferation but not to the same high levels as a classical mitogen. This effect suggests that honey might enhance a variety of immune responses, and be of use in wound healing situations. Further studies using *in vivo* models of impaired healing or chronic wounds are required before the real nature of its effectiveness can be determined.

Chapter Three: The Effect Of Honey On Cytokine mRNA Expression In Whole Blood

SUMMARY

The ability for honey to induce messenger RNA expression for key cytokines involved in wound healing was investigated. Conventional reverse transcriptase-PCR was used to detect the production of mRNA for honey at 0.25% concentrations for various times (0–24 h). The more sensitive molecular technique, quantitative real-time RT-PCR was then used to quantify the abundance of cytokine mRNA transcripts expressed in bovine blood exposed to 0.25% manuka honey as compared with Con A or control cultures. Transcriptional activity of ten genes, IL-1, IL-5, IL-12, IL-18, IFN-γ, HSP70, HSP90, i-NOS, TNF-α, and TGF-β were studied at the mRNA level during a 0–24 h exposure of whole blood to honey. To test for any modulatory effects of honey on gene expression in an inflammatory model, whole blood was exposed to honey at the same time as LPS and the mRNA expression for the genes was measured. The results show that honey up-regulates a wide range of mediators, including TNF-α, IL-1β, and TGF-β, and this supports the hypothesis that honey induces cytokine release. Honey gave a transient and moderate induction of cytokine mRNA compared with a massive and prolonged induction by the mitogens, Con A and LPS. The inclusion of honey with LPS led to a reduced expression of mRNA for key inflammatory mediators, including iNOS and TGF-β, compared with LPS alone. This supports the hypothesis that honey modulates inflammation.

3.1 INTRODUCTION

Cytokines are small messenger molecules secreted by one cell to alter the behaviour of itself or another cell (Arai *et al.*, 1990). They are produced by virtually all cells and have a wide variety of functions, which are dependent on the tissue they are produced in and the type of cytokine (Böcker *et al.*, 2001). However, the principle cytokine producing cell populations in blood are the lymphocytes, monocytes/macrophages and the neutrophils (Arai *et al.*, 1990). Timing of cytokine expression is crucial for the normal wound healing process to occur, and cytokine expression conforms to a predictable sequence in a normal tissue repair model (Enoch and Harding, 2003; Trengrove *et al.*, 2000; Barone *et al.*, 1998; Moore *et al.*, 1997; Mast and Schultz, 1996; Harris *et al.*, 1995). The kinetics of gene expression and the production of several cytokines following stimulation have been studied (Fraser *et al.*, 1993; Arai *et al.*, 1990; Ullman *et al.*, 1990; Crabtree, 1989; Weiss *et al.*, 1986; Kröncke *et al.*, 1985; Efrat *et al.*, 1982). It is when tight regulation of interactions between various growth factors, cytokines and angiogenic mediators breaks down that abnormal healing occurs (Section 1.6).

In inflammation the first cytokines to be produced are IL-1β and TNF-α, by a range of cells involved in the inflammatory response including activated T cells, dendritic cells and macrophages (Arai *et al.*, 1990). In addition to being primal danger signals (Ullman *et al.*, 1990), these cytokines are proinflammatory and perpetuate activation of mononuclear cells through positive feedback amplification (Ullman *et al.*, 1990). Of further relevance to wound healing, these cytokines are essential for neutrophil recruitment (McClenahan *et al.*, 2000), and in the late inflammatory phase play a key role in activation of the fibroblasts and signalling for the formation of the tissue matrix (Clarke, 1996).

Nitric Oxide (NO) plays an important role in tissue repair and the formation of NO by cells during inflammation is important for cell signalling to proceed. The enzyme inducible nitric oxide synthase (iNOS) is a member of the family of isoenzymes responsible for NO production from L-arginine (Liang *et al.*, 2001). Prior to the current study, Al-Waili (2003) had reported that oral ingestion of honey led to an

increase in NO in saliva. Further, it was reported that honey contains different amounts of NO metabolites (Al-Waili, 2003). This suggested honey may have some effect upon mRNA expression of the iNOS enzyme. Though NO has a beneficial role in tissue repair, the high levels of NO which are produced in a model of excessive inflammation, such as that created by incubating blood with LPS, are potentially damaging to tissues through the formation of free radicals.

NO diffuses freely across cell membranes, and is consumed quickly due to the large number of molecules with which it interacts. For this reason, NO only has a localised effect on cells, behaving in both a paracrine and autocrine manner. NO binds to protein receptors in the cell, being either a metal ion in the protein or one of the S atoms of the protein. Once bound to the protein, an allosteric change occurs and a second messenger forms within the cell (*e.g.* when NO binds to guanylyl cyclase cyclic GMP is generated). NO is involved in blood vessel dilation of endothelial cells, with response being effected in seconds. NO also facilitates cell signalling in a number of pathways; chiefly through oxidation of kinase signalling, nitrosation of protein sulfhydryl groups, and release of metal ions (Dedon and Tannenbaum, 2004; Kim and Tannenbaum, 2003). NO enhances the effect of cyclooxygenases and stimulates the production of pro-inflammatory eiconosoids. A number of factors upregulate iNOS, and these are known to include interleukins, IFN-γ, TNF-α and LPS.

Interferon-gamma (IFN-γ) plays an important role in activating macrophages and stimulating their phagocytic capability by increasing their respiratory rate. IFN-γ up-regulates expression of interleukin (IL)-2 receptor on T-cells (Pighetti and Sordillo, 1996). IFN-γ plays a key role in the second phase of wound healing and the expression of constitutive and inducible adhesion molecules necessary for interaction between leukocytes and target cells (Bito *et al.*, 2002). Like TNF-α and IL-1β, IFN-γ can act as both a primal danger signal and perpetuate activation in mononuclear cells through positive feedback amplification (Gallucci and Matzinger, 2001). Of additional note for wound healing, IFN-γ drives Th_1 cytokine production (including the pivotal cytokines TNF-α and IL-1β).

Interleukins act synergistically during inflammation, mediating many of the effects of inflammation including production of nitric oxide synthase. Other Th1 cytokines, IL-12 and IL-18 play an important role in inflammation. IL-12, a key molecule in the differentiation of Th1 $CD4^+$ T cells (Trinchieri, 1995) and augments IFN-γ synthesis (Alluwaimi *et al.*, 2001) in addition to driving the Th_1 cytokine response. IL-18 also plays a role in augmentation of IFN-γ synthesis in bovine lymphocytes (Shoda *et al.*, 2004). Therefore, it was useful to consider the effects of honey on the mRNA expression of each of these key cytokines.

In comparison, the Th2 cytokines (*e.g.* IL-5) play a key role at the end of the inflammatory process and are involved in the dampening down of the immune response. Induction of a mature antibody immune response also involves cytokines of the Th2-type. IL-5 is regarded as a representative of this family (Kourilsky and Truffa-Bachi, 2001) and ultimately leads to activation of the immunoglobulin G pathway through promotion of B-lymphocyte activity and differentiation of eosinophils.

TGF-β, produced by mononuclear cells, plays an important role in activation of fibroblast activity and re-epithelialisation (Cobbold and Sherratt, 2000). As described in Section 3.1, significant production of TGF-β and iNOS over a relatively long time period are not desirable as they are linked to the development of fibrotic disorders (*e.g.* keloid scarring). TGF-β is important in the down-modulation of the immune response when the antigen has gone.

TNF-α, TGF-β and IFN-γ are all examples of cytokines whose actions switch from pro-inflammatory to anti-inflammatory according to timing and tissue context. Self-limited inflammation is normally characterised by decreasing TNF activity, and when this fails to occur, mediators such as eicosanoids and nitric oxide will continue to be amplified by TNF-α. It is noted that other proteins may bind to TNF-α mRNA transcripts, resulting in either increased stability of the transcript or conversely, its breakdown. In arthritis for example, there is a greater proportion of TNF-α mRNA persisting for longer periods of time (Haringman and Tak, 2004). TGF-β produced at

high levels over a relatively long time period has been shown to cause gastric ulceration through its effects on macrophage and neutrophil infiltration and their resultant production of oxidant species (Kulkarni *et al.*, 1993).

Therapeutic agents which may alter cytokine expression may therefore be of use where there is abnormal wound healing. In addition to the wealth of anecdotal reports that honey optimises wound healing, limited investigations have indicated that honey modifies cytokine production by mononuclear cells *in vitro* (Tonks *et al.*, 2003; de Jong, 2003; Tonks *et al.*, 2001). To investigate the possibility that hydrogen peroxide in honey might be responsible for the observed anti-inflammatory effects through induction of heat shock proteins (HSP), these were selected for quantification. HSP are induced by and confer protective effects against further cellular damage, and among these the actions of HSP 70 and HSP 90 in inflammation have been well documented (Gallucci and Matzinger, 2001). While HSP 70 stimulates lysosomal hydrolysis, HSP 90 binds the tyrosine-specific protein kinase and slows the cell cycle.

To investigate the role of honey in induction of mRNA for cytokines, the molecular techniques, agarose gel electrophoresis and quantitative real-time RT-PCR (real-time PCR), were used in the current study to identify whether mRNA coding for key inflammatory proteins had been expressed by blood cells. One of the key advantages of real-time PCR over gel electrophoresis is that real-time PCR provides a sensitive method for detecting expression of mRNA for cytokines and quantifies expression even when abundance is very low (Pfaffl, 2003). The advantage of using whole blood assays to study inflammatory mediator expression is that they retain all blood components and maintain a normal environment for cell-cell interactions, thereby mimicking the natural physiological environment (Mayringer *et al.*, 2000). Con A was used as a positive control for cellular activation, and on this basis others have used Con A to establish an inflammatory model (Mizuhara *et al.*, 1994).

As honey has been shown to be anti-inflammatory (discussed in Section 1.4.4) an additional aim was to test whether honey could modulate the expression of mRNA for proinflammatory cytokines (*e.g.* iNOS) in LPS-treated blood. LPS was used to induce

an inflammatory response and provide a model based on published reports (Tse *et al.*, 2004; Rao, 2001; Hambleton *et al.*, 1996). Primarily a model for infection, LPS is the major outer membrane component of gram-negative bacteria. LPS binds with LPS-binding protein and is then recognised by the CD14 receptor (a GPI-linked protein present on the surface of phagocytic leukocytes) (Gretzer and Thomsen, 2000). LPS can float around in the supernatant and be accessed by CD14. LPS upregulates CD14 expression on non-adherent monocytes after 24–72 h incubation periods (Gretzer and Thomsen, 2000). After being bound to CD14, LPS acts via the Toll-like receptor (TLR)4 coreceptor (1), and ligation of these receptor complexes activates a signal transduction cascade (Belge *et al.*, 2002). On any one cell there are a number of TLRs, which bind to proteins being produced. After mobilisation of transcription factors (*e.g.* NF-κB), cytokines (*e.g.* TNF-α) are expressed.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Alsevers

A 1 L solution of Alsevers was made consisting of; D-Glucose 20.5 g, TriNa Citrate 8g; citric acid 0.55 g, distilled water 1000 ml, and NaCl 4.2 g. These reagents were all purchased from BDH Laboratory Supplies, Poole, England.

Sodium acetate

A 2 M solution was prepared by dissolving 27.22 g of sodium acetate powder (Sigma-Aldrich, cat no# A-8219) in 1 litre of Milli Q water. The pH was adjusted to 4 using 2M acetic acid.

PCR buffer

10 mM Tris-HCl, 50 mM KCl, pH 8.3

Reverse Transcriptase buffer

Purchased in Superscript First Strand Kit (GibcoBRL, Grand Island, NY) containing 200 mM Tris-HCl (pH 8.4) 500 mM KCl.

dNTP mix (10 mM)

Purchased in Superscript First Strand Kit (GibcoBRL, Grand Island, NY) containing 10 mM each dATP, dCTP, dGTP, dTTP.

Concanavalin A (Con A)

Con A stock solutions were prepared as described in Section 2.2.1 to a final concentration of 1mg/ml (Sigma C5275).

Honey solutions

M109 manuka honey, Otago pasture honey and artificial honey were prepared as described in Section 2.2.2.

LPS solutions

A 1 mg/ml stock solution (*E. coli* serotype 0111: B4; Sigma-Aldrich UK; cat no# L4516) was purchased. The LPS was diluted with medium to final concentrations in wells of 10 μg/ml (unless otherwise stated).

3.2.2 Preparation of whole blood

Blood was collected from a Holstein-Friesian steer by tail venipuncture, containing K_3EDTA. The cattle were well-fed, free of diseases and held in quarantine conditions. In the laboratory, 10 ml of blood was mixed gently with 2 ml of RPMI 1640 base medium (GibcoBRL) and 3 ml of Alsevers solution. The blood was transferred to three 50 ml tissue culture flasks (Greiner) in 5 ml aliquots.

3.2.3 Assay design

3.2.3.1 Assay to measure stimulatory effects

Bovine blood was incubated with honey or the mitogen Con A (to provide a positive control) for 24 h prior to RNA extraction. To investigate the effect of honey on mRNA expression of immune-related genes, blood was cultured for 24 h with honey.

The blood was subjected to three treatments, honey, Con A or no stimulation. Con A was added to give a final concentration of 5 μg/ml.

The treatments (honey solutions, Con A or RPMI-1640 basal medium) were added to separate flasks each containing 5 ml of the diluted blood. All treatments were added to flasks in 25 μl aliquots. Control blood suspensions received 100 μl of RPMI-1640 base medium only. The blood was incubated for 24 h (37°C, 5% CO_2, 95% air in a humidified atmosphere) and 1 ml of suspension removed for RNA extraction from each flask at 0 h, 6 h, 12 h, 24 h after addition of the treatments. The cytokines, IL-1β, IL-5, IL-12, IL-18, TNF-α, IFN-γ, TGF-β, iNOS, HSP70, HSP90 were selected for study.

3.2.3.2 Assay to measure modulatory effects

To investigate the effect on cytokine expression when blood was exposed to honey at the same time as LPS, the experiment was set up as described above, with the addition of LPS (final concentration 10 μg/ml). The control flasks were set up. One with no addition and the second with LPS alone (final concentration 10 μg/ml). The experiment continued as described in Section 3.2.3.1. All PCR reactions were performed in duplicate and repeated in time at least twice, according to standard protocols.

3.2.4 Lysis of cells and RNA extraction

The 1 ml aliquot of blood was immediately placed in a 15 ml Sarsted tube containing 4 ml of 5 M Guanidium Thiocyanate (GdnSCN, Roche). The tubes were left at room temperature until the final sub-sample had been collected at 24 h. At this point the tubes were inverted several times and 0.5 ml of 2 M sodium acetate (pH 4) and 2 ml of 100% ethanol were added to each tube to precipitate the nucleic acids, particularly the tRNA. The tube was incubated on ice for 10 min prior to centrifugation for 15 min

(18 547 x *g*, 4°C, Beckman) to pellet the nucleic acids. The supernatant was then removed.

The RNA pellets were resuspended in 1 ml of Trizol reagent (ProGenz) to prevent degradation of mRNA and the suspension transferred to a 1.5 ml Eppendorf tube, and thoroughly mixed by vortexing. Following 5 min incubation at room temperature, 200 µl of chloroform was added and mixed by vortexing for 20 sec, and the tubes incubated on ice for 10 min. The tubes were centrifuged for 15 min (Eppendorf, 572 x *g*, 4°C) and the clear layer at the top of the supernatant was transferred into a new 1.5 ml Eppendorf tube. An equal volume of isopropanol was added to the clear supernatant, and the contents mixed by gentle inversion of the tube prior to incubation for 20 min at -20°C. The tube was then centrifuged for 10 min (Eppendorf, 572 x *g*, 4°C) to pellet the RNA and the supernatant removed. To further precipitate the RNA, 1 ml of 70% ethanol was added to the RNA pellet and mixed by vortex for 10 sec to resuspend. The tube was centrifuged for 5 min (Eppendorf, 572 x *g*, 4°C) and all of the ethanol removed using a pipette, leaving the RNA pellet to air dry. The RNA pellet was dissolved in 20 µl of 10 mM Tris (pH 7.8) 0.66 M $MnCl_2$ and mixed by vortex for 10 sec prior to addition of 1 µl of DNAse (Promega) to remove any residual DNA. The tube was incubated for 30 min in a thermomixer (37°C). DNAse was inactivated by adding 1 µl of EGTA (Promega DNAse Stop Solution) and the tube incubated for 10 min at 65 °C. To check the quality of the extracted RNA, 2.5 µl of RNA from each tube was run in a 2% agarose gel (Section 3.2.4.1) in TAE buffer. The samples electrophoretically separated at 55 volts for 45 min, and the intensity of the bands compared using a Bio-Rad GS-690 Imaging Densitometer. RNA integrity was indicated by the presence of sharp, clear 28S and 18S rRNA bands, preferably with the 28S band twice as intense as the 18S rRNA band. Partially degraded RNA would appear as a low molecular weight smear (not seen in the current study). RNA purity was checked by measuring the absorbance of samples at 260 nm (A_{260}) using a UV/Vis Spectrophotometer (Model U-2001, Hitachi Instruments Inc). Values of 1.9–2.1 were considered acceptable. It was observed that relatively small quantities of RNA were recovered from the bovine blood preparations in comparison with the

volumes recovered from human blood samples (R. T. M. C. Cursons; personal communication).

3.2.4.1 Preparation of a 2% agarose gel

Agarose (2 g) was added to 100 ml TAE buffer (0.04 M Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8) and microwaved on high for 2 min. The agarose was cooled to approximately 65°C by swirling the flask in a cool water bath, and 2 µl of ethidium bromide (10 mg/ml stock solution) was added. Approximately 30 ml was poured into a gel caster (Horizon® 11.14, GibcoBRL), an 8 lane comb was added, and the gel was left to set.

3.2.5 Primers

Specific cytokines that play a key role in the immune response during wound healing were chosen for investigation. Primer pairs were selected based on previous publications (Shoda *et al.*, 2004; Amills *et al.*, 2004; Hunter-Lavin *et al.*, 2004; Schmitz *et al.*, 2004; Eicher *et al.*, 2003; Konnai *et al.*, 2003; Alluwaimi and Cullor, 2001; Leutenegger *et al.*, 2000; Mertens *et al.*, 1996). Care was taken to ensure that the RT-PCR primers spanned intron sequences so that any possible DNA product could be excluded. All primers were purchased from Sigma-Aldrich and are listed in Table 3.1. For each cytokine, primer solutions were prepared by adding 80 µl of TE buffer to 10 µl of forward primer stock and 10 µl of reverse primer stock in an Eppendorf tube, and stored at 4°C .

3.2.6 Conventional RT-PCR Analysis

This technique was chosen to detect whether mRNA for specific proteins of interest had been expressed, as PCR is absolute in terms of whether a template is present. This enabled a number of proteins to be screened for further quantification with real-time PCR.

All steps were carried out in a PCR cabinet. After extraction of purified RNA (given in Section 3.2.4), cDNA was prepared for conventional RT-PCR according to a method adapted from Jobin *et al.* (1998) and Bierhaus *et al.* (1997).

3.2.6.1 First-strand cDNA synthesis

Complementary DNA (cDNA) was synthesised with a Superscript™ First-Strand Synthesis Reverse Transcriptase kit (GibcoBRL, Grand Island, NY, Cat no# 11904-018) following the manufacturer's instructions. Briefly, for each blood sample, 5 µl of RNA (1 µg/ml) was placed in a 1.5 ml Eppendorf tube along with 1 µl Oligo dT (GibcoBRL) and 1 µl 10 mM dNTP (GibcoBRL) mix and the contents mixed by inversion. The tubes were incubated for 5 min at 65°C to separate secondary structures from the RNA. The tubes were then put on ice for 10 min to enable the oligo dT primers to anneal to single-stranded RNA.

In a separate 1.5 ml Eppendorf tube 2 µl 10 x Reverse Transcriptase buffer (GibcoBRL, described in Section 3.2.1) was placed with 5 µl 25 M $MgCl_2$, 2 µl of 0.1 M DTT, and 1 µl of RNaseOUT Recombinant RNase inhibitor (GibcoBRL). The contents of this tube were added to the tube containing the RNA (with oligo dT primers attached) and incubated at 42°C for 2 min prior to addition of 1 µl of SUPERSCRIPT II™ reverse transcriptase (GibcoBRL). The contents of the tube were mixed by inversion and then incubated for 50 min at 42°C.

Table 3.1: List of the forward and reverse primers for the amplification of the interleukin (IL)-1, IL-5, IL-12, IL-18, interferon (IFN)-γ, inducible-nitric oxide synthase (i-NOS), tumour necrosis factor (TNF)-α, and transforming growth factor (TGF-β).

Gene	Primer sequence[a] (forward and reverse, 5'-3')	Length		Optimal conditions				Accession number
		Primer (bp)[b]	Product (bp)	$MgCl_2$[c] (mM)	Anneal temperature (°C)	Acquisition[d] temperature (°C)	Product[e], *Tm* (°C)	Cow (locus)
IL-1β	CAA GGA GAG GAA AGA GAC A TGA GAA GTG CTG ATG TAC CA	19 20	236	4	53	83	85.4	M37211
IL-5	TGG CAG AGA CCT TGA CAC TG TTT TCA CAG CAT CCC CTT GT	20 20	405	3	53	83	64.3	NM_173922
IL-12p40	AAC CTG CAA CTG AGA CCA TT ATC CTT GTG GCA TGT GAC TT	20 20	186	4	55	85	87.3	U11815
IL-18	TTG CAT CAG CTT TGT GGA AA TGG GGT GCA TTA TCT GAA CA	20 20	754	4	60	75	63.9	NM_174091
IFN-γ	ATA ACC AGG TCA TTC AAA GG ATT CTG ACT TCT CTT CCG CT	20 20	218	4	52	82	84.6	M29867
TGF-β_1	AGA GAG GAA ATA GAG GGC TT ATG AAT CCA CTT CCA GCC CA	20 20	262	1.2	60	76	66.6	NM174621
TNF-α	TAA CAA GCC GGT AGC CCA CG GCA AGG GCT CTT GAT GGC AGA	20 21	277	3	62	79.5	69.0	AFO11926
HSP70	TGC TGA TCC AGG TGT ACG AG CGT TGG TGA TGG TGA TCT TG	20 20	184	4	58	72	92	VO947G06
HSP90	TGA CGA GGA TGA CCC CAC TG TGG AGG GAA TGG AGA CAG AGC	20 20	335	4	55	72	92	NM005346
iNOS	ACC TAC CAG CTG ACG GGA GAT TGG CAG GGT CCC CTC TGA TG	21 20	316	4	62	88	66.0	U14640

The reaction was terminated by first incubating the tubes at 70°C for 15 min, and then placing the tubes on ice. To remove the RNA, 1µl of RNase H (GibcoBRL) was added to the tube and the contents gently mixed by inversion prior to incubation of the tube at 37°C for 20 min. The cDNA was stored at 80°C.

3.2.6.2 PCR with HotStar® Taq

The PCR process involves multiple cycles of template denaturation, primer annealing, and primer elongation to amplify DNA sequences. HotStar® *Taq* DNA Polymerase (Qiagen, Valencia, NV) was used for PCR amplifications. An antibody complexed to *Taq* polymerase acts to block polymerase activity until after the first 94°C denaturation step during PCR cycling. Using a Superscript First-Strand Synthesis System for RT-PCR kit (GibcoBRL), amplifications were performed according to the manufacturer's instructions. PCR reactions (20 µl) contained 0.2 mmol/l dNTPs, 200 nmol/l forward primer, 200 nmol/l reverse primer, 0.4 U HotStar ® *Taq* DNA Polymerase, PCR buffer (1 x), 1.5 mmol/l magnesium chloride and 0.8 µl cDNA from the reverse transcriptase reaction (Section 3.2.6.1). A PTC-100 Thermocycler (MJ Research, Reno, NV) was used for PCR thermo-cycling. After pre-incubation for 2 min at 94°C, the target DNA was amplified with 40 cycles, each cycle consisting of a denaturation step at 94°C (20 sec melting), annealing for 20 sec at 55°C (optimised for the specific genes, as presented in Table 3.1), and extension at 68°C for 35 sec.

3.2.6.3 Electrophoresis of DNA

To check whether the cDNA had been successfully synthesised, a 2% agarose gel was run containing 5 µl of 100 bp DNA ladder (Invitrogen, 100 bp DNA) and 10 µl of amplified cDNA for each sample. Agarose gels were prepared as described in Section 3.2.4.1, and DNA samples were premixed with 2 µl of DNA loading dye (40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol). A 100 bp DNA ladder (Invitrogen) was loaded on each gel to enable the base pair size of the DNA products to be determined. Electrophoresis was carried out in Horizon tanks (Invitrogen) containing TAE (1 x) buffer, at 100 v until separation was achieved. DNA was illuminated using UV, and photographed using the Gel-Doc system (Bio-Rad).

3.2.6.4 Quantitative Real-Time RT-PCR

Real-time PCR was used to provide a quantitative measure of the transcription of mRNA for specific proteins of interest. A fluorescent marker is hybridised to the cDNA between two PCR primers for a single gene of interest. As PCR proceeds, the 5' nuclease activity of the Taq polymerase cleaves the probe, releasing the tag such that the fluorescence is proportional to the amount of cDNA present. SYBR Green® (Roche) was used as a fluorescent marker allowing any double-stranded DNA generated during PCR to be detected (Wilhelm and Pingoud, 2003). Specificity of the PCR products was confirmed by dissociation curve analysis and electrophoresis on a 2% agarose gel.

After extraction of purified RNA (Section 3.2.4) it was prepared for real-time PCR according to a method adapted by Dr Ray Cursons and Gina Chu (University of Waikato) from that described by Konnai *et al.* (2003). All steps were carried out in a PCR cabinet.

Real-time (quantitative) PCR was performed using a LightCycler instrument (Roche) to perform the PCR reaction, including fluorescent emission and detection of the signals. Real-time PCR reactions were carried out in a total volume of 25 µl. A reaction master mix was prepared consisting of 15 µl HotStar Taq DNA polymerase (Qiagen), 480 µl of 5 x Qiagen PCR buffer supplemented with $MgCl_2$ to 2.5 mM, 1800 µl of Milli Q water, 0.2 mM dNTPs (Roche), 48 µl Rox reference dye (Invitrogen), 24 µl SYBR Green® dye and 36 µl Triton X-100 (stops dye from binding to the plastic tube). The real-time PCR reaction mixture (10 µl) contained 80 nM forward primer, 80 nM reverse primer, 2 µl Lightcycler FastStart DNA Master^{Plus} SYBR Green® (Roche), and 5 ng reverse-transcribed RNA. To exclude genomic DNA contamination, a negative control was done for every reaction set by performing the RT-PCR reaction with sterile water instead of cDNA.

There were ten genes of interest in total. The samples were analysed for both the target genes and an endogenous control gene, β2-microglobulin (β2M). The expression levels of the target genes were reported relative to the expression levels of β2M. It is

recognised that housekeeping genes are differentially expressed under various experimental conditions (Rogler *et al.*, 2004).

The LightCycler protocol consisted of pre-incubation for 15 min at 94°C followed by amplification of target cDNA with 40 cycles, each cycle consisting of a denaturation step at 94°C for 20 sec, annealing for 10 sec at 55°C, extension at 68°C for 30 sec, and determination of fluorescence at 80°C for 15 sec. The LightCycler detects the point at which the fluorescent signal generated by the PCR reaction is visible above the background fluorescence of the sample.

In addition, melting curve analysis was performed which resulted in single product specific melting temperatures above 80°C for each primer pair, confirming specificity of each primer pair. All real-time amplifications were performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Data capture included the values obtained for PCR stage 2, extension step 4. The data for IL-5 and IL-18 was obtained from the extension step 3 as those reactions were found to have a lower melting point (68°C). All primers were selected to span intron sequences, so that genomic DNA amplification was not possible.

After analysis of the real-time PCR results, an end-product sample of DNA from the reaction for each gene was analysed by gel electrophoresis (Section 3.2.6.3). This enabled the presence of any DNA contamination to be verified and the size of the products to be determined by comparison with a SYBR Green ® ladder. The agarose gel was prepared as described in Section 3.2.4.1 omitting the ethidium bromide due to the presence of the SYBR Green® dye.

A Sequence Detector v. 1.7 of the ABI system in a Mackintosh computer was used to analyse each RT-PCR plate, and the clipped file was exported to Microsoft Excel as fluorescence level per cycle number. Using the LinReg PCR programme, a linear regression line was calculated to fit the log-linear phase of the data points for each sample. A minimum of 4 data points were included in the data analysis for each sample, and the slope was chosen for each data set by taking in combination, the slope closest to the maximum along with the highest possible r^2 value. Only samples with PCR efficiencies above 1.5 were selected. The starting concentration (No) of the cDNA

template of each sample was calculated from the intercept extrapolated from the straight line that fitted best to the included data points which was expressed in terms of fluorescence intensity.

All data values were expressed as the expression relative to the control sample (time 0) and corrected for β2M gene expression (endogenous control gene). To normalise the level of fluorescence of each gene of interest the following equation was used:

Step 1

$$\text{relative expression at time 0} = \frac{\text{concentration } N^0 \text{ value for gene of interest at time 0}}{\text{concentration } N^0 \text{ for } \beta 2M \text{ at time 0}}$$

Step 2

For each time point the relative expression of the gene of interest was calculated by normalising levels of fluorescence to that obtained for β2M at the same time point:

$$\text{relative expression at time x} = \frac{\text{concentration } N^0 \text{ value for gene at time x}}{\text{concentration } N^0 \text{ value for } \beta 2M \text{ at time x}}$$

Final step

The relative expression of the gene of interest at time x (*i.e.* 6 h) was then normalised for the relative expression of the same gene at time 0, by dividing the answer from step 2 by the answer from step 1.

3.2.7 Statistical analysis

Data was gathered using the LinRegPCR programme (Ramakers *et al.*, 2003), quantifying the PCR product in the log-linear phase of the PCR reaction using linear regression analysis. The starting concentration of mRNA and the individual PCR efficiencies were calculated for each sample. The kinetics of cytokine mRNA expression was determined for blood exposed to the various treatments. Results were expressed as relative abundance of target gene mRNA normalised against β2M mRNA. After setting the threshold Ct value, the clipped files showing the fluorescence intensity of each reaction at each cycle were exported in the spreadsheet format of Microsoft Excel (version 97 SR-2 and XP). Real-time PCR reactions were performed in duplicate and were repeated in time at least twice. However, the data presented in this thesis was obtained for duplicate samples, as only samples with the same C_t values could be compared for significance. This is because data was analysed for overall effects of treatment and time by ANOVA ($P<0.05$). A two-way ANOVA was used to analyse data, with timepoint and treatment as the parameters. The two-tailed Students' *t*-test was applied for statistical comparisons between individual groups. Unless otherwise stated, data presented are expressed as means ± SEM (calculated from the LinRegPCR programme).

3.3 RESULTS

In Section 3.2.4 it was noted that prior to PCR, total RNA was electrophoretically separated to check its integrity (Figure 3.1).

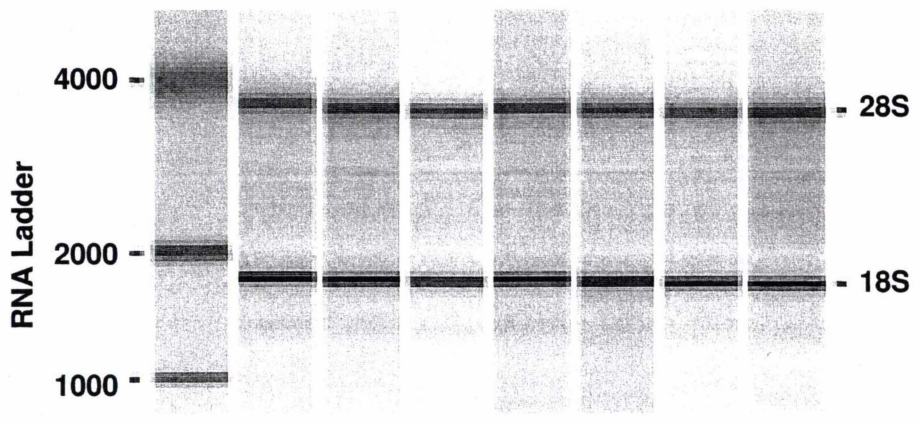


Figure 3.1: 28s and 18s RNA bands of total RNA isolated from blood samples. This is a representative example.

It can be seen from Figure 3.1 that the RNA samples had clear 28s and 18s bands, and that there was no DNA contamination.

As described in Section 3.2.7.3, when using the relative quantification strategy, the increase or decrease of cellular cytokine mRNA levels has to be calculated in relation to a constantly expressed housekeeping gene (β2M). This quantification compensates for minimal changes in the quantity and quality of different cDNA preparations. The amplification of the β2M gene is shown in Figure 3.2.

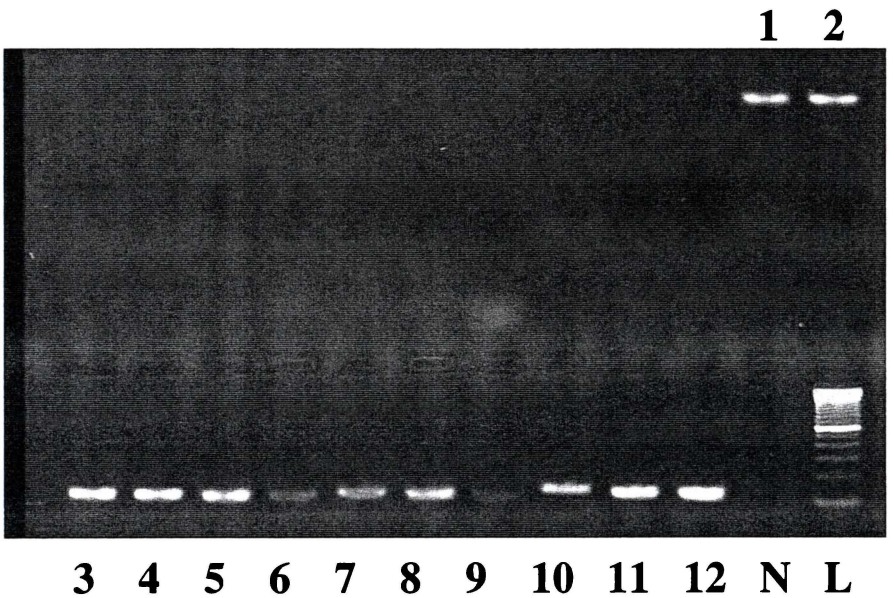


Figure 3.2: Ethidium bromide-stained agarose gel. Amplification of the housekeeping gene B2M in whole blood exposed to honey (lanes 1–4), Con A (lanes 5–8) or maintained in medium alone (lanes 9–12). N= the negative control/water. L=100 bp ladder (B2M product =280 bp). This gel was representative of the results from all of the RT-PCR amplifications (n=8). The data are from replicate experiments.

The results in Figure 3.2 confirm that all of the samples contained cDNA of good quality and that no amplification of β2M had occurred in the absence of cDNA. It is noted that the samples in lanes 6 and 9 had less DNA than others.

3.3.1 The direct effects of honey on the expression of mRNA in bovine blood

As described in Section 3.2.7.3, real-time PCR was used to compare the abundance of amplicon between samples. At various times (ranging from 0 to 24 h), cells were

collected and the RNA purified and reverse transcribed. By normalising the abundance of PCR product to the housekeeping gene product, the total mRNA content was corrected for, so that abundance of the amplicon could be compared among samples. By presenting the results for each cytokine, at each time-point, as a proportion of that expressed on the mRNA from the same initial blood sample at time 0, change in relative cytokine mRNA expression could be shown (Figure 3.3). Each bar on the graph represents up- or down-regulation of a target gene in relation to the endogenous control, β2M. A logarithmic scale was used, and time 0 values are shown by the baseline (y = 1). The effect of blood stimulation on cytokine mRNA expression is shown in Figure 3.3.

The profile and kinetics of mRNA production for cytokines were quantitatively and qualitatively different for blood exposed to honey as compared to Con A or toRPMI medium alone (the control). The mRNA expression obtained for control blood cultures (Figure 3.3) shows that whole blood constitutively synthesised readily detectable levels of mRNA for each of the cytokines studied. Further, all of the studied cytokines, except IFN-γ, were down regulated compared with time 0 basal production levels. At the 24 h measurement, mRNA expression in control cultures for IFN-γ had returned to the same levels as obtained for time 0.

For most of the cytokines, their mRNA expression increased by the 6 h timepoint for honey-treated and Con A-activated cultures (Figure 3.3). By contrast, HSP90 was down-regulated at 6 h of honey or Con A stimulation. In the honey-treated cultures, iNOS was also down-regulated at the 6 h timepoint. Considering the range of cytokines as a complete picture, honey-stimulated cultures have a different kinetics profile to that obtained for Con A-activated cells.

Following Con A stimulation of blood cultures, peak mRNA expression for most cytokines occurred by 6 h post-stimulation. For the Con A-stimulated cultures, peak expression of TGF-β, TNF-α, and HSP 70 occurred between 6–12 h post-stimulation. With the exception of HSP90, which peaked by 24 h post-stimulation, there was a decrease in mRNA expression for Con A-activated cells over the final 12 h in culture.

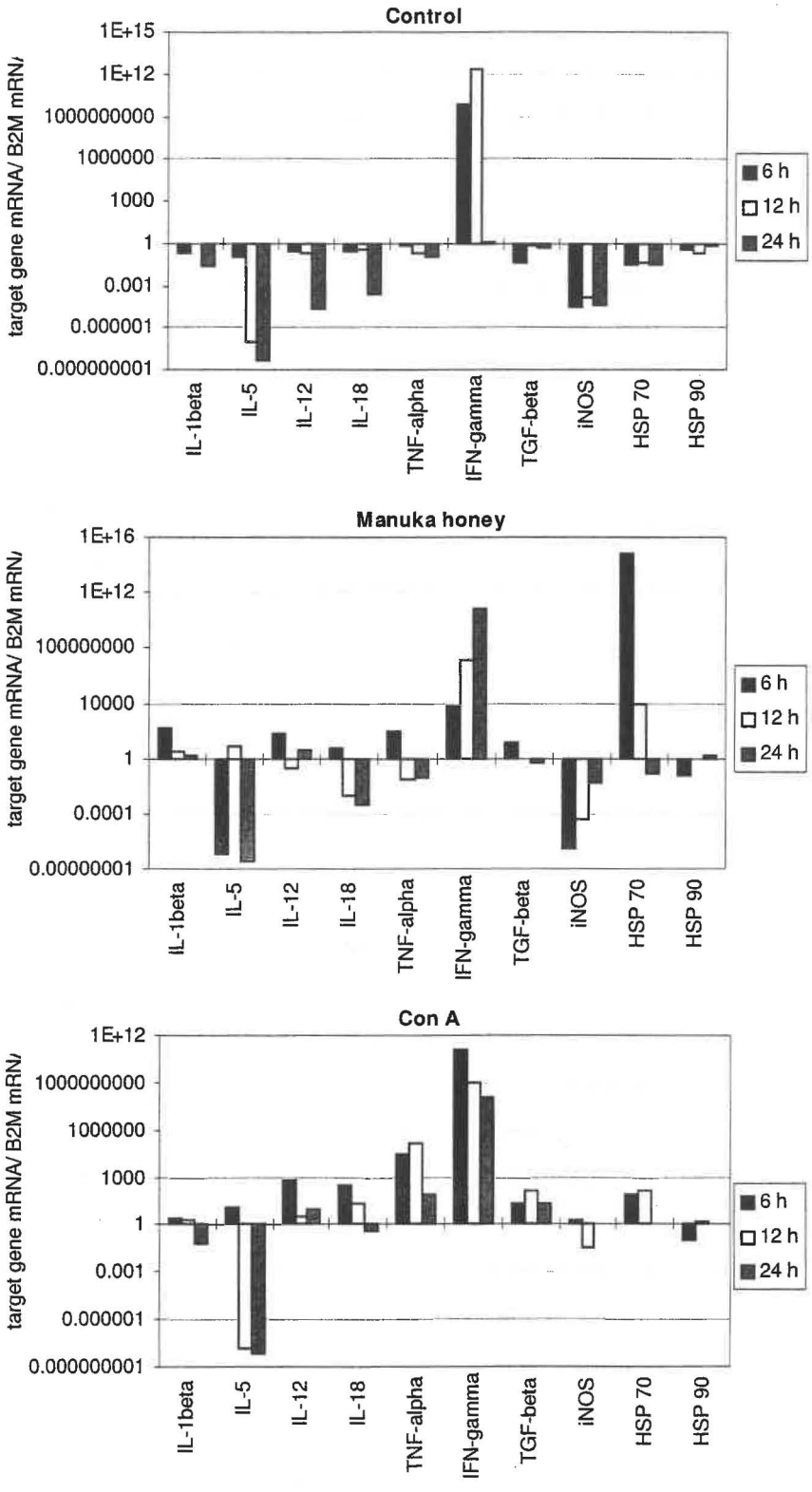


Figure 3.3: Cytokine mRNA expression in blood induced by honey or Con A. Bovine blood was treated with 0.25% manuka honey or Con A (5 μg/ml), as compared with RPMI 1640 alone. RNA was extracted from these blood cells at 0, 6, 12 or 24 h, and analysed by quantitative real-time RT-PCR . The results shown are ratios obtained by dividing concentrations of the PCR products from the cytokine genes by those from the β_2-microglobulin gene and related to time 0 data. Values are representative of single results.

In general, the peak mRNA expression for cytokines in the honey-activated blood occurred by 6 h post-stimulation. However, IL-5 was down-regulated at 6 h and peaked at 12 h post-stimulation. On the basis of two independent PCR analyses, the results for IL-5 and IL-12p40 were found to be real. Also, peak IFN-γ mRNA expression occurred at 24 h post-stimulation for the honey-treated cultures. Another significant difference was that iNOS was down-regulated over the culture period for honey-treated cells. Further, mRNA expression for IL-18 and TNF-α was strongly down-regulated in honey-treated blood after 6 h in culture. With the exception of IL-12p40, IFN-γ and HSP90, expression of mRNA for the cytokines in honey-treated blood decreased over the last 12 h in culture. The expression of mRNA for HSP70 by 6 h after honey stimulation was profound compared with Con A-activated cultures.

In the following figures, a few key cytokine mRNA amplicons on which honey had a significant effect (from Figure 3.3), were selected for further statistical analysis. The following figures show the relative mRNA abundance (incorporating data from Figure 3.3 and a second PCR reaction) expressed as a percentage of time 0 values from RNA abundance (normalised to β2M mRNA). The effect of exposing whole blood to honey or Con A compared with medium alone on expression of mRNA for IL-1β, TNF-α or TGF-β (from Figure 3.3) is shown in Figure 3.4.

The stimulation effect of 0.25% manuka honey on mRNA for IL-1β, TNF-α, and TGF-β was profound. Maximal relative mRNA abundance for each of the cytokines in Figure 3.4 was detected after 6 h of stimulation with honey or Con A. Induction of mRNA for IL-1β was 1.3-fold greater, and for TNF-α was 1.2-fold greater in honey-treated cultures than for Con A-activated blood. In contrast, mRNA expression for TGF-β was 1.5-fold greater for Con A-activated cultures than for honey-treated blood. While the expression of mRNA for TNF-α and TGF-β had decreased to basal levels for honey-stimulated cultures by 24 h post-stimulation, there remained significant expression of mRNA for these cytokines at the 12 h timepoint.

The effect of exposing whole blood to honey or Con A on expression of mRNA for iNOS and HSP70 is shown in Figure 3.5. There were potent differences in the amount of iNOS mRNA expressed for cultures according to stimulation treatment.

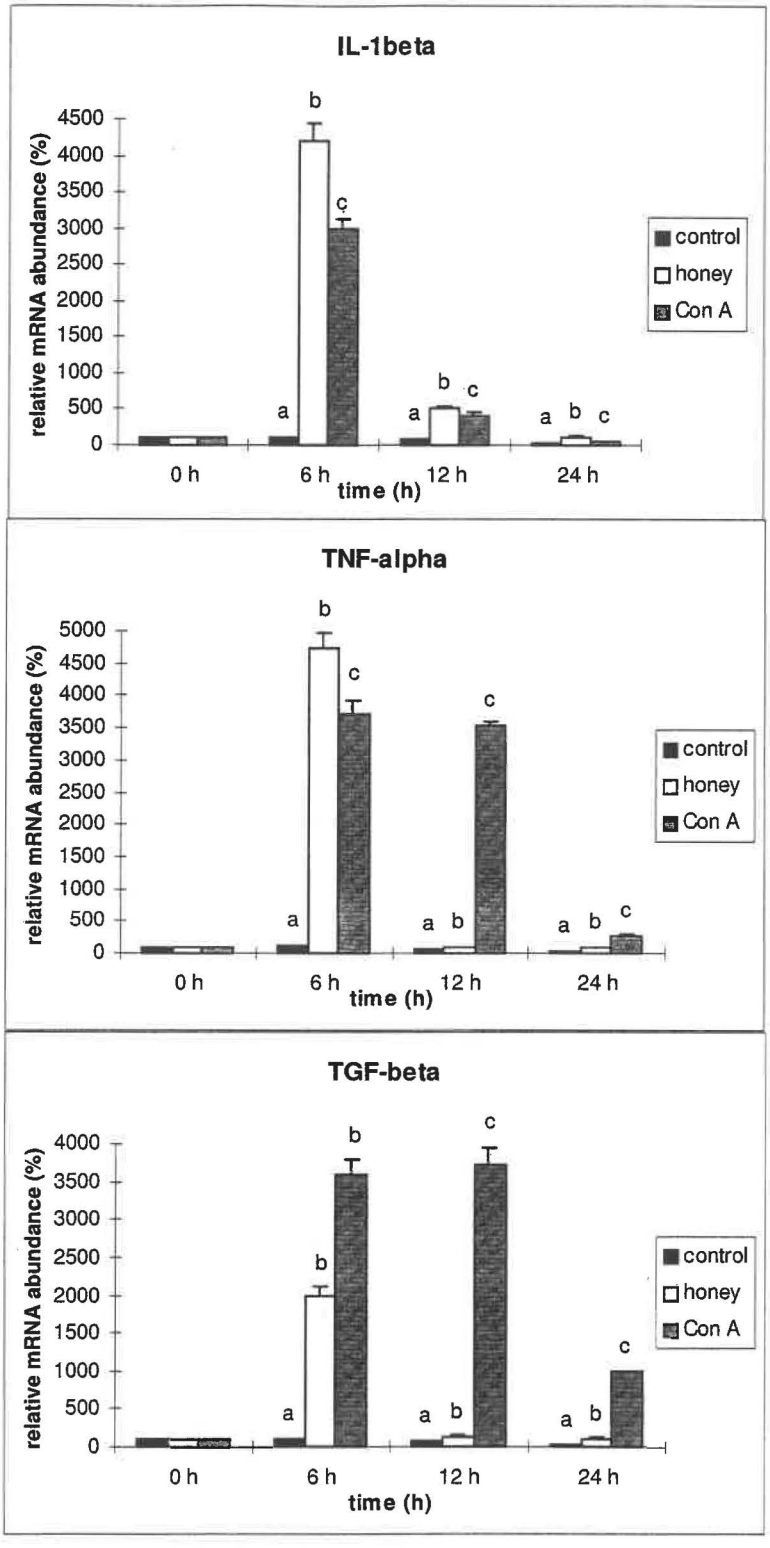


Figure 3.4: IL-1β, TNF-α and TGF-β mRNA expression induced by honey or Con A. Blood was treated with 0.25% manuka honey or Con A (5 μg/ml), compared with RPMI 1640, and mRNA product for 0–24 h, measured by quantitative real-time RT-PCR. The results were expressed as mRNA abundance calculated relative to non-stimulated cells at time 0, after normalisation against the housekeeping gene, B2M. Error bars represent the SEM of two independent experiments. Superscript letters indicate significant differences within a timepoint for individual cytokines, and means not sharing letters are significantly different ($P<0.05$).

While Con A-activated cultures had 1-fold higher expression of mRNA for iNOS at 6 h post-stimulation, expression levels had increased 6-fold by 12 h post-stimulation. HSP70 was up-regulated by 6 h at levels 1.6-fold greater in cultures stimulated with honey than Con A. However, by the 12 h timepoint expression of mRNA for HSP70 had decreased significantly below levels in Con A-activated cultures.

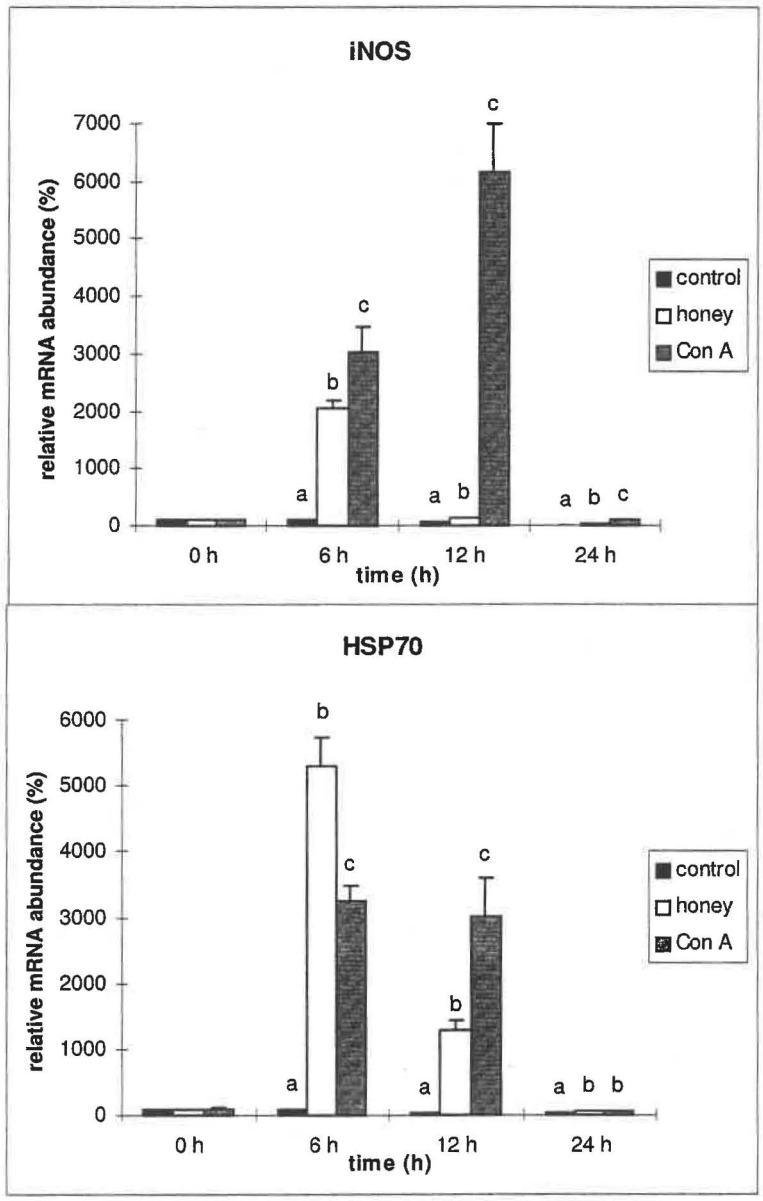


Figure 3.5: HSP70 or iNOS mRNA expression induced by honey or Con A. Blood was cultured in RPMI 1640 (control) or stimulated with 0.25% manuka honey or Con A (5 μg/ml), and mRNA product for 0–24 h, measured by quantitative real-time RT-PCR. The results were expressed as mRNA abundance calculated relative to non-stimulated cells at time 0, after normalisation against B2M. Error bars represent the SEM for two independent experiments. Superscript letters indicate significant differences within a timepoint for individual cytokines, and means not sharing letters are significantly different ($P<0.05$).

3.3.1.1 Electrophoresis results

Use of conventional RT-PCR showed in absolute terms whether a transcript was present (by a band on the gel) or not (no band). In Figure 3.6 three representative gels obtained by conventional RT-PCR are shown.

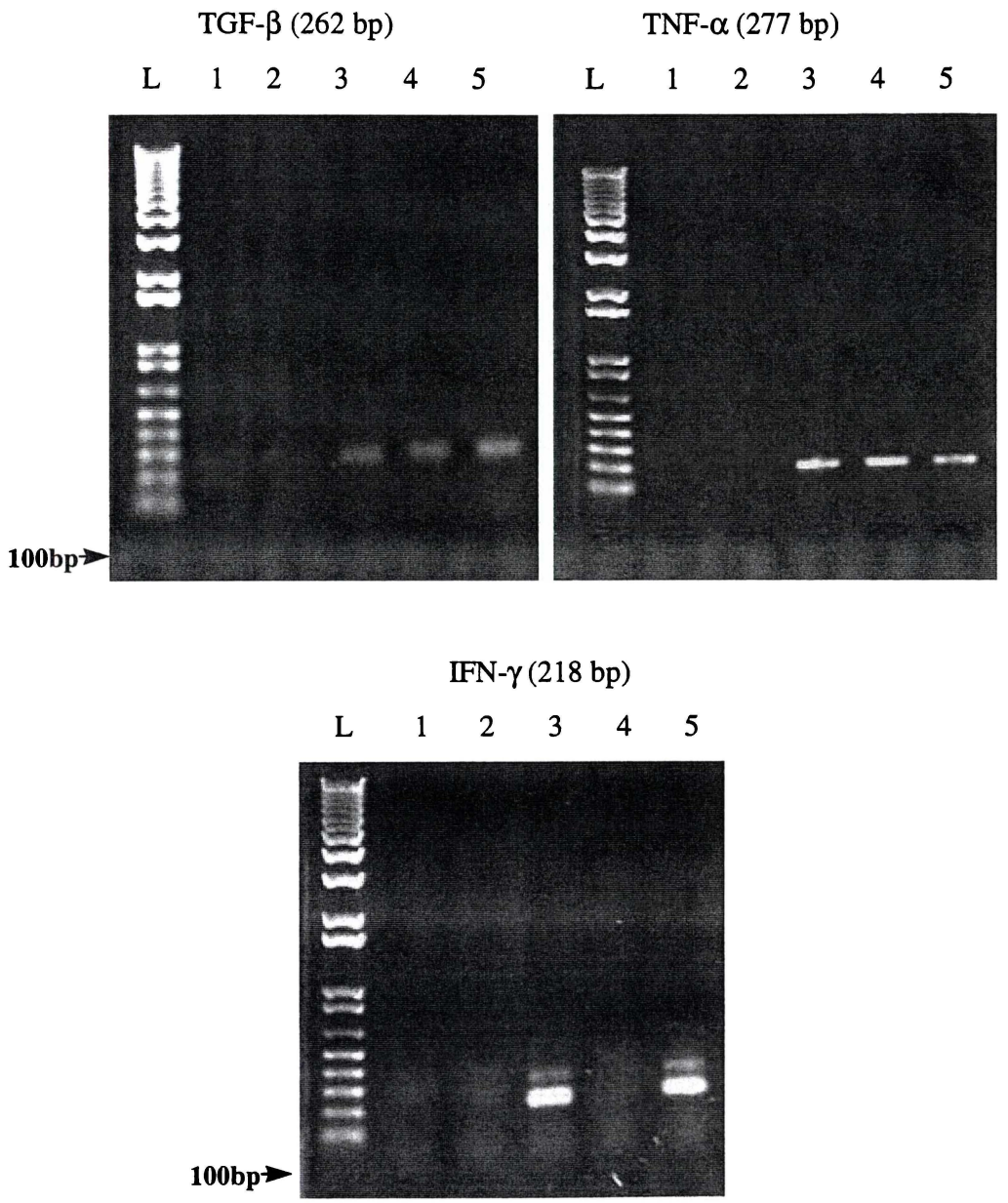


Figure 3.6: The effect of honey on cytokine mRNA expression in whole blood was measured using conventional RT-PCR. (L) 100 bp ladder; (1) no treatment (control); (2) 0.25% artificial honey; (3) 0.25% manuka honey; (4) 0.25% Otago pasture honey; (5) 5μg/ml Con A. Results are representative of three experiments.

As shown in Figure 3.6, there were no detectable bands for any of the cytokines for control blood cultures indicating that any mRNA expression for these cytokines were too low to be detected by conventional RT-PCR. These results confirmed that artificial honey had no effect on mRNA expression beyond the control. These results were consistent with the expression profiles obtained using quantitative real-time RT-PCR.

Differences in honey composition were shown to have an effect on induction of mRNA for IFN-γ. At a 0.25% concentration, manuka honey but not Otago pasture honey induced expression of mRNA for IFN-γ. Gel electrophoresis could not detect any differences in the levels of mRNA expressed for the ten cytokines (listed in Table 3.1) for blood stimulated with Con A alone or at the same time as honey (data therefore not shown). An alternative mitogen, LPS, which acts on monocytes through the CD14/ Toll receptor 4 complex and B cells, was also examined for its effect on cytokine expression by whole blood cultures (Figure 3.7).

The addition of 0.25% manuka honey to LPS-stimulated blood cultures led to a decrease in mRNA expression for some cytokines (Figure 3.7). There was a potent decrease in mRNA expression for IL-12p40 and TNF-α when blood was exposed to honey at the same time as LPS, compared with LPS alone. There is suggestion from the kinetics profile in Figure 3.7 that levels of mRNA expression for iNOS and TGF-β are decreased when blood is co-stimulated with honey and LPS compared with LPS alone. LPS stimulated a rapid increase in mRNA expression as shown by increased expression at 6 h. In four of the seven cytokines tested, expression remained at this level for a further 6 h.

In contrast to cultures stimulated with LPS alone, cultures co-stimulated with honey and LPS had 50-fold higher levels of mRNA expression for HSP70 at 6 h. Further, mRNA for HSP 90 was only expressed in the cultures exposed to both LPS and honey. Of the cytokine mRNA amplicons regulated by honey in combination with LPS (from Figure 3.7), three were selected for further statistical analysis. The following figure shows the relative mRNA abundance (incorporating data from Figure 3.7 and a second PCR reaction), expressed as a percentage of time 0 values for mRNA abundance (normalised to β2M mRNA).

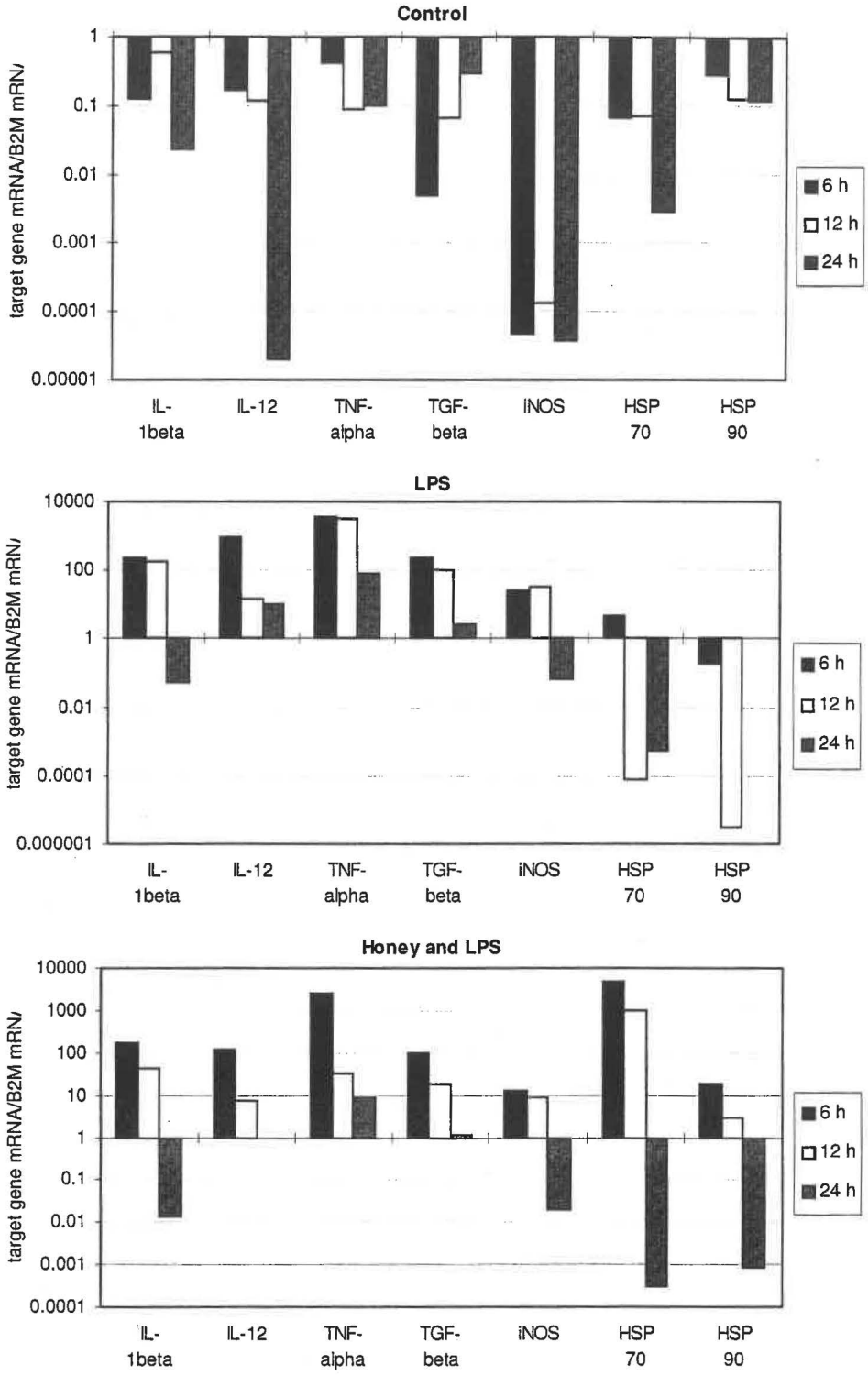


Figure 3.7: Cytokine mRNA expression in blood induced with LPS alone or LPS and honey. Bovine blood was treated with LPS (10 μg/ml) alone or at the same time as 0.25% manuka honey as compared with RPMI 1640. RNA was extracted from these blood cells at 0, 6, 12 or 24 h, and analysed by quantitative real-time RT-PCR . The results shown are ratios obtained by dividing concentrations of the PCR products from the cytokine genes by those from the β_2-microglobulin gene and related to time 0 data. Values are representative of single results.

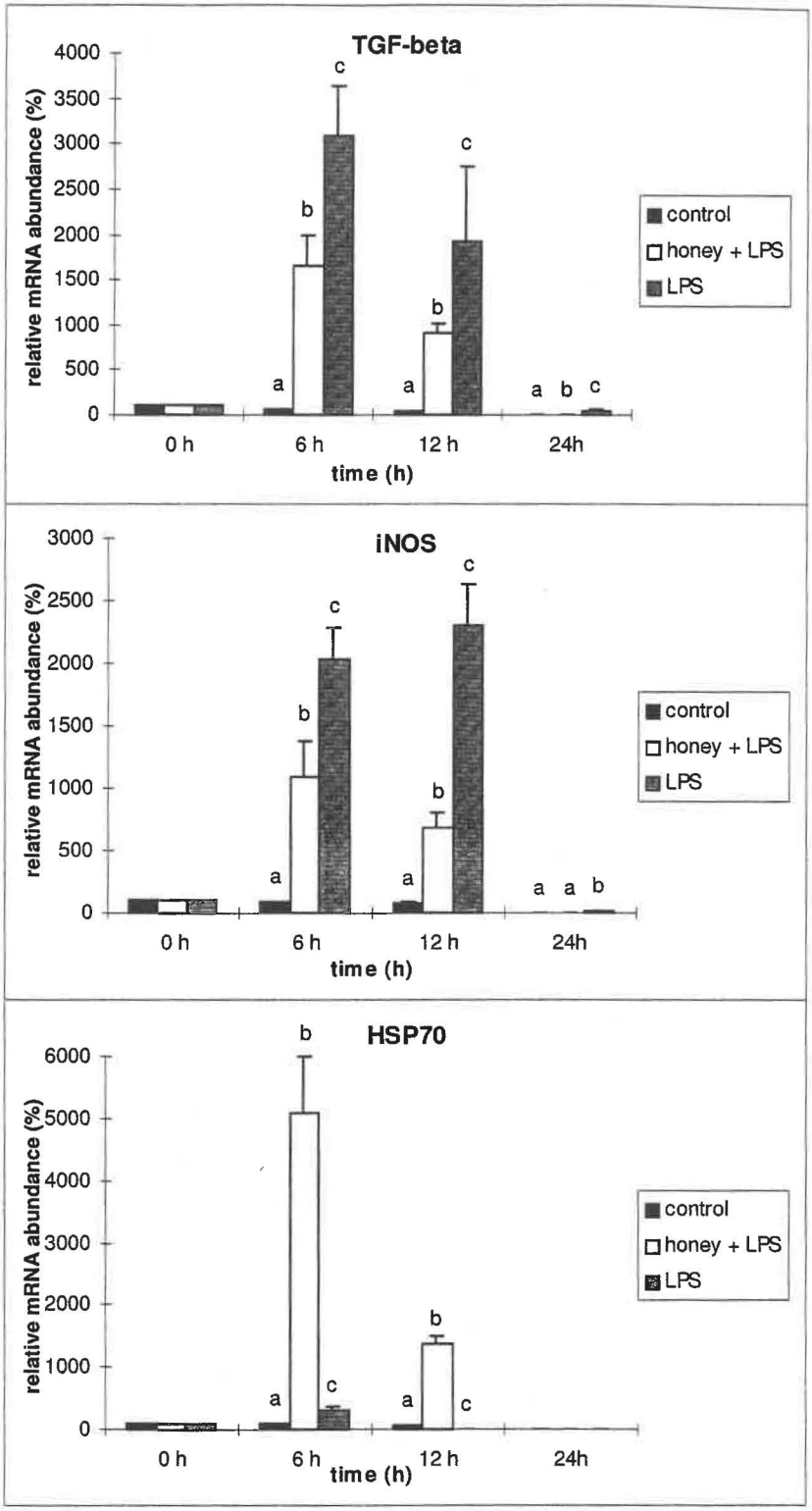


Figure 3.8: TGF-β, iNOS or HSP70 mRNA expression induced by LPS or LPS and honey. Bovine blood was cultured in RPMI 1640 in the presence of LPS (10 μg/ml) alone or with the addition of 0.25% manuka honey, and mRNA product for 0–24 h, measured by quantitative real-time RT-PCR. . The results were expressed as mRNA abundance calculated relative to non-stimulated cells at time 0, after normalisation against B2M. Error bars represent the SEM for two independent experiments. Superscript letters indicate significant differences within a timepoint for individual cytokines, and means not sharing letters are significantly different ($P<0.05$).

It can be seen in Figure 3.8 that the addition of honey when blood was stimulated with LPS altered the mRNA expression for several cytokines. There were significantly higher levels of mRNA expression for TGF-β and iNOS in LPS-stimulated cultures, indicating that honey had decreased induction of mRNA for these cytokines. Relative expression of mRNA for HSP70 was strongly increased for cultures stimulated with both honey and LPS, with a peak at 6 h post-stimulation. In contrast, for LPS-stimulated cultures there was a transient increase in mRNA for HSP70 at 6 h post-stimulation.

3.4 DISCUSSION

The findings in the current study show that honey directly induces mRNA expression for a range of cytokines in whole blood cultures. The expression of cytokine mRNA does not guarantee the production of functional protein. A number of events need to occur before mRNA transcripts are translated into functional proteins. Therefore, it is premature to speculate about how the mRNA profiles observed in the current study translate to functional proteins. However, it is expected that changes in mRNA expression and levels of mature protein would follow a similar trend (Dokter *et al.*, 1993; Entrican *et al.*, 1992; Nordmann *et al.*, 1989).

The observation that low level constitutive expression of mRNA for certain cytokines could be detected in freshly isolated, non-stimulated bovine blood may reflect induction of mRNA during the collection process (Donnelly *et al.*, 1991; Fuhlbrigge *et al.*, 1987). It is likely that an *in vivo* attenuation event may influence the immunological responsiveness of the blood. Leukocytes are sensitive to *ex vivo* handling (Baechler *et al.*, 2004), and this may influence gene expression. Post-transcriptional regulation is also known to interfere with the stability of a mRNA transcript (Tovey *et al.*, 1988) and this may influence the amount of time a message can persist in culture before it degrades and the likelihood of it being detected. When the transcription of a gene is increased, a higher concentration of the corresponding mRNA results. It follows from this that the concentration of mRNA in supernatants at any time will be affected by the stability or half-life of a particular mRNA. Factors

that increase mRNA stability can be expected to raise the concentration of that mRNA molecule, and it is not known how honey affects mRNA stability. A further unknown is how honey might influence the rate of translation of mRNA into functional proteins, and this needs to be investigated.

Worthy of note here is the observation by Tonks *et al.* (2003) that TNF-α protein peaked around 6 h after monocytes were treated with honey, consistent with the peak in mRNA expression for TNF-α by 6 h observed in the current study. TNF-α mRNA has a short half-life (Smart, 1995) and hence, is rapidly translated into TNF protein appearing as early as 30 min after LPS stimulation (Belge *et al.*, 2002). Both mitogens induced a rapid induction of mRNA for pro-inflammatory mediators, and this is followed by a prolonged period of mRNA expression. Honey also induces a rapid expression of key pro-inflammatory genes, but the response is generally transient compared with the mitogens.

Others have reported that production of inflammatory cytokines by LPS-stimulated blood monocytes and dendritic cells peaks after 6 h in culture (Bueno *et al.*, 2001). Due to its nature as an endotoxin, it is expected that LPS would act on different cell types and via different receptors to honey. Feezor *et al.* (2003) have reported that LPS up-regulates genes involved in pro-inflammatory signalling, such as those encoding IFN-regulatory factors, IFN-stimulated proteins, and signal transduction genes (*i.e.* NF-κB). The kinetics profile for LPS-stimulated blood is likely to reflect the blood monocytes being the primary target of LPS (Hambleton *et al.*, 1996). Con A, a T cell mitogen (Miller *et al.*, 1991), is likely to have induced a cytokine mRNA profile which reflects T cell activation. Therefore, the differences which exist for the mRNA profiles in honey-treated cultures and mitogen-stimulated cultures must reflect the cell types which are being activated (Boldrick *et al.*, 2002). The results from Section 2.4 demonstrate that honey has activity on T cells. That honey-treated blood cultures induce a different mRNA profile than Con A-stimulated blood is clear indication that honey also has activity on cells other than T cells. Further, that honey induces a different kinetics profile for mRNA to that obtained for Con A-activated cultures suggests that honey works through a different mechanism to the lectin.

The results presented in the current study demonstrate that honey has potent effects on the Th_1-type cytokine (IL-1β, IL-12 p40, TNF-α and TGF-β), mRNA profile. Further evidence that honey may activate macrophages comes from its ability to upregulate mRNA transcripts for IL-12 p40, produced mainly by macrophages and Natural Killer cells (Collins *et al.*, 1999). Macrophages are also known to produce high levels of TNF-α and IL-1β (Havsteen, 2002). That honey induces expression of mRNA for these proinflammatory signals in the first 6 h is consistent with what is observed clinically for the rapid effect of honey in stimulating wound healing (Tonks *et al.*, 2003; Phuapradit and Saropala, 1992; Efem, 1988). The decrease in mRNA expression levels which occurred for most cytokines in the last 12 h in culture is likely to reflect increased apoptosis and loss of viability due to the *in vitro* environment (Baechler *et al.*, 2004). Decreased expression of mRNA transcripts by honey-stimulated cells may indicate that honey activated an apoptotic mechanism.

The unexpected profile for IL-5 mRNA expression obtained for honey-treated blood may reflect what is known about the susceptibility of mRNA transcripts to individual variations in genetics, environment and handling (Baechler *et al.*, 2004; Radich *et al.*, 2004; Myrianthefs *et al.*, 2003; Whitney *et al.*, 2003). In particular, the expression of IFN-responsive genes are known to vary between individuals (Whitney *et al.*, 2003), and over a time course (Baechler *et al.*, 2004). Radich *et al.* (2004) have demonstrated that some individuals have constitutive expression of IFN-regulated genes and this influences their response to various stimuli such as LPS. To increase the reliability with which a comparison of the amount of target gene expressed can be made with the reference gene, given that differential expression of housekeeping genes may occur (Rogler *et al.*, 2002), a second reference gene could have been included. These variable experimental conditions may include disease state, therapeutic regimens, or tissue source (Mackay, 2004). It has been reported that B2M is not an appropriate reference gene for detecting gene expression in systems induced with TNF-α or IFN-γ as both of these genes have their responsive elements in the promotor of β2M (Ullmannova and Haskovec, 2003). However, this was not of concern here.

That manuka honey induced transcription of mRNA for the heat shock proteins is an important observation. That there was no detectable expression of heat shock protein expression in control cultures confirms that this result was unlikely to be an artefact of blood processing prior to RNA extraction. The component in honey most likely to be responsible for heat shock protein induction is hydrogen peroxide, though the likelihood of this agent exerting these effects in the presence of antioxidants is dubious. The ability for glycated proteins or antigenic complexes formed from a combination of polyphenols and proteins to induce heat shock proteins cannot be ruled out.

The cytokine mRNA profile obtained with honey gives strong support to speculation that hydrogen peroxide may provide the mechanism for cellular activation. Hydrogen peroxide is reported to induce rapid cellular activation (Reth, 2002). To investigate this hypothesis further, it would be useful to track the expression of mRNA for nuclear factor κB (NF-κB), a transcription factor known to be activated by hydrogen peroxide (Reth, 2002). Of potential relevance here, it has been reported that low concentrations of hydrogen peroxide stimulate both IL-1β and TNF-α transcription (Haddad, 2002) and hydrogen peroxide is produced by glucose oxidase in honey (Bang *et al.*, 2003). Transcription of TGF-β is also known to be induced by the presence of oxidant species (such as hydrogen peroxide) and is crucial for recruitment of monocytes to the wound site (Junn *et al.*, 2000). As discussed in Section 3.1, TGF-β plays an important role in fibroblast activation in the later stages of inflammation. A key regulator of cytokine induction is NF κB and hydrogen peroxide has been implicated in the phosphorylation and subsequent removal of the NF κB inhibitor, IκB, thereby activating transcription (Adcock *et al.*, 1994). In light of a report by Xie *et al.* (1994) it is known that activation of NF-κB is necessary for induction of iNOS gene expression by macrophages, further justifying the need to investigate whether honey may have an effect on NF-κB.

The possible role of glycated proteins and anti-oxidants in the immunological response induced by honey cannot be ruled out by the results reported here. Havsteen (2002) has proposed that flavonoids found in honey may play a significant role in the activation of the immune system. Havsteen (2002) proposed that flavonoids modulate inflammation through their effects on P-kinase cascades, most likely at the ATP-binding site. Protein P-kinase cascades play a pivotal role in the production of cytokines. There is evidence that flavonoids stimulate the release of IFN-γ protein by macrophages and this has been attributed to effects on protein phosphokinases (Mitrocotsa *et al.*, 1999; Sen and Lengyel, 1992). In particular, it has been reported that flavonoids stimulate macrophages to produce IL-1 (Havsteen, 2002) a monokine which stimulates T cell activity. In view of the observed potency with which honey stimulates mRNA transcription for IL-1β in whole blood, and IL-1 protein by monocytes (Tonks *et al.*, 2003), this raises the likelihood that flavonoids may play a role. It is surmised that flavonoids can act as antigens since antibodies to them have been found in the human blood (Havsteen, 2002).

Others have demonstrated that antioxidants reduce the mRNA expression of iNOS proteins (Bito *et al.*, 2002; Tsai *et al.*, 1999), and of particular relevance to the current study, tannins have been shown to inhibit iNOS mRNA and iNOS activity (Yokozawa *et al.*, 2002). TGF-β is known to inhibit iNOS expression by decreasing iNOS mRNA stability (Chen *et al.*, 1998). Of further relevance to the current study, Yokozawa *et al.* (2002) reported that low concentrations of tannins modulated cell response to LPS. While others have demonstrated that sugars alone can stimulate transcriptional activity of IL-18 in a variety of epithelial-like cell lines (by Takeuchi *et al.*, 1999) and of IL-1β in PBMC cultures (Shapiro and Dinarello, 1997), sugars alone (artificial honey), at the concentrations tested in the current study, had no effect on mRNA expression.

Evidence from the current study which does not support the hypothesis that hydrogen peroxide is the active component, comes from the observation from gel electrophoresis that manuka honey and not Otago pasture honey at 0.25% concentrations induced the transcription of IFN- γ mRNA. This observation must be

due to the differences in composition between the two honeys and manuka honey has a lower hydrogen peroxide content than the Otago pasture type. It remains to be demonstrated how the levels of IFN-γ mRNA expression correspond to those of functional IFN-γ protein. However, it is noted that the superior antibacterial activity of manuka honey *in vivo* (Molan, 1992b) would be consistent with up-regulated levels of IFN-γ. This is based on the known ability for IFN-γ to increase phagocytosis and also antigen presentation by class II MHC proteins (Hu *et al.*, 2001). Further, the observation that upregulation of bovine IFN-γ in turn up regulates bovine IL-2 receptors (Pighetti and Sordillo, 1996) provides a possible mechanism by which honey was observed to stimulate expression of lymphocyte IL-2 receptors (Section 2.3.1.6). Thus, the effect of honey on expression of IFN-γ protein would be a key area for future investigation.

This increases the probability that an antigenic protein or polyphenol complex, likely to occur at higher concentrations in manuka honey (Section 1.3.1.5), may be the active component. Of further relevance here, Simuth *et al.* (2004) have reported that the presence of royal jelly proteins in various honeys directly stimulate murine macrophages to produce TNF-α.

The antioxidant content of honey was discussed in Sections 1.3.1.4 and 1.4.4.1. Flavonoids in particular have been proposed to have an anti-inflammatory effect on iNOS proteins (Tsai *et al.*, 1999). Like honey itself, there is abundant evidence that flavonoids can have both a stimulatory and modulatory role, and the answer to this apparent dichotomy lies in their ability to interfere with cellular signalling. In Section 1.4.4.1 of the current study, the ability for flavonoids to ameliorate in the inflammatory pathway through effects on free radicals was discussed. Of further note, it has been reported that flavonoids inhibit the activity of IL-5 (Park *et al.*, 1999).

That honey induced a transient response for mRNA transcription, compared with the persistent induction obtained with the mitogens is likely to be beneficial for wound healing. The massive and prolonged expression of pro-inflammatory cytokine mRNA and corresponding proteins obtained with Con A or LPS-stimulation of cultures *in*

vitro, would likely contribute to the pathogenesis of inflammatory disease and tissue damage, and yet carefully regulated production of these cytokines results in tissue healing (Zhang and Morrison, 1993; McCartney-Francis *et al.*, 1993).

In the current study, LPS-stimulated blood provided a model of excessive inflammation. The implications of the observed modulatory effect of honey on cytokine mRNA amplicons in LPS-stimulated cells *in vitro*, for ameliorating inflammatory conditions *in vivo* are unknown. It is noted that in the current study the cytokine mRNA expression of blood cultures was only measured over a 24 h period, and wound healing takes place over a far longer period of time.

Considering the effect of honey on induction of cytokine mRNA, it is hypothesised that initially honey induces some of the pro-inflammatory responses during blood incubation *in vitro*, but later it acts to counter-regulate the hyperinflammatory state by up-regulating anti-inflammatory mediators. It is also possible that honey has dual effects to be able to induce both pro- and anti-inflammatory states at the same time and that this is what contributes to observed accelerated healing with minimal scarring. Therefore, honey may potentially modulate inflammatory states without excessive suppression of the host defence system. The effects on cytokines raise the question of how and in what form and concentrations the "stimulating components" from honey have to the blood *in vivo*.

Chapter Four: Stimulation By Honey Of TNF-α Production By Monocytes

SUMMARY

To investigate whether honey could induce THP-1 monocytes to release TNF-α, bioassays using WEHI cells were carried out to measure TNF-α production after the monocytes were exposed to honey. Honey was found to stimulate release of TNF-α by the monocytes when at a range of concentrations between 0.000025–0.1%, with no differences between the levels produced at the various concentrations of honey. At concentrations from 0.25–1% the TNF-α production decreased as the concentration of honey increased. This may indicate an anti-inflammatory action overrides the stimulatory effect at concentrations greater than 0.25%. Sugar content had no effect upon TNF-α release, as demonstrated by the artificial honey control. There were no differences between honey types (manuka honey and pasture honey) in induction of TNF-α release. Time-course analysis confirmed that a 4–6 h incubation period of cells with 0.25% honey gave maximal TNF-α production. A 2 h minimum exposure period of cells to honey was critical for TNF-α production. Incubation of LPS-stimulated monocytes with honey had no effect on their subsequent TNF-α production. A good correlation was found between the TNF measurements detected by ELISA and the WEHI Bioassay.

4.1 INTRODUCTION

Monocyte production of cytokines and chemokines is crucial in determining whether the outcome of wound healing will be normal. There are a number of pathways which could potentially be modified by exposure of monocytes to honey. The production of TNF-α is of particular relevance to wound healing as it stimulates the inflammatory cascade which is necessary for tissue repair to occur (Belge *et al.*, 2002). However, in high concentrations TNF-α is one of the most harmful endogenous pro-inflammatory cytokines (Tracey and Cerami, 1993) and may lead to tissue destruction (Sommer *et al.*, 1994). In Section 3.4 it was established that honey stimulates blood cells to express cytokine mRNA for TNF-α. However, it was not established which of the cell types cultured expressed the TNF-α mRNA and the objective of the investigations described in this chapter was to measure the production of biologically active TNF-α (or TNF protein) by a monocyte cell line and determine whether TNF production was influenced by honey.

Research conducted by Tonks *et al.* (2001) established that 1% concentrations of manuka and pasture honeys induced low levels of TNF-α (200-500 pg/ml) release by a macrophage-like cell line. The investigations described in this chapter were designed to repeat the assays described by Tonks *et al.* (2001) and confirm their findings using a different cell line. A further aim for the investigations described in the current study was to extend the range of honey concentrations tested to identify where the TNF-inducing activity stopped. The production of TNF-α by monocytes has been studied using a variety of assay types, the most common of which include ELISA (Dewar *et al.*, 2005) and WEHI Bioassays (Carter *et al.*, 1999; Joyce *et al.*, 1997; Khabar *et al.*, 1995; Kurzman *et al.*, 1993; Espevik and Nissen-Meyer, 1986) to detect protein, and RT-PCR (Amills *et al.*, 2004) to detect mRNA expression.

4.1.1 A brief introduction to monocyte biology

In order to understand the function of monocytes in the immune system and to comprehend how changes to this function may be brought about by incubation of the cells with honey, it is necessary to begin with a brief summary of the functional biology of monocytes. Monocytes represent the circulating macrophage population, and use integrin molecules (*e.g.* β_2 integrins) to adhere to ICAM-1 on the endothelial cells. As described in Section 1.5.1, the adhesion molecules utilised by monocytes are now well established. Integrin binding induces activation of multiple transcription factors, the expression of key cytokines (*e.g.* IL-1β) and accumulation of cytokine mRNA (Bocker *et al.*, 2001). Both LPS-binding and integrin-binding to the endothelium are required for translation of cytokine mRNA into functional protein (Bocker *et al.*, 2001). In particular, the production of functional IL-1β is critically interrelated with changes in cytoskeletal structure of the monocyte to enable spreading and extravasation (Bocker *et al.*, 2001). The ability for monocytes to adhere to the endothelium, undergo diapedesis, and migrate out to the tissues is critical for their differentiation into macrophages (Arai *et al.*, 1998; Benjamini *et al.*, 1996). Chemotactic factors (including bacterial breakdown products, complement components, and leukotriene B4) act on the cell membrane and result in cell movement toward injured tissues (Arai *et al.*, 1998). Unlike the lymphocytes, monocytes do not proliferate, but undergo differentiation to become macrophages, potent producers of reactive oxygen intermediates (Martin and Edwards, 1993). Monocytes are created in the bone marrow and only spend a brief time in the blood system, constituting 2-8% of the total leukocytes found in the blood as compared with lymphocytes constituting 20-30%. It is in the tissues that the monocytes change their phenology and become specialised macrophages, for example the Kupffer cells in the liver (Ziegler-Heitbrock, 1989). The monocyte-differentiated macrophages enter the site to replace neutrophils which dominate during the first 24-48 h after tissue injury, removing cellular debris and foreign material through phagocytosis. Monocytes bear Fc and C3b receptors on their surfaces (as do neutrophils) which enable recognition of opsonized materials for phagocytosis (Barbuddhe *et al.*, 1998; Benjamini *et al.*, 1996). They play an important role in host

resistance to pathogens by virtue of their phagocytic and antibacterial activities (Higgenbotham and Pruett, 1994).

Mononuclear macrophages produce hydrogen peroxide and, via a reaction of the phagosome, generate a hydrogen peroxide-myeloperoxidase-halide system, producing reactive oxygen intermediates which kill bacteria (Tonks *et al.*, 2001). The hallmark of chronic inflammation is infiltration of the tissues with mononuclear inflammatory cells (monocytes being among these) resulting in tissue damage from reactive oxygen intermediates produced from leaked hydrogen peroxide.

Endotoxin-stimulated macrophages are the most important source of TNF (Beutler and Cerami, 1988) and the monocyte/macrophage lineage respond to the presence of Gram-negative or mixed Gram-negative/positive lipopolysaccharide (LPS) antigen with a massive production of TNF-α (Kurzman *et al.*, 1993). As described in Section 3.1, LPS binds to CD14, and acts via the Toll-like receptor (TLR)4 coreceptor (1) (Belge *et al.*, 2002). After ligation of these receptor complexes, a signal transduction cascade is activated which mobilises transcription factors (*e.g.* NF-κB) leading to production of cytokines (*e.g.* TNF-α). Given that LPS stimulates TNF-α production by monocytes, it is crucial to determine whether any possible endotoxin contamination *in vitro* culture could contribute to levels of this cytokine. The activity of TNF-α in the innate immune system is only part of its role in wound healing. TNF-α has a broader range of effects on host immune responsiveness, enhancing interactions with other blood cells. TNF-α is considered to be pro-inflammatory because it recruits other cells to fight bacterial infection during inflammation.

TNF-α primes and activates phagocytes (Jiang *et al.*, 1992) and initiate their anti-bacterial and tumoricidal activity, as well as to increase expression of adhesion molecules on the surfaces of endothelial cells (Lefkowitz and Lefkowitz, 2001). Of particular relevance to this study, TNF-α has been reported to stimulate T-cell proliferation, upregulate MHC, and initiate the production of other cytokines by T cells (among them IL-2) (Chaudhri and

Clark, 1989). In many cases TNF-α exerts its cellular effects through IL-1 in a paracrine manner, (Mantovani *et al.*, 1998). Monocytes also produce growth factors along with hydrolases, collagenase, and elastase. It has also been shown to increase phagocytosis by neutrophils (Utreras *et al.*, 2000), and has a role in inducing production of reactive oxygen intermediates by fibroblasts and stimulating their proliferation via bystander activation (O'Toole *et al.*, 2001; Meier *et al.*, 1990). Furthermore, it has been demonstrated to increase permeability of tight junctions (Singh *et al.*, 2001). Monocytes produce the vasoactive amines histamine and serotonin, the classic mediators of vascular permeability. The TNF family includes 19 members of proteins which signal through 29 receptors, and either induce cellular proliferation, survival, differentiation or apoptosis (Aggarwal, 2003). Clearly, the release of TNF-α by monocytes is a key determinant of immune function and plays an important role in lymphocyte activation and immune regulation.

TNF-α has been implicated in a number of inflammatory diseases caused by an abnormal immune response, including rheumatoid athritis (Lefkowitz and Lefkowitz, 2001), Crohn's disease and multiple sclerosis (Sharief and Thompson, 1992). In rheumatoid athritis, TNF-α is essential for the perpetuation of inflammation (Lefkowitz and Lefkowitz, 2001) and this is primarily due to its activating effect on both immune and non-immune cells, such as endothelial cells and osteoblasts, thus amplifying cytokine concentrations (Carter *et al.*, 1999). TNF-α binds with its receptor, triggering the phosphorylation and subsequent destruction of IκB, liberating NF-κB. Once in the nucleus, NF-κB acts as a transcription factor, binding to enhancers and promoters of genes. Indeed, glucocorticoids exert their anti-inflammatory and immunosuppressive effects through enhancing the production of IκB (Gortz *et al.*, 2005; Lefkowitz and Lefkowitz, 2001). NF-κB turns on the genes encoding IL-1 and other key cytokines which induce inflammation, along with the genes needed for cell proliferation, cell adhesion and angiogenesis.

Therefore, the search for therapeutic agents which have activity on TNF-α production specifically are of prime importance to drug discovery. There are now well established anti-TNF biologicals, used in treating inflammatory conditions such as rheumatoid arthritis and Crohn's disease *e.g.* entanercept and infliximab (Singh and Suruchi, 2004). These therapies apply monoclonal antibodies to TNF-α, the IL-6 receptor, and the recombinant soluble TNF-α receptor (Wakamatsu *et al.*, 2005). Monoclonal antibodies to TNF-α block binding of this cytokine with its receptor, and therefore the intracellular pathways activated by TNF-α (*e.g.* mitogen-activated protein kinases such as p38MAPKα and extracellular signal-regulated kinase such as ERK in the synovial membrane) are blocked or inhibited (Gortz *et al.*, 2005).

The THP-1 cell line was selected for studying the effect of honey on TNF-α production as it has been extensively characterised (Wang *et al.*, 2001; Kremlev *et al.*, 1997; Kremlev and Phelps, 1997; Schwende *et al.*, 1996; Auwerx, 1991) and in particular its ability to produce TNF-α in response to LPS (Janic *et al.*, 2003) and retain its ability to synthesise and secrete cytokines (Grandics, 2002) make it a good experimental model.

4.2 MATERIALS AND METHODS

4.2.1 Culture media

RPMI Complete medium

RPMI 1640 with 10% foetal calf serum (as described for T cells in Section 2.2.1).

Bioassay medium

RPMI 1640 with 3% foetal calf serum (as described for T cells in Section 2.2.1). As 10% foetal calf serum was known to interfere with the WEHI Bioassay it was necessary to reduce the serum content. Monocytes require serum in the medium to maintain normal metabolic function. Although 3% serum content is not optimal for monocyte culture, it

has been used successfully by others where assays were sensitive to serum levels (Jiang *et al.*, 1992). Preliminary assays conducted in this study indicated that THP-1 incubated in 3% foetal calf serum produced TNF-α at levels that enabled treatment effects to be detected by the WEHI Bioassay (data not shown).

Trypsin solutions

PBS containing 0.25% trypsin and 5 mM EDTA.

Honey Solutions

Solutions of manuka M109, Otago pasture honey and artificial honey in bioassay medium were prepared as described for Section 2.2.2.

LPS solutions

As described in Section 3.2, 1 mg/ml stock solution (*E. coli* serotype 0111: B4; Sigma-Aldrich UK; cat no# L4516) was purchased. The LPS was diluted to final concentrations in wells of 10 μg/ml (unless otherwise stated). This single concentration was chosen based on pilot studies, as the higher dose was found to induce higher TNF-α production levels.

All glassware was autoclaved and plasticware was purchased new.

4.2.2 Cell lines and subculturing

WEHI 164 var 13

The mouse fibrosarcoma cell line WEHI 164 clone 13 was purchased from American Type Culture Collection (ATCC, VA USA). The cultures were grown in an atmosphere of 5% CO_2, 95% air (37°C), and maintained twice-weekly by seeding a new flask with cells at a density of 1 x 10^6/ml based on the methods described by Khabar *et al.* (1995). The WEHI cells are a mixture of floating and adherent cells. Non-adherent cells were removed from culture flasks and the adherent cells were washed twice with 5 ml of PBS.

Adherent cells were dislodged by incubation with 5 ml of the trypsin stock solution (Section 4.2.1) for 5 min, followed by gentle scraping. The cell suspension was centrifuged (223 x *g*, 5 min) after which time the supernatant was discarded and the cell pellet re-suspended in RPMI complete medium. The same method was used to split the cell cultures 1:10 (with complete medium) 2 days prior to use in assays (as described by Khabar *et al.*, 1995).

THP-1

The human monocyte line, THP-1, was obtained as a gift from J. Fraser, Auckland University, and were used between passages 5 and 40. Cells were cultured in suspension and passaged once a week, to maintain densities between 5 x 10^5 and 1 x 10^6 cells/ml (according to ATCC guidelines). They were grown in RPMI complete medium containing 0.5 μM mercaptoethanol (β-ME) and 10% foetal calf serum (5% CO_2, 95% air, 37°C).

PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from bovine blood using the Ficoll procedure as described in Section 2.2.3.2.

4.2.3 Preliminary Investigations

4.2.3.1 Establishing an osmotic tolerance range of honeys for THP-1 monocytes

Prior to conducting TNF-α assays, it was necessary to establish an osmotic tolerance range for the THP-1 monocytes. THP-1 cells were loaded into wells of a 96-well microtitre plate, at a final density of 2 x 10^6 cells/ml and incubated with various honey concentrations (0.1–3% v/v) for 48 h (37°C, 5% CO_2, 95% air). Honey was diluted with bioassay medium to obtain the final honey concentrations, and treatments were conducted in triplicate. Bioassay medium alone was included as a control. As the sole objective of this assay was to examine the viability of the THP-1 cells, trypan blue staining was used to count the proportion of viable cells (Section 2.2.6.1). Briefly, 100 μl of cell suspension

from a single well was mixed with an equivalent volume of trypan blue, and after 1 min incubation (37°C, 5% CO_2, 95% air), a haemocytometer count was performed.

4.2.3.2 Setting up of depletion cultures to determine whether monocytes in bovine blood were responsible for TNF-α production

In Section 3.4 it was reported that honey induced expression of mRNA for TNF-α in whole bovine blood. In order to test whether monocytes might be responsible for the observed TNF-α production in the presence of honey, depletion cultures were established. Briefly, heparinised bovine blood was centrifuged on a Ficoll Paque (Pharmacia Biotech) gradient (as described in Section 2.2.3.2) and cells from the interphase were collected. To isolate the monocytes from the PBMC fraction, the PBMC were incubated on a plastic Petri dish (Nunc) for 18 h at 37°C (5% CO_2, 95% air). Subsequently, the supernatant was removed and the adherent cells were detached by means of a 0.02% EDTA solution and suspended in RPMI complete medium (10% foetal calf serum). The cells were then recentrifuged on Ficoll gradient (900 x *g*) for 1 h at 4°C. A highly purified fraction of monocytes of more than 98% viability, determined by 0.5% trypan blue exclusion, was used in a WEHI Bioassay.

The experimental design reported in Section 2.2.4.1 was modified slightly so that the purified monocytes were distributed among wells of a 96-well microtitre plate at a density of 2.5 x 10^6 cells/ml in 100 µl of bioassay medium (3% foetal calf serum). Either manuka honey (to obtain the required final concentration), LPS (10 µg/ml) or bioassay medium alone was added in 50 µl aliquots to triplicate wells and incubated for 6 h (37°C, 5% CO_2, 95% air). The supernatants were collected via centrifugation and stored at -80°C for use in a WEHI Bioassay.

4.2.3.3 Establishing whether honey had any osmotic or other direct effects on WEHI cells

Prior to commencing the Bioassay it was necessary to establish whether honey had any osmotic or other direct effects on the WEHI cells. To enable any effects on cell metabolism and viability to be assessed, MTT assays were used (Section 2.2.6.2). WEHI cells were seeded in 100 µl aliquots into wells of a 96-well microtitre plate, at a final density of 2 x 10^4 cells/well in RPMI complete medium. Control wells were set up containing RPMI complete medium only. The plates were incubated for 48 h before the supernatants were removed and either bioassay medium or honey was added. Various honey concentrations were incorporated into bioassay medium. All treatments were conducted in triplicate. The plates were incubated at 37°C (5% CO_2, 95% air) for 48 h, after which time the honey solutions were replaced with 100 µl of bioassay medium, and 10 µl of MTT (50 µg/ml, Roche Molecular Biochemicals, Cat. No. 1 465 007) added to each well before returning the plate to the incubator. After 4 h (37°C, 5% CO_2, 95% air) 100 µl of 10% SDS (in 0.01 M HCL, Roche Molecular Biochemicals) was added to each of the wells. The plates were left to incubate at 37°C (5% CO_2, 95% air) overnight, to allow all of the tetrazolium crystals to be solubilized. The optical density was determined at 570 nm (Bio-Tek instruments, as in Section 2.2.6.1).

4.2.4 WEHI Bioassay

TNF-α has a cytotoxic effect on the murine fibrosarcoma cell line WEHI 164 (clone 13). The detection limits for this Bioassay have been reported as 0.029 ng/ml (Carter *et al.*, 1999) and less than 0.1 ng/ml (Joyce *et al.*, 1997). WEHI were seeded in 96-well plates at 2 x 10^4 cells/well, in RPMI complete medium. Cell control wells received only RPMI complete medium. The plates were left for 48 h before the supernatants were replaced with those obtained from honey-treated monocytes or the assay standards. Each well of the WEHI-containing microtitre plate was loaded with 50 µl of the supernatant and 50 µl

of bioassay medium containing 1 μg/ml actinomycin-D (Sigma) as described in the methods of Carter *et al.* (1999) and Khabar *et al.* (1995).

For control of LPS contamination, contamination cultures were set up with and without polymyxin B (PMB; Pfizer, Karlsruhe, Germany) at 10 μg/ml. Monocytes in the contamination cultures were exposed to polymyxin B for 30 min at 37°C (5% CO_2, 95% air), prior to adding treatments, to inactivate LPS. All glassware and metal spatulas were autoclaved. Only sterile and endotoxin-free culture flasks, microtitre plates, and pipette tips were used. No LPS contamination was detected.

4.2.4.1 *Quantification of TNF-α release*

After a further 48 h incubation (37°C, 5% CO_2, 95% air), the test fluid in the wells was removed by flicking, in one careful movement. The adherent WEHI cells were washed 3 times using phosphate-buffered saline (room temperature), to ensure all traces of the test supernatants were removed. One hundred microlitres of bioassay medium was added to each well followed separately by 10 μl of MTT. The microtitre plate was incubated for 4 h (37°C, 5% CO_2, 95% air) to enable reduction of the tetrazolium salts by WEHI cells. To stop the reaction, 100 μl of SDS was added, and the plate returned to the incubator overnight (37°C, 5% CO_2, 95% air). The plate was then agitated on a plate shaker (30 min) to ensure homogeneity of the purple dye through the solution. Optical density at 570 nm was measured using a Bio-TEK plate reader. A standard curve was generated using the recombinant TNF standard by plotting optical density against concentration (Appendix 1). WEHI cells cultured in bioassay medium alone were taken to represent 100% viability. The optical density readings from the blank wells were subtracted from the other readings to allow for any colour from MTT not arising from metabolism.

4.2.4.2 *Standards*

To allow quantification of TNF-α production, four standards were used with final TNF-α concentrations of 1ng/ml, 0.5 ng/ml, 0.1 ng/ml and 0.05 ng/ml. To make the standards, a 2 ng TNF-α stock solution (2 µl of 10 µg TNF-α stock in 10 ml of bioassay medium) was diluted with bioassay medium containing actinomycin-D (5 µl of actinomycin-D, Sigma, St Louis, MO, at a concentration of 1 mg in 1 ml DMSO, added to 9.905 ml of bioassay medium) giving a final concentration in all wells of 0.5 µg/ml actinomycin-D. The standards were added in triplicate (100 µl) to wells containing the WEHI cells. The standard curves showed a linear relationship existed between the OD readings obtained for WEHI cells and the TNF-α concentration of the standards (Appendix 1a). The upper detection limit of the WEHI Bioassay is 1 ng/ml (1000 pg/ml) of TNF, as survival of the WEHI is 100% at these levels. As Tonks *et al.* (2003; 2001) had reported that honey induced small levels of TNF production (less than 500 pg/ml) this was not deemed to be a problem. Results are expressed as nanograms (ng) of TNF-α per millilitre of supernatant fluid.

4.2.5 TNF-α ELISA

To confirm the results obtained using the WEHI Bioassay, a commercial enzyme-linked immunosorbent assay (ELISA) was used to measure the TNF-α content of supernatant fluids which had been stored at -80°C (human TNF-α ELISA Cat # BMS223HS, Bender MedSystems, Austria) with a detection limit of 0.13 pg/ml. All of the reagents and antibodies were provided in the kit, and the manufacturers instructions were adhered to. Briefly, the basic principle of the ELISA was the quantitative solid-phase sandwich enzyme immunoassay technique in which a monoclonal antibody specific for human TNF-α was used to coat microtitre plates (Nunc). All reagents, antibodies and supernatants were added 100 µl/well, and all washing steps were performed 4 times with PBS (1% Tween 20). The supernatants were diluted 1:4 with diluent prior to the assay as

determined by preliminary testing. After incubation (2 h, room temperature) the microwells were washed, blotted, and the test supernatants (and controls) were added in triplicate to wells of the plate, enabling the protein to bind to the antibodies adsorbed to the microwells. After a further 2 h incubation (room temperature) a biotin conjugated antibody (anti-TNF-α polyclonal rabbit) was added to the wells and bound to the TNF-α protein captured by the monoclonal (first) antibody. Following 2 h incubation (room temperature) the wells were washed and streptavidin-HRP added and bound to the polyclonal antibody. After a 20 min incubation (room temperature) the wells were washed and loaded with tetramethyl-benzidine (with 0.02% hydrogen peroxide). After incubation (30 min, room temperature) 50 µl of 1M phosphoric acid was added to each well to stop the reaction. The optical density was measured immediately at 450 nm against a reference wavelength of 540nm (Bio-Tek).

The average absorbance values for each set of duplicate standards and samples were calculated, and a standard curve was produced (Appendix 1b). The standards were supplied in the ELISA kit. A standard curve was prepared for each plate assayed. Results are expressed as nanograms (ng) of TNF-α per millilitre of supernatant fluid.

4.2.6 Experiments

4.2.6.1 Measuring the effect of honey on TNF-α release by resting monocytes

After 3 to 4 days of growth, THP-1 cells were harvested, and distributed among wells of a 96-well microtitre plate at a density of 2.5 x 10^6 cells/ml in 100 µl of bioassay medium. Honey was serially diluted from an original 25% stock solution and added in 50 µl aliquots to triplicate wells to obtain final concentrations of 0.000025–0.1%. As indicated in Section 2.2.2, the dilution steps were adjusted to allow for the volume of other reactants in the assay mixture.

After the monocytes had been incubated for 4 h (37°C, 5% CO_2, 95% air) with the honey, the plates were centrifuged (Sorval 503 x *g*, 15 min) and the supernatant removed. All treatments and controls were conducted in triplicate wells and the experiment was replicated three times. Supernatant fluids were stored at -80°C for later assays.

4.2.6.2 Characterising the active fraction of honey responsible for inducing TNF-α release

M109 manuka honey was fractionated by dialysis into high and low molecular weight fractions using a membrane with a 10 kDa cut-off (as described in Section 2.2.2.1). The dialysed honey fractions were serially diluted so that final concentrations of 0.000025–0.1% were obtained (as described in Section 4.2.7.1) and incubated with THP-1 cells for 4 h (37°C, 5% CO_2, 95% air) prior to centrifugation (Sorval 503 x *g*, 15 min). After collection of the supernatants, they were stored at -80°C. Artificial honey and bioassay medium alone were included in addition to blanks (as previously described in Section 4.2.7). All treatments and controls were conducted in triplicate wells and the experiment was run three times. The supernatants were then assayed for presence of TNF using the Bioassay described.

4.2.6.3 Time-series analysis of effect of honey on TNF-α production of THP-1 cells

The levels of TNF-α have been observed to rise and peak within 90 min after LPS has been added to monocytes, and fall to basal levels around 4–6 h later (Tracey and Cerami, 1993). In the present study it had already been established that a 2 h minimum incubation period was required for honey to have activity on T cells (Section 2.3). Investigations by Tonks *et al.* (2003) demonstrated that a 2 h incubation period was sufficient for honey to have effects upon MM6 cells but that a 4–6 h period gave maximal TNF-α production. In order to find the incubation time needed for honey to induce maximal TNF-α production by THP-1 cells, a range of pre-incubation times were chosen between 0–24 h. THP-1 cultures (2.5 x 10^6 cells/ml in a 96-well microtitre plate) were incubated with 0.25% honey (artificial, M109 or Otago pasture) at 37°C (5% CO_2, 95% air) for 0, 2, 4, 6, 8, 12

or 24 h prior to collection of the solutions. Cells incubated with bioassay medium alone for these time periods were included as a control. All treatments and controls were conducted in triplicate wells and the experiment was replicated three times. As has been previously described (Section 4.2.7), the plates were centrifuged (Sorval 503 x *g*, 15 min) to obtain the supernatants. The supernatants were then assayed for TNF using WEHI cells (2 x 10^4 cells/well). A single plate was used for each time point and the supernatants were stored at -80°C until the Bioassay.

4.2.6.4 Investigation to determine whether honey can modulate TNF-α release by LPS-stimulated monocytes

LPS stimulates TNF-α production by monocytes, achieving maximal levels when used at a minimum of 10 to 100 ng/ml and up to a maximum of 20 μg/ml (Sipe *et al.*, 1992). The modulatory effect of honey solutions on lymphocyte response to Con A was demonstrated in Section 2.3.2, and based on those results, an assay was conducted to measure whether honey could interfere with TNF production of LPS-stimulated monocytes. Briefly, monocytes were delivered to a 96-well microtitre plate at a density of 2.5 x 10^6 cells/ml in 100 μl of bioassay medium. Samples were treated with LPS (10 μg/ml) or left untreated. After 24 h incubation (37°C, 5 % CO_2, 95% air) serial dilutions of honey were added in 50 μl aliquots to triplicate wells to obtain final concentrations of 0.000025–0.1%. After a further 24 h incubation (37°C, 5 % CO_2, 95% air), the supernatants were obtained after centrifugation and stored at -80°C. The experiment was repeated twice.

4.2.6.5 Comparison of the measurements obtained for TNF in supernatants from the WEHI Bioassay with those obtained using a commercially available ELISA

Briefly, THP-1 cultures were stimulated with either LPS (10 μg/ml), manuka honey (0.025–1 %) or bioassay medium alone for 4 h (37°C, 5 % CO_2, 95% air) and the

supernatants harvested by centrifugation and stored at -80°C. TNF-α content in the supernatants was then determined for duplicate samples and standards by both the WEHI Bioassay and a TNF-α ELISA.

4.2.6.6 Statistical analysis

Values were expressed as the mean of triplicates ± SEM, and each experiment was replicated at least twice (n=6). For multiple group comparisons, the data were subjected to one-way analysis of variance (ANOVA) to determine overall difference between the group means. The amount of TNF-α produced was the between group effect. Significant effects were further analysed using Tukey's honestly significant difference for pair-wise differences for within group comparisons. Significance was accepted if $P<0.05$. For the comparison between ELISA and WEHI Bioassay determinations, a one-way ANOVA was used to analyse significance in results obtained. Significance was determined using Tukey's honestly significant difference and was accepted if $P<0.05$. Systat (version 7) was used for all analyses.

4.3 RESULTS

4.3.1 THP-1 viability

To establish a range of honey concentrations which could be tested for TNF-activating effects on THP-1 cells, it was first necessary to assess the osmotic tolerance range of the cells. The effect on THP-1 viability following incubation with honey is shown in Figure 4.1. This experiment determined that natural honeys had no effect on viability at 1% concentrations, but that equivalent concentrations of artificial honey reduced viability of the THP-1 cells. Beyond 1%, viability of the monocytes was reduced, indicating that the maximum honey concentration to be included for use in the TNF assays was 1%. Further, these viability assays indicate that while sugars alone at 1% reduced viability, when the equivalent concentration of sugars were present in honey, there was no detectable effects

on monocyte viability. That the osmolarity of the natural honey solutions did not differ significantly from artificial honey (sugars alone) was established in Section 2.2.2.3.

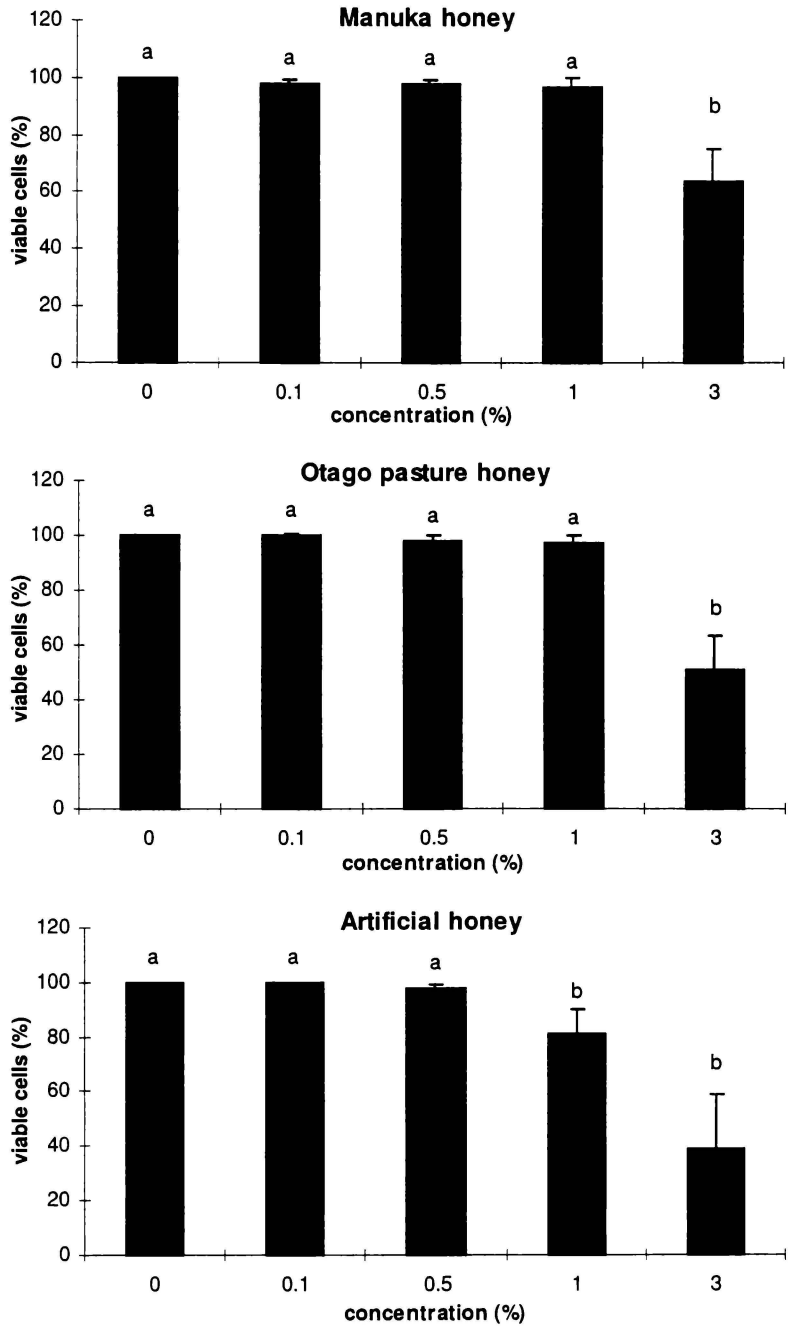


Figure 4.1: The direct effect of honey on THP-1 cell viability. Cells were incubated with various honeys (manuka, Otago pasture, or artificial) for 48 h and compared with THP-1 cultured in bioassay medium alone (100% viable). Data are presented as the mean of triplicates ± SEM (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within a graph are significantly different ($P<0.05$).

4.3.2 Depletion cultures to identify the contribution of monocytes in a whole blood fraction to TNF production

Prior to conducting TNF-α measurements in a monocytic cell line, it was important to test the assumption that the TNF-α detected in the blood cultures (Section 3.3.1) came from the monocytes. Purified monocytes from bovine blood were incubated with either 0.25% manuka honey, LPS (10 μg/ml) or bioassay medium for 6 h and the TNF content of the supernatants measured using the WEHI Bioassay (Figure 4.2).

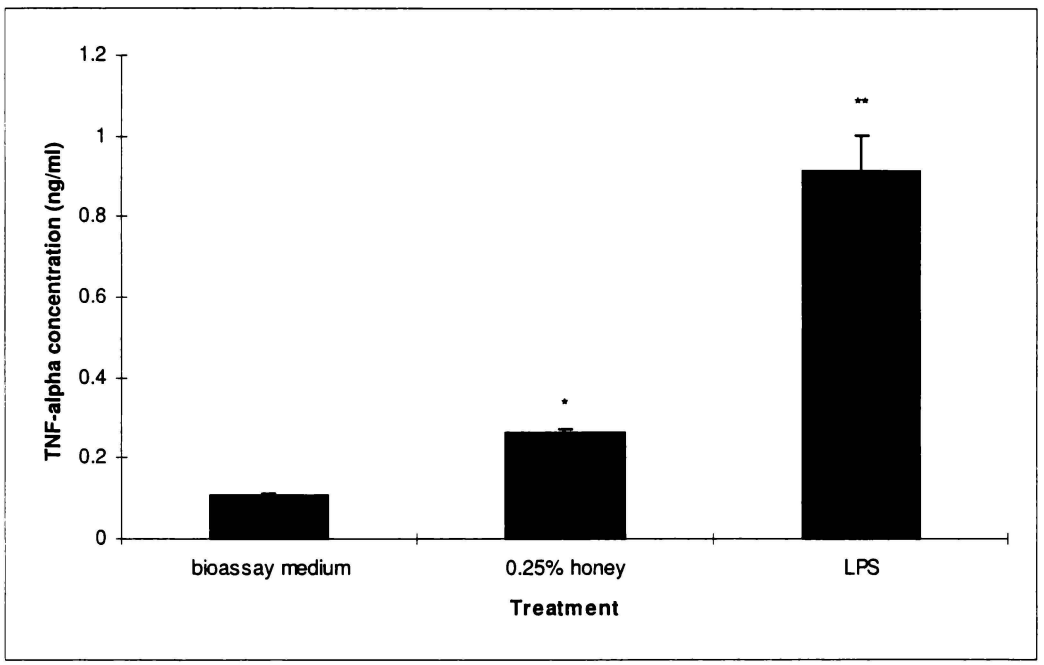


Figure 4.2: TNF-α production by monocytes derived from bovine blood after incubation with LPS or manuka honey. Bovine monocytes were incubated for 6 h with LPS (10 μg/ml), manuka honey at 0.25%, or bioassay medium alone. Supernatants were measured in a WEHI Bioassay. Differences between the means are indicated by *, where means not sharing * are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n=6).

As shown in Figure 4.2, monocytes derived from bovine blood released significant amounts of TNF-α after incubation for 6 h with 0.25% manuka honey compared with the control group (medium alone). Monocytes treated with LPS produced twice as much TNF-α as the honey-treated cells during the same period. These results justified further

investigation to determine how honey activates monocytes and more specifically, production of cytokines involved in tissue repair.

4.3.3 Direct effects of honey on WEHI cells

To ensure that honey had no deleterious effects on WEHI cell survival following incubation with honey, MTT assays were performed as shown in Figure 4.1. Honey alone had no direct effect on WEHI cell viability (and metabolism) at concentrations tested in these assays (0–1% v/v) as determined by optical density. This experiment confirmed that honey would not cause direct effects upon WEHI cells (a fibrosarcoma cell line) and interfere with their use in a Bioassay.

WEHI Bioassays were conducted to measure the amount of TNF-α produced by THP-1 monocytes exposed to various honey concentrations. The THP-1 monocytes were incubated with a range of honey concentrations which were shown to stimulate PBMC activity (Section 2.3.1). The amount of TNF-α produced by the monocytes was determined with the WEHI Bioassay. The effect on TNF-α production of exposing monocytes to various honey concentrations is shown in Figure 4.4.

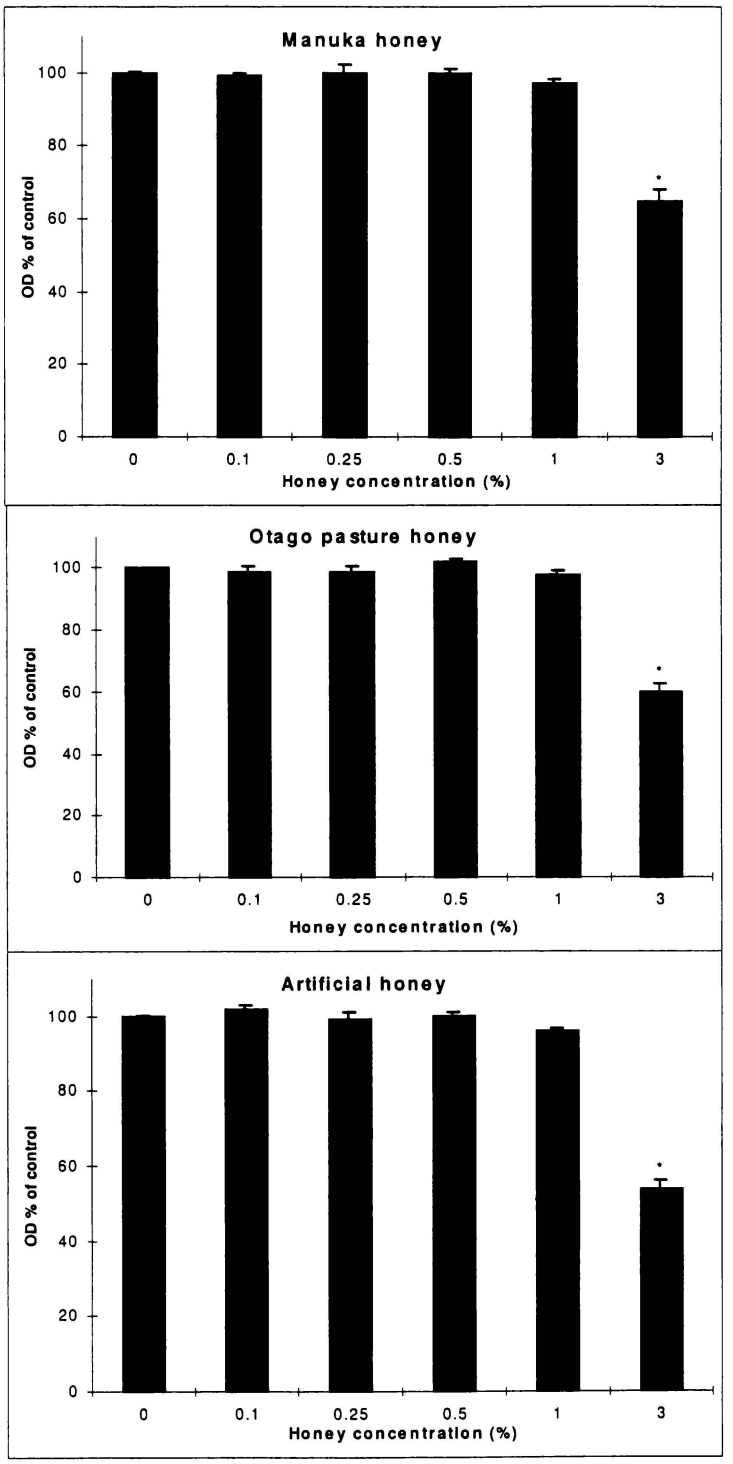


Figure 4.3: The direct effect of honey on WEHI metabolism and viability. WEHI fibrosarcoma cells were incubated with various honeys (manuka, Otago pasture, or artificial) for 48 h and compared with WEHI cultured in bioassay medium alone (100% viable), using an MTT assay. Data are presented as the mean of triplicates ± SEM (n=9). Differences between the means within graphs are indicated by * ($P<0.05$).

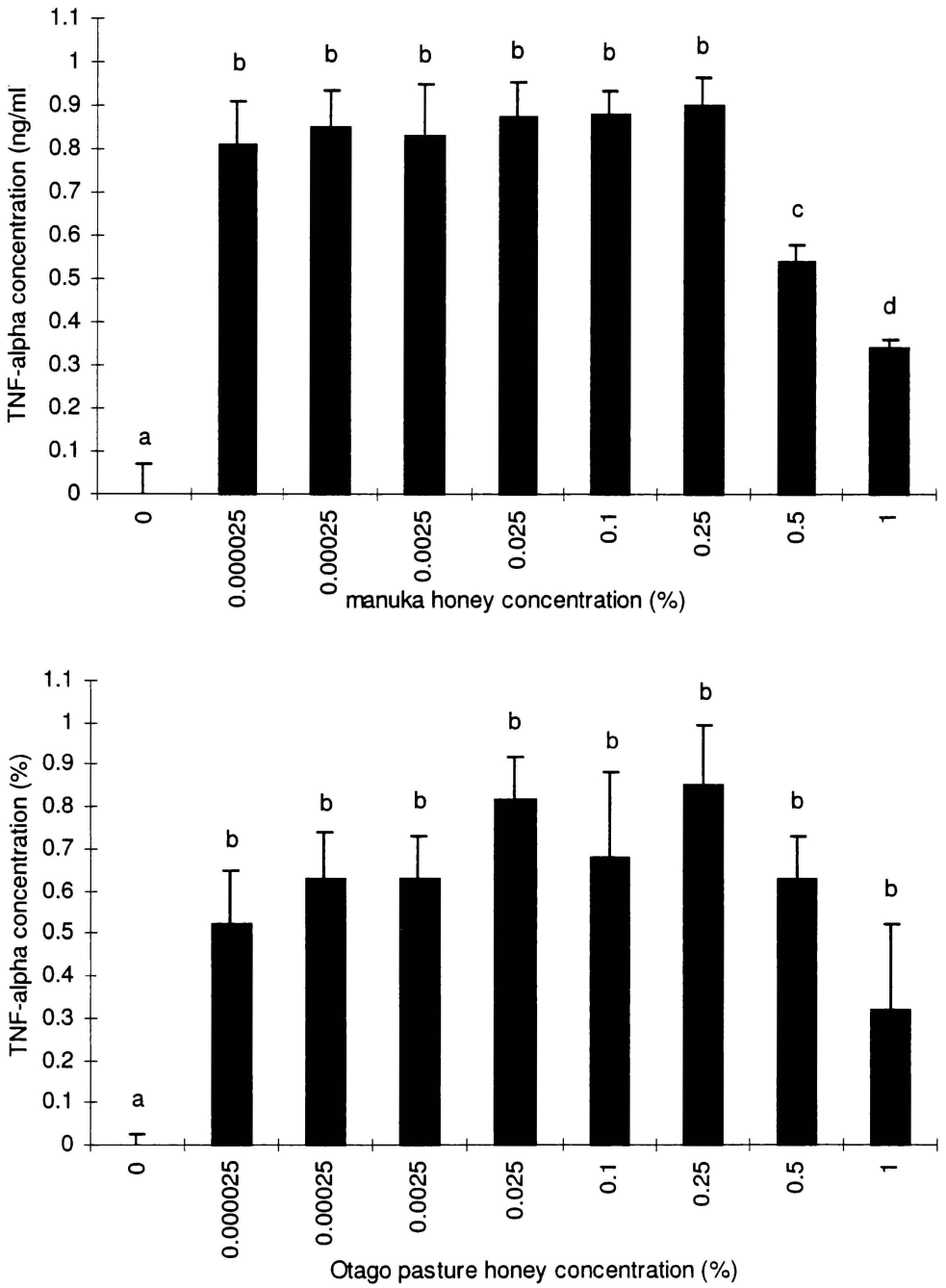


Figure 4.4: Levels of TNF-α produced by THP-1 monocytes exposed to various honey concentrations. THP-1 were incubated for 4 h with various honeys (manuka or Otago pasture) or bioassay medium alone. Supernatants were measured in a WEHI Bioassay. Differences between the means are indicated by superscript letters, where means not sharing common letters within a graph are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from two independent experiments (n=6).

4.3.4 Effect of honey on TNF-α production by resting monocytes

The THP-1 cell line was selected for use in the assays. Both natural honey types elicited TNF-α production by the monocyte cell line beyond that of the bioassay medium alone. In contrast artificial honey had minimal stimulation activity on the monocytes, with levels less than 0.5 ng/ml at all concentrations tested, 0.000025–0.1%.

Maximal TNF-α production was obtained for monocytes incubated with 0.25% manuka honey. Concentrations greater than 0.25 % had decreased TNF-α production. The effects of honey at lower concentrations (0.000025–0.0025%[1]) varied between experiments so that there was no statistical difference between the TNF levels stimulated by this range of honey concentrations. Investigation of the osmotic tolerance range for THP-1 monocytes had detected slight reduction in cell viability when incubated with 1% honey, but not to the same extent as with PBMCs (Section 2.2.7.1). However it was observed that cells still produced significant TNF-α levels despite the slight osmotic problem although less than at 0.25%.

Figure 4.4 shows that Otago pasture honey induced TNF-α production by monocytes at all of the concentrations tested, but that the amounts of TNF-α produced were not significantly different from each other. The size of the error bars indicates that there were reproducibility problems for the amounts of TNF-α produced by monocytes exposed to Otago pasture honey between assays replicated in time. Therefore it was impossible to distinguish any effects between honey concentrations, and yet activity was still exerted beyond medium alone.

The optimal stimulatory dose was determined to be 0.25% honey. A direct comparison of manuka, Otago and artificial at 0.25% concentrations is shown in Figure 4.5.

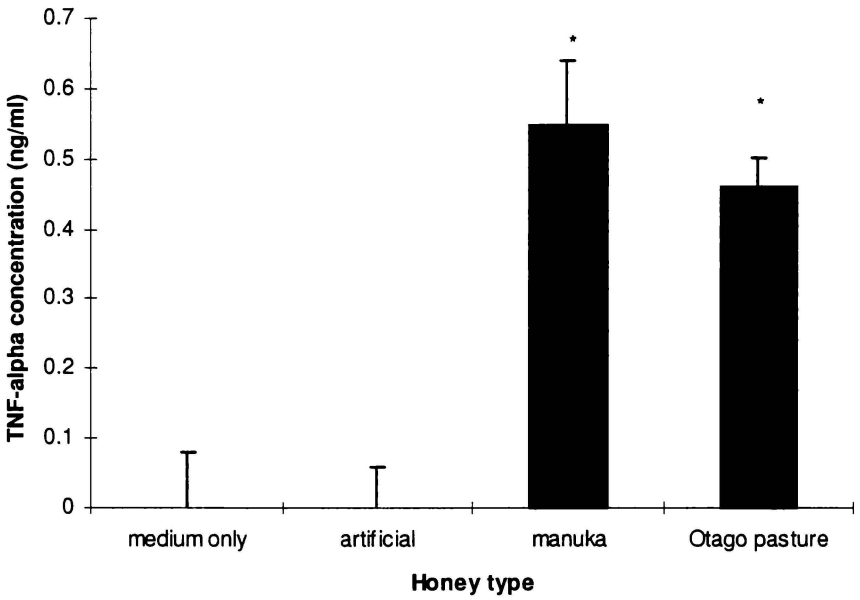


Figure 4.5: Levels of TNF-α produced by THP-1 monocytes exposed to 0.25% honeys. THP-1 were incubated for 4 h with 0.25% concentrations of various honeys (manuka, Otago pasture or artificial) or bioassay medium only. Supernatants were collected and assayed in WEHI Bioassay. Differences between the means are indicated by *, where means not sharing * are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from three independent experiments (n=9).

Monocytes incubated with 0.25% concentrations of manuka honey or Otago pasture honey were activated to produce TNF-α beyond those levels obtained for bioassay medium. Artificial honey had no activating effect. No significant differences were detected between manuka honey and Otago pasture honey for their stimulation of TNF-α production at 0.25%.

In order to identify the fraction of honey responsible for this stimulatory effect, honey was fractionated by dialysis. The effect on TNF-α production of exposing monocytes to fractions from dialysis of manuka honey are shown in Figure 4.6.

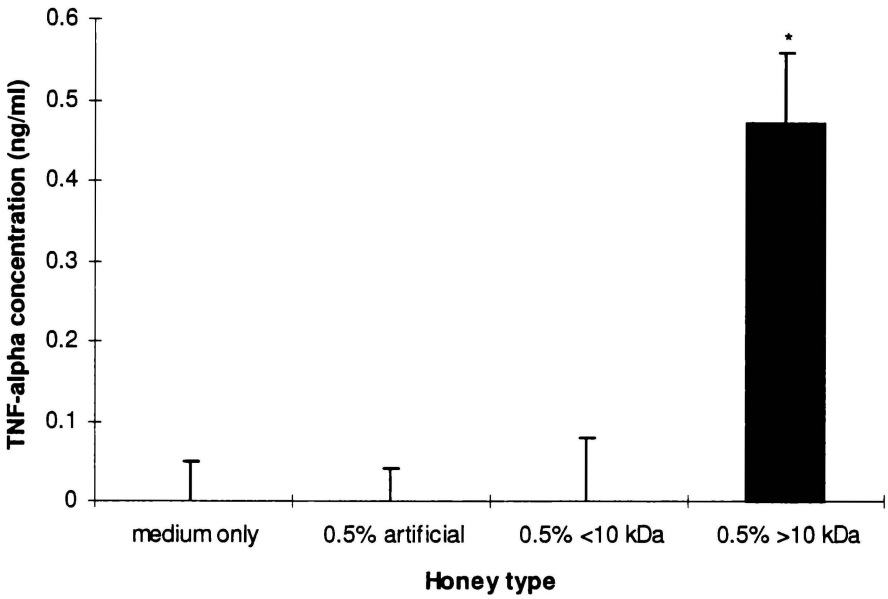


Figure 4.6: Levels of TNF-α produced by THP-1 monocytes exposed to dialysis fractions of manuka honey equivalent to 0.5% honey. THP-1 were incubated for 4 h with fractions of low molecular weight (<10 kDa) or high molecular weight (>10 kDa) manuka honey, artificial honey or bioassay medium only. Supernatants were collected and measured in a WEHI Bioassay. Differences between the means are indicated by *, where means not sharing * are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from three independent experiments (n=9).

While 0.25% concentrations of whole honey were optimal for induction of TNF-α release, 0.25% honey fractions had no activity beyond the bioassay medium alone. Therefore, the range of concentrations of the honey fractions were extended. Incubation of monocytes with the high molecular weight fraction of manuka honey (0.5%) had stimulatory activity, whereas no activity was detected for the equivalent low molecular weight fraction. Artificial honey had no activity. No activity was detected for equivalent fractions of Otago pasture honey beyond medium alone. The concentration of TNF-α was less than 0.1 ng/ml in the control cultures.

As the samples were dialysed and the sugars were removed from the high molecular weight fractions, higher concentrations did not create osmotic problems. The range of concentrations tested were therefore extended for the high molecular weight fractions.

The effect on TNF-α production of exposing monocytes to higher concentrations of dialysis fractions of manuka honey are shown in Figure 4.7.

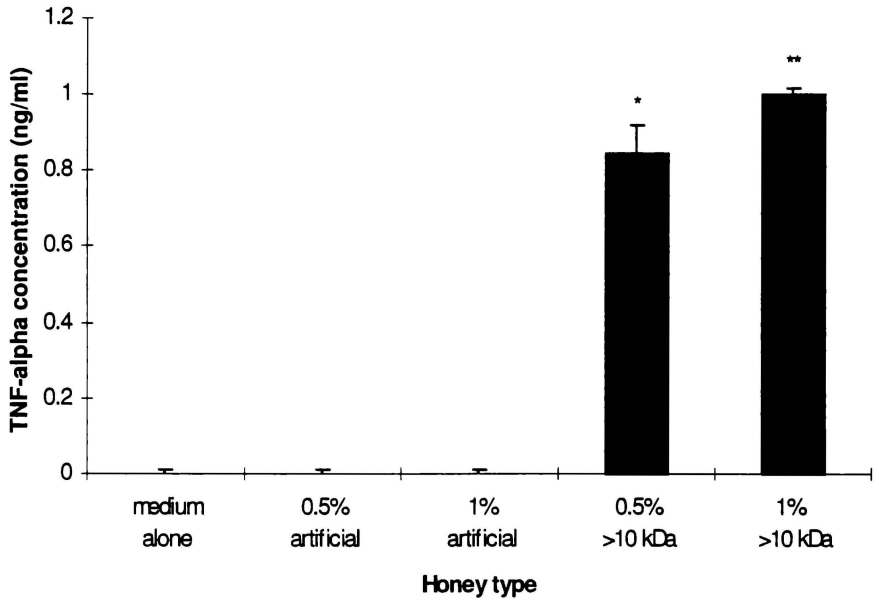


Figure 4.7: Levels of TNF-α produced by THP-1 monocytes exposed to fractions of dialysed manuka honey equivalent to 0.5% and 1% honey. THP-1 were incubated for 4 h with fractions of high molecular weight (>10 kDa) manuka honey, artificial honey or bioassay medium only. Supernatants were collected and measured in a WEHI Bioassay. Differences between the means are indicated by *, where means not sharing * are significantly different ($P<0.05$). Treatments were conducted in triplicate and these means are derived from three independent experiments (n=9).

Both 0.5% and 1% high molecular weight fractions of manuka honey had a stimulatory effect on TNF-α production by monocytes beyond bioassay medium alone. Artificial honey had no activating effect at either concentration tested.

Contrary to the results obtained for whole honey, increased concentration of the fraction led to increased TNF-α production. There were significant differences detected between the 0.5% and 1% high molecular weight fractions, with the 1% fraction exhibiting the greatest stimulatory activity. No activating effect was detected for the low molecular weight fractions of manuka honey at either concentration tested beyond the bioassay

medium alone (0.5% < 10 kDa 0.022 ng/ml ± 0.012, 1% <10 kDa 0.01 ng/ml ± 0.026, bioassay medium alone 0.01 ng/ml ± 0.018).

To study the time-dependent effects of different honeys on TNF-α production, monocytes were exposed to 0.25% concentrations of various honeys for 0–24 h (Figure 4.8).

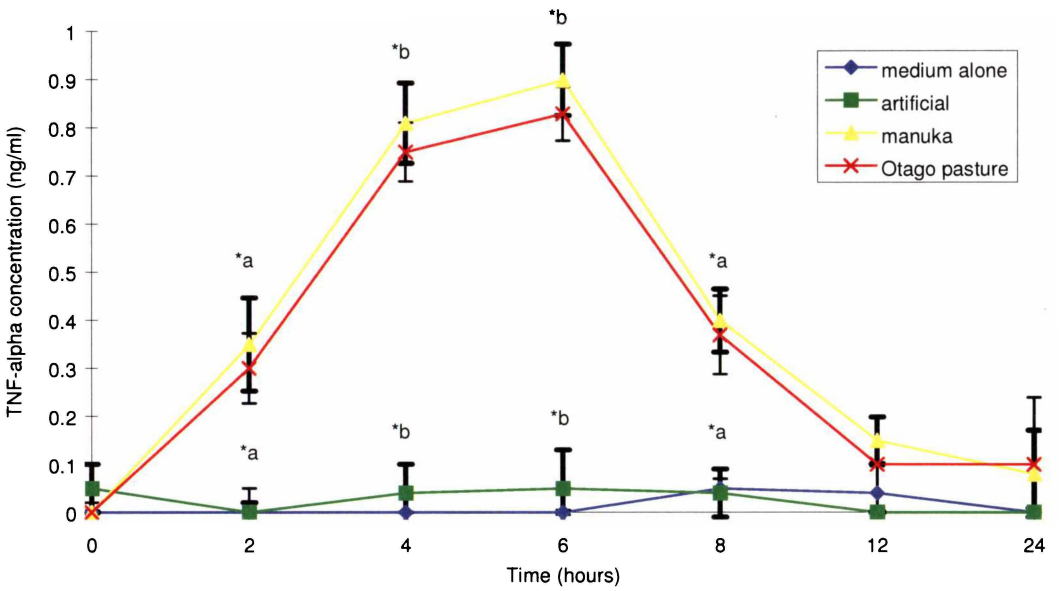


Figure 4.8: Effect of different incubation periods of 0.25% (v/v) honeys with THP-1 monocytes on TNF-α production. THP-1 were incubated with 0.25% honey (artificial, manuka or Otago pasture types) or bioassay medium alone for various periods. Supernatants were collected and measured in a WEHI Bioassay. Each point represents the mean for each treatment (conducted in triplicate). Error bars show SEM (n=9). Significant differences between the results at each time point and at the starting time (*) and between types of honey at each time point (indicated by superscript letters where means not sharing significant letters are significantly different) are shown (P<0.001 analysed by ANOVA and Tukey's pair-wise comparisons).

Incubation of monocytes with either manuka or Otago pasture honeys (at 0.25% concentrations) for times between 2–8 h, significantly increased their TNF-α production beyond bioassay medium alone. After 12 h exposure to 0.25% honeys, the levels of TNF-α detected in the supernatants had returned to baseline levels. TNF-α production peaked when the monocytes had been incubated with the honeys for 4–6 h. Sugars alone had no activating effect on TNF-α production at any time. The activity of manuka honey and Otago pasture honey was not significantly different from each other at any time-point.

4.3.5 Effect of honey on TNF-α production by LPS-stimulated monocytes

To examine whether honey can modulate TNF-α production by monocytes after LPS-stimulation, cultures were exposed to various manuka honey concentrations for 4 h following 24 h incubation with LPS (Figure 4.9). TNF activity in LPS-stimulated monocyte cultures exceeded immunoassayable TNF-α in the WEHI Bioassay (>1 ng/ml). This meant that treatment effects of honey on LPS-stimulated monocytes could not be determined using the WEHI Bioassay, and therefore a commercially available TNF ELISA was used, and the assay repeated.

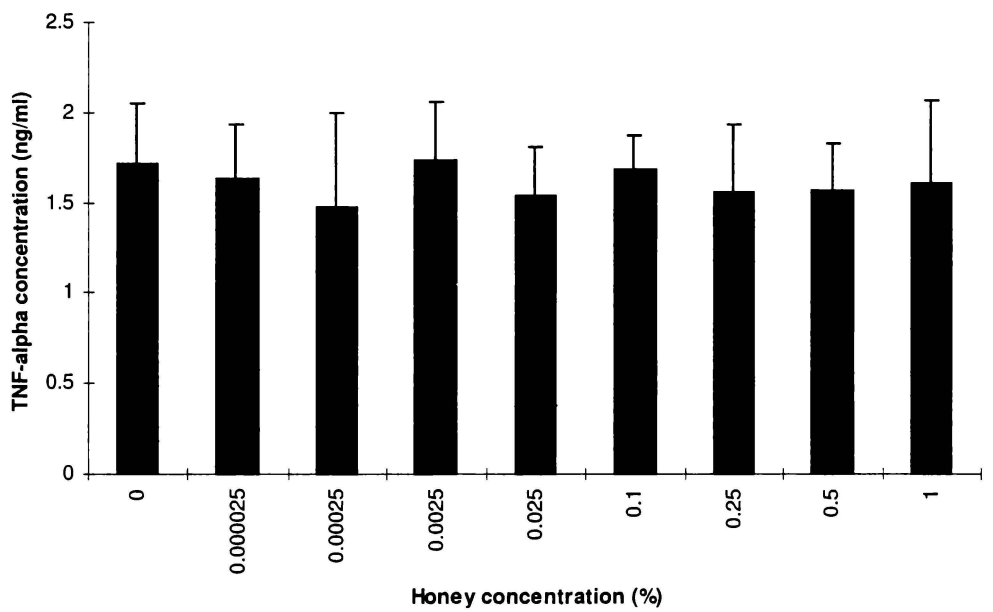


Figure 4.9: Effect of exposing LPS-stimulated THP-1 monocytes to various manuka honey concentrations on TNF-α production. THP-1 were incubated with LPS (10 μg/ml) for 24 h prior to various manuka honey concentrations or bioassay medium alone and the cells incubated for a further 4 h. Supernatants were collected and measured in a TNF-α ELISA. Each point represents the mean for each treatment (conducted in triplicate). There were no significant differences between the means ($P<0.05$). These means are derived from two independent experiments (n=6).

The results in Figure 4.9 show honey had no measurable effect on the amount of TNF-α produced by monocytes after they had been pre-exposed to LPS for 24 h. Assays were then conducted to investigate whether manuka honey (0.25% and 0.5%) could interfere with the TNF production by LPS-stimulated monocytes when cell exposure time to LPS was reduced (2–24 h). There was no significant difference in the effect of honey on the TNF response by LPS-stimulated monocytes when exposure time with LPS was reduced (data not shown). Honey could not modulate the production of TNF protein after the cells were pre-exposed to LPS for a minimum of 2 h. The effect of increasing the amount of time LPS-stimulated monocytes were exposed to manuka honey (0.25% and 0.5%) from 2–6 h had no effect on TNF-α production. Of further note, a comparison of Figure 4.4 and Figure 4.5 with Figure 4.9, demonstrates that LPS stimulated TNF-α production more strongly than honey had done.

4.3.6 Validation of TNF-α determinations from the WEHI Bioassay by ELISA

The purpose of these assays was to compare the determinations from the WEHI Bioassay with those obtained using a commercially available ELISA. Briefly, THP-1 cultures were stimulated with either LPS (10 μg/ml), manuka honey (0.025–1%) or bioassay medium alone for 4 h and the TNF protein content in the supernatants was then determined for duplicate samples and standards by both the WEHI Bioassay and a TNF-α ELISA (Table 4.1).

The data presented in Table 4.1 demonstrate that a good correlation was found between the ELISA and WEHI Bioassay when TNF-α was measured in supernatants. This confirms the observations which have been reported in the current study.

Table 4.1: Comparison of total TNF (ng/ml) detected in supernatants from monocytes exposed to various treatments using a commercial TNF ELISA and a WEHI Bioassay. THP-1 cultures were stimulated with either LPS (10 μg/ml), manuka honey (0.025–1%) or bioassay medium alone for 4 h and the TNF protein content in the supernatants was then determined using both assay methods. Treatments were conducted in duplicate and the data represent the mean (± SEM) of two separate assays. (>1 means the levels were beyond the detection limits of the Bioassay).

Treatment	ELISA (ng/ml)	WEHI BIOASSAY (ng/ml)
LPS	0.97 ± 0.08	>1 ± 0.04
1% manuka	0.32 ± 0.001	0.38 ± 0.07
0.5% manuka	0.54 ± 0.02	0.57 ± 0.004
0.25% manuka	0.48 ± 0.001	0.51 ± 0.08
0.025% manuka	0.65 ± 0.09	0.67 ± 0.05
0.025% manuka + polymyxin B	0.59 ± 0.02	0.53 ± 0.003
Bioassay medium	0.16 ± 0.003	0.21 ± 0.004

4.4 DISCUSSION

The physiological relevance of the investigations described here were twofold; to see whether honey could stimulate release of TNF-α in resting monocytes, a key cytokine involved in the early phase of tissue repair (Belge *et al.*, 2002). Further, to investigate whether honey could modulate the production of TNF-α by LPS-stimulated monocytes with view to its potential use as a mediator of excessive TNF production as occurs in the pathogenesis of some inflammatory diseases (Lefkowitz and Lefkowitz, 2001).

The observation that 1% honey elicits low levels of TNF-α release by monocytes (0.2–0.5 ng/ml) corroborated the earlier findings by Tonks *et al.* (2003; 2001). However, the current study is the first to demonstrate that very low concentrations of honey can induce

TNF-α release by resting monocytes (2.5×10^{-5}–1×10^{-1}). These results have important consequences for the potential use of honey.

Of further note, the results presented in the current study show that the 1% honey concentration tested by Tonks *et al.* (2003; 2001) was not optimal for TNF induction which was obtained at a 0.25% concentration. It is possible that removal of cytotoxic/osmotic effects would have occurred with progressive dilution of honey below 1%. Reduction in glucose content due to dilution of honey would contribute to removal of osmotic effects. The observation that natural honeys did not cause osmotic damage to THP-1 monocytes at 1%, unlike an equivalent concentration of artificial honey (sugars alone), despite having the same osmolarity, may indicate the presence of protective agents in honey. The existence of both an anti-inflammatory and a stimulatory component in honey and each exerting their effects at opposite ends of the dilution range (0–1%) cannot be ruled out at this stage. Further fractionation of honey to isolate the stimulatory agent needs to be undertaken so that this can be resolved.

Timing of measurement for TNF content in the supernatants is known to be critically linked to the amount of cytokine which will be free in the suspension. After monocytes had been cultured with honey for 6 h the levels of TNF protein detected in the supernatants decreased. It is likely that the TNF is either taken up by TNF receptors on the monocyte cells or broken down by proteases. Further, TNF is a fragile cytokine with a short half-life, breaking down quickly after production and cannot be detected in culture after a fairly short time (up to 80 min) (Smart, 1995). Various factors such as temperature at which the medium is maintained will have further impacts on the half-life of this cytokine (Smart, 1995). If monocytes continually respond to the honey stimulus a equilibrium level of TNF-α will be reached where the rate of breakdown becomes the same as the rate of synthesis, and this may explain the peak in TNF-α production observed after 4–6 h incubation. Of further note here, in Section 3.3 it was observed that transcription of mRNA for TNF-α was significantly down regulated after blood had been exposed to honey for 12 h. In Section 3.3, the implications for blood handling *ex vivo*

prior to performing any assay were discussed. A study by Baechler *et al.* (2004) reported that TNF-related genes and TNF itself is critically sensitive to *ex vivo* handling of cells and specifically, incubation time.

The time-dependent effects of honey reported in the current study confirm those observed by Tonks *et al.* (2003) and indicate that a 4-6 h incubation period of monocytes with honey gives maximal TNF-α levels. This optimal stimulation period may relate to cell-cytokine mechanisms only, and have no bearing on the nature of the stimulatory agent in honey. Saitoh *et al.* (2003) have reported that the induction of TNF-α is rapid due to its preformed existence in mast cells and its release by T cells and monocytes, early arrivals to the site of tissue damage, but that its induction is only transient. It has been reported that delivery of hydrogen peroxide by honey decreases over time and the rate of this decline is dependent on the rate at which catalase breaks down hydrogen peroxide (Bang *et al.*, 2003). However, it is not yet understood what the implications of these observations made in higher concentrations of honey (optimal at 50%) are for the very low concentrations shown to have immunological activity in the current study. Maximum levels of hydrogen peroxide are produced after 30 min when samples are incubated at 35°C, and production is reported to decrease steadily after this time (Kerkvliet, 1996). Even when ideal conditions are maintained, the activity of the glucose oxidase enzyme declines to a low, basal rate after 24 h (Buntting, 2001). Further, White and Subers (1964) have reported that the glucose oxidase enzyme operates most efficiently within a narrow range of pH (with an optimum pH of 6), and this is likely to be altered through changes in medium composition over time, though monitoring pH during the current study did not detect any pH fluctuations. White blood cells themselves produce catalase, with levels produced ranging from high amounts by neutrophils to very little by monocytes (Sagone *et al.*, 1984). The consequences of this activity are unclear, but may have implications for the research described here if hydrogen peroxide was indeed shown to play an important role in monocyte function.

The absence of LPS in contamination cultures as demonstrated by culturing cells in the presence of polymyxin B confirms that observed stimulation of TNF release from monocytes by honey cannot be due to endotoxin content. The role of sugars alone (represented by artificial honey) on stimulation of TNF-α production was also excluded in the current study.

The levels of TNF which are required for tissue healing to be initiated *in vivo* are not readily transferable to *in vitro* culture. The role of TNF-α in tissue repair has been demonstrated *in vivo* and is known to accelerate wound epithelialization and neovascularization (Frank *et al.*, 2003). The mechanisms by which TNF promotes tissue repair include; mitogenic effects on fibroblasts, stimulation of collagen synthesis and capillary formation possibly through synergistic actions with platelet-derived growth factor (Fu *et al.*, 1996). Further, a single dose of exogenous TNF to an experimental wound was found to increase both the RNA and DNA content of regenerative tissue (Fu *et al.*, 1996). That low concentrations of honey induce monocytes to produce TNF-α increases the likelihood that honey may optimise the damaging and killing activity of activated macrophages in damaged tissues.

The observation that the *in vitro* effect of exposure to honey on monocyte TNF production was clearly distinct in its magnitude from that observed after exposure to LPS, an agent involved in endotoxic shock. The observation that honey has no additive effect on LPS-stimulated TNF-α production is important for its potential use as a therapy, as TNF-α contributes to the pathogenesis of a range of inflammatory diseases when produced at high levels (Joyce *et al.*, 1997). That honey stimulates the release of modest, though statistically significant amounts of TNF-α compared with LPS, means that it is unlikely to contribute to production of the cytokine at levels which lead to pathogenesis. Yet, the significant levels of TNF-α released by honey-stimulated monocytes may be of considerable significance if sufficient to induce tissue repair processes.

Although the active agent has not been identified, it is characterised by its presence in a high molecular weight fraction exceeding 10 kDa. The observation that fractionated honey did not produce activity in the same manner as whole honey has implications for the identity of the active agent. In the high molecular weight fraction, sugars have been removed and it is likely the cells will respond differently to these fractions compared with whole honey. It may be a consequence of interrupting some synergy existing within whole honey. The observation that there was much less activity in the fractions of 0.25 % honey than in the original 0.000025% honey concentration raises several possibilities for the nature of the active component in the honey. The >10 kDa fraction would have contained the glucose oxidase enzyme (which breaks down glucose to produce hydrogen peroxide) but not the glucose substrate. However, the bioassay medium, containing RPMI-1640, had a glucose content of 2000 mg/l meaning that some of its substrate would have been available when the >10 kDa fraction was diluted with medium in the wells. However, the availability of the glucose substrate would be greater for the enzyme contained within whole honey due to its glucose content. A further possibility may be that the FAD cofactor for glucose oxidase (needed for the enzyme to function) had been dialysed out. It is not currently known how the FAD cofactor is bound to the glucose oxidase enzyme contained in honey, and therefore the ease with which it may be removed during fractionation processes (P. C. Molan; personal communication).

Catalase is present in foetal calf serum and acts to metabolise hydrogen peroxide, with the potential to negate the tissue-stimulatory effects produced by the presence of hydrogen peroxide in honey. Hydrogen peroxide has been demonstrated to modify the redox status of various cells and cause corresponding changes in the activation of the transcription factor NF-κB, leading to changes in cellular activity and function (Los *et al.*, 1995). Therefore, it is very likely that hydrogen peroxide could activate monocytes. That natural honey promotes TNF-α release by unprimed monocytes fits well with what is observed clinically, the optimisation of wound healing when honey is used.

It was noted in Section 2.4 that various cells, including monocytes, possess receptors for glycated proteins. Binding of glycated proteins has been reported to trigger the release of cytokines by monocytes (Brownlee, 1995; Wolff *et al.*, 1991) and it is likely that honey contains these. As the dialysis retentate fraction had a potent stimulatory effect on monocytes, it would therefore be useful to examine the possible contribution of protein content to induction of TNF. Of further note, the equivalent retentate fraction of pasture honey did induce TNF release, and this is likely to reflect the difference in composition of the two honeys, (such as manuka honey having more protein).

The results of the study undertaken here have highlighted a multitude of investigations which need to be performed in order to fully understand the implications of these results. The extent to which the *in vitro* model used here differs from the *in vivo* situation means that the results of this study are not readily transferable in the absence of further testing. However, there is no evidence that these *in vitro* results could not be realised *in vivo*.

Chapter Five: Effects Of Honey On NO Production By PBMCs And Monocytes

SUMMARY

To test whether honey could modulate LPS-stimulated NO production by THP-1 monocytes and bovine peripheral blood mononuclear cells, Greiss assays were performed. Both manuka and Otago pasture honey at 0.5% and 1% concentrations suppressed LPS-induced nitrite release in a dose-wise manner, indicating modulation of nitric oxide production. Manuka honey had a more potent modulating effect on LPS-driven nitrite production than pasture honey, and maintained activity at 0.25%. Sugars alone had no effect. High molecular weight dialysis fractions of either honey contained the activity, but some of the activity was lost by fractionation. An ether extract of manuka honey led to the greatest modulation of nitrite production by LPS-stimulated monocytes.

5.1 INTRODUCTION

It has been established by the current study that honey downregulates mRNA expression for the inducible nitric oxide synthase enzyme (iNOS) by LPS-stimulated blood cells (chapter 3), and the investigations described here were undertaken to determine whether this also correlated with decreased production of nitric oxide (NO) protein. The critical role played by NO in the pathogenesis of inflammatory diseases (*e.g.* colitis) is well established (Childress and Stechmiller, 2005; Rizk *et al.*, 2004; Bansal and Ochoa, 2003; Bilsel *et al.*, 2000; Chen *et al.*, 1998; Grimble, 1994; Ochoa *et al.*, 1991; Marletta, 1988), and therapeutic agents which can ameliorate NO production are of immense value.

NO has a diverse range of activites, both anti and pro-inflammatory depending on the context in which it operates and the interactions undertaken with other mediators. NO is known to impair wound healing and lead to tissue damage by increasing vascular permeability (ligand mediated and flow dependent), thereby enabling infiltration of tissues by leukocytes (Rizk *et al.*, 2004; Schwentker and Billiar, 2003). NO inhibits leukocyte adhesion to vascular endothelium, inhibits platelet adhesion to vascular endothelium (anti-thrombotic), and anti-proliferative

Once present in tissues, hydrogen peroxide released by leukocytes during their host defense response gives rise to the superoxide radical, which in turn may combine with NO to form peroxynitrite (Zhu *et al.*, 1992). Peroxynitrite is a highly unstable free radical which is reported to cause loss of normal cell function due to damaging effects on DNA (Rizk *et al.*, 2004; Patel *et al.*, 1999; Inoue *et al.*, 1993).

In Section 3.1, the involvement of NO in cell signalling was outlined. The substrate for NO is L-arginine, and after being transported into the cell it is acted upon by nitric oxide synthase (NOS), forming NO and citrulline (Rizk *et al.*, 2004). NO is continuously produced by constitutive NO synthase (cNOS), and the activity of cNOS is modulated by calcium. Substances such as bradykinin, histamine, insulin and substance P stimulate

release of calcium when ligands are bound to their receptors, and therefore, stimulate NO production by this mechanism. Shear forces acting on the luminal surface of vascular endothelium also stimulate calcium release and therefore, increased cNOS activity. The inducible form of NO (iNOS) is not calcium-dependent, but is stimulated by cytokine activity (*e.g.* IFN-γ, TNF, interleukins such as IL-1), is controlled by transcription factors such as NF-κB, IFN-regulatory factor-1, and bacterial endotoxins (*e.g.* LPS) (Rizk *et al.*, 2004; Bansal and Ochoa, 2003; Chen *et al.*, 1998). Induction of iNOS occurs over several hours and produced 1,000-fold more NO than by cNOS. However, cytokines such as TGF-β, IL-4 and IL-11 have been found to suppress stimulated iNOS expression in macrophages (Rizk *et al.*, 2004; Chen *et al.*, 1998). After diffusion across cell membranes, NO is rapidly consumed due to its high reactivity with a diverse range of molecules. NO binds to protein receptors in the cell, being either a metal ion in the protein or one of its S atoms, and this triggers an allosteric change in the protein and the formation of a second messenger (*e.g.* cyclic GMP, cGMP) (Rizk *et al.*, 2004; Bansal and Ochoa, 2003; Chen *et al.*, 1998). The cGMP then acts to initiate cytokine release.

As noted in Chapter 3, cytokines do not operate in isolation and when production of a single cytokine changes, this will have profound effects on cellular activation and the inflammatory response of an individual ((Rizk *et al.*, 2004; Bansal and Ochoa, 2003; Chen *et al.*, 1998; Lamas *et al.*, 1991; Ferrante *et al.*, 1988). For this reason, when NO is produced at high levels it is associated with the onset of chronic inflammation (Cobbold and Sherratt, 2000). It should be noted however, that NO is necessary for wound healing to occur, playing a vital role in host defense (Wink and Mitchell, 1998), and in tissue repair (Witte and Barbul, 2002). NO is needed for collagen deposition, cell proliferation and wound contraction (Witte and Barbul, 2002). Total inhibition of NO would have dire consequences for immune function (Patel *et al.*, 1999) and tissue repair (Witte and Barbul, 2002).

There is some evidence that honey can modulate NO production. A study by Bilsel *et al.* (2002) tested the anti-inflammatory effect of 50% honey on experimentally induced

colitis in rats. Applied as an enema, the honey had an effect on colitis through prevention of free radical release and a decrease in NO production. In addition to anecdotal observations that honey reduces inflammation (Molan, 2004), Tonks *et al.* (2001) reported that levels of reactive oxygen intermediates were reduced by addition of honey to mitogen-stimulated monocytes. This would suggest that honey could decrease the formation of peroxynitrite, and that this might account for anecdotal observations that honey alleviates tissue damage. Therefore, the following investigations were designed to test the hypothesis that the ability for honey to modulate NO production is key to the mechanism by which honey has anti-inflammatory effects. Many different cell types produce NO, and among these the macrophages constitute a key group (Wallace *et al.*, 2000). To investigate whether honey could modulate NO production, LPS-stimulated THP-1 monocytes were used to provide an established inflammatory model *in vitro* of tissue macrophages (Jagannath *et al.*,1998; Chen *et al.*, 1996; Mautino *et al.*, 1994). To determine whether honey may have any effect on NO production by immune cells it was necessary to consider both; (a) how much NO was being produced and (b) how much NO was actually being removed by various agents in an *in vitro* system, by using Greiss assays.

5.2 MATERIALS AND METHODS

Nitrite and nitrate are both formed from the reaction of NO with oxygen and water, so that quantification of nitrite levels by the Greiss reagent provides a reliable measure for NO (Martin and Edwards, 1993). Therefore, use of the Greiss reagent to measure nitrite (a stable metabolite of NO) enabled the effect of honey on NO levels in PBMC culture to be investigated. Prior to adopting the Greiss method, the more sensitive method described by Turner (2001), enabling quantification of the conversion of NO_3 to NO_2, was utilised. Unfortunately several attempts to follow this method established that honey distorts the modified Greiss assay, and this was abandoned for the less sensitive Greiss method. For the purposes of this thesis however, a simple demonstration of the effects of honey on nitrite levels in PBMC cultures and THP-1 monocytes was deemed adequate.

The method used was adapted from Martin and Edwards (1993). NO production was determined as the amount of stable end product (nitrite) in the PBMCs or THP-1 culture medium. This is because NO is very labile, with a half-life of only seconds (Marletta *et al.*, 1988).

5.2.1 Cell cultures

Preparation of the PBMC cultures was performed as described in Section 2.2.3.2. The THP-1 monocytes were maintained according to the procedures described in Section 4.2.2.

In separate experiments PBMCs or THP-1 monocytes were placed in 96-well plates at a density of 1 x 10^6/ml. The cell densities were adopted from the methods described by Tonks *et al.* (2003) and Jungi *et al.* (1996). The cell suspension contained complete medium. Cell viability (determined by trypan blue exclusion) was >98% throughout the culture period.

5.2.2 Reagents

LPS solutions

As described in Section 3.2.1, a 1 mg/ml stock solution (*E. coli* serotype 0111: B4; Sigma-Aldrich UK; cat no# L4516) was purchased. The LPS was diluted to final concentrations in wells of 10 μg/ml (unless otherwise stated).

Concanavalin A (Con A)

As described in Section 2.2.1, a 500 μg/ml stock solution was prepared. Unless otherwise stated, the Con A stock solution was further diluted to final concentrations of 5 μg/ml in wells.

Sodium nitrite ($NaNO_2$)

A 100 mM stock was prepared by dissolving 6.9 mg of $NaNO_2$ in 1 ml of RPMI medium.

Greiss reagent

The reagent was freshly prepared by mixing equal parts of 0.2% N-[naphthyl]ethylenediamine dihydrochloride in H_20 and 2% sulphanilamide in 5% phosphoric acid (H_3PO_4) (Wako Fine Chemicals).

5.2.3 Honey solutions

It had been established in Section 4.3.1 that THP-1 monocytes had a different osmotic tolerance from PBMCs (Section 2.2.7). At the 0.5% (v/v) honey concentration, the sugars contained in honey begin to have negative effects on PBMCs, and at 1% (v/v) the osmotic effects on the cell population is significant. Visual inspection of the cell populations revealed that a high proportion of lymphocytes, monocytes and macrophages had osmotic damage. Although some signs of osmotic stress were observed for lymphocytes at the 0.5% concentration, only slight cell damage was noted for monocytes and macrophages at this honey concentration. However, THP-1 cells tolerate the higher 1% (v/v) honey concentration. With this in mind the honey concentrations tested in the Greiss assays described were restricted to a maximum of 0.25% (v/v) for PBMC and 1% (v/v) for THP-1 monocytes. Solutions of M109 manuka honey, Otago pasture honey, and artificial honey in RPMI complete medium were prepared as in Section 2.2.2. Dialysed fractions of honey were prepared in RPMI complete medium as in Section 2.2.2.1. An ether extract of manuka honey (containing the ether-soluble components of the honey) was made as described in Section 2.2.2.1. The pH of the honey solutions was measured using a pH meter (Mettler Toledo) and did not differ significantly from medium alone.

5.2.4 Measurement of NO levels

5.2.4.1 Preparation of Greiss calibration curve

To enable the quantification of nitrite in suspension, a calibration curve was prepared for each assay by diluting the $NaNO_2$ standard within the range 0–100 μmol/L. The RPMI complete medium alone provided the blank (0 ng/ml $NaNO_2$). For each standard, 75 μl was loaded in duplicate to wells of a 96-well flat bottom plate, and 25 μl of the Greiss reagent added. The plate was incubated for 10 min at room temperature, protected from light, and the absorbance read spectrophotometrically at 550 nm (BioTek) within 30 min. The average absorbances were calculated for each of the standards, and the average absorbance from the blank wells subtracted. The mean absorbance values for each standard dilution were then plotted against the standard concentration of $NaNO_2$ (as given in Appendix 2). The nitrite concentration of each sample could then be determined by interpolation from the standard curve. The assay had a lower limit of detection of 1.56 μM.

5.2.4.2 Determination of NO content in cell suspensions

After incubation of the cells, the plates were centrifuged (Sorvall, 503 x *g*, 15 min) to pellet the cells and 75 μl of each supernatant was transferred to a separate well of a sterile 96-well flat bottom plate. All samples were loaded in triplicate. Samples were diluted with diluent so that detectable nitrite fell within the sensitivity range of the assay. The Greiss reagent was added in 25 μl volumes to the supernatant and the plate was incubated for 10 min at room temperature. Optical density was measured at 550 nm (BioTek) and the control values subtracted from treatment wells. The total nitrite produced following treatment was then determined from a standard curve.

5.2.5 Statistical Analyses\

Values were expressed as the mean of triplicate wells ± SEM. All data presented have been adjusted for background absorbance. Each experiment was replicated in time at least twice (n=6). For multiple group comparisons, the data were subjected to one-way analysis of variance (ANOVA) to determine overall differences between the group means. The group effect was the amount of NO produced. Significance was further analysed using Tukey's honestly significant difference for pair-wise differences for within group comparsions. Results were accepted as statistically significant when $P<0.05$. Systat (version 7) was used for all analyses.

5.2.6 Preliminary experiments

Comparison of THP-1 and PBMCs for NO production

To establish whether the THP-1 monocytic cell line would provide a more sensitive model for NO measurement than bovine blood-derived PBMCs, the Greiss assay was performed for both cell types. This had the additional benefit of assessing whether the results obtained from using a cell line had physiological relevance, by comparing them with results obtained from primary cells (PBMCs). Preliminary assays showed that adding LPS alone to PBMCs did not trigger a sufficient response for sensitive detection of nitrite by the Greiss method. Addition of Con A stimulates T-cell proliferation by initiating cytokine production and subsequently, bystander activation (Miller *et al.*, 1991; Young *et al.*, 1994). Prior stimulation of the THP-1 monocytes with Con A before addition of the treatments was not required.

To induce production of NO, the cells were incubated with Con A, LPS or both. Suspensions of PBMCs or THP-1 monocytes were loaded in 980 µl aliquots into wells of a 24-well plate at final densities of 1 x 10^6/ml. Triplicate wells of PBMCs received 10 µl of Con A solution (500 µg/ml) and incubated for 24 h (37°C, 5% CO_2, 95% air), prior to 10 µl of a LPS solution (10 µg/ml). THP-1 monocytes only received 10 µl of LPS

solution (10 μg/ml). All cells were incubated for 24 h (37°C, 5% CO_2, 95% air) with LPS. Control wells for both cell types received 10 μl of RPMI complete medium only. The OD values obtained for control wells after a Greiss assay was performed were subtracted from OD values obtained for treatment wells.

The effect on NO production of incubating PBMCs and THP-1 cells with LPS are shown in Figure 5.1.

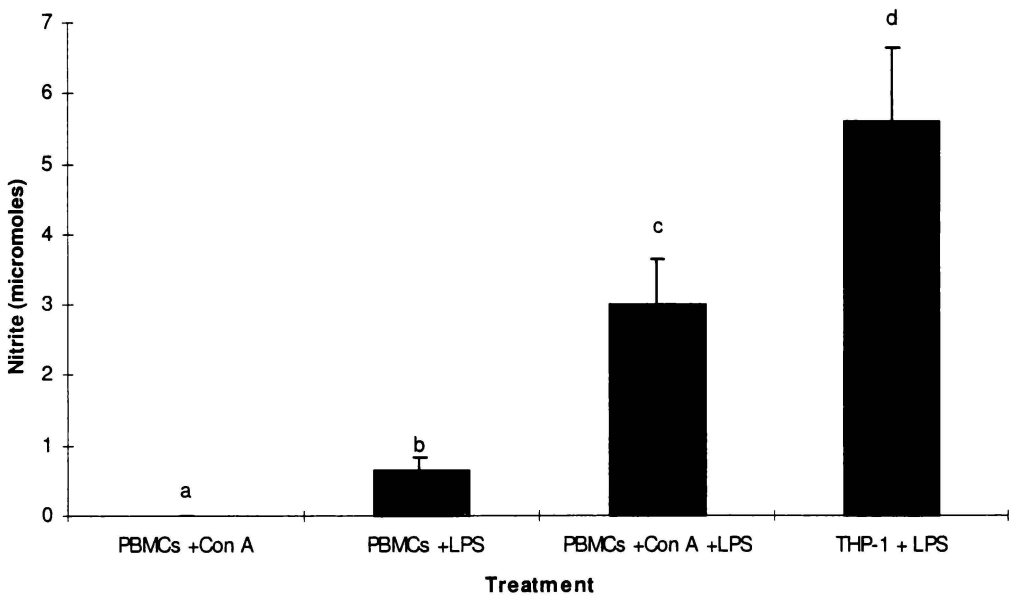


Figure 5.1: Nitrite concentration of supernatants from stimulated PBMCs and THP-1 cell suspensions. PBMCs were each incubated with Con A (5 μg/ml) for 24 h prior to addition of LPS (10 μg/ml) and the cells incubated for 18 h (37°C, 5% CO_2, 95% air). THP-1 monocytes were incubated with LPS (10 μg/ml) alone for 18 h (37°C, 5% CO_2, 95% air). Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P<0.05$). These means are derived from three independent experiments (n=9).

LPS-stimulated THP-1 monocytes produced significantly higher levels of nitrite than any of the stimulated PBMC cultures. Exposure of the PBMCs to LPS alone led to production of nitrite beyond levels obtained when cells were incubated with RPMI complete medium

alone. However these levels were significantly lower than those obtained for LPS-stimulated THP-1 cells. Exposure of the PBMCs to Con A for 24 h prior to LPS-stimulation increased nitrite production beyond that obtained for PBMCs incubated with LPS alone, but at significantly lower levels than for THP-1 cells with LPS alone. There were no detectable levels of nitrite in the supernatants from PBMCs incubated with Con A alone, which confirmed that Con A was not inducing nitrite production. As THP-1 monocytes produced higher levels of NO and did not require pre-incubation with Con A, all future nitrite measurements were performed using these cells. However, key assays were repeated using PBMC to verify that results obtained were not restricted to the THP-1 cell line.

5.2.7 Experimental design

5.2.7.1 Measuring the direct effect of honey on NO production by mononuclear cells

To measure the direct effect of honey on NO production by PBMC and THP-1 monocytes, cells were incubated with various honey concentrations for 24 h prior to conducting a Greiss assay. Suspensions of PBMCs or THP-1 cells in RPMI complete medium were loaded in 950 µl aliquots into wells of a 24-well flat bottom plate at final densities of 1 x 10^6/ml. Cells were incubated with honey (artificial, manuka, Otago pasture) at 0.25% (v/v) for PBMCs and 1% concentrations (THP-1). The honey concentrations were added to triplicate wells of the 24-well plates in 50 µl aliquots and the cells incubated for 24 h (37°C, 5% CO_2, 95% air). Controls of cells incubated with RPMI complete medium only were included as a basis for comparison, and the OD values obtained for the controls were subtracted from OD values obtained for treatment wells. Background absorbance was determined by the inclusion of wells containing the range of honey concentrations and no cells.

To determine whether components of honey react with nitrite and thus remove it or whether the protein in honey would interfere with the assay, the Greiss reaction was run

with the range of nitrite standards with and without 1% manuka honey present. Honey was found to have no effect on the concentration of nitrite standards, indicating that any effects observed for treatment wells were not due to honey removing nitrite from solution directly and were due to an effect on the cells.

5.2.7.2 Measuring the effect of honey on NO production by LPS-stimulated monocytes

To measure the effect of honey on NO production by LPS-stimulated THP-1 monocytes, cells (1 x 10^6/ml) were added to a 96-well flat bottom plate in 940 µl volumes. To each well either 50 µl of honey solution (artificial, manuka or Otago pasture, serially diluted to obtain final concentrations of 0.1–1%), or RPMI complete medium, was added along with 10 µl of LPS solution (10 µg/ml). Cells were the incubated for 18 h (37°C, 5% CO_2, 95% air) prior to performing a Greiss assay.

5.2.7.3 Characterising the fraction of honey which modulates NO production by LPS-stimulated monocytes

Assays were repeated as described in Section 5.2.7.2, to test the effect of honey fractions on NO production by LPS-stimulated monocytes. Testing of honey fractions was undertaken to characterise the active component in honey. Based on the optimised dose range established by the assays performed in Section 5.2.7.2, dialysis fractions of either honey type (manuka or Otago pasture) and an ether extract of manuka honey were tested at a concentration equivalent to 1% honey. As before, honey was added at the same time as LPS. Cells were then incubated for 18 h (37°C, 5% CO_2, 95% air) prior to performing a Greiss assay.

5.3 RESULTS

To determine whether honey stimulated cells directly to produce NO, THP-1 cultures were incubated with honey at various concentrations. No nitrite could be detected in any of the supernatants, indicating that NO levels were below the detection threshold (less than 1.56 μM, data not shown).

To examine the immunomodulatory activity of honey on the NO production of LPS-stimulated monocytes, honey was added to cells at the same time as LPS. Both natural honey types at higher concentrations (0.5–1% v/v), significantly reduced nitrite levels below those obtained for cells stimulated with LPS alone. There was a dose-wise inhibitory effect on NO production with increasing honey concentration (0.5–1%), but this was only just significant ($P<0.05$). Incubating LPS-stimulated monocytes with 1% concentrations of either natural honey type led to the greatest inhibitory effect on NO production. Only manuka honey had a significant effect on NO levels at a 0.25% concentration, but no effect at 0.1% concentrations. An inverse relationship existed, with NO production by LPS-stimulated monocytes decreasing with increasing honey concentration (Figure 5.2).

To compare a monocytic cell line with a complex population of cells, PBMC were then incubated with the same range of honey concentrations (Figure 5.3).

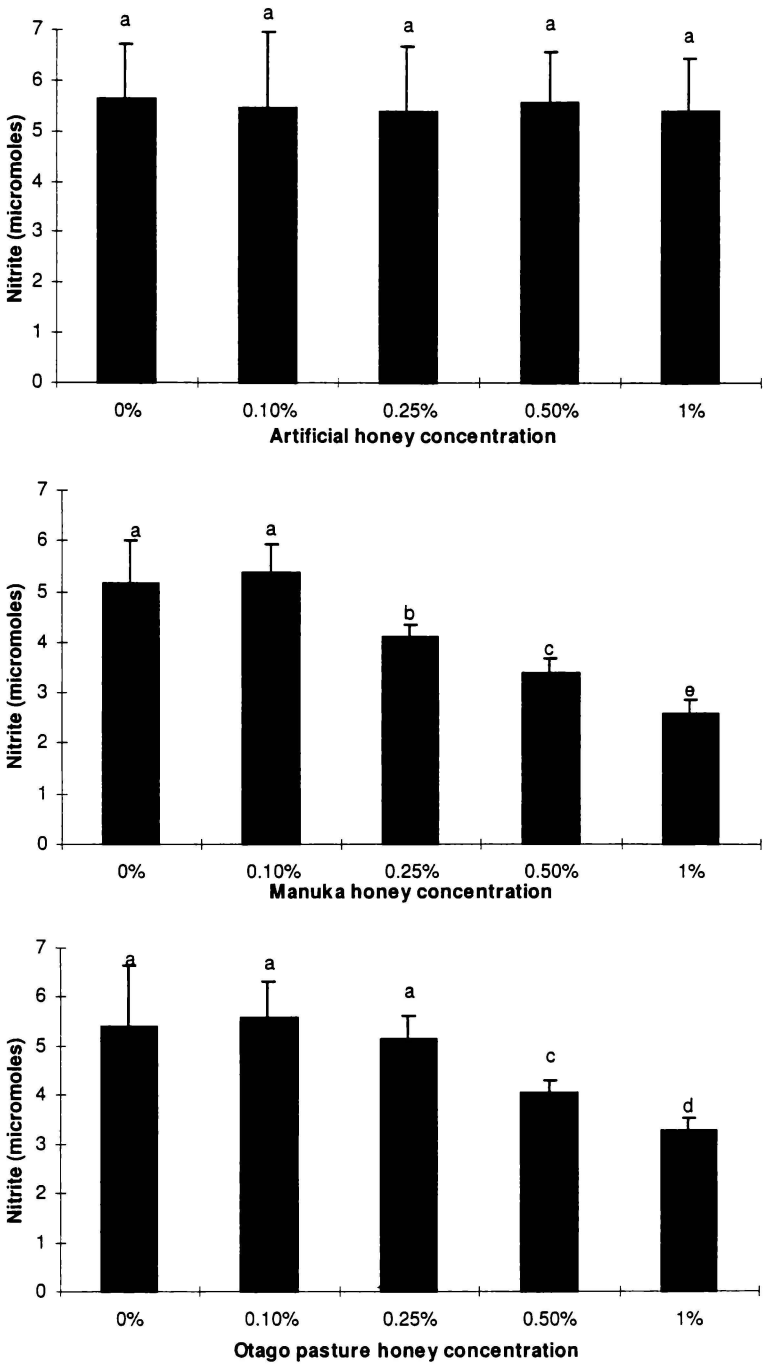


Figure 5.2: Effect of treating LPS-stimulated THP-1 monocytes with concentrations of various honeys. Monocytes were incubated with honey (artificial, manuka or Otago pasture) at concentrations 0.1–1%, or RPMI complete medium, and LPS (10 μg/ml). The cultures were incubated for 18 h (37°C, 5% CO_2, 95% air) and nitrite determined using the Greiss reagent. Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$). These means are derived from two independent experiments (n=6).

The results in Figure 5.3 show that incubation of LPS-stimulated PBMCs with honeys at 0.5% concentrations, suppressed nitrite production indicating that 0.5% honey modulated NO production as compared to positive controls (cells with LPS alone). Visual inspection of the cultures indicated that some cell damage had occurred in the cultures incubated with 0.5% concentrations of honey and the sugar syrup (cell viability at this concentration with artificial honey was 85.1% ± 3.2, manuka honey 88.3% ± 2.6, and Otago pasture honey 91.4% ± 4.6). Artificial honey had no effect on NO production by LPS-stimulated PBMC. This indicated that the effect of the 0.5% natural honeys on nitrite levels could not be attributed to sugars alone. Only manuka honey had a modulatory effect on NO production at a 0.25% concentration. At 1% concentrations, both honeys and the sugar syrup lead to less NO production by LPS-stimulated PBMC than the positive control (as indicated by measurable nitrite), but also a decreased cell viability (artificial honey 55.1% ± 8.4, manuka honey 63.6% ± 7.1, and Otago pasture honey 67.3% ± 9.6). After the effects of sugars alone on NO production were removed, the effect of 1% natural honeys on NO production were indistinguishable from 0.5% concentrations. Therefore, both manuka honey and Otago pasture honey had a modulatory effect on NO production by LPS-stimulated PBMC at 0.5% and 1% concentrations but not in the dose-wise manner as had been observed for THP-1 monocytes.

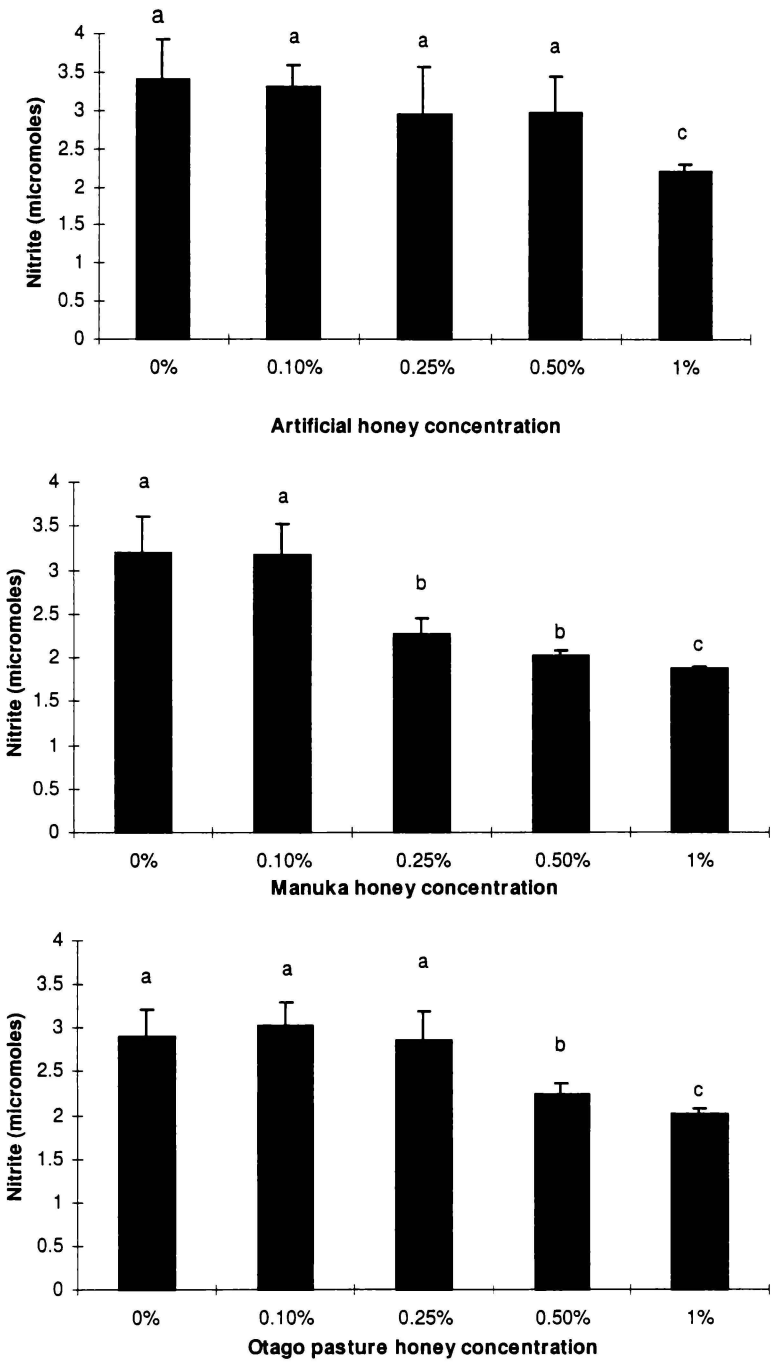


Figure 5.3: Nitrite concentration of supernatants from PBMC suspensions incubated with various honeys and LPS. PBMCs were exposed to Con A (5 μg/ml) for 24 h, and then incubated with LPS (10 μg/ml) and various honeys (artificial, manuka or Otago pasture) at 0.1–1% concentrations or RPMI complete medium alone. Cells were then incubated for 18 h (37°C, 5% CO_2, 95% air) prior to conducting a Greiss assay. Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$). These means are derived from two independent experiments (n=6).

The effect on nitrite production of incubating LPS-stimulated monocytes with dialysis fractions at a concentration equivalent to 1% honey are shown in Figure 5.4.

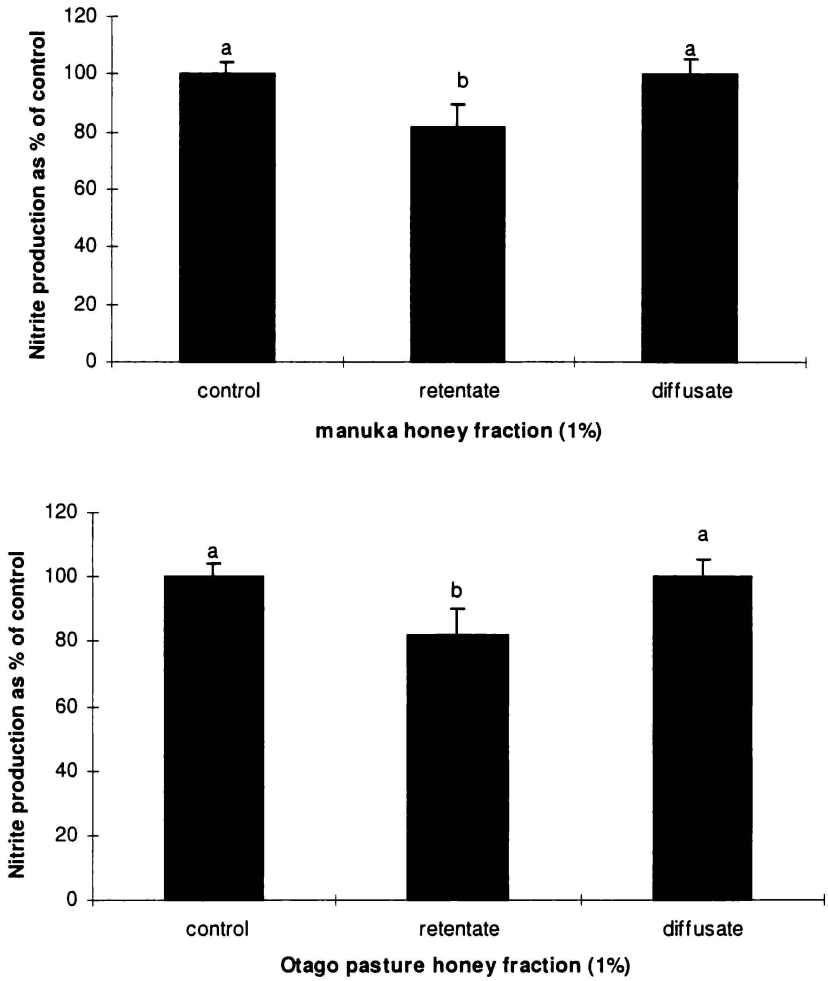


Figure 5.4: The effect of dialysed honey fractions on nitrite production by LPS-stimulated THP-1monocytes. THP-1 (1 x 10^6 cells/ml), were incubated with LPS (10 μg/ml) and dialysis fractions at a concentration equivalent to 1% Otago pasture honey or manuka honey. The cells were incubated for 18 h (37°C, 5% CO_2, 95% air), prior to determination of the suspension nitrite levels using the Greiss reagent. Data is presented as a proportion of that from cells incubated with LPS alone (4.32 micromoles ± 0.71). Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means within graphs not sharing common letters are significantly different ($P<0.05$). These means are derived from three independent experiments (n=9).

Retentate fractions (>10 kDa) of both honeys obtained from dialysis, at a concentration equivalent to 1%, decreased nitrite production. The diffusate fractions (<10 kDa) obtained from dialysis of either honey had no effect on NO production.

To compare the activity present in dialysed retentate fractions of honey with the original unfractionated stock, LPS-stimulated monocytes were incubated with 1% honeys (manuka or Otago pasture) or retentate fractions of these honeys (at concentrations equivalent to 1% honey). The effect of honey fractions compared with original stock solutions on NO production by LPS-stimulated monocytes is shown in Figure 5.5.

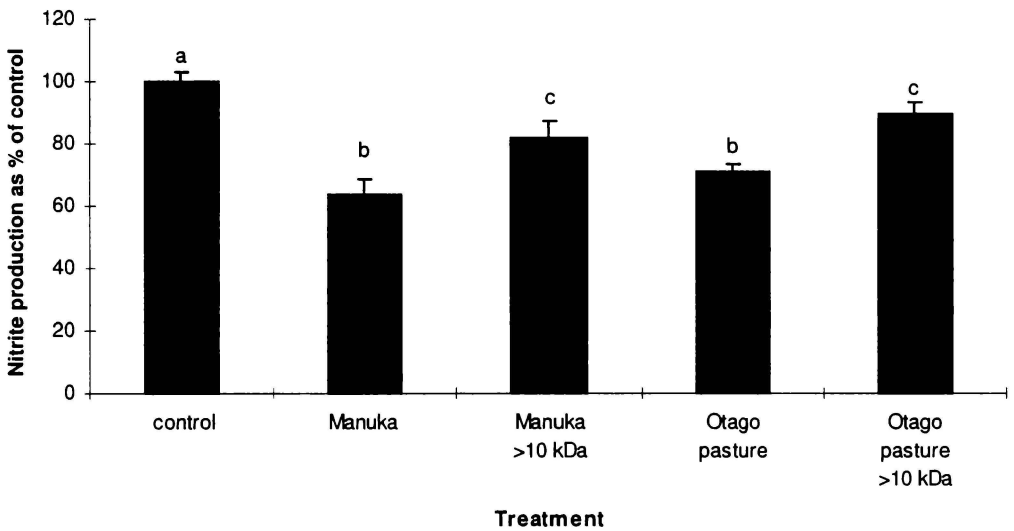


Figure 5.5: The effect on nitrite production by LPS-stimulated THP-1 monocytes, of high molecular weight fractions of honey compared with unfractionated honey stocks. THP-1 (1 x 10^6 cells/ml), were incubated with LPS (10 μg/ml) and 1% honeys (manuka or Otago pasture) or retentate fractions of these honeys obtained from dialysis (at concentrations equivalent to 1% honey). The cells were incubated for 18 h (37°C, 5% CO_2, 95% air), prior to determination of the suspension nitrite levels using the Greiss reagent. Data is presented as a proportion of that from cells incubated with LPS alone (5.27 micromoles ± 0.23). Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P<0.05$). These means are derived from three independent experiments (n=9).

Fractionation of honey by dialysis leads to a loss of some of the modulatory activity found in natural honey. While the retentate fractions inhibited NO production compared to the control (cells stimulated with LPS alone), less inhibition was obtained than with the original honey stock. There were no significant differences between the honey types in modulatory effects on NO production by LPS-stimulated monocytes.

The effect on nitrite production of incubating LPS-stimulated monocytes with either 1% whole manuka honey, or fractions of this obtained from dialysis or an ether extract (at concentrations equivalent to 1% honey) are shown in Figure 5.6.

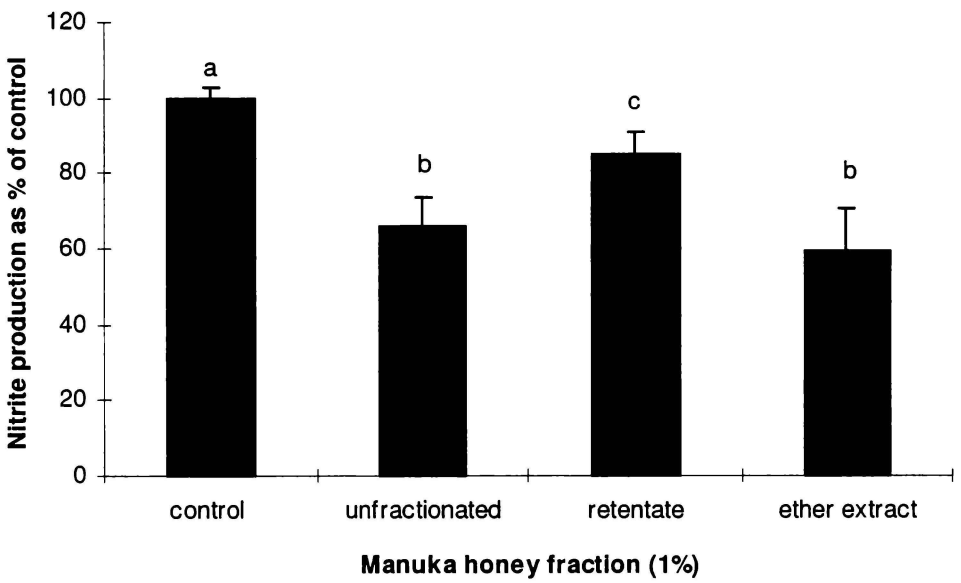


Figure 5.6: The effect on nitrite production by LPS-stimulated THP-1 monocytes, of an ether extract of manuka honey, compared with a high molecular weight fraction or the unfractionated stock. THP-1 (1 x 10^6 cells/ml), were incubated with LPS (10 μg/ml) and either 1% manuka honey, or fractions of this honey stock (retentate fractions of this honey obtained from dialysis or an ether extract) both at concentrations equivalent to 1% honey. The cells were incubated for 18 h (37°C, 5% CO_2, 95% air), prior to determination of the suspension nitrite levels using the Greiss reagent. Data is presented as a proportion of that from cells incubated with LPS alone (4.87 micromoles ± 0.36). Each point represents the mean for each treatment (conducted in triplicate). Differences between the means are indicated by superscript letters, where means not sharing common letters are significantly different ($P<0.05$). These means are derived from three independent experiments (n=9).

The modulatory activity present in the manuka honey stock solution was retained in the ether extract with similar levels of inhibition obtained with an ether extract at a concentration equivalent to 1% natural honey as the honey source at 1%.

5.4 DISCUSSION

The current study presents evidence that honey modulates, but does not completely inhibit, the production of NO by LPS-stimulated monocytes. LPS-stimulated monocytes were used to provide an inflammatory model *in vitro* of tissue macrophages. In Section 5.1, it was noted that NO plays a crucial role in host defense but is cytotoxic and pro-inflammatory at high concentrations. A tightly regulated cellular redox state is necessary for proper cell functioning. A number of *in vivo* studies have shown that iNOS inhibitors exacerbate infectious diseases (Evans *et al.*, 1993; Chan *et al.*, 1995), indicating that NO plays a crucial role in host defense. One of the key problems with glucocorticoid use is that they totally inhibit NO production, preventing the physiologically important actions of NO from being effected (Yokozawa *et al.*, 2002) which in itself contributes to undesirable side-effects. Indeed, NO has an important role as an intercellular signal molecule, perpetuating inflammation through interactions with cytokines and growth factors, and therefore plays a key role in the tissue repair process (Wink and Mitchell, 1998). Release of NO is an important part of the macrophage anti-bacterial repertoire, and has also been shown to be cytotoxic to both tumour cells and parasites, thus benefiting the host (Patel *et al.*, 1999). Therefore, it is important that honey does not inhibit production of NO.

There are two aspects of the effect of honey on NO production which need to be considered here. The first is the direct effect of honey on NO production by resting cells. In Chapter 3, incubation of whole blood with 0.25% manuka honey was shown to induce upregulation of iNOS mRNA beyond medium alone, but not to the same levels obtained with a mitogen. However, in the current study, incubation of THP-1 monocytes or PBMC with honey alone did not produce detectable levels of NO compared with medium alone. This result is likely to be due to the lack of sensitivity of the Greiss assay with a lower

detection limit of 1.56 μM. Others have reported that honey directly activates the nitric oxide system resulting in low levels of NO production (Al-Waili, 2003; Al-Waili and Boni, 2003; Ali, 1997). Of further note, Al-Waili (2003) has reported that honey ingestion only had a slight inducing effect on NO levels in saliva. In addition, Al-Waili (2003) proposed that different honeys contain various concentrations of nitric oxide metabolites and that this may influence the capacity of honey types to activate tissue healing. The direct effect of honey on NO production by resting cells was not the focus of this chapter, however, this could be investigated further using western blotting and antibodies against iNOS protein. Further, it has been demonstrated that NF-κB is a transcription factor essential in regulation of iNOS expression after LPS-stimulation (Xie *et al.*, 1994). Therefore, it would be useful to examine the expression of NF-κB in relation to iNOS expression and production of NO protein.

The second point for consideration here is the base level of NO necessary to enable normal inflammatory processes to occur, and whether honey might reduce these to sub-optimal levels. Clearly this is not what is observed anecdotally, where honey is consistently associated with enhanced tissue repair along with alleviation of inflammation (Molan, 1999; 1998). Critical levels of NO are required for angiogenesis, inflammation, cell proliferation, matrix deposition and remodelling, and specifically for the functioning of collagen and collagenase (Witte and Barbul, 2002). Diabetic wound healing impairment is one of the best-known chronic wound situations and is the result of sub-optimal NO levels (Luo and Chen, 2005). Murrell (1997) reported that NO levels needed to increase 5-fold from base levels over the 7-day period following injury for a tendon to heal. Though not readily transferred to the inflammatory model *in vitro*, this would indicate that NO would need to be increased above base levels for the necessary inflammatory response to occur, and this is what is seen with honey. It has been established in the current study that LPS-stimulated monocytes exposed to honey still produce more NO than resting cultures in medium alone.

In view of the observed ability for honey to stimulate cytokine production (Chapters 3 and 4) and the knowledge that cytokines interact with NO to stimulate immune function (Childress and Stechmiller, 2005; Rizk *et al.*, 2004; Bansal and Ochoa, 2003; Bilsel *et al.*, 2000; Chen *et al.*, 1998; Grimble, 1994; Ochoa *et al.*, 1991; Marletta, 1988) the observation that honey alone has only a slight stimulatory effect on NO production compared with a mitogen at an optimal concentration is important. As honey has been shown to modulate the cytokine profile of immune cells, and therefore modulates activity within the inflammatory cascade, it could be speculated that honey might interfere with the induction of NO by immune cells. NO is induced primarily by activated immune cells, and the amount of NO produced is directly controlled by the activity of the NOS isoenzymes, their activity in turn controlled by the production of inflammatory cytokines (Liang *et al.*, 2001; Jiang *et al.*, 1998).

Of particular relevance to wound healing, NO has been implicated in keloid scarring. Macrophages produce NO (Schaffer *et al.*, 1997b) and NO stimulates fibroblasts to produce collagen (Schaffer *et al.*, 1997a). Fibroblasts themselves produce NO leading to a self-amplification loop for collagen synthesis (Schaffer *et al.*, 1997a). While NO is essential for neovascularisation (Lisids *et al.*, 1997) and re-epithelialisation (Schaffer *et al.*, 1997b), when produced at high levels it is thought to stimulate excessive collagen production by fibroblasts and result in keloid and hypertrophic scarring (Cobbold and Sherratt, 2000). The proven ability for honey to downregulate iNOS expression in an inflammatory model (Section 3.3) and to modulate NO production in an inflammatory model provides a mechanism by which honey reduces scarring in burn patients (as observed by Subrahmanyam, 1991).

The total levels of NO obtained for the THP-1 monocyte cultures in the current study are low compared with other published investigations using murine macrophages (Mautino *et al.*, 1994). This is likely to be a feature of the THP-1 cell line (Jagannath *et al.*, 1998; Nathan and Hibbs, 1991). NO production by primary monocytes is known to vary widely among individuals (Mautino *et al.*, 1994). Therefore, it is difficult to assign concentration

thresholds for NO production, but rather it is the change in kinetics that appears to be important.

That subtle differences have been observed for osmotic tolerance and production of nitrite between the PBMCs and THP-1 cultures is reflective of their different population structures and origins. Unlike the THP-1 monocytic cell line which was of human origin and immortal, PBMCs were derived from bovine blood *ex vivo*, and contained a range of cell populations. It has been established that macrophages are key producers of NO (Jungi *et al.*, 1996), and therefore the proportion of macrophages in culture will have significant effects on NO levels and thus, measurable nitrite. It is encouraging that honey modulated NO production by both LPS-stimulated PBMC and LPS-stimulated THP-1, indicating that the activity could potentially be realised *in vivo*. The investigations presented in the current study now need to be extended to *in vivo* models.

The lack of substantial differences between the effects of the two honeys despite likely variation in the content of antioxidants, hydrogen peroxide and protein of each (Molan 1992b) makes it difficult to speculate about the active component. Artificial honey was found to have no activity beyond medium alone, indicating that sugars alone could not account for modulatory effects. Trypan blue exclusion confirmed that cell death was not a major cause of the decrease in NO production. This suggests that LPS stimulation of the THP-1 cells does not affect cell sensitivity to the cytotoxic effects of honey.

The neutralising effect of medium on the acidity of honey, and the resulting lack of differences in pH between the different honey solutions and medium alone, indicates that pH cannot be responsible for the observed differences. The current study has characterised the modulatory agent as being primarily of a high molecular weight, noting that some of the activity was lost during fractionation by dialysis. Further, it was demonstrated that the active component was present in an ether extract of manuka honey. In addition, it was observed that manuka honey still had significant modulatory effects on NO production by LPS-stimulated monocytes at a lower concentration than Otago

pasture honey (0.25%). Drawing these observations together, it is clear that the modulatory component is present at a higher concentration in manuka honey.

With reference to what is currently known about the composition of manuka honey (Section 1.3.1.5) it is likely that a higher content of proteins and/or antioxidants in manuka honey may account for having activity at lower concentrations. The nature of the active compound must therefore be both ether soluble and of high molecular weight. Honey is known to contain polyphenols bound to proteins (Gheldof *et al.*, 2002). Phenols are characterised as anti-oxidant compounds due to their ability to scavenge peroxyl radicals and their ability to reduce and chelate ferric ion which catalyse lipid peroxidation (Yao *et al.*, 2004; Gheldof *et al.*, 2002). The possibility that tannins may bind iron and prevent formation of radicals and therefore modulate activation of NF-κB was introduced in Section 2.4.2.1.

Ether extract has been characterised as a concentrated source of polyphenols and flavonoids (both antioxidants) and would also contain the aromatic groups present in honey (Arts *et al.*, 2002). This knowledge raises the likelihood of antioxidant participation in the observed modulatory effects of honey on nitrite content. The ability of antioxidants to sequester metals and to trap radicals by their propensity for electron sharing is well understood (Grimble, 1994). The significance of this is that the presence of antioxidant species (such as the aromatic structures contained in honey) form electron bonds with peroxynitrite and this prevents the formation of free radicals. Chelation of ferric ion prevents the reaction of NO with transition metals to form damaging metal-NO adducts. Honey has been demonstrated to scavenge free radicals (Frankel *et al.*, 1998). Confirmation that honey had no effect on nitrite standards indicates that honey must interfere with NO production by the cells rather than remove nitrite from the suspension medium. A number of studies have investigated the mechanisms by which various antioxidant drugs interfere with NO production by immune cells *in vitro*. There is overwhelming evidence that flavonoids inhibit the induction and activity of the NOS isoenzymes responsible for NO production from L-arginine (Ielpo *et al.*, 2000; Liang *et*

al., 2001; Heller *et al.*, 1999; Jiang *et al.*, 1998). In Section 3.3 it was shown that exposure of whole blood to honey prior to LPS leads to downregulation in iNOS mRNA expression, and this is evidence that honey interferes with enzymic production of NO.

It is not currently known how antioxidants interact directly with NO, however in addition to their effect on NO production, antioxidants have been shown to stop the production of peroxynitrite by inhibiting superoxide generation through electron sharing with free radicals (Keaney *et al.*, 1999). As described in Section 3.4.2, Yokozawa *et al.* (2002) reported that tannins inhibited iNOS mRNA and iNOS activity. Of particular relevance to the current study, Yokozawa *et al.* (2002), reported that very low concentrations of tannins were sufficient to suppress iNOS production.

Uric acid is an endogenous antioxidant and has been shown to inhibit peroxynitrite-related chemical reactions (Spitsin *et al.*, 2002). Of relevance to the current study, Al-Waili (2003) reported that honey increases the uric acid level in blood serum indicating that honey augments the antioxidant status of the body.

Evidence from the current study that honey reduces NO production by LPS-stimulated monocytes is in accordance with anecdotal evidence that honey reduces inflammation and the production of free radical species (Tonks *et al.*, 2001), on the basis that NO participates in the formation of peroxynitrite. There is evidence that NO contributes to the development of pathological conditions including the onset of insulin-dependent *diabetes mellitus* through generation of peroxynitrite, reported to induce apoptosis and DNA damage in a variety of cells (Patel *et al.*, 1999; Inoue *et al.*, 1993). The serious side-effects associated with free radical tissue damage during the inflammatory phase of wound repair highlight the need for therapies which can modulate the content of oxidant products in the cellular environment (Grimble, 1994). This may be achieved by mopping up radicals and their intermediates in suspension through metal sequestration or trapping, or by regulating their release from cells via feedback inhibition (Bourdon *et al.*, 1999; Grimble, 1994; Thomas, 1992; Zawadzki *et al.*, 1991). Furthermore, the observation that

honey decreases NO production but does not totally stop its production means that it may reduce inflammation but will not prevent tissue repair. This raises the possibility that honey might modulate NO production *in vivo*.

The results presented in the current study demonstrate a clear effect of honey on NO production by LPS-stimulated monocytes, thus providing a valuable contribution to an understanding of the mechanism by which honey is anti-inflammatory. The results of the investigations performed here suggest the possible therapeutic use of honey to alleviate tissue damage caused by persistent inflammation. It is premature to speculate about the targeted use of honey to reduce NO levels during inflammatory disease in the absence of *in vivo* testing. Considering the bigger picture, these results demonstrate that the role of antioxidants in honey justify further attention.

Chapter Six: The Effect of Honey on Neutrophil Efficacy

SUMMARY

To investigate whether honey has an effect on phagocytosis, whole blood was incubated with honey and the ability of neutrophils to take up fluorescent-labelled bacteria was measured using the Phagotest® assay. The artificial honey control provided clear evidence that low concentrations of honey (optimal at 0.25%) induce phagocytosis by neutrophils due to the supply of sugars. Manuka honey had an additional opsonizing effect on bacteria, which enhances the phagocytic response beyond that seen with sugars alone.

6.1 INTRODUCTION

Phagocytic leukocytes play a key role in the immune response to tissue damage functioning to remove foreign matter and damaged host tissue from the wound site. A common therapeutic use of honey is application to wounds (Molan, 2004), thereby creating a physiological environment where phagocytes are in contact with honey at the local wound site. With this interaction in mind, there is potential for honey to have an effect on phagocyte efficacy. The investigations described here measured the effects of honey on phagocytosis, when honey was applied in an *in vitro* model analogous to a fresh wound.

The potential for honey to modify neutrophil behaviour has been investigated by Abuharfeil *et al.* (1999) who demonstrated that low concentrations of Jordanian wildflower honey led to a slight increase in phagocytosis of heat-killed bacteria. This published account, taken in conjunction with anecdotal reports that honey promotes both elimination of infectious agents from wounds and reduces the need for artificial debridement, presented strong justification to investigate the direct effect of honey on phagocytes. Phagocytes include polymorphonuclear cells and macrophages, with neutrophils constituting up to 70% of the total leukocyte population in blood (Benjamini *et al.*, 1986). Therefore, due to their high density in blood, any impact on neutrophils could be expected to have profound effects on wound healing, and these specific phagocytes were selected for study.

The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. Neutrophils are produced in the bone marrow and are released into the circulation in a terminally differentiated state (Benjamini *et al.*, 1996). Once present in the blood, the neutrophils are ready to respond to any immune challenges. The neutrophils engulf debris and micro-organisms, providing the first line of defense against infection. Neutrophil migration ceases after the first few days post-injury if the wound is not contaminated. If this acute inflammatory phase persists, due to wound hypoxia, infection, nutritional deficiencies (Brosche and Platt, 1995), medication use, or other factors related to the patient's immune response, it can interfere with the late inflammatory phase.

Neutrophils have a repertoire of mechanisms to effect host defence and clean up injured tissue sites, and the current study focuses on the role of neutrophils in wound healing. In order for wound healing to occur, neutrophils need to perform an essential tissue debridement role, removing dead and dying cells, and digesting both the fibrin mesh and clot (Lorena *et al.*, 2002; Dierich *et al.*, 1987). To enable tissue debridement to occur, fibrinolytic enzymes and lysosomal enzymes are produced by the neutrophils (Dierich *et al.*, 1987; Benjamini *et al.*, 1996). Neutrophils release cytokines, growth factors, and nitric oxide, and induce nearby keratinocytes to migrate across the wounded epithelium. In addition, neutrophils produce lymphokines which are chemotactic for lymphocytes (Benjamini *et al.*, 1996). Further, the lactic acid produced as an end product of phagocytosis stimulates proliferation of fibroblasts in the proliferation phase of tissue repair which follows initial inflammation (Section 1.5).

There are several stages of phagocytosis, and these have been well characterised (Benjamini *et al.*, 1996). Receptors on neutrophil cell membranes enable them to respond to gradients of chemicals released by various cells (chemotaxis), and this directs movement to sites of tissue injury. Once at the origin of the chemotactic factor, Fc receptors on the neutrophil enable them to adhere to micro-organisms via antibody interactions. Cellular debris and micro-organisms are taken into the cell by pseudopodia, enabling the lysosomal contents (a combination of chemicals including myeloperoxidase, hydrogen peroxide and chloride) to fuse with the phagosome and digest the contents (Benjamini *et al.*, 1996). Macrophages are drawn to the site of tissue injury in response to myleoperoxidase released by neutrophils (Lefkowitz and Lefkowitz, 2001), themselves removing cellular debris including apoptotic neutrophils (Cox *et al.*, 1995).

In order to function efficiently neutrophils require an adequate supply of nutrients and oxygen (Taylor *et al.*, 1992). Phagocytes consume oxygen during a respiratory burst and this is converted to hydrogen peroxide (Dierich *et al.*, 1987). The possibility that topical application of honey to wounds may improve the supply of these substances to tissues has been suggested (Gupta *et al.*, 1992). Importantly, disease states associated

with impaired or excessive wound healing can be attributed to defects in these responses. Two key examples of impaired wound healing where neutrophil populations are themselves known to be impaired occur in patients with diabetes mellitus (Pierce, 2001) and undergoing chemotherapy (Stark *et al.*, 2005). The majority of the neutrophil population is stored within the bone marrow where a reservoir is maintained as a buffer against neutrophil depletion. The toxic action of the chemotherapeutic agents appears mainly in the myeloid lineage (neutrophils), leading to severe and sustained neutropenia (Stark *et al.*, 2005). Diabetes has been shown to interfere with chemotaxis so that neutrophils cannot migrate into damaged tissues in adequate numbers (Pierce, 2001). Burns patients also exhibit poor wound healing, and it has been established that neutrophils present in the burned wound site do not ingest tissue debris and micro-organisms efficiently, interfering with tissue debridement (Kaufman *et al.*, 1990; Crogan, 1976).

Phagocyte activity is also known to be affected by the nutritional status of the host (Brosche and Platt, 1995; Taylor *et al.*, 1992). One example for which overwhelming evidence exists, is that zinc deficiency can alter the chemotactic response of neutrophils, leading to reduced phagocytosis and an inability to debride the wound site (Taylor *et al.*, 1992). These observations raise the possibility that including nutrient-dense substances in the diet (*e.g.* honey) may have significant implications for phagocyte efficacy.

To enable comparison with known potentiators of the phagocytic response, a range of substances were used to provide positive controls, and these are considered briefly here. Before they can engulf pathogens, neutrophils must first bind them. A range of priming agents such as TNF-α and LPS are known to dramatically increase the phagocytic performance of leukocytes (Condliffe *et al.*, 1998). TNF-α has particular physiological relevance as an early activating signal, as it is released by mast cells upon damage to blood vessels (Wu *et al.*, 1999), and regulates cellular adhesion molecules on a number of leukocytes including neutrophils (Lefkowitz and Lefkowitz, 2001). Further, TNF-α is released constitutively by mast cells upon injury, thus does not provide a model only relevant to an infected wound. The classical stimulating agents LPS and fMLP, derived from bacteria, were included on the basis of their

established ability to promote phagocytosis. LPS has been reported to induce significant levels of phagocytosis when neutrophils have been cultured *in vitro* (Zhao *et al.*, 2003), and specifically for bovine neutrophils (Ducusin *et al.*, 2001). The mode of action by which LPS activates neutrophils to take up particles is well understood. Diez-Fraile *et al.* (2002) have reported that the presence of LPS leads to upregulation of neutrophil surface receptors, specifically CD18 and CD11b, and it is the expression of these that enables the cells to bind to the particles.

Formyl-methionyl-leucyl-phenylalanine (fMLP) is a low level physiological agonist, known to induce neutrophil accumulation in skin (Colditz and Movat, 1984). N-Formyl peptides are derived from proteolytic breakdown of bacterial and mitochondrial proteins, and is associated with inflammation in the gut region (Nguyen and Pei, 2005).

Finally, phorbol-12-mystritate-13-acetate (PMA), a direct activator of protein kinase C was used to provide a high level stimulus for the intracellular signalling pathway (Benjamini *et al.*, 1996).

As blood flow slows near the infection site, neutrophil membrane sialic acid-containing carbohydrate called sialyl Lewis^x tether the neutrophils to the endothelium via the P- or E- selectins expressed on the endothelial surface. Neutrophils roll along the endothelial surface until chemokines (*e.g.* IL-8), complement C5a, and platelet-activating factor (PAF) bind receptors on the neutrophil, and signal the neutrophil to express integrins (Simon and Green, 2005). Integrin activation and upregulation are separate processes and can be independently induced by separate stimuli. Upregulation does not necessarily imply an active adhesion molecule. To enter the inflamed/ injured tissues, neutrophils adhere to the vascular endothelial cell walls via their surface integrin receptors (Simon and Green, 2005). Neutrophil recruitment is directed by transendothelial gradients of IL-8, synthesised by the endothelium in response to exogeneous TNF-α and IL-1β, or from cells resident in the inflamed tissues and diffusing through the endothelium (Marshall *et al.*, 2003). It has been shown that binding of IL-8, released by endothelial cells, is essential for neutrophil transendothelial migration as it binds the neutrophils to the endothelium (Marshall *et*

al., 2003). Further, plasminogen activator inhibitor-1 (PAI-1) released by platelets and endothelial cells stabilises IL-8 gradients to facilitate neutrophil recruitment (Marshall *et al.*, 2003) and make the neutrophils more toxic. IL-8 exerts its chemoattractant function via binding with its cognate receptor CXCR2 (Zhou *et al.*, 2005). VEGF (Vascular Endothelial Growth Factor) also plays an important role in the recruitment of neutrophils into tissues, being chemotactic and stimulates a rapid Mac-1(an inducible β_2 integrin) expression as well as a LFA-1 expression so that the neutrophils bind with activated vascular endothelial cells (Garbacki *et al.*, 2005). LFA-1 and Mac-1 both enable neutrophils to interact with ICAM-1and ICAM-2 expressed on endothelium, along with neutrophil expression of PECAM (Simon and Green, 2005). The integrins facilitate diapedesis through more tight bonding. Neutrophils then secrete proteolytic enzymes to penetrate the basement membrane.

Recent evidence challenges the notion that neutrophils are terminally differentiated, short lived phagocytes, and infact perform diverse tasks including, phagocytosis, antigen presentation and immunoregulation (Zhou *et al.*, 2005). This study aimed to extend the limited observations by Abuharfeil *et al.* (1999) that honey stimulates phagocytosis, by examining the effects of a range of honey concentrations and types on phagocyte activity. Further, this study aimed to characterise the active component in honey, and to use several different assay techniques to investigate the mechanism by which honey has been reported to induce phagocytosis.

6.2 MATERIALS AND METHODS

Reagents

The Phagotest® diagnostic kit was purchased from Orpegen Pharma (Heidelberg, Germany). Phorbol myristate acetate (PMA), recombinant TNF-α and n-formyl-

methionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma Chemical Co., (St. Louis, MO). Giemsa stain and LPS were obtained from Sigma-Aldrich (UK).

Honey solutions

M109 manuka honey, Otago pasture honey and artificial honey were freshly prepared as described in Section 2.2.2. Unless otherwise stated, honey stocks were diluted in RPMI 1640 basal medium (GibcoBRL) to create v/v solutions. Dialysis fractions at concentrations equivalent to that found in manuka honey (0.25%) were prepared as described in Section 2.2.2.1, and diluted in RPMI 1640 basal medium.

6.2.1 Blood collection

Bovine blood was collected into 50 ml Nunc tubes containing heparin solution (5 IU/ml) from the jugular vein of a freshly slaughtered heifer at the AgResearch abbatoir. Whole blood was used for the Phagotest® assays.

6.2.2 Measurement of phagocytosis

A commercial Phagotest® kit was purchased to measure phagocytosis. The proportion of neutrophils which had ingested bacteria was obtained, and phagocyte efficiency was calculated based on the number of bacteria ingested per cell (as described by Wimmer *et al.*, 2004; Gastaldello *et al.*, 2000; Carulli *et al.*, 1998). The Phagotest® assays were performed according to the manufacturer's instructions (Orpegen Pharma).

Heparinised whole blood was transferred in 100 µl volumes to 12 x 75 mm tubes. All assays were performed within an hour of receiving the blood. After the blood had been incubated (37°C, 5% CO_2, 95% air) with 10 µl of either; various treatments (honey, artificial honey, LPS) or RPMI basal medium, all tubes were placed on ice for 10 min to cool them down. Each tube then received 20 µl of pre-cooled Fluorescein

isothiocyanate labelled (FITC)-*E. coli* at a concentration of 10^9 cells/ml (ratio of bacteria:leukocytes was 25:1). The tubes were vortexed at low speed for 3 secs, and then the samples were placed in a shaking waterbath (Julabo, Labortechnik GMBH, Germany) for 10 min at 37°C, and agitated constantly at 120 rpm to allow phagocytosis. Duplicate baseline control tubes were prepared for each treatment, with identical preparation to the corresponding treatment samples, but were placed on ice (0°C) just prior to addition of bacteria, and were maintained on ice during the assay period. All treatments were conducted in triplicate. At the end of the incubation period, samples were placed on ice to stop phagocytosis. To each tube (samples and controls), 100 µl of ice-cold quenching solution was added, and each tube gently vortexed. The tube contents were each washed twice with 3 ml of washing solution (centrifuged at 250 x *g* for 5 min at 4°C). To each tube, 2 ml of lysing solution was added (pre-warmed to room temperature), and the tube contents vortexed. After a 20 min incubation at room temperature, the tubes were centrifuged (250 x *g* for 5 min at 4°C). Finally the samples were washed twice (3 ml washing solution followed by centrifugation at 250 x *g* for 5 min at 4°C), and 100 µl of propidium iodide was added for 10 min at 0°C to stain the DNA.

6.2.2.1 Preparation of counting slides and counting procedure

To analyse phagocytosis, 100 µl of stained blood was transferred from individual tubes to a haemocytometer. For each haemocytometer count, the total number of neutrophils present in 5 squares from the centre set of 25 squares was obtained according to standard methods. The counts were performed within an hour after the staining procedure, protecting the blood from light at all times.

Using the same 5 squares, 3–6 visual fields were scanned under 400 x magnification, and 100 neutrophils randomly chosen for examination (Leica TCS-NT Epifluorescence with a blue filter block; BA 520; excitation of 465–495 nm). The number of neutrophils with engulfed bacteria and/or adherent bacteria were expressed as a proportion of total neutrophils. Phase-contrast in bright field was used where necessary to clarify visual counts.

The quenching solution enabled the discrimination of attached and internalised bacteria, by suppressing the fluorescent green colour of the adherent bacteria and not the colour of internalised bacteria. The propidium iodide stain enabled discrimination of leukocyte nuclei from bacterial aggregates, and from each other based on the morphology of the nuclei. Where bacteria were both attached to the cell surface and engulfed, the cell was placed in the 'engulfed' category. Cells with bacteria attached to the surface or engulfed bacteria were both defined as phagocytosing neutrophils.

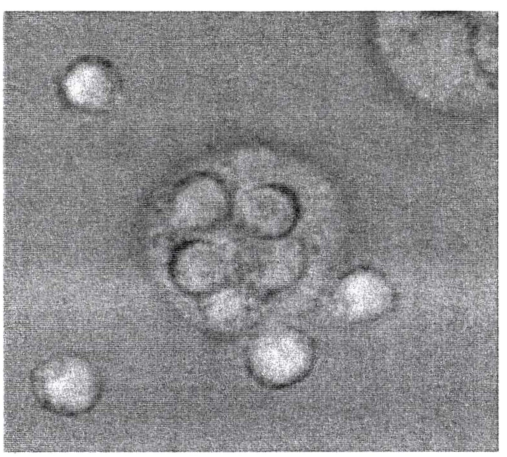


Figure 6.1: Position of fluorescent beads in a bovine blood-derived neutrophil (from preliminary assays). Use of quenching solution, and phase-contrast under bright field microscopy (400 x), enabled discrimination between attachment and internalisation of bacteria.

6.2.3 Statistical analysis

All tests were performed using triplicate samples and repeated at least twice (n=6 independent observations). The mean % values obtained for the duplicate baseline control tubes (maintained on ice throughout the assay period), were subtracted from the corresponding treatment means. A two-way analysis of variance (ANOVA) was used to test the significance of the phagocytosis curve, with the parameters, timepoint and activating agent. Significant effects were further analysed using Tukey's honestly significant difference for pair-wise differences for within group comparisons. The

paired student's *t*-test was used to compare the effects of different activating substances on phagocytosis with the effects of RPMI basal medium alone. For multiple group comparisons, the data were subjected to one-way analysis of variance (ANOVA) to determine overall difference between the group means. Significance was accepted for $P<0.05$. Systat software version 7.0 (SPSS Inc.) was used for all analyses.

6.3 EXPERIMENTAL DESIGN

6.3.1 Preliminary experiments

Preliminary experiments were conducted to optimise conditions. Initially neutrophils were isolated from bovine blood, and flow cytometry was used to measure the uptake of FITC-labelled beads. However, the difficulties associated with preparation of the purified neutrophil suspensions, requirement for optimised serum content and opsonization of beads, and lack of physiological relevance of the purified cell suspensions to the wound *in vivo* meant that the earlier assays were abandoned.

The decision to use a microscope and count neutrophils in a blood smear necessitated time spent optimising counting procedures. Initially the precision with which neutrophils could be recognised using the Phagotest® assay was determined from counts obtained by haemocytometry (propidium iodide-stained cells) and from standard slides (Giemsa-stained cells).

6.3.1.1 Calculation of average neutrophil numbers in a bovine blood smear and precision of counting

According to the Phagotest® assay protocol, only leukocytes were present in the counting slides (as red blood cells were lysed and removed). To prepare Giemsa-stained slides, after lysing the blood cells (Section 6.2.2), the cell pellet was resuspended in 200 µl of PBS, and 10 µl of the suspension smeared across a standard microscope slide. The remaining suspension was stained with propidium iodide, for haemocytometer counts. Standard procedures were then followed for preparation of methanol-fixed Giemsa stained slides. Briefly, the smear was fixed in absolute methyl alcohol for 5 min, and coloured with Giemsa stain (20 min) according to the manufacturer's instructions (Sigma-Aldrich). The number of neutrophils was counted among a total of 100 cells on each slide (Nikon Eclipse TS100) using 3 visual fields under 400 x magnification. These counts were compared with those obtained from

haemocytometry (Section 6.2.2.1). The morphology of leukocyte nuclei was checked against a reference guide (Wheater *et al.*, 1987).

For the counting precision tests, slides were prepared from five different tubes of bovine blood (all obtained from the same animal). Each tube of blood was subsampled for haemocytometry and the Giemsa-stained slide counts. The average number of neutrophils per 100 cells was 59 ± 4. In addition, these results indicated that a minimum of 3 visual fields would need to be viewed to obtain 100 neutrophils for counting. In order to ensure that all counts were representative of treatment differences, two slides were prepared from each tube of blood, and the average of the two readings used in the statistical analysis. The number of neutrophils obtained from each count were found to be comparable. This indicated that staining nuclei with propidium iodide could be used reliably to discriminate between leukocytes, based on the morphology of their nuclei.

6.3.1.2 Optimising the positive controls

Positive controls were included to provide a baseline for differences in the activity of resting cells compared with primed cells. Positive controls were chosen according to published reports, and included LPS, fMLP, PMA, or recombinant TNF-α (Table 6.1). To optimise the response of phagocytes to activation by the positive controls, a series of assays were conducted based on the conditions described by published reports (Zhao *et al.*, 2003; Rivas *et al.*, 2002; Ducusin *et al.*, 2001; Crowley and Raffin, 1991), and are not shown here.

Incubation period with positive controls

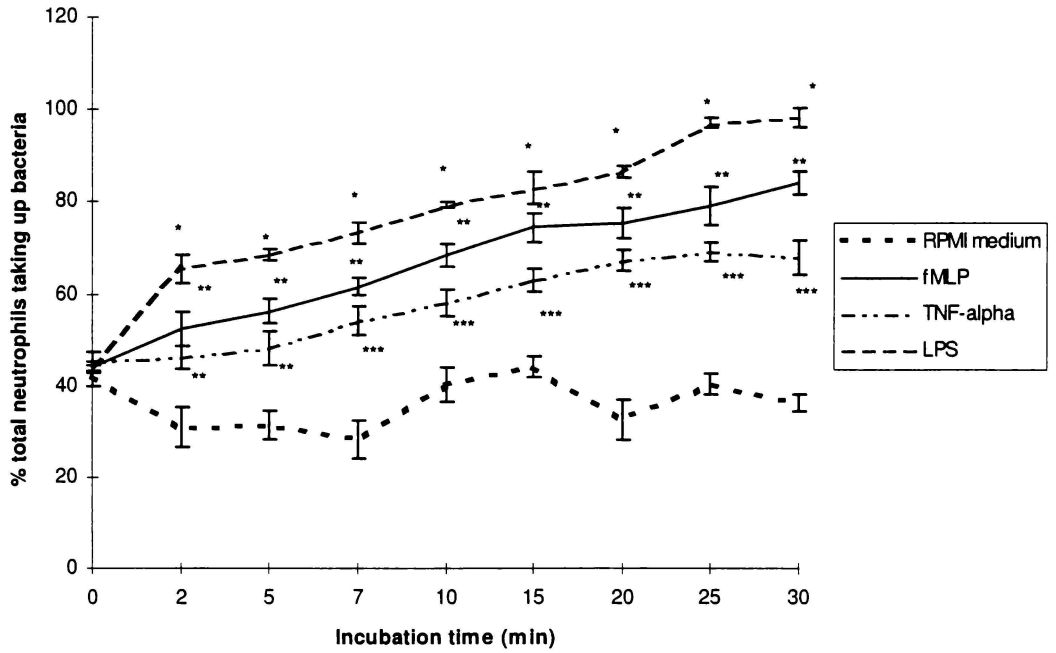
One hundred microlitres of heparinised blood was loaded into 12 x 75 mm tubes, and either fMLP, LPS, PMA or TNF-α at the stated final concentrations, were added as summarised in Table 6.1. To triplicate blood samples, a 10 µl volume of a single activating agent or RPMI basal medium alone was added to each tube, and two further tubes also prepared to act as baseline controls. After the blood had been incubated

(37°C, 5% CO_2, 95% air) for the indicated times (0–30 min), bacteria were added according to the Phagotest® assay protocol, first placing the baseline control tubes on ice. The baseline controls were maintained on ice throughout the assay period, and the mean values obtained from these controls were subtracted from the corresponding treatment samples. The proportion of neutrophils which had taken up bacteria by phagocytosis (out of total neutrophils) were calculated (described in Section 6.2.2) and means for each time-period (conducted in triplicate) were compared (Figure 6.2).

Table 6.1: Priming regimes for positive controls

Primary stimulant	Conc.	Incubation time (min)	Secondary stimulant	Incubation time (min)	Reference
TNF-α	10 ng/ml	0–30	NIL	0	O'Flaherty *et al.*, 1991
TNF-α	10 ng/ml	0–15	fMLP (0.01 mmol)	15	Heine *et al.*, 2000; Crowley and Raffin, 1991
LPS	10 ng/ml	0–30	NIL	0	Ducusin *et al.*, 2001
LPS	10 ng/ml	0–15	fMLP (0.01 mmol)	15	De Leo *et al.*, 1998
fLMP	100 ng/ml	0–30	NIL	0	Zhao *et al.*, 2003
PMA	10 ng/ml	0–30	NIL	0	Zhao *et al.*, 2003; Gastaldello *et al.*, 2000

A.



B.

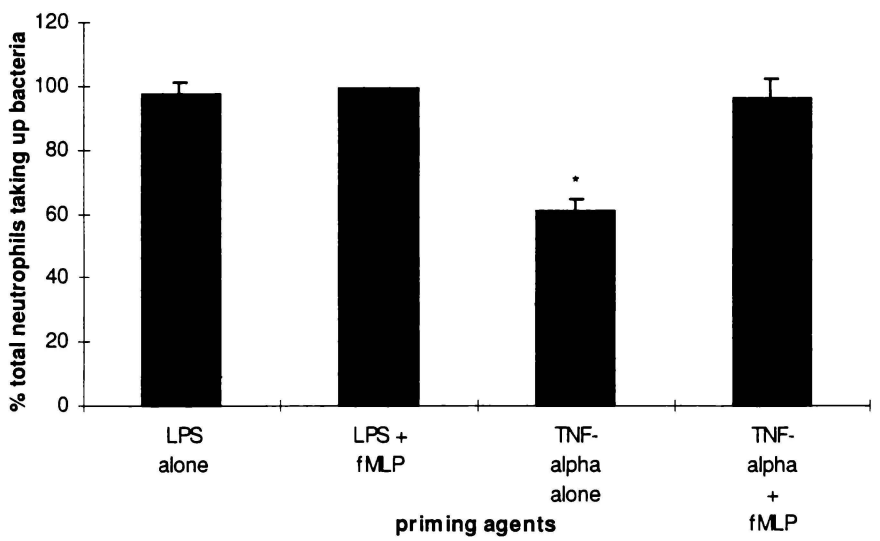


Figure 6.2: Effects of different priming agents on the phagocytosis of fluorescent-labelled bacteria by bovine neutrophils. A. Effect of length of incubation with either; fMLP (100 ng/ml), TNF-α (10 ng/ml), LPS (10 ng/ml), on the % neutrophils taking up bacteria compared with the effects of RPMI basal medium alone. * $P<0.05$, for each timepoint, means not sharing * are significantly different from each other. Data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6). B. Effect of different activating agents; LPS alone (10 ng/ml), LPS (10 ng/ml) for 15 min prior to addition of fMLP (100 ng/ml) for 15 min, TNF-α alone (10 ng/ml), or TNF-α (10 ng/ml) for 15 min prior to addition of fMLP (100 ng/ml) on % neutrophils taking up bacteria. * $P<0.05$, means not sharing * are not significantly different from each other. Data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6).

Priming blood with either LPS alone, LPS for 15 min followed by 15 min incubation with fMLP, PMA, or TNF-α prior to fMLP, induced a maximal phagocytic response. TNF-α alone, induced a lower uptake of beads than the other priming agents over the assay period. These results indicated that priming blood for 30 min was sufficient to produce significant differences in the proportion of phagocytising neutrophils between resting and treated blood cultures. In addition, these results confirmed that while TNF-α alone induced significant neutrophil activity compared with the control, the presence of bacterial peptides potentiated a stronger response.

6.3.1.3 Viability of leukocytes following incubation with honey

To ensure that the honey solutions did not have detrimental effects on neutrophil viability, blood was incubated with various concentrations of honey for 30 min prior to trypan blue staining. Briefly, 100 µl of heparinised bovine blood was loaded into a 12 x 75 mm tube along with 10 µl of a serially diluted honey solution (manuka, Otago pasture or artificial, to obtain final concentrations 0.1–1% v/v) and incubated (37°C, 5% CO_2, 95% air), for 30 min. Blood with 10 µl RPMI basal medium was included as a control. According to the methods described by Gorman *et al.* (1997), an equal volume of typan blue was added to each tube and the contents incubated for 1 min at 37°C. Using haemocytometry under bright field (Olympus, 40 X), the proportion of viable leukocytes was established by observing 100 leukocytes in 3–6 fields of view. The effect on cell viability of exposing leukocytes to honey concentrations are presented in Figure 6.3.

Honey at concentrations between 0.1–0.5% (v/v), of all three types, had no effect on leukocyte viability. Earlier assays had established that neutrophils constituted an average of 59 ± 4% of total leukocytes in a typical bovine blood sample. At the 1% concentration there was a significant decrease in cell viability with all honeys indicating that sugars were causing osmotic stress. Based on these results, assays were restricted to testing honey concentrations within the established osmotic tolerance range 0–0.5% (v/v).

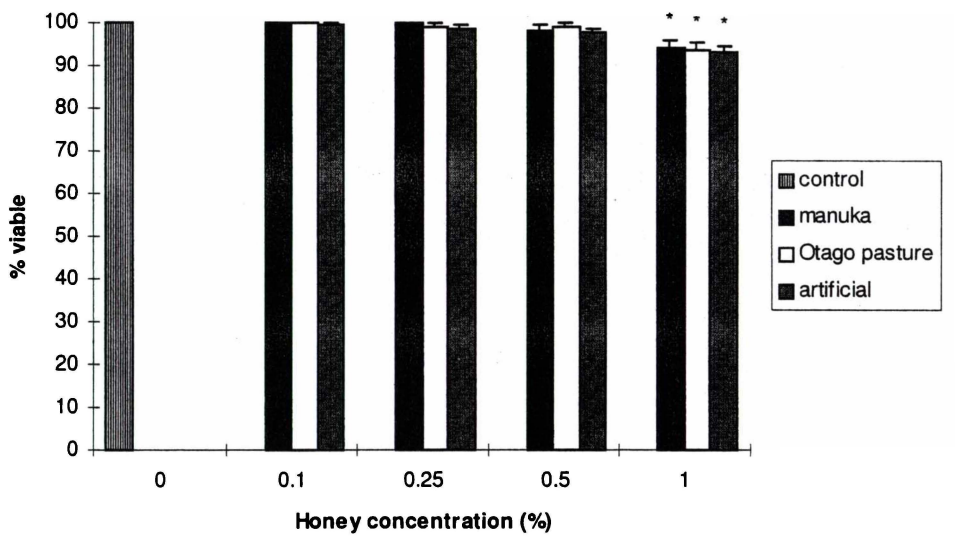


Figure 6.3: The effect of honey concentration on leukocyte viability. Bovine blood was incubated with and without honeys (manuka, Otago pasture or artificial) at various final concentrations (0.1–1%) for 30 min and assessed for viability using trypan blue staining. Data points represent the mean of triplicate counts ± SEM and the assay was replicated twice (n=6). Each mean % was compared with the mean of the blood control (RPMI basal medium alone). * $P<0.05$, where means not sharing * are significantly different from each other.

6.3.2 Evaluation of the direct effect of honey on neutrophils

To determine whether honey has a direct effect on phagocytosis by neutrophils, blood was incubated with honey concentrations prior to addition of bacteria.

6.3.2.1 Identification of honey concentrations with an activating effect on uptake of bacteria by neutrophils

Blood was dispensed into triplicate tubes, following the protocol described in Section 6.2.2. Honeys (artificial, manuka or Otago pasture) were added in a 10 µl volume to give final concentrations of 0.025–0.5% (v/v). Blood incubated with 10 µl of RPMI basal medium was included as a control. The proportion of neutrophils which had taken up bacteria (out of total neutrophils) was calculated (Section 6.2.2).

6.3.2.2 Comparison of the activating effect of manuka honey stock and dialysed fractions at concentrations equivalent to 0.25% on uptake of bacteria by neutrophils

To characterise the stimulatory component in honey, blood was incubated with 0.25% concentrations of honeys (Section 6.3.2.1), in addition to dialysed manuka honey retentate (with sugars removed, at a concentration equivalent to 0.25%). The proportion of neutrophils which had taken up bacteria (out of total neutrophils) was calculated (Section 6.2.2).

To exclude the possibility that endotoxin contamination could account for the additional stimulation seen with honey (beyond sugars alone), a contamination assay was run. Both honey solutions and LPS solutions (10 ng/ml) were incubated with polymyxin B (PMB; Sigma), at a concentration of 10 µg/ml (37°C, 5% CO_2, 95% air) for 60 min prior to adding to blood. As before, blood was loaded in triplicate tubes and incubated with 10 µl volumes of either; PMB treated honey, untreated honey, or PMB treated LPS for 30 min prior to conducting the assay (as described in Section 6.2.2.). Blood incubated with either RPMI basal medium, or untreated honey, was maintained under the same conditions and were included as controls.

6.3.2.3 Investigation to determine whether priming blood with both honey and TNF-α would augment phagocytosis

The aim of the current study was to determine whether honey would have effects on aspects of cell function pertinent to tissue repair. When honey is applied to a fresh wound, it will be present at the same time as TNF-α, released initially by mast cells (Wu *et al.*, 1999). This assay was performed to determine whether honey would augment the activating effect of TNF-α on phagocytes. The possibility that honey might augment phagocytosis was based on the observations by Condliffe *et al.* (1998), that neutrophils show an enhanced response when several agents act in concert. Recombinant TNF-α (10 ng/ml) and diluted manuka honey (to obtain a final concentration of 0.25%) were added to blood in triplicate tubes, according to two regimes; TNF-α alone for 15 min followed by honey, or honey and TNF-α added at the same time. By adding the TNF-α prior to the honey, this was simulating the release of TNF-α by mast cells at the site of a fresh wound. The protocol was then followed as described in Section 6.2.2.

6.3.3 Evaluating the effect of honey on opsonization of bacteria

These assays were designed to measure the indirect effect of honey solutions on neutrophil activity. Opsonizing beads and bacteria by pre-coating them with various agents has been demonstrated to have significant effects on their uptake by phagocytes (de la Fuente *et al.*, 1998; Volle *et al.*, 2000; Ducusin *et al.*, 2003). By priming the bacteria with honey prior to adding the bacteria to blood, the effect of honey on the opsonization of bacteria could be measured. Nonopsonized bacteria (with FITC-label) were used instead of opsonized bacteria (at the same ratio as described in Section 6.2.2). Bacteria opsonized with 1% BSA were included as a positive control (as described by Ducusin *et al.* 2003).

6.3.3.1 Measuring the effect of honey on the opsonization of bacteria with BSA

Opsonization of the bacteria was adapted from the methods described by Nagata *et al.* 2003 and Volle *et al.* 2000. The *E. coli* bacteria were added to either: 1 ml of phosphate-buffered saline (containing 1% BSA), 1 ml of phosphate-buffered saline alone (providing the non-opsonized control), or 1 ml of honey (artificial or manuka at 0.25%), giving a final concentration of 1 x 10^9 bacteria/ml (according to the manufacturer's instructions; Phagotest, Orpegen Pharma), and incubated at 37°C for 60 min prior to assay use. The blood received no treatments prior to the opsonized bacteria, and the phagocytosis assay was performed according to the protocol described in Section 6.2.2.

Though phagocytes would have been exposed to honey when the bacterial suspension was added to the tubes, the resulting hundred-fold dilution meant that the honey was at concentrations shown previously to have no effect (0.025%; Figure 6.3).

6.4 RESULTS

The proportion of neutrophils in blood, which had taken up bacteria by phagocytosis after priming with honeys (manuka, Otago pasture or artificial) at various final concentrations (0.025–0.5%) were examined. The results are presented in Figure 6. 4, and show the proportion of phagocytosing neutrophils as a proportion of total neutrophils.

All the honeys tested stimulated significantly more phagocytosis by neutrophils at final concentrations between 0.1–0.5% than obtained with RPMI basal medium alone. There were no differences between the effect of Otago pasture honey and artificial honey on the cells, indicating that the observed effects were due to sugars alone. At a 0.25% concentration, however, manuka honey had an optimum effect on phagocytosis, not seen with the other honeys. This cannot be accounted for by sugars alone. At a 0.5% concentration, manuka honey had no activity beyond artificial honey at the same concentration.

Since 0.25% honey showed a stimulatory effect on phagocytosis, a contamination control experiment was undertaken adding PMB, which prevents LPS binding to its

receptor. While the stimulating effect of LPS on phagocytosis was completely inhibited by the addition of PMB, there was no effect on the amount of phagocytosis induced by honey. This indicates that the stimulatory effect of honeys on neutrophils cannot be attributed to endotoxin content (Figure 6.5).

To determine how the levels of phagocytosis stimulated by the honeys compared with known priming agents (using TNF-α-fMLP as a model for an infected wound, and TNF-α alone to simulate inflammatory signals from injured tissues), the number of neutrophils taking up bacteria were calculated as a proportion of total neutrophils for each treatment. Blood was either primed with TNF-α (10 ng/ml; 15 min) followed by fMLP (100 ng/ml; 15 min), TNF-α (10 ng/ml; 30 min) or an optimal concentration of each honey (diluted to a final concentration of 0.25%; 30 min) and compared with resting blood (Figure 6.6).

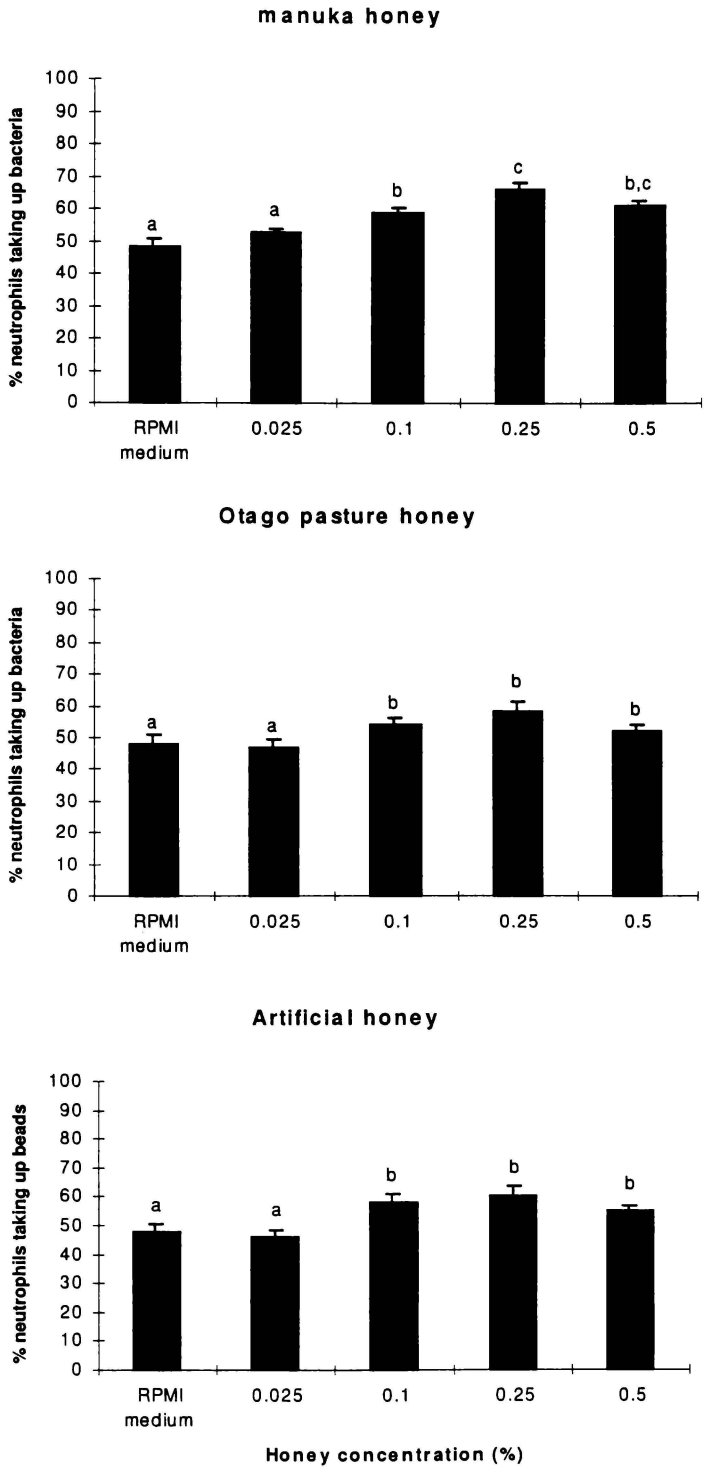


Figure 6.4: Effects of different honey concentrations on the phagocytosis of fluorescent bacteria by bovine neutrophils. Blood was incubated with and without honeys (manuka, Otago pasture or artificial) at various final concentrations (0.025–0.5%), prior to addition of bacteria. A Phagocytosis assay was conducted and the proportion of neutrophils taking up bacteria calculated. At each concentration, the paired student's *t*-test was used to compare the mean % neutrophils taking up bacteria in blood exposed to honey compared with the control (RPMI basal medium) (* $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6).

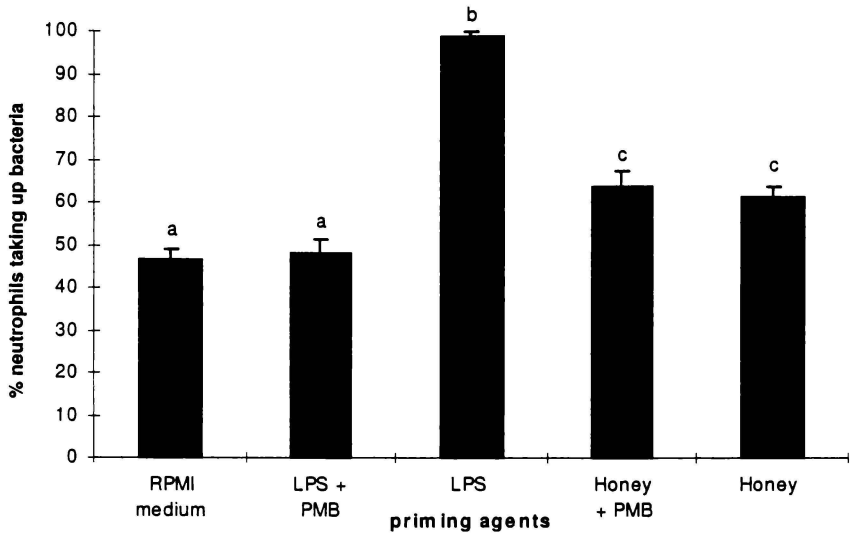


Figure 6.5: Control experiments to investigate the role of endotoxin in the honey-stimulated phagocytosis of fluorescent bacteria by bovine neutrophils. Both manuka honey (giving a final concentration of 0.25%) and LPS (10 ng/ml) were incubated with polymyxin B (10 μg/ml , 37°C, 5% CO_2, 95% air) for 60 min prior to adding to blood, and the samples incubated for 30 min (37°C, 5% CO_2, 95% air) prior to addition of bacteria. Blood incubated with RPMI basal medium prior to addition of bacteria was included as a control. A Phagocytosis assay was conducted and the proportion of neutrophils taking up bacteria calculated. At each concentration, the paired student's *t*-test was used to compare the mean % neutrophils taking up bacteria in blood exposed to honey compared with the control (RPMI basal medium) (* $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6).

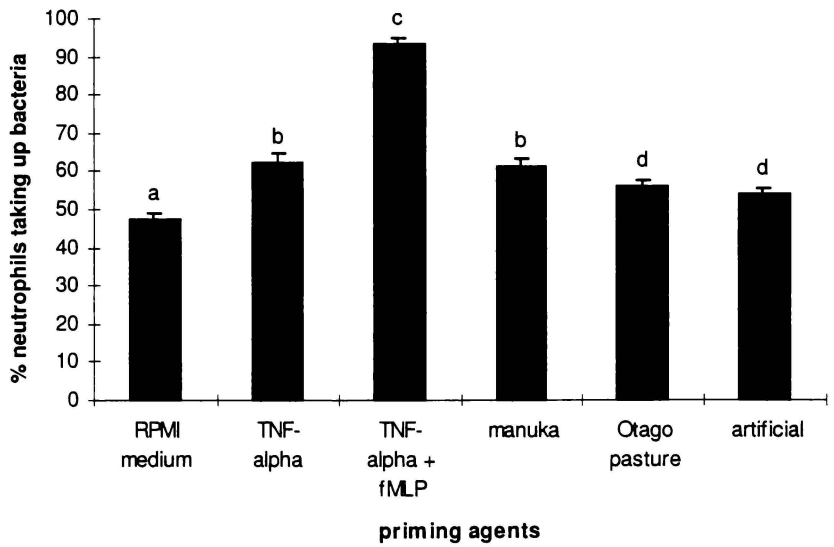


Figure 6.6: Effects of honey compared with TNF-α on the phagocytosis of fluorescent bacteria by bovine neutrophils. Blood was incubated with and without either: TNF-α (10 ng/ml; 15 min) followed by fMLP (100 ng/ml), TNF-α (10 ng/ml; 30 min), or honey diluted to obtain an optimal final concentration (artificial, manuka or Otago pasture 0.25%; 30 min), prior to addition of bacteria. Each mean was compared with the control (blood with RPMI basal medium). * $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6.

Priming blood with TNF-α-fMLP had a maximal effect on the proportion of neutrophils undergoing phagocytosis (96.3% ± 3.1%). TNF-α alone had a similar stimulatory effect on the proportion of neutrophils taking up bacteria to manuka honey at a final concentration of 0.25%. Both artificial honey and Otago pasture honey (at final concentrations of 0.25%) induced a higher proportion of neutrophils to take up bacteria than seen when RPMI medium was added to blood, but this activating effect was lower than that obtained with manuka honey at the same concentration.

To characterise the active component in manuka honey (at the optimal concentration, 0.25%), which could not be accounted for by sugar content alone, blood was primed with fractions of manuka honey obtained from dialysis. Dialysis removed sugars from the high molecular weight fraction of honey. Blood was primed with manuka honey stock, dialysed fractions of the same manuka honey (at final concentrations equivalent to 0.25% honey), and compared with artificial honey as a control for sugar content alone. As before, the number of neutrophils taking up bacteria was calculated as a proportion of total neutrophils for each treatment (Figure 6.7).

Both manuka honey stock, and both fractions of this stock, had stimulatory activity on phagocytosis beyond that obtained when RPMI medium was added to blood. There was no significant difference in the uptake of bacteria when blood was primed with fractions of manuka honey or artificial honey. Only manuka honey stock had a significant stimulatory effect beyond that seen with an equivalent concentration of artificial honey. As the retentate fraction had sugars removed by dialysis, these results must indicate that some high molecular weight compound in honey has an activating effect on neutrophils (Figure 6.7). When present together, the high molecular weight compound augments the stimulation induced by the sugars. The high molecular weight fraction of Otago pasture honey at an equivalent final concentration (0.25%), had no stimulatory effect on phagocytosis beyond RPMI basal medium. This indicates that the non-sugar stimulatory component, is likely to be unique to manuka honey.

The possibility that honey was merely sticky, and that bacteria were non specifically binding to the cell surface but not being taken up, was addressed by examining location of the bacteria. TNF-α alone was included as a positive control for

comparison with honey as it had previously been shown to induce a similar level of activation. Blood was primed with and without TNF-α, manuka honey or artificial honey (both at 0.25% final concentrations), and the proportion of neutrophils with adherent or engulfed bacteria calculated (Figure 6.8).

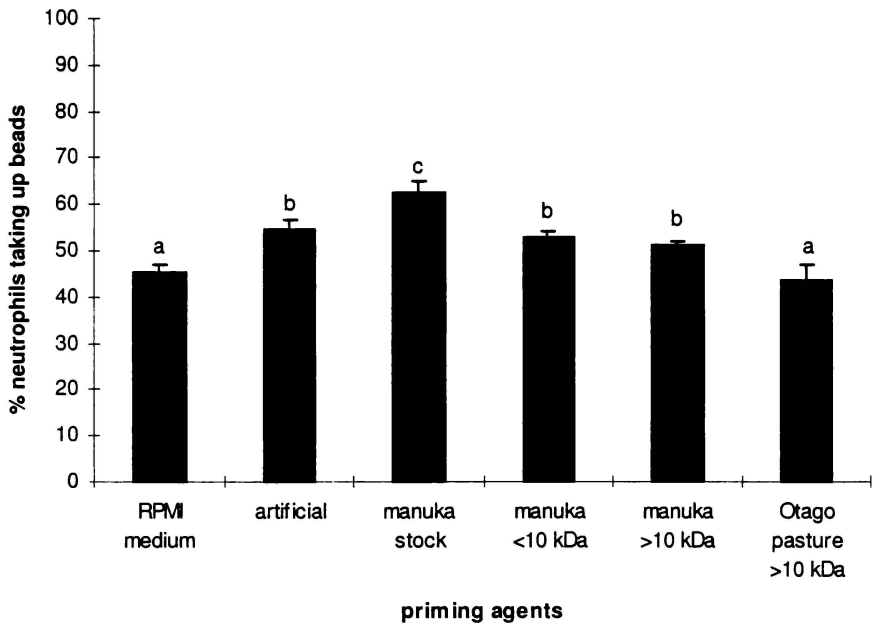


Figure 6.7: Effects of sugar content on the phagocytosis of fluorescent bacteria by bovine neutrophils. Blood was primed with or without either: manuka honey (stock and dialysed fractions of the stock at final concentrations equivalent to 0.25% honey), a high molecular weight fraction of Otago pasture honey (0.25%), or artificial honey (0.25%) prior to addition of bacteria. A Phagocytosis assay was conducted and the proportion of neutrophils taking up bacteria calculated. Each mean was compared with that obtained for artificial honey and for RPMI basal medium. * $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6.

Despite the likelihood that these agents have different stimulatory mechanisms, the proportion of adherent and engulfed bacteria were similar when blood was primed with either TNF-α, manuka honey or artificial honey. There were significantly more engulfed bacteria when blood was exposed to the priming treatments than to RPMI basal medium alone, and the control had more bacteria adhered to the neutrophil surface than bacteria actually engulfed.

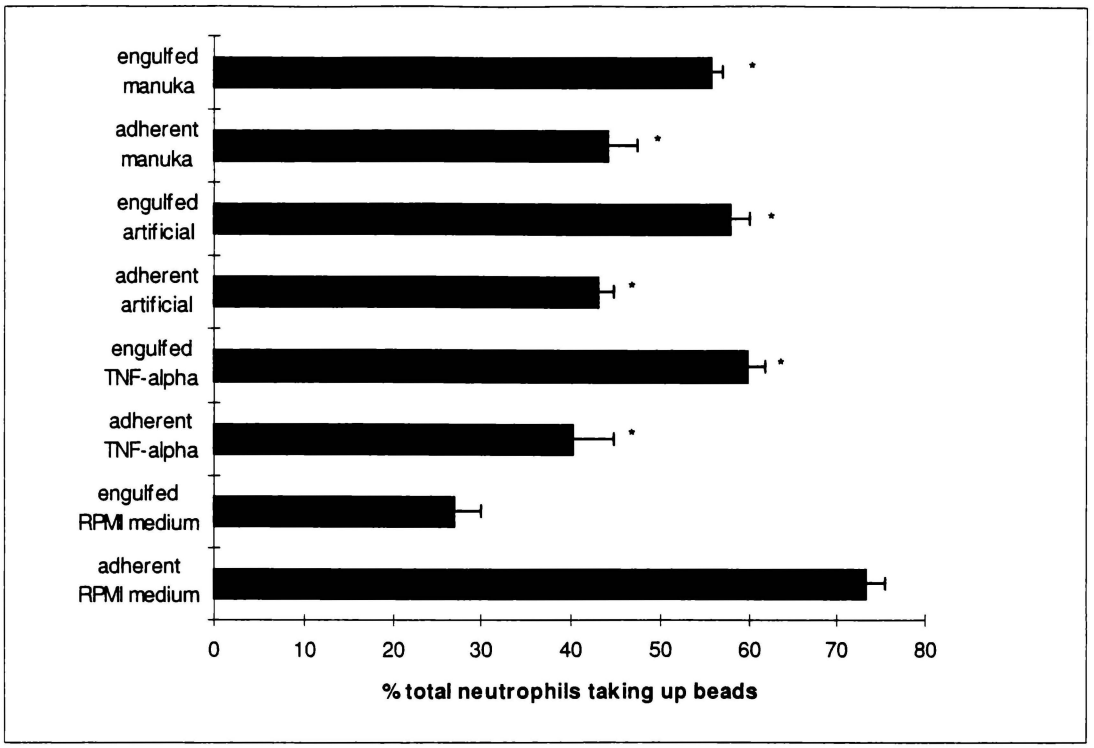


Figure 6.8: Effects of honeys compared with TNF-α on the proportion of adherent versus engulfed bacteria in neutrophils from bovine blood cultures. Blood was incubated with and without TNF-α (10 ng/ml; 30 min) or honey diluted to obtain optimal final concentrations (artificial or manuka; 0.25%; 30 min), prior to addition of bacteria. A phagocytosis assay was conducted and the number of neutrophils with adherent bacteria compared with engulfed bacteria were calculated as a proportion of total neutrophils taking up bacteria. Means for each treatment were compared with that obtained for the control (blood with RPMI basal medium), separately for adherent versus engulfed means. * $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6.

To test the possibility that honey might augment the stimulatory effect of TNF-α on phagocytosis, blood was primed with either TNF-α for 15 min followed by addition of honey for 15 min (as a model for application of honey to a wound after TNF-α signalling by local cells had begun), or with TNF-α and honey added at the same time (as a model for honey added immediately following surgery). The activating effect was compared with TNF-α alone. The number of neutrophils taking up bacteria was calculated as a proportion of total neutrophils for each treatment. Honey did not augment the TNF-α stimulus when added at the same time as TNF-α, or following prior incubation of the blood with TNF-α (null result, data not shown). These results indicate that when TNF-α is present in blood cultures *in vitro*, it has a maximal effect on neutrophil activity which honey cannot modulate. However, of prime importance here, honey did not exacerbate the stimulatory effect of exogenous TNF-α on neutrophils *in vitro*.

A further possibility to be tested was that honey may opsonize bacteria, and that this could account for the additional stimulation observed for blood primed with manuka honey (at a final concentration of 0.25%). Nonopsonized bacteria were primed with and without 1% BSA or 0.25% honeys (artificial or manuka), and the number of neutrophils taking up bacteria was calculated as a proportion of total neutrophils (Figure 6.9).

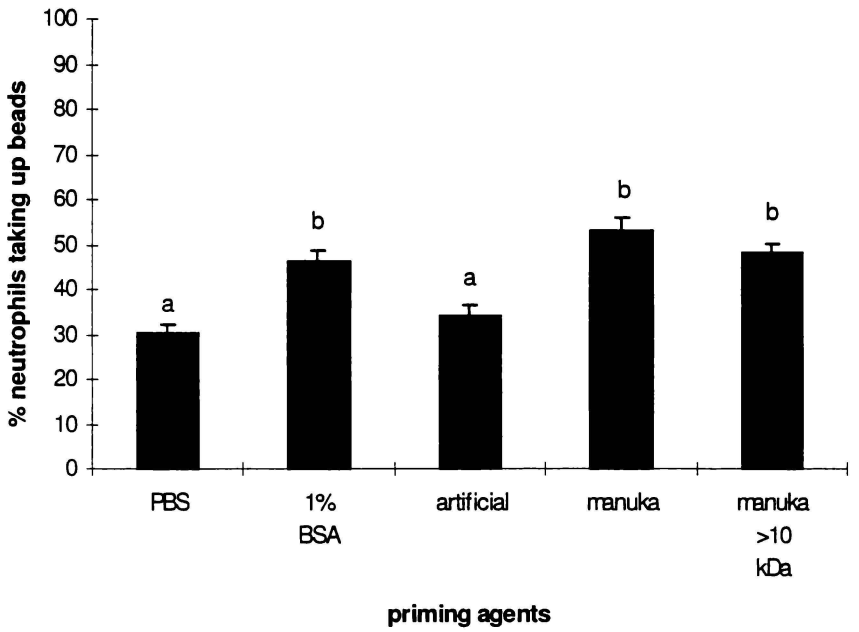


Figure 6.9: Effects of opsonizing bacteria with honey compared with 1% BSA on the phagocytosis of bacteria by bovine neutrophils. Bacteria were incubated with phosphate-buffered saline +/- 1% BSA or 0.25% concentrations of artificial or manuka honeys for 60 min (37°C, 5% CO_2, 95% air) prior to addition to blood cultures. A phagocytosis assay was conducted, and the number of neutrophils taking up beads were calculated as a proportion of total neutrophils. Each mean was compared with that obtained for the control (blood with non-opsonized beads). * $P<0.05$, data are expressed as the mean ± SEM of 2 independent experiments, conducted in triplicate; n=6.

The proportion of neutrophils taking up bacteria was significantly higher when bacteria were incubated with either: manuka honey, a high molecular weight fraction of manuka honey (both at 0.25%), or phosphate-buffered saline containing 1% BSA, prior to conducting a Phagocytosis assay than with phosphate-buffered saline alone. Incubation of bacteria with artificial honey had no effect on phagocytosis beyond phosphate-buffered saline alone. This result indicates that sugars alone cannot account for the opsonizing effect observed for bacteria incubated with manuka honey. That

bacteria opsonized with a high molecular weight fraction of manuka honey (with sugars removed), induced similar levels of bacteria uptake to the manuka stock, indicates the component acting as an "opsonin" must be of a high molecular weight. It was observed that background levels of phagocytosis were lowered when non-opsonized bacteria were used in a Phagocytosis assay (compared with Figures 6.2, 6.4–6.8). Though stimulating uptake of bacteria beyond the control, opsonizing bacteria with manuka honey did not induce the same levels of phagocytosis as had been seen when neutrophils were exposed to equivalent concentrations of manuka honey prior to conducting an assay with BSA-opsonized bacteria (Figures 6.4–6.8). Further, these results suggest that the additional stimulation of phagocytosis observed for manuka honey-primed blood (Figures 6.4–6.9), may be due to some opsonization effect on bacteria.

6.5 DISCUSSION

The prime objective of the current study was to model *in vitro,* the physiological environment which is created when honey is applied to a fresh wound site, and to investigate whether honey has an effect on phagocytosis. While limited studies had demonstrated that 0.1% honey could stimulate low levels of phagocytosis (Abuharfeil *et al.*, 1999), this was the first to investigate the mechanisms by which honey has this stimulatory effect.

The current study provides clear evidence that low concentrations of honey (optimal at 0.25%) induce phagocytosis by neutrophils due to the supply of sugars. The increased availability of sugars to the phagocytes gained by adding low concentrations of honey to cell suspensions increases cell activity. These conclusions fit well with the observations by others (Abuharfeil *et al.*, 1999; Efem, 1988). While there is substantial evidence that low levels of sugars activate the respiratory burst of macrophages (Gelderman *et al.*, 1998; Lefkowitz *et al.*, 1997 and Lincoln *et al.*, 1997) and neutrophils (Cohn and Morse, 1960), it has been established that neutrophil functions are rapidly downregulated once an optimal dose of sugars is exceeded (Candlish, 1993). It was noted that the stimulatory effects of sugars in the current

study were minimal compared with the positive controls. The assays described in the current study were conducted under ideal culture conditions, and sugars were unlikely to be lacking. This may mean that there is potential scope for greater stimulatory effects to be obtained when the cellular environment is suboptimal (*i.e.* during illness).

It was noted, as has been reported by others (Ducusin *et al.*, 2001), that there is a high background level of phagocytosis in blood when no priming agent is present (Figures 6.2, 6.4–6.9). A key observation in this study was that when blood is primed with some potentiating agent, the proportion of engulfed bacteria increases in relation to the proportion of bacteria merely adhered to the cell surface. This must indicate that the priming agent is stimulating the neutrophils to actively ingest bacteria, and therefore induces phagocytosis at levels beyond the background activity, which fits with numerous reports by others (Ducusin *et al.*, 2001; Cohen *et al.*, 2001; Heller *et al.*, 2001; Benjamini *et al.*, 1996). The observation that similar proportions of bacteria adhered to the neutrophil surface as were ingested by neutrophils, regardless of the priming stimulus, suggests that once initiated phagocytosis is an all-or-nothing response. In this study, the potential for honey to play a role in the chemotactic response of neutrophils to bacteria was not investigated, as it was assumed that the high ratio of bacteria:cells adopted (according to the Phagotest® protocol) was such that neutrophils would not need to seek out bacteria. Based on the demonstrated ability for honey to activate phagocytosis *in vitro*, it is suggested that a series of investigations could be designed to test whether honey affects chemotaxis, though this was beyond the scope of the objectives stated in this study.

At an optimal concentration (0.25%), manuka honey induces a similar level of phagocytosis as exogeneously applied TNF-α, an artificial model for one of the first mediators released at the site of tissue injury. In thermal injury, TNF-α release is often compromised by damaged tissues (Kaufman *et al.*, 1990; Crogan, 1976), and this directly contributes to the low numbers of neutrophils present in burnt tissues. Inadequate numbers of phagocytes lead to impaired debridement and defense functions (Kaufman *et al.*, 1990; Crogan, 1976), and consequently, tissue repair.

These results suggest a mechanism by which honey may have a place in treatment of burns, and help to explain what is observed anecdotally (Subrahmanyam, 1991).

The observation that manuka honey at an optimal concentration (0.25%) had no additional activating effect on neutrophils beyond TNF-α is itself a useful observation. While induction of phagocytosis by a stimulus is a necessary response, high levels of neutrophil activity have been associated with tissue damage due to the production of free radical species (Flohé *et al.*, 1985). In the current study, maximal concentrations of LPS and fMLP were demonstrated to induce a potent phagocytic response (96.3% ± 3%), which was 35% higher than phagocytic activity elicited by manuka honey. It is likely that the small stimulatory effect induced by manuka honey would be sufficient to initiate phagocyte activity in a non-infected wound, as it potentiated the same levels of activity as TNF-α. It is noted that the complexities of the non-infected wound *in vivo* are different from the simple *in vitro* model adopted here, and it would be useful to extend these observations to an animal model.

Interactions between receptors on the neutrophil cell surface and chemical signals produced by the target are essential for phagocytosis to occur (Benjamini *et al.*, 1996; Brown, 1995). Evidence that supports the likelihood that the additional stimulation obtained with manuka honey may be due to protein content, comes from reports that glucose-modified proteins act as attractants for neutrophils (Cohen *et al.*, 2001). Glucose-modified proteins have been demonstrated to modulate neutrophil chemotaxis, glucose uptake and consequently, phagocytosis (Cohen *et al.*, 2001; Cohen *et al.*, 2000). Much of the evidence that elevated levels of glycated proteins inhibit the respiratory burst of neutrophils comes from diabetes modelling (Candlish, 1993). A number of reports have established that glycated proteins are created when D-glucose reacts with serum albumin (Wu *et al.*, 1996) and that the resultant glycated proteins may have profound effects on various cells (Jensen *et al.*, 2003; MacCarthy *et al.*, 1994 and Takata *et al.*, 1988) and especially the phagocytes (Cohen *et al.*, 2001; Cohen *et al.*, 2000; Thornalley, 1999; Makita *et al.*, 1994; Makita *et al.*, 1991; Kirstein *et al.*, 1990; Seow *et al.*, 1987; Johnson *et al.*, 1983).

Only manuka honey (0.25%) acted as an opsonin for bacteria. It has been established that manuka honey has a higher protein content than pasture honey types and this may help to explain these observations (Weston, 2000). It is reasonable to assume that the ability of manuka honey to act as an opsonin indicates that the active compound must manipulate the recognition by neutrophils of ligands on the phagocytosed beads. Phagocytosis may only be achieved when specific receptors on neutrophils recognise ligands on their target (Brown, 1995). Opsonizing beads with Bovine Serum Albumin acts through an indirect binding mechanism as serum contains complement (Volle *et al.*, 2000). The interaction of complement and AGE has been identified as an important factor in increased macrophage-activation by glycated proteins (Vlassara *et al.*, 1985). Perhaps the most convincing evidence in support of this study comes from the observation that macrophages have receptors for AGE and uptake beads coated with AGE-linked to Bovine Serum Albumin (Takata *et al.*, 1988). Of relevance here, Wu *et al.* (1996) reported that incubation of serum albumin with D-glucose creates glucose-modified proteins which have profound effects on various cells, including the phagocytes.

Protein glycation and AGE are associated with increased free radical activity (Ahmed, 2005). AGE acting through receptors such as the receptor for advance glycation end products (RAGE, along with AGE-R1, AGE-R2, AGE-R3, sRAGE, macrophages scavenger receptor) induce NF-κB activation and cytokine production (Ahmed, 2005; Pullerits *et al.*, 2005; Hein and Franke, 2002). In addition to effects on intracellular signalling, gene expression, and release of pro-inflammatory molecules, the AGE-RAGE association leads to the production of free radicals (Ahmed, 2005). AGE receptors are found in a range of immune cells, and the stimulation of NF-κB through AGE-RAGE interaction leads to a more prolonged activation than NF-κB stimulation through cytokines (Hein and Franke, 2002). Sustained receptor expression of RAGE has been demonstrated to create a positive feedback loop (Pullerits *et al.*, 2005). There is evidence that a soluble RAGE receptor (sRAGE) can block the ligand-RAGE interaction through direct binding with leukocyte β2-integrin Mac-1 (Pullerits *et al.*, 2005). The implication for the sRAGE being a competitive receptor with RAGE is

that upregulation of sRAGE will reduce infiltration of neutrophils into tissues. Cell-bound RAGE function as a counter-receptor for leukocytes, resulting in an amplification of the inflammatory response (Pullerits *et al.*, 2005

However, the total concentration of proteins in manuka honey is low (National Honey Board USA, 2003; Molan, 1992b), and this casts doubts on the feasibility of their likely potency. Evidence that supports the possibility that the antioxidant content of manuka honey may account for its additional stimulatory activity comes from reports that phagocytosis is augmented when phagocytes are exposed to antioxidants (Heller *et al.*, 2001; Linden *et al.*, 1988). The high molecular weight fractions of dialysed manuka honey would contain large polyphenols (Molan, 1992b).

A direct nutritive effect of honey on cells cannot be ruled out. A number of reports have proposed that the availability of trace levels of zinc and copper influences phagocyte activity (Lentsch *et al.*, 2001; Chandra and Chandra, 1986) and that marginal deficiencies in copper cause markedly impaired functions of both neutrophils and lymphocytes. This is due to the requirement of key enzymes for copper to act as a cofactor. Honey contains copper, and a study by Al-Waili (2003) reported that oral ingestion of small amounts of honey was sufficient to markedly increase serum copper concentrations. Honey was also found to give a slight increase in serum zinc levels (Al-Waili, 2003). Therefore, it is possible that elevation of copper and zinc levels in phagocyte cultures obtained by addition of honey may have enhanced neutrophil activity. The feasibility of such low concentrations of honey having this effect in the current study are unknown (though the demonstrated ability for oral ingestion of small amounts of honey to have significant effects on serum content reported by Al-Waili, 2003, make it likely).

While observations from the phagocytosis assays offer insight into the mechanisms by which honey has been observed to promote tissue repair in a variety of wound types (Molan, 2004; Wood *et al.*, 1997; Harris, 1994; Efem, 1988), no anti-inflammatory activity was identified. Due to the limitations of an *in vitro* system, the use of an animal model will be necessary to shed light on the effects on neutrophil functions *in vivo*.

Chapter Seven: The Effect Of Honey On Tight Junction Integrity

SUMMARY

The effects of honey on tight junction (TJ) resistance were assessed for MDCK cell monolayers subjected to an EGTA challenge. It was found that manuka honey and pasture honey have protective effects on TJ following the challenge, and enhance post-challenge recovery of transepithelial resistance. Manuka honey had greater modulatory activity on TJ with increased concentration from 0.1–1%, and 1% concentrations of both honeys gave the greatest protective effects. Manuka honey appeared to have greater protective effects than pasture honey. Application of manuka honey (at 1% final concentrations) to both the apical and basolateral sides of the MDCK cell monolayer significantly enhanced TJ tightness beyond the control. Dialysis of the honey confirmed that the high molecular weight fraction contained the active component. Diffusate fractions from either honey type had no effect on TJ. Artificial honey had no effect.

7.1 INTRODUCTION

While this thesis had identified a number of points at which honey had been shown to stimulate activity in a range of cell types, limited evidence for the anti-inflammatory activity of honey had been found. Yet, there is abundant evidence from both clinical trials and from experimental animal models, that honey has anti-inflammatory activity in a range of therapeutic applications including: tissue repair after burn injury (Postmes *et al.*, 1997; Burlando, 1978) and disruption of the gastric mucosa (Bilsel *et al.*, 2002; Gharzouli *et al.*, 2001; Ali *et al.*, 1997). Inflammation can only proceed if activated leukocytes can leave the circulation and undergo a number of cellular adhesion interactions, enabling them to pass through permeable tight junctions, and reach the tissues (Mowery *et al.*, 2004). The main focus of the investigations described here was to test whether honey effected any changes in tight junction (TJ) permeability.

In order for leukocytes to pass through the cell layers between the vascular system and the target tissues, diapedesis must occur, whereby leukocytes must adhere first to the vascular endothelium, and then squeeze through tight junctions. This series of events is initiated by a sequence of interactions between selectins on leukocytes, and activated endothelial cells and connective tissues (Ebnet and Vestweber, 1999).

After adhesion of leukocytes to the endothelium has occurred, diapedesis and transmigration is regulated by the permeability of TJ in the endothelium and surrounding tissues. The TJ (zonula occludens), is part of the plasma membrane surrounding endothelial or epithelial cells near their apical domain, and has two key functions; acting as a barrier by regulating the paracellular transport of molecules and ions across cell sheets, and as a fence, separating the apical and basolateral domains of the plasma membrane (Schneeberger and Lynch, 1992). By uncoupling the barrier and fence functions, Singer *et al.* (1994) demonstrated that the two functions are distinct from each other. Therefore, TJ provide a barrier function, regulating movement of cells between the tissues (Singer *et al.*, 1994; Schneeberger and Lynch, 1992).

The structural integrity of the TJ is dependent on the localization of key TJ proteins, occludin, claudin-1, zonula occludens-1 (ZO-1), junction adhesion molecule-1 (JAM-1, A, B, C) and molecules such as VE-cadherin (Wittchen *et al.*, 2003; Hopkins *et al.*, 2003). VE-cadherin is a calcium reliant adhesion molecule, and therefore removal of calcium will prevent integrins from functioning. If calcium is removed, monocytes will not adhere to plastic surfaces due to the inability of MAC-1 to function. Where the assembly of proteins in the TJ is disrupted, the barrier function breaks down. When the barrier function is breached, such as occurs during the inflammatory response, the TJ become permeable to leukocytes, enabling them to accumulate in tissues and creating inflammation as a result. As this study used a pulmonary epithelial cell line to study the effect of honey on tight junctions it is appropriate to consider what is currently known about the role of tight junctions in immune exclusion of bacteria and viruses. Enteric pathogens either alter cytoskeletal structure or specific tight junction proteins (Berkes *et al.*, 2005). There is divergence in the mechanisms and resultant signalling pathways by which different microorganisms disrupt the epithelium. *Clostridium perfringens*, an anaerobic bacterium causing food borne gastrointestinal illness, has an endotoxin (*C perfringens* endotoxin, CPE), which binds to the epithelial cell surface and increases permeability (Berkes *et al.*, 2005). The 200 kDa CPE complex contain the TJ protein occludin, and induce TJ fibrils and remove claudin-4 from TJ, disrupting the TJ structure (Berkes *et al.*, 2005). Other TJ proteins known to function as receptors for micro-organisms include junctional adhesion molecules, to which reovirus bind, along with both the coxsackievirus and the adenovirus receptor (Berkes *et al.*, 2005). A further example of how micro-organisms disrupt TJ integrity comes from a documented study on *Escherichia coli* cytotoxic necrotizing factor-1 (CNF-1) (Hopkins *et al.*, 2003). CNF-1 changes the polarization of key TJ proteins, occludin, ZO-1, and JAM-1 and displacement of the proteins leads to profound reductions in TJ gate function (Hopkins *et al.*, 2003).

Epithelial mechanisms to protect against penetration by micro-organisms include the expression of cytokines, adhesion molecules, and MHC class II molecules Berkes *et al.*,

2005) after bacterial adherence or invasion. Secretion of IL-8 by basement membrane in response to transendothelial migration regulates the migration of neutrophils (Marshall *et al.*, 2003). Both invasive and non-invasive enteric pathogens trigger activation of NF-κB (Berkes *et al.*, 2005), and this transcription factor controls the expression of most of the proinflammatory cytokines, chemokines, immune receptors and cell surface adhesion molecules. Several cytokines involved in IBD and Crohn's disease act through activation of the STAT family (Musso *et al.*, 2005). Leukocytes are recruited into intestinal tissues by chemotactic substances and adhesion molecules (*e.g.* ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), mucosal addressin cell adhesion molecule 1 (MAdCAM-1) and E-selectin) on the surface of endothelial cells lining blood vessels (Miller *et al.*, 2005). Leukocytes bind with the endothelial cell adhesion molecules according to the expression of their respective integrin counter-receptors. ROS have been shown to upregulate the expression of cell adhesion molecules on endothelium (Segui *et al.*, 2004). The oral ingestion of honey will bring it into contact with the gut mucosa, and may have implications for inflammatory gut conditions.

After inflammation has been initiated it proceeds according to two key phases, cellular adhesion followed by diapedesis (Figure 7.6). Briefly, cellular adhesion is the phase incorporating capture of the leukocytes via interactions with activated endothelium. Selectins and their carbohydrate ligands initiate rolling of the leukocytes along the endothelium so that integrin molecules on the immune cells bind with counter-receptors on the endothelial cells (creating firm adhesion) (Kruidenier *et al.*, 2003). Once the flattened leukocytes are firmly adhered, diapedesis is facilitated by some members of the immunoglobulin gene family (*e.g.* ICAM, VCAM), enabling the cells to squeeze through the endothelial sheet and move toward chemotactic gradients in the target tissues. At the target tissues, leukocytes may have to migrate through another epithelial layer. It is this second key phase of inflammation that is the focus of the investigations described in this chapter. Cell emigration from the circulation and into the target tissues depends on permeability of TJ. Consequently, if TJ are too tight the cells will not cross the barrier, and inflammation will not proceed. Movement is not just a consequence of TJ integrity,

but also a combination of factors including the expression of appropriate adhesion molecules and counter-receptors for the integrins. As well a re-distribution of molecules such as VE-cadherin, the junctional adhesion molecules (JAM-A, -B, and -C), PECAM and CD99 in endothelium.

The investigations described in this chapter were based on studies performed by Stelwagen and Ormrod (1999; 1998), which focused on the development of a cell-based assay for TJ integrity to evaluate and characterise the anti-inflammatory activity of hyperimmune milk. Likewise, the *in vitro* model described herein was designed to mimic the barrier function of TJ as occurs in both the gut and the skin.

In persistent inflammation, TJ remain leaky thereby maintaining swelling and associated white blood activity in the affected site, resulting in tissue damage from excessive free radical production (Lichtenberger, 2001). It follows from an anti-inflammatory perspective that it is desirable to restore TJ integrity quickly after an inflammatory challenge, to prevent persistent inflammation from causing further tissue damage. TJ leakiness has also been associated with administration of various anti-inflammatories, such as steroids and NSAIDs, which cause loss of the intestinal barrier function and result in the abnormal passage of blood components into the gut (Lichtenberger, 2001). However, the observed increase in gut permeability accompanying administration of NSAIDs (such as Indomethacin) result from cell damage and resulting reduction in cell cover rather than changes to TJ themselves (Hollander, 1999).

Across polarised cell layers a small potential difference exists but only when TJ are intact (Stelwagen *et al.*, 1994). *In vitro* it is possible to measure electrical resistance across a cell monolayer, which changes according to the permeability of the TJ, decreasing when they become leaky and increasing as the integrity is restored (Stelwagen *et al.*, 1999). The electrical resistance can be measured directly, and changes in resistance can be mapped through time, in the presence or absence of bioactive substances, following an inflammatory challenge. It was this technique that enabled Stelwagen *et al.* (1999) to

investigate the effects of hormones on the molecular structure of TJ in mouse mammary cells (Stelwagen *et al.*, 1999) and the anti-inflammatory activity of HIMF (Stelwagen and Ormrod, 1998).

In order to understand how honey may have an impact on restoration of TJ integrity, it is necessary to consider how the composition of the TJ is affected by known damaging agents, (such as EGTA), and protective agents, (such as HIMF). TJ are dynamic structures. There are many known agents which increase permeability across the TJ, these include: endocrine factors, cytokines, bacterial products, and specific toxins such as *Vibrio cholerae* (called Zonula Occludens Toxin) (Hollander, 1999; Nguyen and Neville, 1998).

The primary objective of this chapter was to investigate if honey acts at this level. While evaluation of the effects of honey on white blood cell function provides an indication of bioactivity at the earliest stages of inflammation, measuring the impact of honey on TJ integrity enables examination of a downstream effect. The observation that topical application of honey led to a reduction in the total neutrophil count at the site of inflammation (Postmes *et al.*, 1997), may reflect some modulatory effect on adhesion and diapedesis. There are a number of reports which demonstrate that honey conveys a protective effect to the gastric mucosa (Bilsel *et al.*, 2002; Gharzouli *et al.*, 2001; Ali *et al.*, 1997) but these have not measured any effects on TJ. Addition of honey to the apical side of the cell lawn in the TJ assay is analogous to both topical application of honey to skin or oral ingestion of honey. Application of honey to the basal side of the cell monolayer in the TJ assay is analogous to honey present in the circulating bloodstream.

It was therefore useful to extend the scope of this study to investigate whether honey has a significant effect on inflammation at this stage of the process, by measuring its effect on TJ integrity when cells were exposed to an inflammatory challenge. Addition of EGTA to MDCK cells results in chelation of calcium ions and the subsequent disruption of the paracellular barrier both *in vitro* and *in vivo* (Stelwagen *et al.*, 1995; Pitella *et al.*, 1993;

Neville and Peaker, 1981). Both *in vivo* and *in vitro* experiments have clearly demonstrated the requirement of TJ for adequate extracellular calcium levels (Stelwagen *et al.*, 1995; Pitellea *et al.*, 1993). These extracellular calcium levels are necessary to ensure sufficient intracellular calcium, in turn required for TJ assembly and maintenance (Cerijido *et al.*, 1993). EGTA was used in this study to challenge TJ in the presence or absence of honey. If any activity was identified, a further objective was to perform a simple fractionation of honey by dialysis and attempt to characterise the molecular weight of the bioactive component.

7.2 MATERIALS AND METHODS

7.2.1 Reagents

DMEM culture medium

Culture medium for MDCK was comprised of: Dulbecco's modified Eagle's medium (DMEM; containing 1.5 g/L sodium bicarbonate and 4.5 g/L glucose,GibcoBRL, Cat # 12 100-046); 10% Foetal Calf Serum (GibcoBRL, Cat # 10 091-148); 1% Penicillin-Streptomycin (GibcoBRL, Cat # 15140-122).

Phosphate-buffered saline

Dulbecco A; Oxoid BR14; 1 tablet in 100 ml of distilled water.

Trypsin-EDTA

To a trypsin stock solution (25 g/l trypsin, GibcoBRL) was added 20% EDTA.

Honey solutions

Honey solutions were prepared as described in Section 2.2.2. Two honey types were selected for testing, M109 manuka honey and Otago Pasture honey, along with dialysis fractions of each (prepared as described in Section 2.2.2.1). Artificial honey was used to control for the osmotic effects of sugar.

7.2.2 Cells and culture

Madin-Darby canine kidney cells (MDCK, strain II) were supplied by AgResearch (Ruakura). They are an epithelial cell line that forms an adherent cell monolayer. Cells were used between passage numbers 82 and 93. Cells were passaged every 2 days, at which time cells could be removed for experimental use.

Maintenance

MDCK cells were passaged every two days, seeded (1 x 10^6 cells/ml) in Nunc culture flasks (160 ml, Life Technologies), and incubated at 37°C (5% CO_2, 95% air). To split the cells, the growth medium was poured off, the cell lawn washed once with 4 ml of phosphate-buffered saline and 4 ml of trypsin-EDTA (Gibco) added. The flask was placed in an incubator (37°C, 5% CO_2, 95% air) for 10 min after which time the flask was tapped to loosen any cells which remained adhered to the base. The trypsin-EDTA was inactivated by adding 5 ml of DMEM medium across the cell lawn and any loose cells washed off the flask wall and collected at the bottom of the flask. If a significant number of cells remained adhered to the wall of the flask, a cell scraper was used to remove these. The cell suspension was then transferred to a 15 ml centrifuge tube (Nunc). The cells were centrifuged (Mistral) at 223 x *g* for 3 mins, and the supernatant discarded. The cell pellet was re-suspended in 2 ml of DMEM medium, and 1 ml containing cells split for passage in a sterile flask with the other cells set aside for experimentation.

7.2.3 Setting up a TJ assay

To prepare sheets of MDCK cells for testing the effects of anti-inflammatory activity on TJ, a series of chambers were seeded with cells and the change in transepithelial resistance (TER) monitored following the addition of various treatments.

7.2.3.1 Seeding the inserts

To prepare the cell monolayers, one insert (Nunc Anapore inserts,10 mm, 0.02 μm pore size; Life Technologies) was placed in to each well of a 12-well culture plate (Nunc). Into each of 11 inserts 500 μl of cell suspension (at a density of 3 x 10^6 cells per insert) was added.

A haemocytometer was used to establish viable cell density, counting nuclei stained with crystal violet (16 mg crystal violet, 4 ml 1 M citric acid, 36 ml Milli Q, Sigma-Aldrich).

Staining of the cells was achieved by placing 100 µl of the cell suspension (the cell pellet re-suspended in 5 ml of DMEM medium), in an Eppendorf tube, vortexed for 10 secs with 900 µl of crystal violet.

The 12th well was reserved as a background well and received only 500 µl of DMEM medium (no cells). Once the inserts were loaded, 300 µl of extra DMEM medium was loaded into each well (so that wells contained a final volume of 800 µl). The plate was incubated overnight (37°C, 5% CO_2, 95% air). After 24 h, formation of TJ was assessed by TER measurement across cell monolayers using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA, USA). Each of the inserts was removed from the culture plate and placed in an Endohm-12 chamber (World Precision Instruments, Sarasota, FL, USA). TER values were recorded as $\Omega.cm^2$. Readings were taken when values remained unchanged on the voltohmmeter for at least 5 sec. The TER of the background well (with DMEM medium and no cells) was subtracted from TER measurements. TER were deemed to have formed when TER readings were >700 $\Omega.cm^2$. The cell preparations were then ready for addition of the honey solutions.

7.2.3.2 Exposure of the cell monolayers to honey solutions

To deliver the honey solutions, it was first necessary to remove the DMEM medium. A standard sequence was adopted for removal of the medium, first aspirating the medium from the plate wells and then from the inserts, taking care not to touch the membrane. The medium was refreshed every 24 h. This sequence for aspirating medium ensured minimum disruption to the apical side containing the cell monolayer. The inserts were each loaded with 500 µl of honey solutions (diluted in DMEM medium to final volumes). Controls received 500 µl of DMEM medium only. The preparation of honey stock solutions was according to the method described in Section 2.2.2. For each treatment, there were five replicates (five seeded inserts) per assay run. The plate was left to incubate for a further 24 h to enable the cells to adjust to their culture conditions before being subjected to an EGTA challenge. Further, exposure of the cell monolayers to honey

solutions prior to the EGTA challenge enabled any protective effects on TJ conferred by the treatments to be detected. The inclusion of background wells containing 800 µl of honey solution (at the same final concentration as used in treatments) and no cells provided a baseline TER measure to compare the conductivities of the different solutions.

7.2.3.3 The EGTA challenge

Preparation of the honey solutions containing EGTA

To measure the protective effect of honey against damage by EGTA, the cells were subjected to an EGTA challenge. Before commencing the EGTA challenge the TER of the cells was measured again exactly 24 h after the previous measurement. EGTA was added at a final concentration of 3 mM.

Administering the EGTA challenge

Immediately prior to the EGTA challenge, TER measurements were taken (time 0 data). The background readings were always taken first, followed by the control and the honey solutions beginning with the lowest concentration. To enable the EGTA-containing honey solutions (and control solution) to be loaded, the medium was aspirated from the inside of inserts and replaced by treatment solutions. No EGTA was loaded into the background insert. The plate was incubated for 60 min, after which TER of each insert was measured. The plate was placed in the incubator for a further 60 min and a second reading conducted 60 min after the first had been performed (time 2 data). Once the final reading had been performed, the contents of the wells and inserts were changed to honey containing medium without EGTA. The plate was returned to the incubator for a further 24 h (i.e. post-challenge recovery period).

During the recovery period, TER measurements were recorded to determine the effect of honey solutions on the ability of TJ to regain their original TER at time 0 (prior to the EGTA challenge). TER readings were repeated 24 h and 48 h after the EGTA had been

replaced with honey solutions (or DMEM medium only). No further solution changes occurred during this period.

7.2.4 Preliminary Experiments

Establishing whether honey could interfere with TER measurements across the insert membrane

To investigate the possibility that honey might be adhering to the well insert, thereby creating an artificial barrier, honey solutions (artificial, manuka and Otago pasture honeys, at 0.1, 0.5, 1, and 3%) or DMEM medium alone were loaded into unseeded inserts, and incubated in the absence of cells. The TER measurements were tracked daily over a three day period. There were no significant differences in TER for inserts containing honey solutions beyond DMEM medium alone (all were in the range of 35-44$\Omega.cm^2$ corrected for 0.6 cm^2 area which is similar to normal background readings) and this established that the presence of honey would not have any direct effects on TER measurements.

Establishing a tolerance range for MDCK cell osmolarity

To identify honey concentrations which damage MDCK cells, 500 µl of suspension containing 3.6 x 10^6 cells per ml was loaded in to wells of a 24-well microtitre plate (Nunc). After 24 h the DMEM medium was removed by aspiration and replaced with 500 µl of various dilutions of honey to obtain final concentrations (0.1, 0.5, 1 and 3% artificial honey, manuka honey and Otago pasture honey) each in triplicate. The plates were incubated at 37°C (5% CO_2, 95% air) for 24 h. Prior to removal of the honey solutions, the appearance of the cell membranes was noted (shrivelled membranes indicate osmotically stressed cells). After incubation, honey solutions were removed and replaced with 100 µl of trypsin (2%) before returning the microtitre plate to the incubator for 10 mins to dislodge the cells from the plate. After addition of 100 µl of DMEM medium (to neutralise the trypsin) the cell suspensions were removed and an equal volume of phosphate-buffered saline added. After centrifugation (223 x *g* 3 min), the

cells were re-suspended in 1 ml of phosphate-buffered saline and viability assessed using trypan blue staining.

The effect on cell viability of exposing MDCK monolayers to honey concentrations are shown in Figure 7.1. The results in Figure 7.1 show that direct incubation of MDCK cells with 1% final concentrations of artificial honey (sugars alone) caused significant osmotic damage. At 3% concentrations, all honey types caused significant osmotic damage.

A further osmotic tolerance assay was conducted to examine whether osmotic effects of honey on cells would be different when a freeflow of sugars exists (as is the case in the TJ assay). Using the TJ assay, inserts were seeded with MDCK cells at a density of 3 x 10^6 cells per insert (as described in Section 7.2.3.1) and after 24 h inserts were loaded with honey solutions (manuka honey, Otago pasture honey or artificial honey at final concentrations ranging from 0.1–3%) or DMEM medium alone as described in Section 7.2.3.2. After 24 h incubation the honey solutions were aspirated from the inserts, leaving the cell monolayer intact. To each insert, 200 µl of trypsin-EDTA was added, and the plate returned to the incubator for 10 min (37°C, 5% CO2). After incubation, 200 µl of DMEM medium was added to each insert and the cell suspensions retrieved and a viability count performed using trypan blue stain (as before). The objective of this experiment was to test whether higher concentrations of honey would pose osmotic problems when used in a system enabling freeflow of sugars.

There was no osmotic damage to MDCK cells incubated with the honey solutions at any of the concentrations tested when the cells were exposed to honey in the TJ assay set-up. However, it was noted that the volume of solution in the insert had increased significantly, indicating that the DMEM medium in the well chamber had been drawn up through the insert membrane. The volume contained in the insert was near the top of the walls after 24 h (pilot assays testing the osmotic effects of higher concentrations of honey

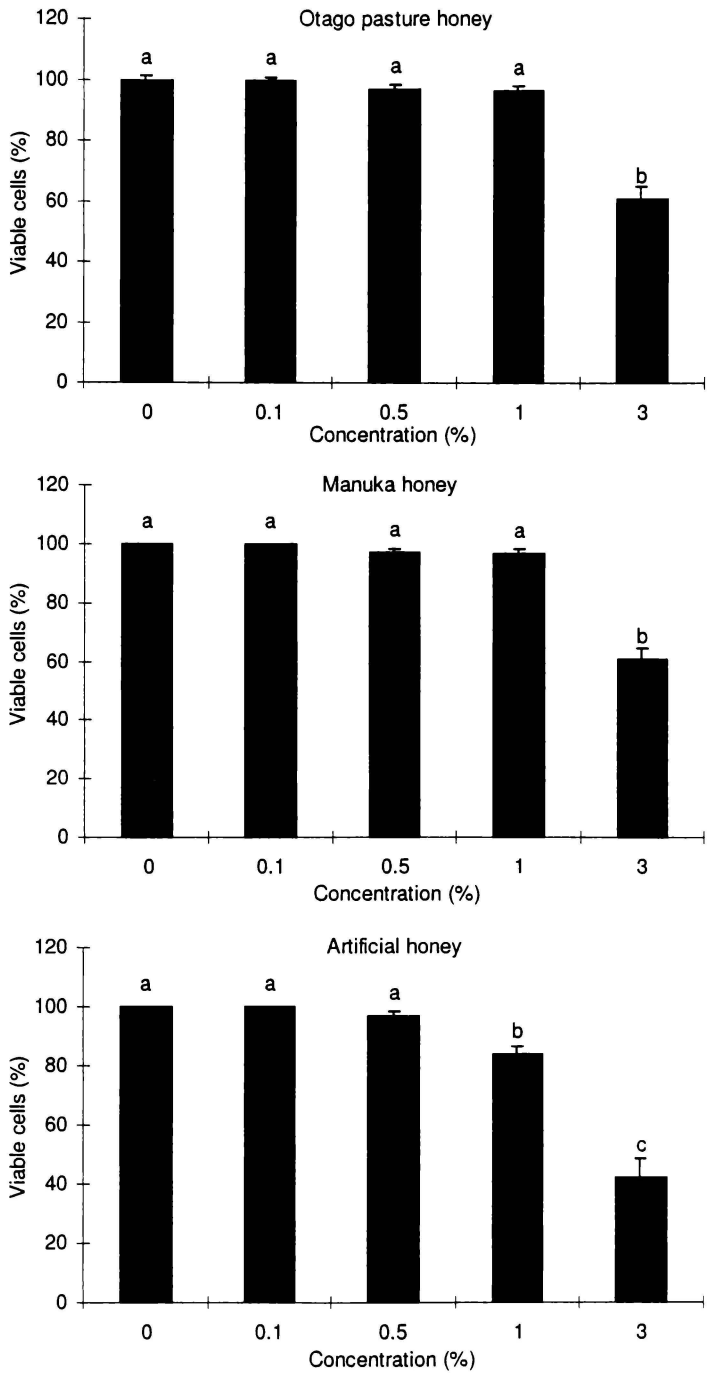


Figure 7.1: The direct effect of honey concentration on MDCK cell viability. Cells were incubated for 24 h with various concentrations of honeys (Otago pasture, manuka honey or artificial) or DMEM medium alone. Cell viability was assessed using trypan blue exclusion. Data points represent the mean of five wells ± SEM and the assay was replicated twice (n=10). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

>3% had shown that after 24 h the contents of the insert were spilling over the insert walls back into the plate well, therefore altering the experimental conditions). These results demonstrate that when the cell monolayers were exposed to honey-free medium on one side and honey on the other side, that osmotic flow through the cells would prevent osmotic damage from occurring (analogous to honey on an open wound). However, the problems created by drawing of the medium into the insert meant that testing would be restricted to honey concentrations below 3% in this assay system. Although 3% honey had posed osmotic problems in a closed system (Figure 7.1), these problems did not occur in the freeflow environment of the TJ assay, and was therefore included as the upper concentration tested.

7.2.5 Experimental Design

7.2.5.1 Establishing the direct effect of honey on TJ resistance

Inserts were seeded with MDCK cells at a density of 3 x 10^6 cells per insert (as described in Section 7.2.3.1) and after 24 h inserts were loaded with honey solutions (manuka honey or artificial honey at 0.1 and 1% final concentrations) or DMEM medium alone as described in Section 7.2.3.2. TER measurements were recorded at 24 h intervals over a 72 h period. No EGTA challenge was undertaken. Manuka honey was used as a representative example of natural honey.

7.2.5.2 Establishing whether honey has activity on TJ resistance during and following an EGTA challenge

Inserts were seeded with MDCK cells at a density of 3 x 10^6 cells per insert (as described in Section 7.2.3.1) and after 24 h, inserts were loaded with honey solutions (manuka, Otago pasture, or artificial honey at final concentrations ranging from 0.1–3%) or DMEM medium alone as described in Section 7.2.3.2. The MDCK cells were then exposed to an

EGTA challenge (as described in Section 7.2.3.3) and recovery of TJ integrity monitored by taking TER measurements 1 h, 24 h and 48 h after the challenge.

This assay was repeated using dialysis fractions of manuka honey and Otago pasture honey at final concentrations equivalent to 1% honeys.

7.2.5.3 Establishing whether honey has different effects on TJ resistance when added to the basal side of the insert compared with the apical side

MDCK cell monolayers were prepared as described for Section 7.2.3.1. As described for Section 7.2.3.2, manuka honey, Otago pasture honey or artificial honey (at 1% final concentrations) was added to the insert and was present on the apical side of the monolayer, being analogous to contact of honey with either the gut mucosa or the skin. To other inserts, manuka honey, Otago pasture honey or artificial honey (at 1% final concentrations) was added to the plate well instead of DMEM medium. This created a basal application which was analogous with circulation of honey in the bloodstream. The control contained DMEM medium on both sides of the monolayer.

7.2.6 Statistical Analysis

Consistency in measurement was maintained throughout the assays. The insert was centred on the electrode in the chamber, and each time the probe was introduced to the insert it was ensured that the probe was immersed in liquid. The conductivity of the electrical charge is optimised by total surface contact with fluid.

Data were analysed with the GLM procedure of SAS (SAS System for Windows, Release 6.11, 1996; SAS Institute Inc., Cary, NC, USA), and differences between means were considered significant at $p<0.05$. Standard error bars represent SEM. The data were expressed as mean TER values taken as a proportion of those obtained for each corresponding treatment prior to the EGTA challenge (after 24 h incubation with honey

solutions). TER values of insert membrane and medium (*i.e.* no cells, medium alone), and membrane surface area (*i.e.* 0.6 cm^2) of the insert were used to correct TER measurements, and TER is expressed as $\Omega.cm^2$. The model used the pre-experiment TER measurement as a covariate to adjust for the starting cell density of the inserts. Differences between treatments were analysed by ANOVA. Replicates were blocked to correct for replication effects. Means for each treatment represent TER values obtained for five replicates, and the assays were repeated twice (n=10).

7.3 RESULTS

The effect on TER measurements of exposing MDCK cells to honey solutions compared with those obtained with DMEM medium alone are shown in Figure 7.2. The results in Figure 7.2 show that after 24 h incubation with 1% manuka honey, TER values were significantly higher than obtained for cells incubated with either concentration of artificial honey or medium alone. An increase in TER values was coupled to an increase in TJ tightness. These results indicate that after 24 h, 1% manuka honey increased the resistance of the MDCK cell TJ. The lower concentration of manuka honey (0.1%) had no effect on TJ resistance beyond DMEM medium alone. After a further 24 h (at the 48 h time-point) there were no significant differences between the TER measurements obtained for any of the honey treatments.

The effect on TJ resistance during and following an EGTA challenge of incubating MDCK cells with various honeys (manuka, Otago pasture or artificial) or DMEM medium alone at concentrations in the range from 0.1–3% are shown in Figure 7.3.

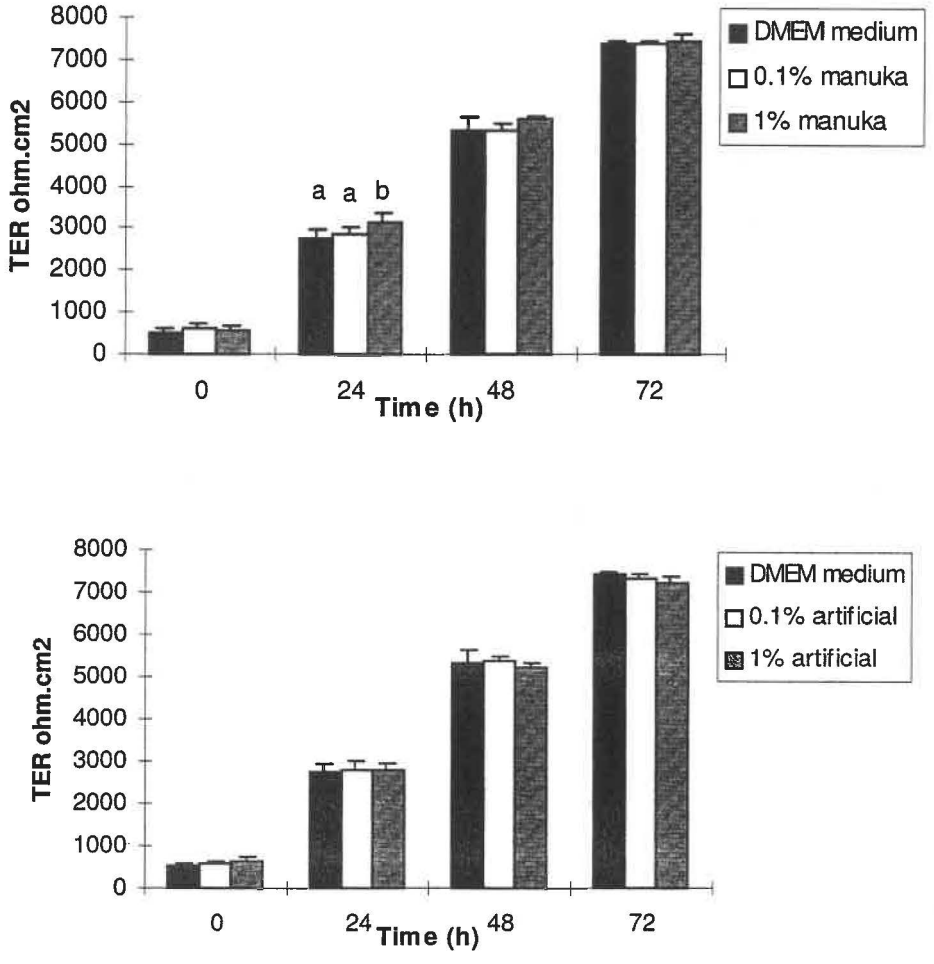


Figure 7.2: The direct effect of honey on TJ resistance in kidney cells (MDCK). Cells were maintained for 72 h in either medium or honey solutions (manuka or artificial at 0.1 or 1% final concentrations). Honey was added at 0 h and TER was measured at this time-point and again at 24 h intervals. The data are expressed as the mean of five replicates ± SEM and the assay was repeated twice (n=10). Differences between the means are indicated by superscript letters, where means not sharing common letters within a timepoint are significantly different ($P<0.05$).

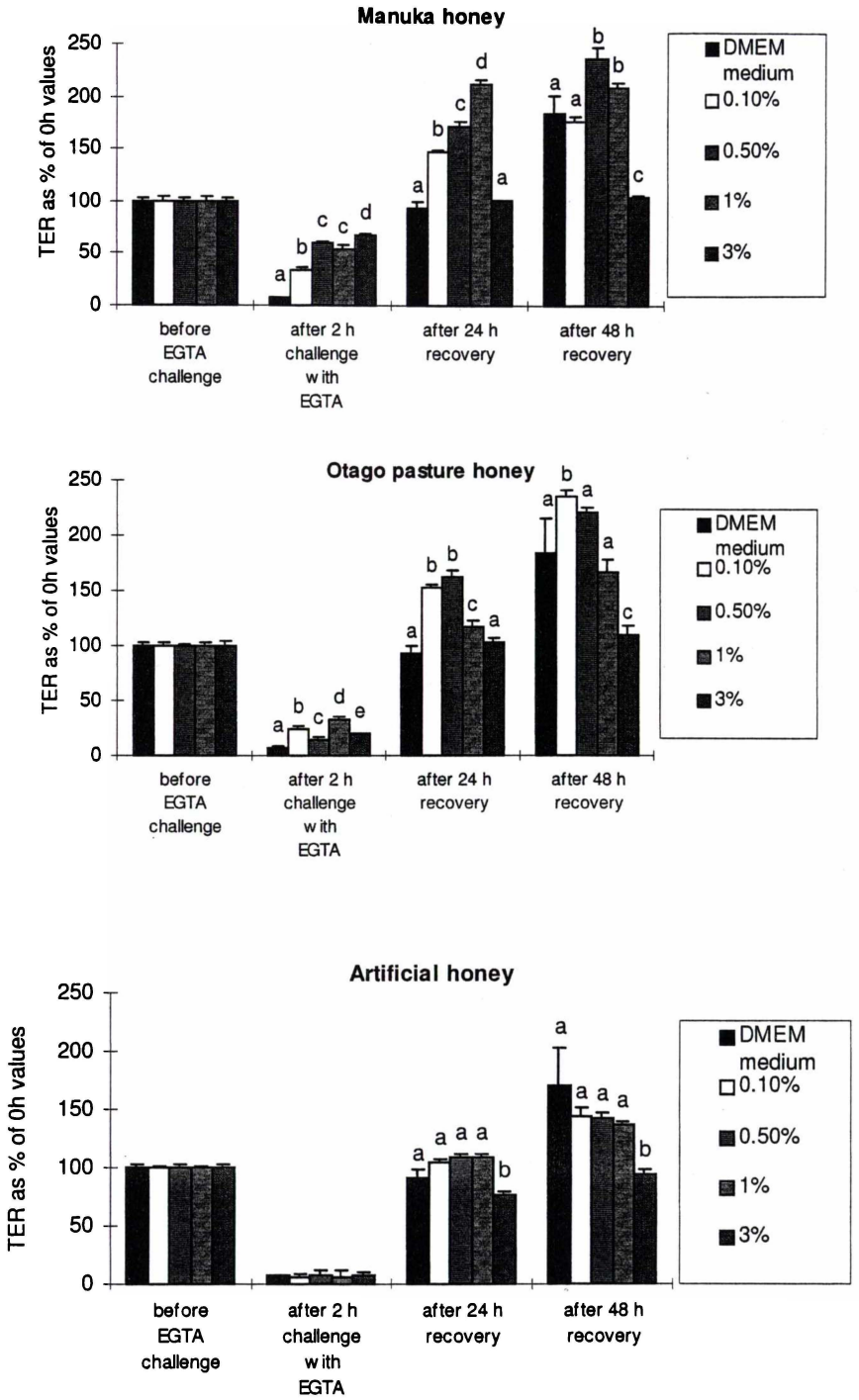


Figure 7.3: Effect of an EGTA challenge on loss of transepithelial resistance (TER) in honey-treated kidney cells (MDCK). Cells were maintained for 24 h with or without honeys (manuka, Otago pasture or artificial at 0.1, 0.5, 1 or 3% final concentrations) prior to a 2 h challenge with 3mM EGTA (in honey solutions). Data points represent the mean of five replicates ± SEM and the assay was repeated twice (n=10). The data are expressed as mean TER values, taken as a proportion of those obtained for each corresponding treatment prior to the EGTA challenge. Differences between the means are indicated by superscript letters, where means not sharing common letters within a timepoint are significantly different ($P<0.05$).

The results in Figure 7.3 show that both manuka and Otago pasture honey exerted protective effects on TJ after challenging the cells for 2 h with EGTA, as TER values did not drop as low as occurred with DMEM medium alone. Artificial honey did not show this protective effect, indicating that sugars alone cannot be responsible. Manuka honey appeared to convey a greater protective effect to TJ integrity during the EGTA challenge than Otago pasture honey.

Both manuka honey and Otago pasture honey significantly enhanced restoration of TER 24 h after addition of EGTA. At this timepoint, TER values were highest for cells incubated with 1% manuka honey. Therefore, manuka honey at 1% enhanced the ability of TJ to recover their TER as a proportion of the values recorded prior to the inflammatory challenge. Artificial honey had no effect on TJ recovery above that obtained for DMEM medium alone (Figure 7.3). The effect on TJ resistance during and following an EGTA challenge of incubating MDCK cell monolayers with dialysis fractions of manuka honey and Otago pasture honey (both at concentrations equivalent to 1% honeys) or DMEM medium alone are shown in Figure 7.4.

The results in Figure 7.4 show that retentate fractions (equivalent to 1% concentrations as found in manuka honey or Otago pasture honey) showed protective effects on TJ as indicated by the TER values, which did not drop as low as with DMEM medium alone during the EGTA challenge. The diffusate fractions had no significant effect. After 24 h recovery, the cells exposed to the retentate fractions had significantly higher TER values than those obtained with DMEM medium alone. By the 48 h recovery time-point, there were no significant differences in TJ resistance for cells incubated with retentate fractions and DMEM medium alone.

The effect on TJ resistance during and following an EGTA challenge of incubating MDCK cell monolayers with manuka honey at 1% or DMEM medium alone applied to the basal side of the monolayer compared with the apical side are shown in Figure 7.5.

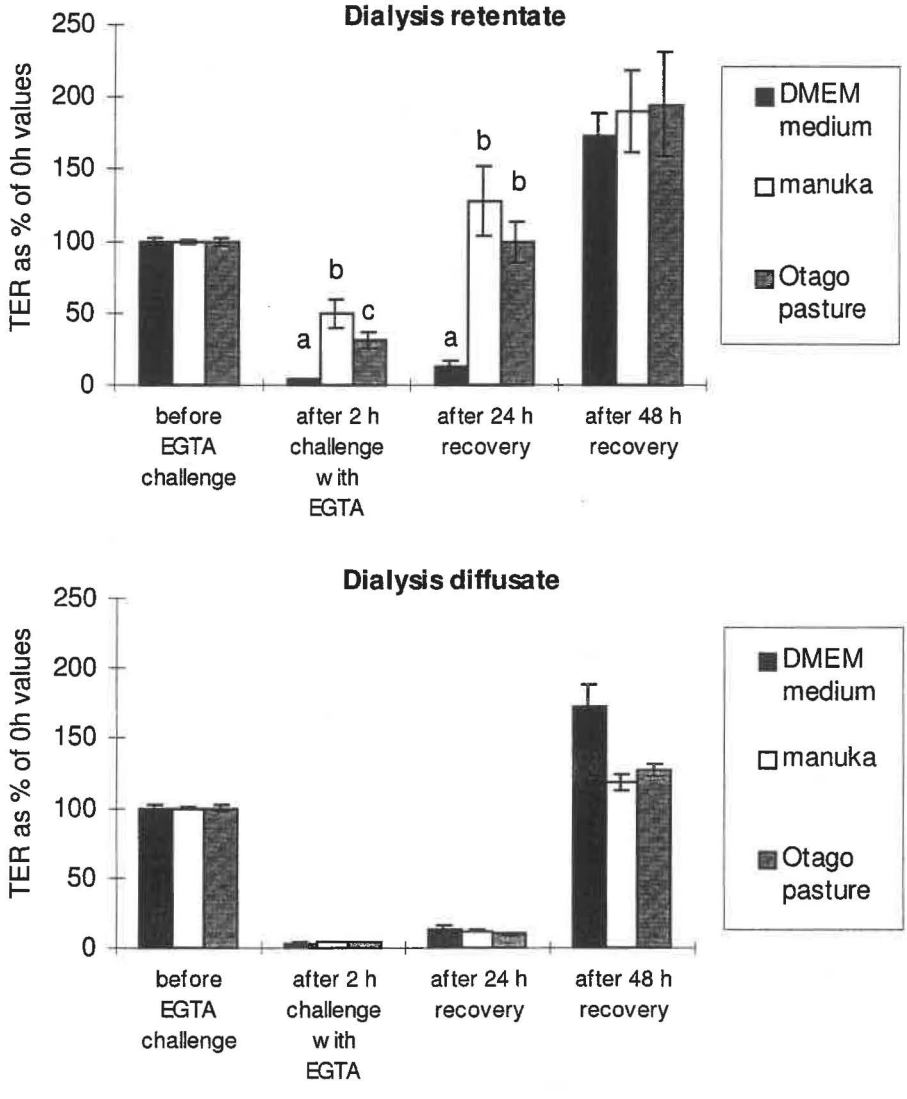


Figure 7.4: Effect of an EGTA challenge on loss of transepithelial resistance (TER) in dialysed honey-treated kidney cells (MDCK). Cells were maintained for 24 h with or without honey (retentate or diffusate fractions of manuka or Otago pasture honeys at final concentrations equivalent to 1%) prior to a 2 h challenge with 3mM EGTA (in honey solutions). Data points represent the mean of five replicates ± SEM and the assay was repeated twice (n=10). The data are expressed as mean TER values, taken as a proportion of those obtained for each corresponding treatment prior to the EGTA challenge. Differences between the means are indicated by superscript letters, where means not sharing common letters within a timepoint are significantly different ($P<0.05$).

The results in Figure 7.5 show that manuka honey (1%), conveyed a protective effect to TJ independently of the side of the cell monolayer to which it was applied. Though manuka honey significantly increased TJ resistance during the 24 h recovery period compared with DMEM medium, the effects were lost by the 48 h timepoint.

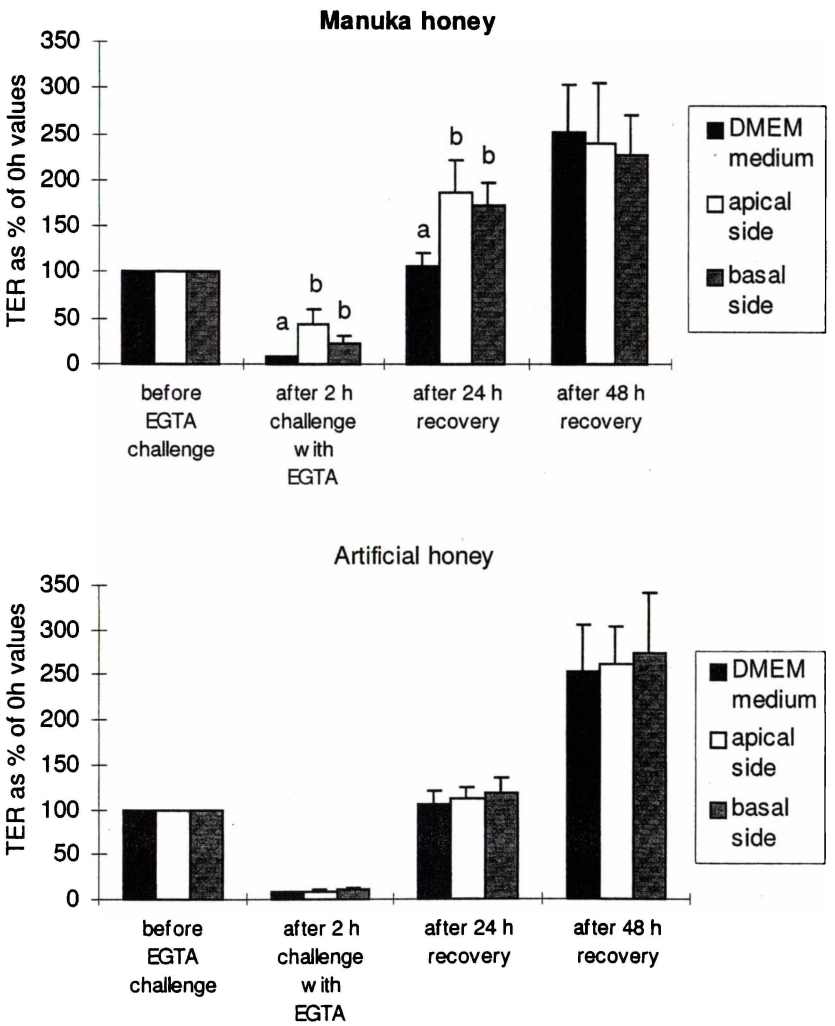


Figure 7.5: Effect of an EGTA challenge on loss of transepithelial resistance (TER) in honey-treated kidney cells (MDCK), when honey was applied apically versus basally to cell monolayers. Cells were maintained for 24 h with or without honey (manuka or artificial honeys at 1% final concentrations) applied to either the apical or basal side of the cell monolayers, prior to a 2 h challenge with 3mM EGTA (in honey solutions). Data points represent the mean of five replicates ± SEM and the assay was repeated twice (n=10). The data are expressed as mean TER values, taken as a proportion of those obtained for each corresponding treatment prior to the EGTA challenge. Differences between the means are indicated by superscript letters, where means not sharing common letters within a timepoint are significantly different ($P<0.05$).

7.4 DISCUSSION

TJ provide a crucial protective barrier in tissues such as the intestine, which can be altered by a variety of physiological, pathological and pharmacological stimuli. An EGTA challenge was adopted in the current study to provide a model to challenge TJ integrity by chelating extracellular calcium. It is noted that EGTA has a different mode of action to *in vivo* factors such as cytokines, growth factors, bacterial products and specific toxins, all of which open up TJ to enable infiltration of the leukocytes into damaged tissues (Lee *et al.*, 1991; Hochman and Artursson, 1994), as these do not chelate calcium. These agents act through a diverse range of pathways but the outcome is that they all act to open up the TJs. In the current study honey has been demonstrated to ameliorate some EGTA-induced TJ disruption (compared with the control) and to increase recovery of TJ tightness during the 24 h recovery period following an EGTA challenge.

The choice of EGTA to create a model for tight junction disruption is questioned due to its mode of action as a calcium chelator. In Section 7.1, the necessity for integrins to have calcium in order to function was discussed. EGTA will disrupt a range of adhesion molecules. An additional key function for calcium is to influence cell signalling through binding to various proteins. With hindsight, LPS may have been a more appropriate model for tight junction disruption as applied to the gastric mucosa.

Although most of the evidence that honey is anti-inflammatory comes from studies on wounds in the skin (Gupta *et al.*, 1992; Kumar *et al.*, 1993; Oryan and Zaker, 1998) and especially from clinical use on burns (Postmes *et al.*, 1997; Burlando, 1978) the anti-inflammatory effect of honey on the gastric mucosa has also been reported (Bilsel *et al.*, 2002; Gharzouli *et al.*, 2001; Ali *et al.*, 1997; Haffejee and Moosa, 1985; Salem, 1981). While the effects of honey on TJ integrity in an inflammatory model have been considered in isolation in the current chapter, the TJ functions within a complex signalling pathway, and the proven ability for honey to have effects at other points in this pathway will have profound implications for TJ functioning *in vivo*. Briefly, studies on

the loss of the barrier function of the intestinal mucosa in Irritable Bowel Disease (IBD) have established that over-expression of NO plays a key role in peroxynitrite-mediated protein nitration, leading in turn to abnormally high neutrophil infiltration and excessive inflammation (Kruidenier *et al.*, 2003). In some cases an altered epithelial barrier is the proximate cause of the disease, while in other cases, loss of barrier function is a secondary consequence of the disease process (Bjarnason and Peters, 1996).

Although epithelial tight junctions provide a tighter barrier than those found between endothelial cells, they both share the same function role. As specific tight junction proteins vary among tissues along with factors regulating tight junction permeability (Gon *et al.*, 2005), the effects of honey should be investigated for various tissues and caution needs to be employed regarding statements about general effects.

There are a number of points in the inflammatory cascade at which honey might modulate the first stage of inflammation (Figure 7.6), indirectly or directly. It is possible that honey may enhance tissue health due to optimisation of nutrient supply (Al-Waili, 2003; Subrahmanyam, 1996). While it is noted that sugars alone had no activity on TJ, honey contains many nutrients in addition to sugars and manuka honey differs in specific content of these from Otago pasture honey (White, 1975a). The observation that manuka honey may increase the tightness of TJ when cells were not exposed to EGTA lends support to honeys ability to support tissue health (and therefore functioning). Further, it should be noted that Gharzouli *et al.* (2001) observed that both natural honey and a complex sugar mixture conveyed protective effects against ethanol-induced gastric mucosal damage in rats. The ability for honey to increase the uric acid content of serum has been suggested as a possible mechanism by which antioxidants may reduce inflammation in the gastric mucosa (Al-Waili, 2003), and should be mentioned here.

Though the molecular basis for the effect is not known, there are several possibilities for the mechanisms by which honey may have an anti-inflammatory effect on TJ disruption during exposure of the cells to EGTA, and in the subsequent recovery period. The

possibility that a high molecular weight component in honey interferes with the chelation of calcium induced by EGTA cannot be ruled out. Calcium mobilisation is a widely used signalling mechanism and influences NF-κB activation (Watson *et al.*, 2001) so if honey manipulates calcium mobilisation in some way then this will have implications for the inflammatory response. When calcium is removed by chelation, E-cadherin (an adhesion molecule which enables TJ to form) is re-distributed along with TJ proteins (e.g. claudin and occludin) causing TJ to open up and cells to separate from each other (Clarke *et al.*, 2000; Citi, 1994).

The possibility that honey acts on adhesion molecules, or on the mobilisation of the TJ proteins needs to be investigated further. A further possibility which needs to be explored is the possible role of honey on stabilising actin filaments. Actin filaments are necessary for the opening and closing of TJ, and stabilisation of the actin filaments restricts the actions of calcium chelators (Phillips *et al.*, 1989). If proteins can chop actin this may destabilise TJ.

The observation that honey increases the resistance of TJ (and therefore TJ intactness) during the 24-h recovery period beyond that obtained by the control suggests that a high molecular weight component in honey has interfered with EGTA-induced disruption of the TJ. If TJ were not completely disrupted, the resistance across the monolayer could be restored in a relatively shorter time than for cells in medium alone which had no buffer against disruption. When the EGTA solutions were removed, TER was re-established after 24 h for MDCK cells maintained in medium alone compared with a significant increase in resistance beyond the original TER values (prior to addition of EGTA) after 24 h for cells exposed to 1% honey solutions. The possibility that honey increases the health of the cells may also apply here. Ali *et al.* (1997) have proposed that honey may contain a 'sucralfate-like' substance which reduces the vascular permeability of the gastric mucosa. In future experiments it would be useful to conduct calcium-EGTA titration assays to ensure that the influence of any calcium in honey is eliminated.

The role of components of honey in cell signalling may be of importance to the ability for honey to act as a TJ modulator. Hydrogen peroxide has been shown to have direct action on NF-κB (as discussed in Section 2.4) through oxidation involving free radicals formed by the Fenton reaction (Cross *et al.*, 1987). Components in honey bind up iron to stop it catalysing the Fenton reaction (Buntting; 2001). Tannins, likely to be present in honey, bind iron, and complex with proteins and thus would be present in the retentate fractions obtained by dialysis. A number of studies have shown that agents which activate protein kinase C cause TJ disruption (Mullin *et al.*, 1992; Ellis *et al.*, 1992; Balda *et al.*, 1991) and it is possible that an antioxidant in honey may interfere with this activation. The observation that the high molecular weight fractions of honey contain the active component confirm that the size of the agent must exceed 10 kDa. The observations that 1% concentrations of manuka honey directly increases the tightness of MDCK cell TJ (independent of an EGTA challenge) after a 24 h incubation period, and that manuka honey has a greater effect than Otago pasture honey suggest that the active component occurs at higher concentrations in manuka honey. The demonstrated effects of antioxidants to remove intercellular oxidant species and exert anti-inflammatory actvity through this mechanism has been discussed (Section 1.4.4.1). Differences in antioxidant content of the honey types may explain observed differences in potency, being significantly higher for the manuka honey. However, it should be noted that the high molecular weight fraction does not contain a complete anti-oxidant profile, as some of the antioxidant compounds would likely have been removed in the diffusate (P. C. Molan; personal communication). Along with tannins, polyphenols would be bound to proteins in the retentate fractions. Various chemical classes of antioxidants have been demonstrated to regulate cell adhesion through modulation of specific signal transduction pathways (Sen and Roy, 2001).

The lack of an established antioxidant profile for the dialysis fractions makes it difficult to draw any conclusions about the role of antioxidants in the observed effects of honey on TER. The fact that Otago pasture honey contains such low levels of antioxidants and yet has activity on TER levels beyond the control means it is unlikely these compounds could

be responsible for all of the observed effects. Further, the likelihood that the diffusate fraction contains antioxidants and yet has no effect on TER beyond the control also casts doubt on the possible contribution of these compounds to the observed effects.

Two factors make it unlikely that hydrogen peroxide plays a significant role in the observed anti-inflammatory effects: the first is that manuka honey has a more potent effect than the pasture type of honey and yet a far lower hydrogen peroxide content; the second is that the activity was present in the high molecular weight fraction so that the glucose oxidase should have been deprived of its substrate, removed in the diffusate. It has been pointed out however, that the glucose content of the medium would have acted as a substrate source (Section 2.4). The optimum concentration of glucose for honey glucose oxidase activity is known to be high, but the level in the medium was probably quite low. That there were no significant differences in the effect of retentate fractions and stock solutions on TJ suggests that glucose oxidase activity was not involved (and therefore hydrogen peroxide itself was not responsible for the observed activity).

The concept of the inducible heat shock proteins as molecular chaperones to protect against cellular injury (*e.g.* calcium chelators such as EGTA) was introduced in Section 2.4.2.1. It is possible that exposure of the MDCK monolayers to honey solutions may have induced low level oxidative stress due to the presence of hydrogen peroxide, and that heat shock proteins may have been synthesised which conveyed a protective effect during the inflammatory challenge. Of relevance to the current study, Bailey *et al.* (2004) reported that the heat shock proteins (HSP 27 and 70) were induced in retinal pigment epithelial cells to protect from the disruption of the TJ barrier function. MDCK cells have been demonstrated to express mRNA for HSP 70 (Cowley *et al.*, 1995). The induction of heat shock proteins after exposure to sub-lethal doses of hydrogen peroxide has been observed for a number of cells which form TJs. In particular, Shivers *et al.* (1988) identified the synthesis of a recovery protein in endothelial cells from the brain capillaries which they proposed to speed up reassembly of TJs and the recovery period after an inflammatory challenge. Further evidence comes from reports by Rokutan (2000) and

Rokutan *et al.* (1998) which demonstrated the key role that heat shock proteins (such as HSP70) play in gastric mucosal defence, interfering with apoptosis through suppression of JNK activation (a transcription factor) and through protection of mitochondria. Of additional interest here, with regard to the function of TJ in the intestinal mucosa, it has been reported that trefoil factors are expressed by epithelial cell monolayers which convey both protective effects to the monolayers during an inflammatory challenge and promotes rapid re-establishment of mucosal integrity after injury (Kindon *et al.*, 1995; Mashimo *et al.*, 1996). This raises the possibility that honey may have an effect on trefoil expression.

In Chapters 3 and 4, the ability of honey to induce both mRNA expression for cytokines, release of TNF-α protein by resting monocytes, and to reduce NO availability in mitogen-stimulated cell suspensions, has demonstrated that it has activity at the

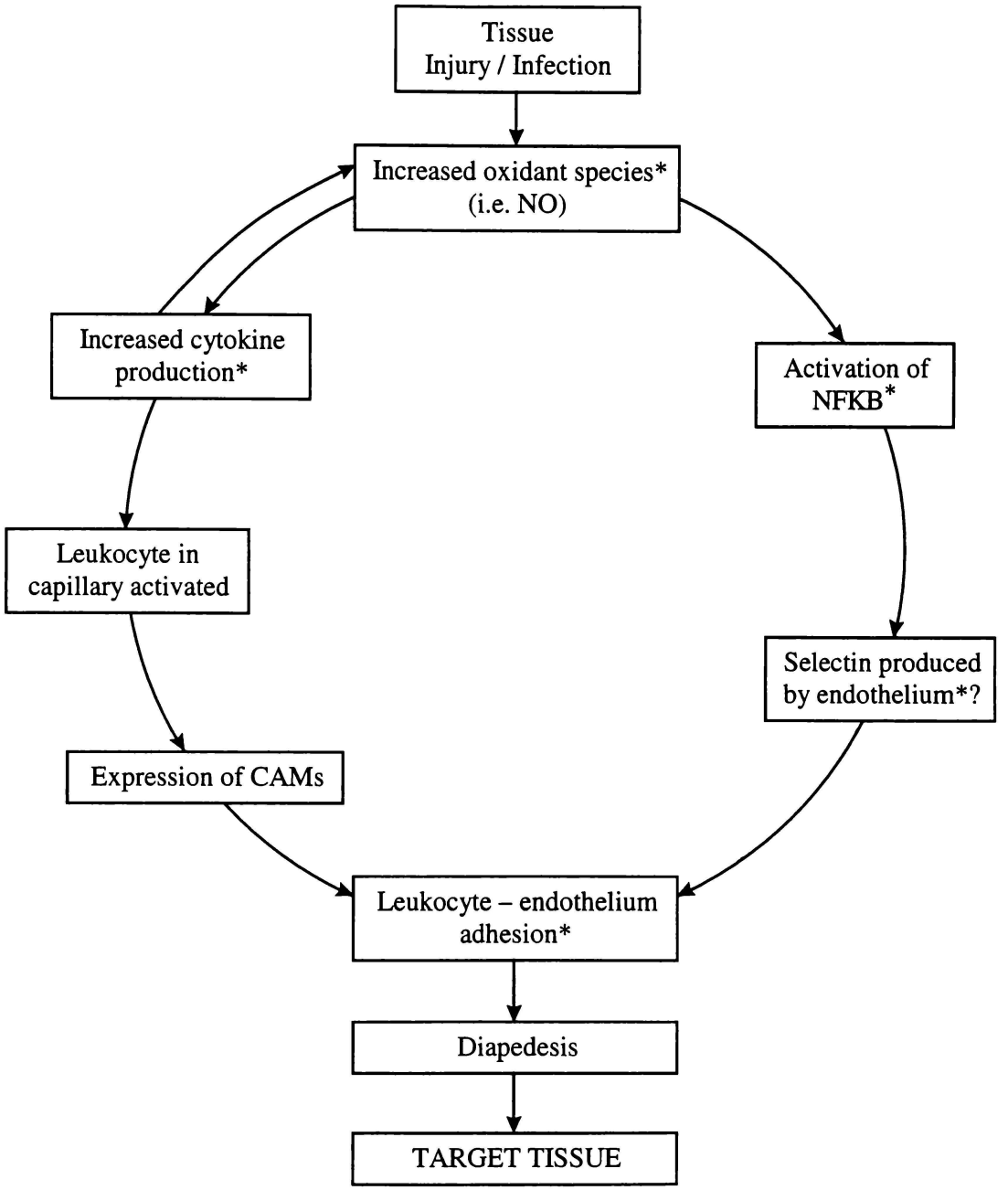


Figure 7.6: Conceptual diagram to explain the movement of leukocytes from the blood to injured tissues. Generation of oxidant species along with cytokines by platelets and phagocytes activate blood leukocytes to migrate to blood/tissue borders. The expression of CAMs (cell adhesion molecules) on leukocytes and selectin by the endothelium enable the blood cells to plug the capillaries, and cytokines increase the permeability of tight junctions (Hollander, 1999), enabling the cells to enter the tissues via diapedesis. Points at which honey has been hypothesised to act on this first stage of inflammation are indicated by *. The effects of honey on selectin production are not yet known, and are denoted *?, to indicate the validity in investigating these in future experiments.

recruitment step of inflammation. The results discussed here suggest that honey has additional activity at the TJ permeability step. This may be further evidence of honey manipulating cytokine production, which has been demonstrated to determine TJ permeability (Hollander, 1999). This is discussed in further detail in Chapter 9. Although the effect of honey on the leukocyte-endothelium binding step has not been investigated in this study, the results obtained here indicate that this would be a worthwhile area for future research. These results also raise questions about whether epithelial cell growth may have occurred and potentially masked the effect of tight junction recovery, or vice versa. In Section 1.4.1 the proven ability for honey to act as a cell growth stimulant was discussed. Of further interest, it would be useful to examine whether honey was having effects on tight junction protein expression (*e.g.* occludin) at the molecular level.

The observation that manuka honey has both protective and restorative effects on TJ when applied to either the apical side or the basolateral side of the MDCK monolayer suggests that the anti-inflammatory activity can be delivered through both oral (through the gut wall), and topical applications as well as through the systemic circulation.

The implications of honey having activity as a TJ modulator to the overall inflammatory response are that recovery from inflammatory events may be quicker. Evidence that replacement of standard electrolyte solutions with honey solutions during oral rehydration after bacterial diarrhoea shortened the period of gut disturbance (Haffejee and Moosa, 1985) may be due, at least in part, to restoration of TJ integrity and therefore, normal barrier function to the gut wall. The demonstrated ability for honey to alleviate redness and swelling (inflammation) from burns (Burlando, 1978) may also be attributed to the recovery of the TJ barrier function and the cessation of leukocyte infiltration to the tissues. A potential danger for use of an agent which prevented TJ from opening up would be that leukocytes could not reach damaged tissues and the necessary inflammatory response could not proceed. However, while honey was demonstrated to ameliorate some of the disruption caused by EGTA, the actions of the chelator were not prevented and this is analogous with expected activity *in vivo* when a bacterial infection occurs. Further, the

results of the current chapter raise the possibility that oral delivery of honey along with classical steroids may help to alleviate some of the disruption caused by gut permeability (through effects on TJ).

Chapter Eight: The Effect Of Honey On Fibroblast Proliferation

SUMMARY

The effect of various honey concentrations on the proliferation of the 3T3-L1 fibroblast cell-line *in vitro* was investigated using MTT proliferation assays. Both manuka honey and pasture honey (0.25%) increased fibroblast proliferation. Artificial honey had no effect on fibroblast proliferation, indicating sugars have no role in mitogenic activity. This suggests that honey contains factors which directly promote cell division in fibroblasts.

An *in vitro* model was used to test whether honey-induced factors produced by peripheral blood mononuclear cells (PBMC) could activate fibroblast proliferation. These assays were performed to examine whether honey could have an indirect stimulatory effect on fibroblasts. Incubating fibroblasts with supernatants derived from honey-stimulated PBMCs (at 0.025% concentrations of honey) led to significant fibroblast proliferation. Low concentrations of honey (less than 0.1%) do not directly stimulate fibroblast proliferation, therefore factors produced by honey-stimulated PBMCs must promote fibroblast proliferation. A high molecular weight fraction of manuka honey obtained from dialysis contained the active component. The diffusate obtained by dialysis (containing sugars) had no activity.

To investigate whether honey could modulate the response of fibroblasts to an inflammatory agent, fibroblasts were exposed to honey for various times prior to and at the same time as IL-1β. Honey did not augment fibroblast proliferation when added at the same time as IL-1β. Prior incubation of fibroblasts with honey (0.25–1%) for 2 h before IL-1β-stimulation modulated the cell response, and this active component was of a high molecular weight.

8.1 INTRODUCTION

The link which is forged between the inflammatory process and the repair of tissue itself by fibroblasts was introduced in Chapter 1 and is the focus of this chapter. Anecdotal evidence and experimental animal models attest to the ability of honey to optimise wound healing and reduce scar formation (Rozaini *et al.*, 2004; Al-jady *et al.*, 2000; Suguna *et al.*, 1993; Suguna *et al.*, 1992; Molan, 1991), as a consequence of effects on fibroblast activity (Rozaini *et al.*, 2004; Lusby *et al.*, 2002; Al-jady *et al.*, 2000; Suguna *et al.*, 1992). In numerous reports on the clinical useage of honey it has been noted that honey promotes the formation of granulation tissue (Dunford *et al.*, 2000; Taks, 2000; Farouk *et al.*, 1998; Dumronglert, 1993; Efem, 1993; Subrahmanyam, 1991; Subrahmanyam, 1998; Efem, 1988; Wadi *et al.*, 1997; Braniki, 1981; Armon, 1980; Cavanagh *et al.*, 1970; Hutton, 1966), granules of fibroblasts growing where new capillary beds form by budding out from blood vessels. It is these fibroblasts that produce the scar tissue that fills in the damaged area and over which epithelial cells glide to provide new skin cover. In experimental wounds in animals honey has been shown to stimulate the synthesis of collagen (Suguna *et al.*, 1992), and other connective tissue components (Suguna *et al.*, 1993). The objective of the investigations described here was to establish whether honey has any effect on fibroblasts *in vitro*, with a view to possible effects on wound healing.

As described in Chapter 1, a key first step for the mechanism by which fibroblasts knit a wound closed is for them to proliferate. They perform this function in response to chemical messages (cytokines) produced by cells involved in the inflammatory phase of wound repair (Witte *et al.*, 1998). Scar formation is a necessary step in tissue repair but excessive scarring results from persistent inflammation. This is thought to be caused by abnormal cytokine gradients (particularly of IL-1β) produced by overactive leukocyte activity, leading to subsequent fibroblast overactivity and formation of raised scars (Ghahany *et al.*, 1995; Lynch *et al.*, 1989). Conversely, abnormally low cytokine signalling by leukocytes (such as occurs in an immune-compromised patient) leads to

insufficient fibroblast multiplication and ultimately, tissues fail to be repaired (Dow *et al.*, 1999).

In view of the observation that honey is associated with optimal wound healing and that fibroblast proliferation is a crucial step in this process, investigations were performed to test the effect of honey on proliferation of fibroblasts *in vitro*. A pre-adipocyte cell line, 3T3-L1, was selected for use in proliferation assays as they are accepted to provide a model on which to measure inflammatory responses (Edward Wood, University of Leeds, personal communication), and they respond to IL-1β (Kimball *et al.*, 1988). However, it was noted that there are distinct differences between the Swiss 3T3-L1 fibroblasts used in this study and primary skin fibroblasts, in collagen synthesis, growth rate, and response to growth factors (Oda *et al.*, 1990; Vande Berg *et al.*, 1989; Buckley-Sturrock *et al.*, 1989). However, the positive results from this study justify further studies being carried out using wound-derived fibroblasts. Stimulation of the fibroblasts with IL-1β was used to provide a positive control for activation of fibroblast cultures, and provide a model of excessive inflammation on which to test the effect of honey on. This model had particular physiological relevance to inflammation *in vivo* as IL-1β produced by mononuclear cells is an important switch in the induction of fibroblast activity, and particularly in excessive scar formation (Wahl, 1988). The ability for honey to stimulate fibroblast directly was also tested.

8.2 MATERIALS AND METHODS

8.2.1 Reagents

DMEM complete medium

Complete medium was made up to 100 ml containing: DMEM, Dulbecco's modified Eagle's medium (containing 1.5 g/L sodium bicarbonate and 4.5 g/L glucose,GibcoBRL, Cat # 12 100-046); 10% foetal calf serum (GibcoBRL, Cat # 10 091-148), and 1% Penicillin-Streptomycin (GibcoBRL, Cat # 15140-122).

Where the serum content was reduced in individual medium preparations, this has been indicated in the experimental design sections.

Trypsin

2.5% trypsin stock solution (25 g/l trypsin, GibcoBRL), containing 1% EDTA and phosphate-buffered saline (17.6 ml).

Phosphate-buffered saline

Dulbecco A tablets (Oxoid BR14; 1 tablet in 100 ml distilled water)

Honey solutions

Solutions of manuka honey (M109) and Otago pasture honey were prepared as described in Section 2.2.2. Dilutions of stock solutions were made to allow for the 1:20 dilution factor of honey by reactants in the assay to obtain stated final concentrations. Artificial honey was made as described in Section 2.2.2. Complete medium (containing 0.05% foetal calf serum) was used to dilute the honey stock solutions. The pH of final cultures containing honey was checked and not found to differ significantly from cultures containing DMEM medium alone.

8.2.2 Cell Sub-culturing

The Swiss Albino 3T3-L1 fibroblast cell line (AgResearch, Ruakura) were maintained in DMEM complete medium. To prevent differentiation into adipocytes from occurring in the culture flasks it was necessary to split the cultures when the cells were just confluent (every 3 days). Experiments were carried out with cells at passage numbers between 5 and 15, as some authors have noted that cells lose their responsiveness with successive passages (Wahl, 1988). As fibroblasts are adherent cells, trypsin was used to detach the cells from the flask by adding 4 ml trypsin solution after the medium had been removed and the cell surface washed with 20 ml of phosphate-buffered saline. The trypsin was left

in the flask for 2 min (37°C, 5% CO_2, 95% air) after which time an equal volume of DMEM complete medium was added, and the supernatant removed by centrifugation (Sorval, 223 x *g*, 3 min). The pellet was re-suspended in 5 ml of DMEM complete medium, and 1ml of suspension added to 29 ml of DMEM complete medium in a new culture flask. The flasks were incubated at 37°C (5% CO_2, 95% air). Cells were grown in DMEM complete medium and seeded in to 96-well flat-bottom plates at 2.5 x 10^4 cells/ml for 48h (37°C, 5% CO_2, 95% air). During this period, the mouse derived fibroblasts adhered to the bottom of the wells. It was important to ensure that the fibroblasts were not overconfluent in the wells, as contact inhibition reduces sensitivity of the cells to stimulation by mitogens (Wahl, 1988). After 48 h, the medium was removed from the wells by flicking, and the cells washed with phosphate-buffered saline before the medium was replaced with 100 µl of treatment solution.

8.2.2.1 Establishing a non-confluent fibroblast monolayer

To establish non-confluent monolayers in a 96-well microtitre plate, 500 µl of fibroblast cell suspension (2.5 x 10^4 cells/ml diluted in DMEM complete medium) was loaded into each well and the cells incubated for 24 h (37°C, 5% CO_2, 95% air). After 24 h the supernatant was removed by gently flicking the plate, and the cells washed twice to remove any serum by adding 100 µl of phosphate-buffered saline to each well and centrifuging the plate (223 x *g*, 3 min).

8.2.3 Analysis of cell activity using MTT assay

Proliferation levels obtained for each treatment regime were determined using the MTT assay described in Section 2.2.6.2. A standard curve was constructed to determine the relationship between absorbance reading (OD) at 570 nm, and cell density (Appendix 3).

8.2.3.1 Statistical analysis

Unless otherwise stated, analyses of difference in fibroblast proliferation between treatments and experiments was done by one-way ANOVA using Systat software version 7 (SPSS Inc.). The between group effect was the amount of proliferation. Significance was further analysed using Tukey's honestly significant difference (HSD) for pair-wise differences for within group comparisons. All tests were performed in triplicate wells and repeated at least twice (n=6 measurements), and each datapoint represents the mean ± SEM of optical density (OD) measurements for two experiments. As the overall conversion of tetrazolium salts varies each time, data were normalised by taking the mean for triplicate treatment wells as a proportion of the mean values obtained for triplicate control wells. SEM for the control wells were calculated on raw data and multiplied by the same proportion as used to convert the control to 100%. Systat was used for all analyses. Results were accepted as statistically significant when $P<0.05$.

8.2.4 Experimental Design

In order to find the maximum concentration of honey which could be added to fibroblast monolayers without reducing cell viability, initial assay were performed to establish an osmotic tolerance range. In order to measure the direct effect of honey on fibroblasts, monolayers were exposed to honey concentrations. As well as having direct effects on fibroblasts at the wound site, honey would have indirect effects on fibroblasts through its activity on PBMCs and their production of cytokines which drive fibroblast activity (Wahl, 1988). It had already been established that honey directly induces mRNA expression for cytokines in whole blood (Chapter 3), and production of TNF protein by THP-1 monocytes (Chapter 4), suggesting that honey might have indirect effects on fibroblasts. Therefore, assays were undertaken to test if honey has activity on fibroblasts partly or wholly via stimulation of cytokine production in leukocytes. To increase

physiological relevance to the real wound healing situation, supernatants derived from honey-stimulated PBMCs were incubated with fibroblast monolayers to measure any indirect effects of honey on fibroblast proliferation.

In the inflammatory situation, fibroblasts proliferate in response to growth factors and cytokines produced by immune cells. IL-1β is a known mitogen which has been shown to direct fibroblast cells into the S phase of the cell cycle, and has been used by various authors to model the effect of inflammation on fibroblast activity (Wahl, 1988). Modelling experiments by Kimball *et al.* (1988) have determined that 3T3 fibroblasts proliferate in a dose-dependent fashion in response to interleukin-1 (IL-1). As anecdotal reports have suggested that honey minimises scar development, and associated recovery from tissue damage, it was useful to examine whether honey had any effect on the response of fibroblasts to a mitogen. To test whether honey could modulate fibroblast proliferation in response to a mitogen, monolayers were exposed to honey prior to and at the same time as IL-1β. To identify the active component in honey, assays were repeated to test for stimulatory activity and modulatory activity in honey fractions

Establishing the osmotic tolerance range of fibroblasts

To identify honey concentrations which damage fibroblast cells, 500 µl of suspension containing 2.5 x 10^4 cells/ml (in DMEM complete medium) was loaded in to wells of a 24-well microtitre plate. After 24 h the medium was removed via aspiration and replaced with 500 µl of honey diluted to obtain final concentrations (0.1, 0.5, 1 and 3% in DMEM complete medium containing 0.05% foetal calf serum) added to triplicate wells. The plates were incubated at 37°C(5% CO_2, 95% air) for 24 h. Prior to removal of the honey solutions, appearance of the cell membranes was noted to identify osmotically stressed cells. Honey solutions were then removed and replaced with 100 µl of trypsin before returning the microtitre plate to the incubator for 2 min to dislodge the cells from the plate. The trypsinised cell suspensions were removed via autopipette, drawing the solution up and down to ensure the cells were retrieved. The contents from each triplicate series of wells were placed in a separate 15 ml centrifuge tube and diluted with an equal

volume of phosphate-buffered saline. The tubes were centrifuged (223 x *g*, 3 min) and the pellet resuspended in 1 ml of phosphate-buffered saline. Into an Eppendorf tube was placed 100 µl of cell suspension and 100 µl of trypan blue and mixed by vortexing the tube contents. Then a haemocytometer count was performed to determine the total number of cells for each treatment along with the proportion of viable cells.

8.2.4.1 Testing whether honey has a direct stimulatory effect on fibroblast proliferation

To treatment wells, 100 µl of honey solution (at final concentrations of 0.1, 0.25, 0.5, or 1% v/v, diluted in DMEM complete medium containing 0.05% foetal calf serum) was added. All treatments and controls were conducted in triplicate. Control wells received 100 µl of DMEM complete medium containing 0.05% foetal calf serum added to the cell monolayer. Background wells containing only honey solutions were included to allow for any background absorbance from the honey, and the mean OD value for these was subtracted from treatments and controls. The plates were incubated for 72 h (37°C, 5% CO_2, 95% air) after which time cell density was assessed using the MTT assay (as described in Section 2.2.6.2).

8.2.4.2 Testing whether honey may have indirect stimulatory effects on fibroblasts through factors produced by honey-stimulated PBMCs

To measure the indirect effects of honey on fibroblasts, PBMCs were exposed to 0.025% final honey concentrations, then the supernatants from these were incubated with fibroblast monolayers to test the effects on proliferation. The 0.025% concentrations of honey had been shown to both, stimulate lymphocyte proliferation and not affect viability in Section 2.3. This experiment was based on the method described by Wahl (1988) which clearly demonstrated that lymphocyte- and monocyte-derived fibroblast growth factors can be harvested from PBMCs and will drive proliferation when added to fibroblasts (Agren *et al.*, 1999; Mendez *et al.*, 1998; Phillips *et al.*, 1998).

PBMCs were isolated from bovine blood as described for the T-cell assays in Section 2.2.3. Into each well of a 24-well microtitre plate, 950 µl of cell suspension (2.5 x 10^6 cells/ml) was loaded along with 50 µl of honey solutions (serial dilutions of manuka, Otago pasture or artificial honey 50% stock solutions) or DMEM complete medium alone. The cells were incubated for 24 h (37°C, 5% CO_2, 95% air). Supernatants were collected by centrifugation of the plates (503 x *g*, 15 min, Sorval) and the supernatants were passed through a 0.2 µm filter (Supor® Membrane Acrodisc®, Pall Corporation) to remove any residual cells.

As serum interferes with fibroblast proliferation assays, it was necessary to reduce the serum content of the suspensions and this was achieved by diluting each treatment 1:4 with serum-free DMEM medium (Wahl, 1988). This reduced the amount of serum to which fibroblasts were exposed, to ensure that the sensitivity of the assay was maintained. The limitations of diluting the PBMCs-derived supernatants were that any factors produced by the cells would be diluted. The objective of this assay was to establish whether honey could stimulate PBMCs to produce factors which would have activity on fibroblast proliferation. As it was confirmed that honey had no effect on fibroblast proliferation at concentrations below 0.25% (Figure 8.2) the honey at 1/5 of 0.025% would not have a direct effect on fibroblasts. Non-confluent fibroblast monolayers (2.5 x 10^4 cells/ml) were established, and washed as described in Section 8.2.3.1. To triplicate wells containing the washed fibroblasts 100 µl of the diluted supernatants from the PBMCs were added. Blanks were included to account for the absorbance due to honey solutions alone and the mean OD value obtained for these were subtracted from all values. The plates were incubated (37°C, 5% CO_2, 95% air) for 48 h before the cell density was measured by the MTT assay (as described in Section 2.2.6.2).

8.2.4.3 Testing whether honey augments IL-1β -stimulation of fibroblast proliferation

To test whether honey would augment IL-1β effects if added to cultures at the same time, honey was delivered to monolayers at the same time as IL-1β (100 ng/ml). Briefly, non-confluent fibroblast monolayers (2.5 x 10^4 cells/ml) were established, washed (Section 8.2.3.1), and each well received 100 µl of either honey (0.25–1%final concentrations) containing IL-1β, or DMEM complete medium containing IL-1β and the cells incubated for 48 h (37°C, 5% CO_2, 95% air). Blanks were included to account for the absorbance due to honey and the mean OD values obtained for these were subtracted from all values. After 48 h, the cell density was determined by means of the MTT assay (as described in Section 2.2.6.2).

8.2.4.4 Testing whether exposure of fibroblasts to honey before IL-1β -stimulation has any effect on proliferation

There are two cases where fibroblasts could potentially be exposed to honey prior to an activating stimulus, in people who consume honey as a preventative, and when honey is applied to a fresh wound. Therefore, testing whether prior exposure of fibroblasts to honey does have physiological application. After establishing non-confluent fibroblast monolayers (2.5 x 10^4 cells/ml), they were washed (Section 8.2.3.1), and each well received 100 µl of either honey (0.1–1%) or DMEM complete medium. Preliminary data had established that a 2 h exposure of fibroblasts to honey prior to IL-1β was the minimum time necessary to obtain any effects on proliferation, and extending the pre-incubation period gave no further effects. After 2 h incubation (37°C, 5% CO_2, 95% air), the honey solutions were removed, the cells washed with 100 µl of phosphate-buffered saline, and the plate centrifuged (223 x *g*, 3 min). To each well was added either, 100 µl of a honey solution, or DMEM complete medium (with 0.05% foetal calf serum) containing IL-1β (at a final concentration of 100 ng/ml), and the cells incubated for 48 h (37°C, 5% CO_2, 95% air). Blanks were included to account for the absorbance due to

honey and the mean OD values obtained for these were subtracted from all values. After 48 h, the cell density was determined by means of the MTT assay (as described in Section 2.2.6.2).

8.2.4.5 Characterising the components in honey with activity on fibroblasts

The aim of these assays was to characterise the components of honey responsible for stimulatory and modulatory effects on fibroblast proliferation. Manuka honey and Otago pasture honey were dialysed using a 10 kDa membrane (as described in Section 2.2.2.1). As mentioned in Section 2.2.2.1, according to the size of the components in honey, glycosylated proteins and anti-oxidants were collected in the high molecular weight fraction (>10 kDa retentate) and sugars in the low molecular weight fraction (<10 kDa diffusate). It was possible that some sugars were retained in the retentate fraction due to the larger size of the long chained oligosaccharides. While the polyphenols bound to proteins would have been retained in the retentate due to their size, the smaller antioxidants would have been in the diffusate fraction (P.C. Molan; personal communication). As the sugars had been removed from the high molecular weight honey fractions, the concentration range was extended to include 3%, as this would potentially increase the dose of any active component present.

Non-confluent fibroblast monolayers (2.5 x 10^4 cells/ml) were established and washed as described in Section 8.2.3.1. To these were added 100 µl of the honey fractions (in DMEM complete medium containing 0.05% foetal calf serum). The cells were incubated for 48 h prior to MTT assay. After exposing the fibroblasts to the honey fractions for 2 h the supernatants were replaced with of 100 µl of fresh honey solutions containing IL-1β (at a final concentration of 100 ng/ml). Blanks were included to account for the absorbance due to honey solutions alone, and the mean OD values obtained for these were subtracted from all values. The cells were incubated for 48 h with the honey fractions prior to measurement of density using the MTT assay (as described in Section 2.2.6.2).

8.3 RESULTS

To assess the effect of honey solutions on fibroblast viability, trypan blue staining was used to examine the proportion of live cells after a 24 h exposure to various concentrations of honey. Both artificial honey (containing sugars alone) and natural honeys significantly reduced cell viability above 1% final concentrations (Figure 8.1). Therefore, concentrations used to test the effects of crude honey on fibroblast activity were restricted within the range 0–1%.

To test the direct effects of honey on fibroblast proliferation, cell monolayers were incubated with honey for 72 h prior to an MTT assay. Manuka honey and Otago pasture honey increased fibroblast proliferation at a single concentration, 0.25%, and the effects were lost either side of this optimal concentration (Figure 8.2). Artificial honey had no significant effects on proliferation.

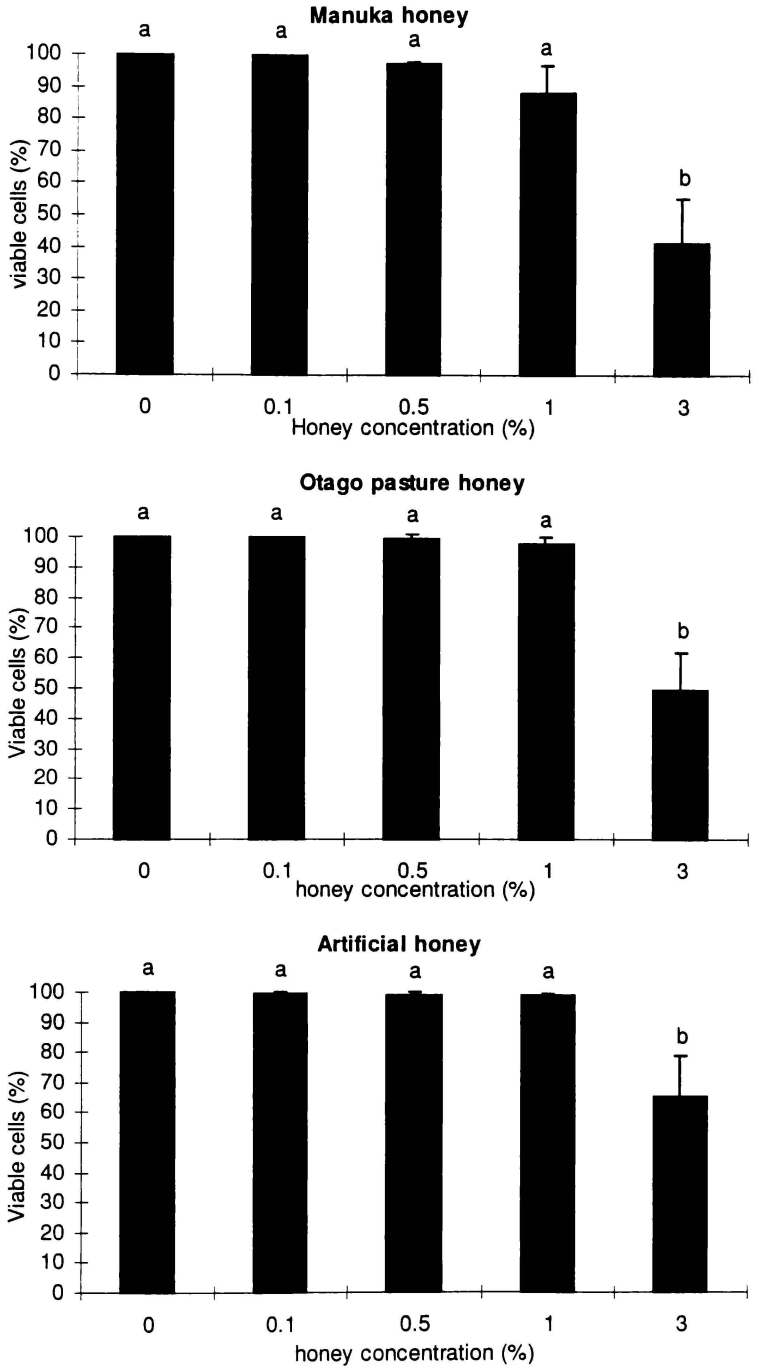


Figure 8.1: The effect of honey concentration on fibroblast cell viability. Cells were incubated with honey (manuka, Otago pasture or artificial) at final concentrations ranging 0.1–3% or DMEM complete medium (containing 0.05% foetal calf serum) alone for 24 h. Viability was then assessed by trypan blue exclusion. Data points represent the mean of triplicate wells ± SEM and are derived from two independent assays (n=6). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

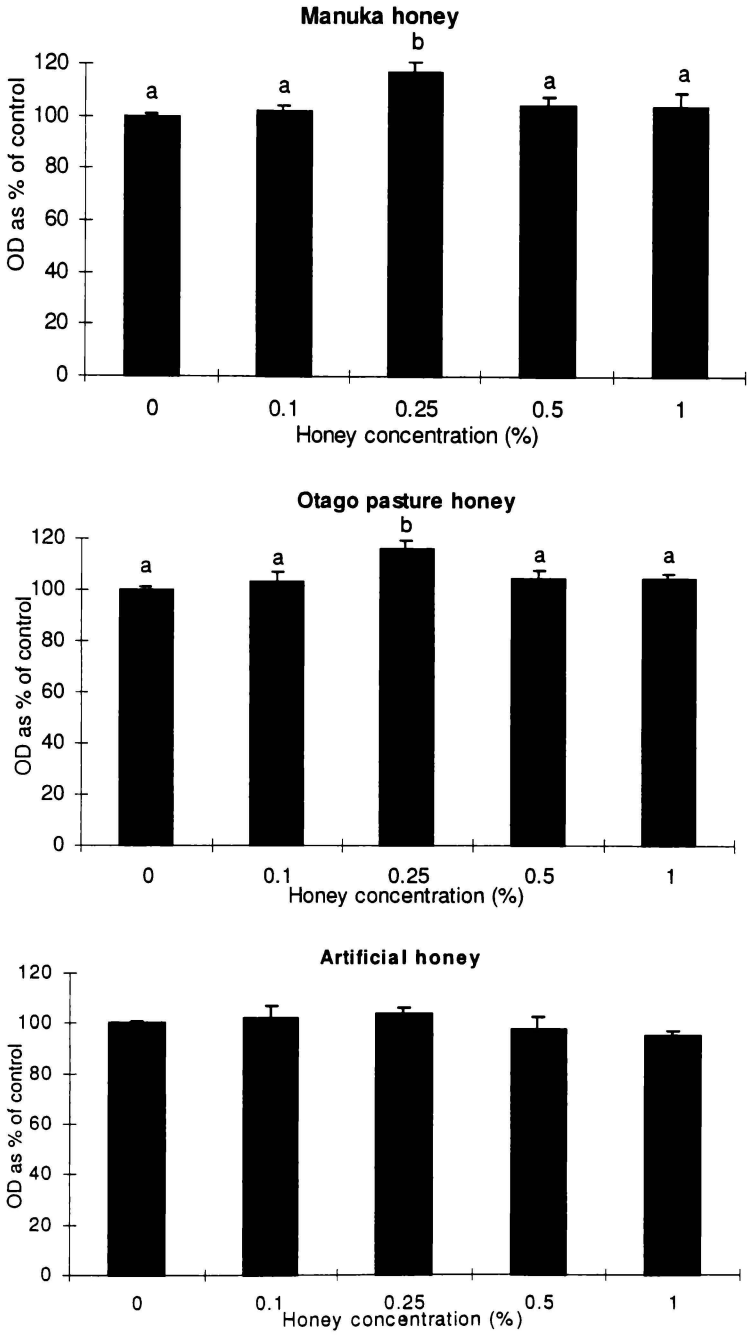


Figure 8.2: Direct effect of honey at various concentrations on fibroblast proliferation. 3T3-L1 fibroblasts were exposed for 72 h to honey (manuka, Otago pasture or artificial) at various final concentrations in the range from 0.1–1% or DMEM complete medium (containing 0.05% foetal calf serum) alone. After 72 h, cell density was determined by the MTT assay. Data are expressed in proportion to the control (fibroblasts exposed to complete medium containing 0.05% foetal calf serum). Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

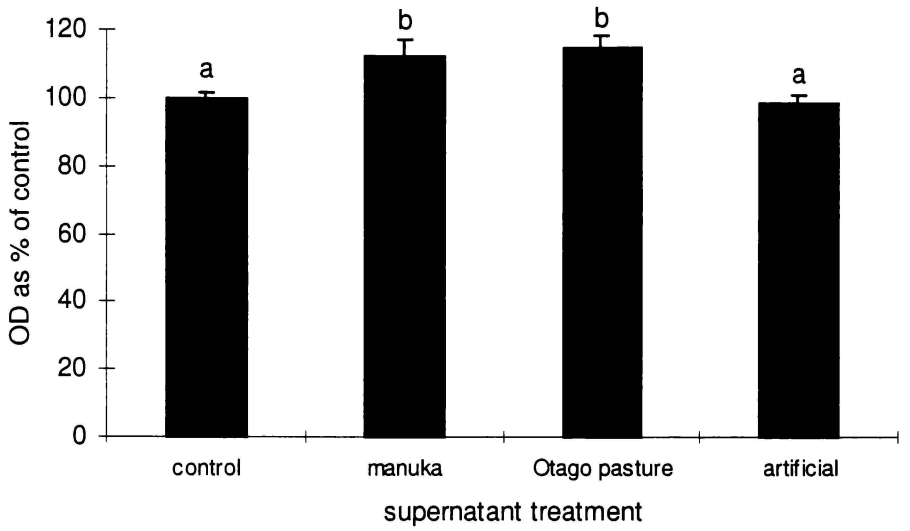


Figure 8.3: The effect on fibroblast proliferation of supernatants obtained from PBMCs exposed to honeys. Fibroblasts were incubated for 48 h with five-fold diluted supernatants obtained from PBMCs exposed to 0.025% final concentrations of honey (manuka, Otago pasture or artificial) or DMEM complete medium alone. Cell density was determined by the MTT assay. Data are expressed in proportion to the control (supernatants from PBMCs exposed to medium alone). Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters where means not sharing letters are significantly different ($P<0.05$).

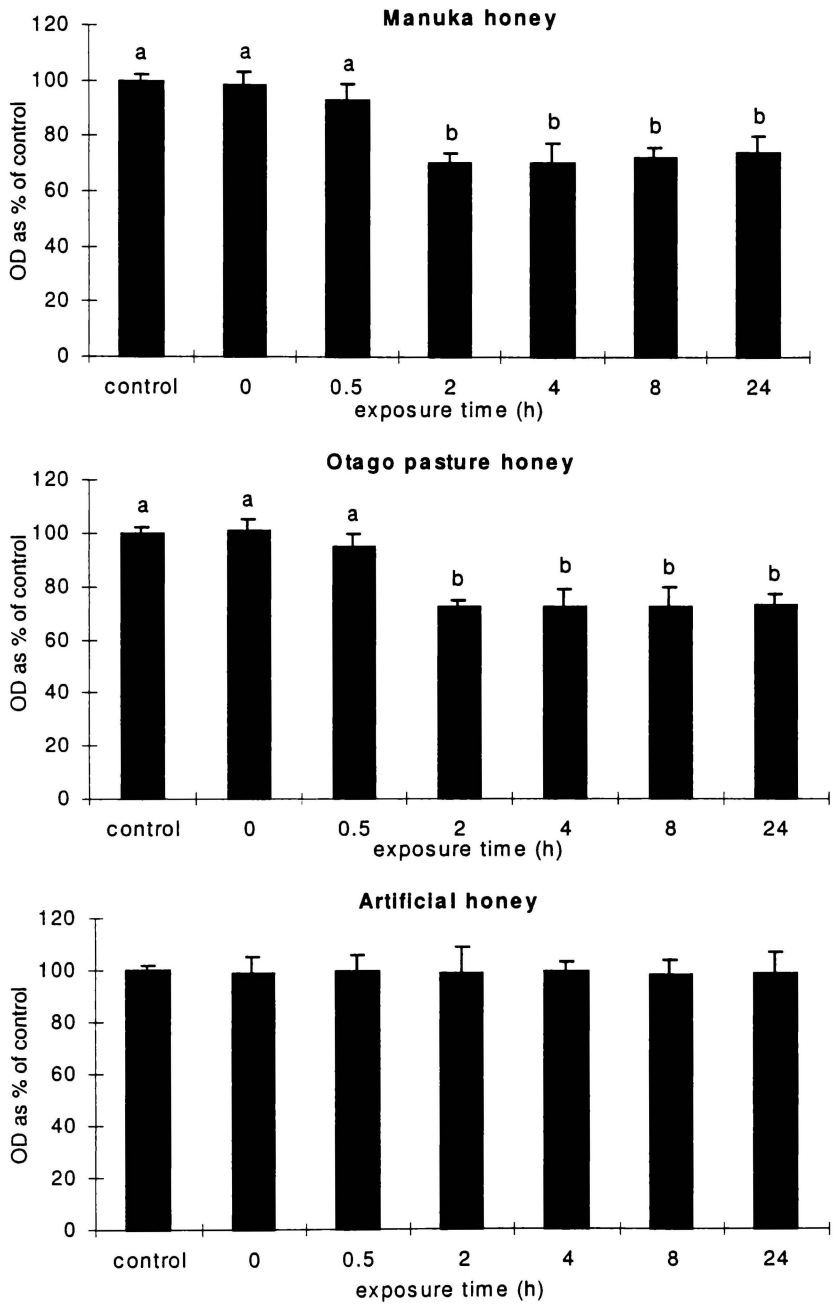


Figure 8.4: The effect on proliferation of exposing fibroblasts to honeys for various times prior to stimulation with IL-1β. 3T3-L1 fibroblasts were exposed to honey at 0.25% final concentrations (manuka, Otago pasture or artificial) for various times (0–24 h) prior to addition of IL-1β (100 ng/ml). After 48 h cell density was determined by the MTT assay. Data are expressed in proportion to the control (fibroblasts stimulated with IL-1β alone for 24 h). Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

To investigate the possibility that honey could have indirect effects on fibroblast proliferation, supernatants were obtained from honey-stimulated PBMCs and added to fibroblast monolayers. Supernatants obtained from artificial honey-stimulated PBMC had no activating effect on fibroblasts (Figure 8.3). This indicated that sugars alone at a 0.025% concentration do not induce PBMC to produce fibroblast-activating factors. However, both natural honeys had an indirect stimulatory effect on fibroblast proliferation, indicating that at 0.025% concentrations, both natural honeys tested must induce the release of fibroblast-activating factors by PBMC.

To test whether honey could potentially exacerbate fibrosis, honey was added to fibroblasts at the same time as IL-1β and proliferation measured. Honey had no effect on resultant fibroblast proliferation, indicating that at all concentrations of natural honey tested (0.25–1%) honey did not augment IL-1β-stimulation. This also indicated that when added at the same time as a high dose of IL-1β (100 ng/ml), honey did not modulate fibroblast proliferation at the concentrations tested (0.25–1%). The positive effect of IL-1β (100 ng/ml) was demonstrated by a mean absorbance reading of 0.358±0.0114 compared with cells maintained in medium alone (0.1793±0.0099). As there was no effect, data is not shown, although observations for 0.25% honey concentrations were included in Figure 8.4.

To establish whether pre-exposure of fibroblasts to honey could modulate their subsequent response to IL-1β, fibroblasts were exposed to honey for various times prior to addition of IL-1β (Figure 8.4). The 0.25% concentration was selected as it had been shown to be optimal for inducing cellular effects in earlier assays. Artificial honey (and therefore, sugars alone) had no effect on resultant proliferation, indicating sugars cannot modulate cytokine-stimulated proliferation. After a minimum 2 h pre-exposure period, both natural honeys had a modulatory effect on IL-1β-stimulated proliferation. Increasing the time cells were pre-exposed to honey had no further effect on proliferation.

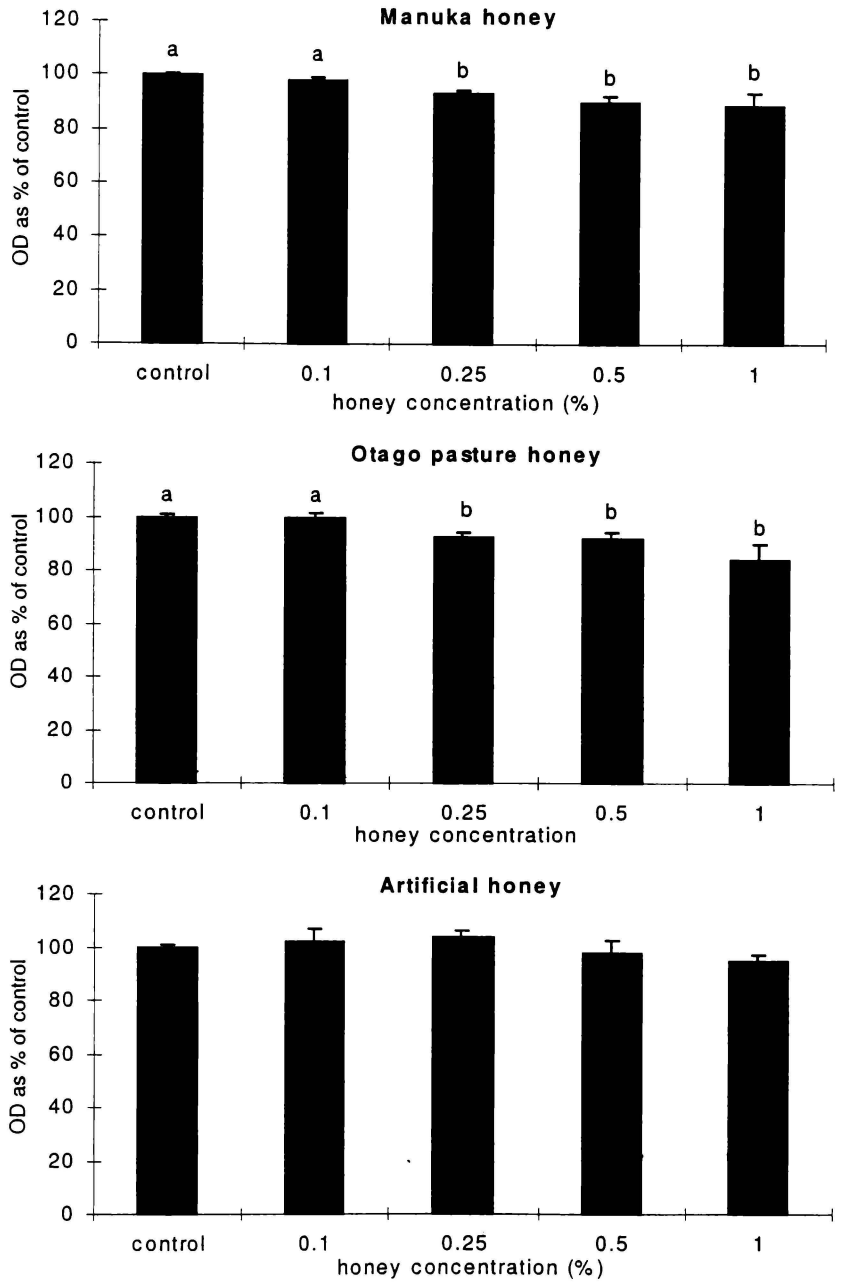


Figure 8.5: The effect on proliferation of exposing fibroblasts to various honey concentrations prior to stimulation with IL-1β. 3T3-L1 fibroblasts were incubated with final concentrations (0.1–1%) of honey (manuka, Otago pasture or artificial) or DMEM complete medium for 2 h prior to addition of IL-1β (100 ng/ml). After 48 h, cell density was determined by the MTT assay. Data are expressed in proportion to the control (fibroblasts stimulated with IL-1β alone). Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

To establish whether pre-incubating cells with a different dose of honey might have a more potent modulatory effect on IL-1β-driven proliferation, monolayers were exposed to a range of honey at final concentrations (0.1–1%) prior to addition of IL-1β. While natural honeys had no effect on IL-1β-driven proliferation at concentrations below 0.25%, they suppressed proliferation at concentrations between 0.25–1%. However, the slight modulatory effect did not differ significantly between these doses (Figure 8.5). Visual assessment of the cell cultures indicated that this result could not be due to osmotic stress.

Although only a 0.25% concentration of honey had been demonstrated to have a stimulatory effect on fibroblasts, there were no indications to why the single dose alone had activity. In an attempt to characterise the active component, honey was fractionated using dialysis, and tested at a 0.25% concentration. The retentate fractions obtained from dialysis (>10 kDa, at a final concentration equivalent to 0.25%), of both honeys, significantly increased fibroblast proliferation beyond medium alone. Comparison of the levels of proliferation obtained for fibroblasts exposed to retentate fractions, compared with unfractionated honey, indicates that the active component is totally in the retentate fractions. The diffusate fractions (<10 kDa) of both honeys had no significant effect on fibroblast activity beyond medium alone (Figure 8.6).

To characterise the modulatory component, honey was fractionated using dialysis, and tested at a 1% final concentration. Upon removing the sugars from the high molecular weight fraction, the possibility that this may have removed any masking effect of the modulatory component needed to be explored, and therefore a 1% concentration was tested. At 1% concentrations, retentate fractions of both honeys modulated the effect of IL-1β-driven fibroblast proliferation, and indicated that all of the activity is in the retentate (Figure 8.7). As before, diffusate fractions (<10 kDa) of both honeys had no effect on fibroblast activity beyond that of the medium alone.

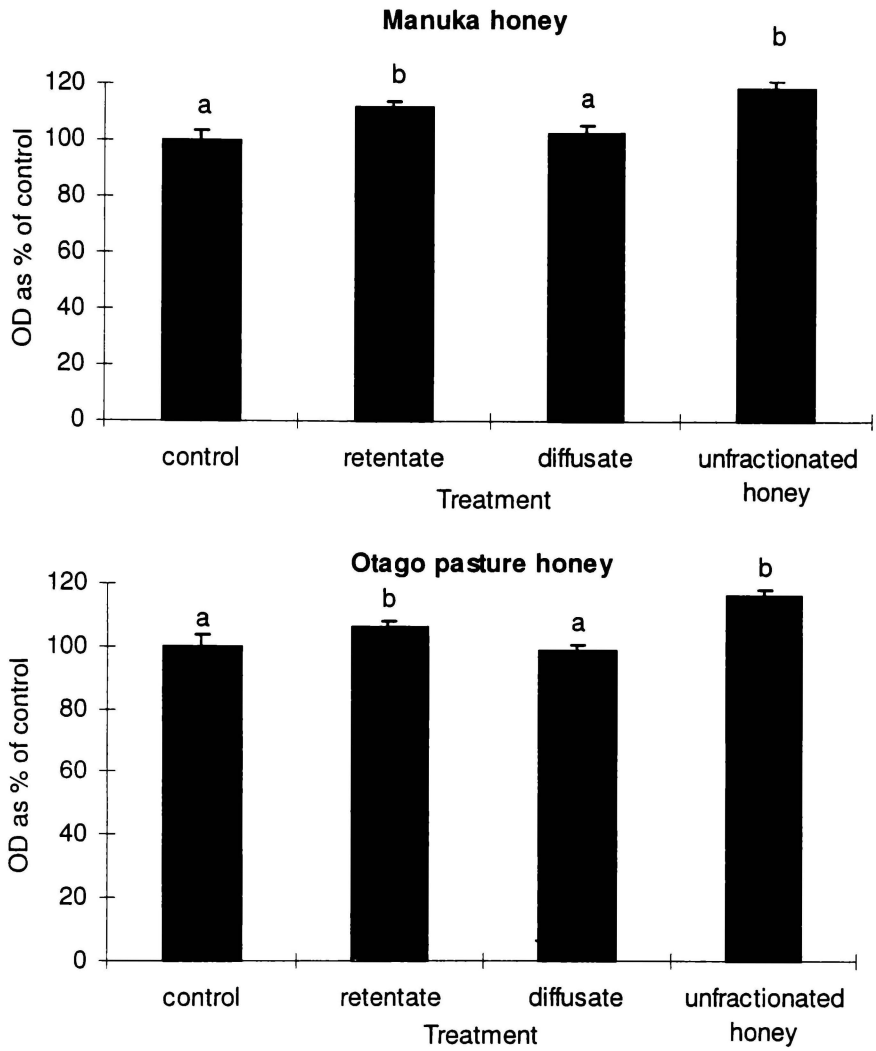


Figure 8.6: The effect of dialysed honey fractions on fibroblast proliferation. 3T3-L1 fibroblasts were incubated with dialysis fractions of manuka honey or Otago pasture honey at a final concentration equivalent to 0.25% honey and compared with cells incubated with DMEM complete medium alone or 0.25% unfractionated honey. After 48 h, cell density was determined by the MTT assay. Data are expressed in proportion to the control (fibroblasts exposed to DMEM complete medium containing 0.05% foetal calf serum). Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

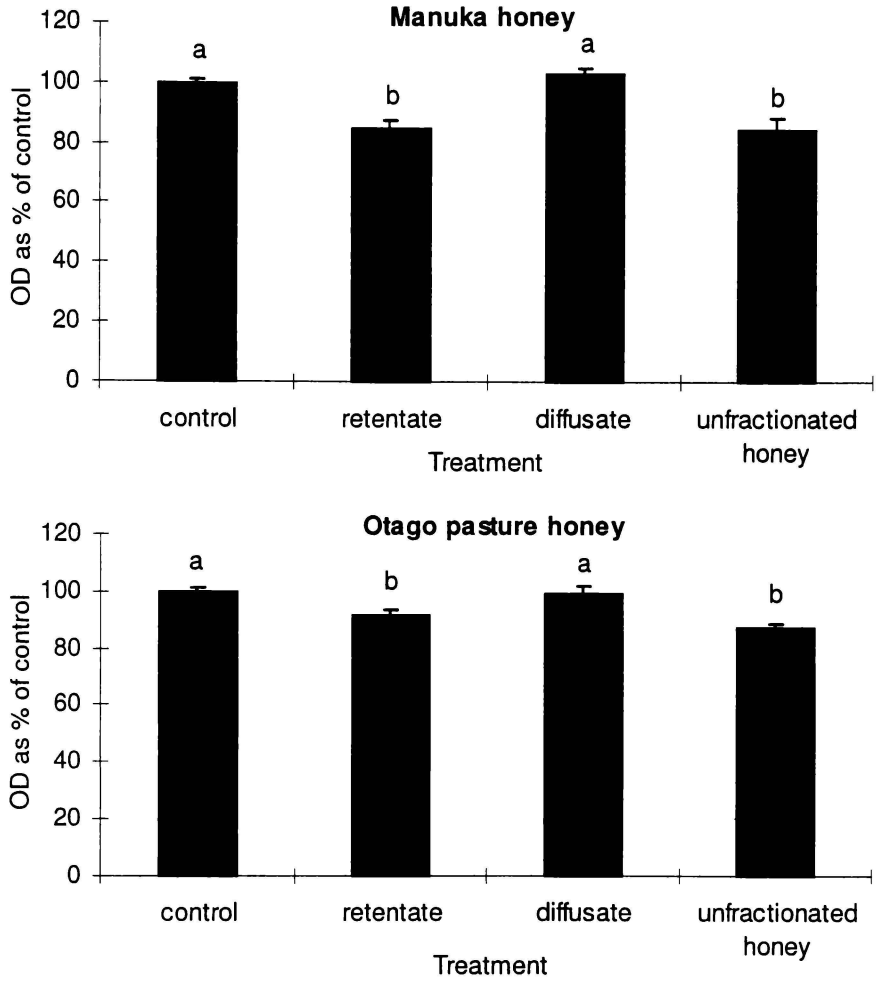


Figure 8.7: The effect on proliferation levels of exposing fibroblasts to dialysis fractions of honey prior to stimulation with IL-1β. 3T3-L1 fibroblasts were incubated with dialysis fractions of honey at a final concentration equivalent to 1% manuka honey, Otago pasture honey or 1% unfractionated honey for 2 h prior to addition of IL-1β (100 ng/ml). After 48 h, cell density was determined by the MTT assay. OD values as a proportion of those cells exposed to IL-1β alone. Data are expressed as the proportion of the control. Data points represent the mean of triplicate wells ± SEM and are derived from three independent assays (n=9). Differences between the means are indicated by superscript letters, where means not sharing common letters within graphs are significantly different ($P<0.05$).

8.4 DISCUSSION

The current study provides evidence that honey has a direct stimulatory effect on fibroblast activity but not at the same levels as obtained for stimulation with the pro-

inflammatory cytokine, IL-1β. Further, proliferation was obtained after exposure of fibroblasts to honey-treated PBMC suspensions, indicating that honey has an indirect effect on fibroblasts through activation of cytokine and growth factor production. Conversely, where fibroblasts had been exposed to honey for a minimum of 2 h prior to stimulation with IL-1β at high levels, as would likely be the case when honey is consumed as a preventative, IL-1β-driven proliferation was modulated. The serious health consequences for wound healing in patients when fibroblast activity is aberrant highlight the need for therapies which can optimise tissue repair processes. Further, it was shown in the current study that honey does not augment IL-1β-driven proliferation. The observations made in the current study are in agreement with clinical evidence which suggests that honey modifies fibroblast activity (Rozaini *et al.*, 2004; Lusby *et al.*, 2002; Al-jady *et al.*, 2000; Suguna *et al.*, 1992).

Incubation of fibroblasts with low concentrations of either type of natural honey at 0.25% concentrations stimulated proliferation. This indicates that honey must contain factors which directly promote cell division in fibroblasts. Viability testing confirmed that the decrease in stimulatory activity which occurred as the honey concentration exceeded 0.25% was not due to any osmotic stress created by sugars. Therefore, some other component in honey must have been exerting inhibitory activity on the stimulatory agent as honey concentration increased.

The stimulatory effect on growth of fibroblasts may be by way of the hydrogen peroxide produced in honey, as hydrogen peroxide has been found to stimulate the proliferation of fibroblasts (Chung *et al.*, 1993). There is a large amount of evidence for hydrogen peroxide being involved in many cell types in the body as a stimulus for cell multiplication, acting at various points in the mechanisms of the cells that control the cycle of cell growth and division (Burdon, 1995), and it has been proposed that low concentrations of hydrogen peroxide might be used to stimulate wound healing, in place of the expensive cell growth factors used for this purpose (Postmes, 1999; Burdon, 1995). However, it has been pointed out that this is only feasible only if the concentration could

be carefully controlled to avoid tissue damage (Chung *et al.*, 1993). This is possible with the controlled sustained release of hydrogen peroxide that occurs in honey. Extrapolating from reported values for the likely concentration of hydrogen peroxide which could be produced by honey at 0.25%, 0.05 mM (Bang *et al.*, 2003), hydrogen peroxide would be at lower levels than those shown to cause oxidative stress in fibroblasts (0.2 mM) (Wang *et al.*, 2001). However, at 0.05 mM levels, hydrogen peroxide would be present at concentrations well above the minimum threshold concentration necessary to induce fibroblast activation (0.2–0.1 nmol)(Murrell *et al.*, 1990).

The lack of substantial differences in stimulatory effect between the two honeys, despite the likelihood that hydrogen peroxide production would be very different (Molan, 1992b) suggests that activity may be due to some factor other than hydrogen peroxide. It was established that medium neutralised the acidity of the honey, so that pH differences between the honey-treated cells and control cultures could not account for stimulatory effects.

That honey contains proteins which are likely to be modified by sugars was discussed in sections 1.4.3 and 2.4.1.1. There is strong evidence that glycated proteins bind to cellular receptors in a similar way to lectins and activate a number of cell types to proliferate, including fibroblasts (as discussed in Section 2.4.1.1).

Evidence reported by Pani *et al.* (2000) that the shifting balance between antioxidant concentrations and the availability of exogenous hydrogen peroxide controls thymocyte proliferation (discussed in Section 2.4.2.1) is of relevance here. The decrease in proliferation reported in the current study as honey concentration increased may reflect the higher concentration of an inhibitor or an antioxidant compound which over-rides the stimulatory effect. Fractionation of the honeys by dialysis confirmed that the active stimulatory components were present in the retentate fraction. No stimulatory activity was seen in the low molecular weight diffusate fraction.

Incubation of fibroblasts with supernatants derived from PBMCs exposed to honey induced proliferation. As it was established that low concentrations of honey (less than 0.1% concentration) had no direct effect on fibroblast proliferation, factors produced by PBMCs must promote fibroblast mitosis. Support for the role of PBMC-derived factors in fibroblast proliferation comes from the observation that PBMCs maintained in medium alone had no effect on fibroblast activity. However, it was important to note that significant activating effects were obtained from the PBMC supernatants after they were diluted 1:4 with medium. These results raise questions about the magnitude of cell activation which might have been achieved with undiluted supernatants and justify future *in vivo* investigation.

Likely candidates for the fibroblast-activating factors from honey-stimulated PBMC include Platelet Derived Growth Factor-α (PDGF-α) a cytokine that causes differentiation and proliferation of fibroblasts (Ghahany *et al.*, 1995; Lynch *et al.*, 1989). PDGF-α is released from macrophages during wound healing, and acts as a chemoattractant and mitogen for fibroblasts.

The significance of this result is that it provides evidence that honey has activity on many different cells in the inflammation pathway and that its actions are perpetuated by cellular interactions. That honey induced expression of mRNA for cytokines (specifically IL-1β and TGF-β) in whole blood cultures (Section 3.3), supports the observation here that honey has indirect stimulatory effects on fibroblasts. Both TGF-β (Cobbold and Sherratt, 2000), and IL-1β (Ghahany *et al.*, 1995; Lynch *et al.*, 1989), are key cytokines involved in the activation of fibroblast activity and re-epithelialisation.

The present study has characterised the stimulatory agent on fibroblasts as being of high molecular weight. While it is known how IL-1β stimulates fibroblast proliferation, it is not known how honey has this effect. IL-1β is known to drive fibroblast activity by stimulating prostaglandin E2 synthesis, collagenase synthesis, plasminogen activator synthesis and breakdown of proteoglycans and fibronectin in extracellular matrix by

fibroblasts (Kahari *et al.*, 1987). In particular, IL-1β is characterised as a potent stimulant of free radical formation in fibroblasts (Kahari *et al.*, 1987).

The growth rate of the cells would be maximal just before confluence, as contact inhibition occurs when the monolayers become confluent. The maximum scope for proliferation using the 96-well plate format was not determined, and it is not known whether greater differences between treatments would have been obtained if confluency was not a limiting factor. Therefore, it is not known if the observed differences provide a true picture of the full potential of honey to have activity on fibroblast cultures. Future *in vitro* assays could be conducted using beads rather than wells as the adherent surface as these would extend the scope for monolayer expansion.

That honey can reduce IL-1β-driven fibroblast proliferation, and does not augment IL-1β-stimulation, is an important observation because excessive inflammation is known to contribute to a number of fibrotic disorders (Dinarello, 1996), and specifically keloid scarring (Ghahany *et al.*, 1995; Lynch *et al.*, 1989). This may suggest that the presence of honey in the systemic circulation, as would likely occur when honey is consumed as a preventative, may ameliorate excessive inflammatory responses. There is evidence from a study by AL-Waili (2003) that routine consumption of honey can modulate the NO content in the circulation, and therefore, every likelihood that honey could modulate other cytokines when regularly consumed.

It is also important that honey does not inhibit proliferation as this would prevent wound healing from occurring. Artificial honey had no modulatory effect on the cell response to IL-1β, indicating that sugars were not responsible for the modulatory action observed with natural honeys. The present study has characterised the modulatory agent as being of high molecular weight. The difference in antioxidant profile and protein content for the honeys was introduced in Chapter 2. That Otago pasture honey has a low antioxidant content and yet interferes with IL-1β-stimulated proliferation reduces the likelihood that antioxidant content alone is responsible for the observed effects. Exposure of fibroblasts

to natural honey (0.25–1%) prior to stimulation with IL-1β led to a 10–15% reduction in proliferation levels as compared to fibroblasts exposed to IL-1β alone. How this slight modulatory effect translates to *in vivo* fibroblast proliferation is not known, though the observation that honey reduces the incidence of hypertrophic scars after burn injury (Subrahmanyam, 1998), suggests that it would. One of the consequences of burn injury is the production of pro-inflammatory cytokines, including IL-1β (Tanaka *et al.*, 1995; Kaufman *et al.*, 1989), at high levels.

This study provides evidence that honey stimulates fibroblast proliferation. The findings made here are in agreement with clinical observations that honey has an activating effect on growth of granulation tissue, and thus contributes to the promotion of tissue healing. This clearly indicates that the promotion of formation of granulation tissue by honey may be through direct stimulation of growth of fibroblasts rather than (or as well as) stimulation of angiogenesis. The observation that manuka honey only had a significant stimulatory effect at a single concentration (0.25% v/v), needs to be investigated further. While this study has identified the optimal honey concentration for stimulation of fibroblast activity *in vitro*, it is not yet known how this can be transferred to the situation *in vivo*. An attractive possibility is that honey could be used in wound management products to stimulate healing.

Chapter Nine: General Discussion And Future Experiments

In the preceding chapters of this thesis, the effect of honey on a variety of cells active at different steps in the inflammatory response have been investigated. In this section the implications of these results for the effect of honey on the overall inflammatory response, and potential benefits for therapeutic application are discussed in light of published reports.

The starting point of this thesis was to repeat some of the key observations described in the few published reports on honey in tissue culture systems (Tonks *et al.*, 2001; Abuharfeil *et al.*, 1999). This enabled the published reports to be corroborated and extended the testing of their observations to both, cells and honeys from different origins. Further, only a few concentrations of honey had been tested for cellular effects prior to this thesis, and therefore, the range of concentrations tested was extended. Far from being a simple repeat of what had previously been shown, this study was the first to report that very low concentrations of honey had stimulatory effects on T cell proliferation, and the production of TNF-α by monocytes, in addition to mRNA for key cytokines involved in wound healing. This study was the first to report the dual ability for honey to stimulate proliferation in quiescent fibroblasts, and to modulate their proliferation in response to a potent proinflammatory cytokine, IL-1β. This study was the first to demonstrate that honey conveys protective effects to tight junction integrity. In addition to demonstrating that the sugars in honey increase phagocytosis by neutrophils, this study was the first to show that manuka honey has an opsonizing effect on bacteria. Further, this study was the first to show in vitro, that honey modulates the production of NO by LPS-stimulated mononuclear leukocytes. Finally, this study was the first to screen for the effect of honey on a wide range of cells involved in the inflammatory response of wound healing, and draws these together to suggest several mechanisms by which honey enables tissue repair to proceed normally.

9.1 JUSTIFICATION FOR CELL TYPES AND ASSAY METHODS SELECTED

The investigations described in the current study were performed using cell-based assays in tissue culture systems. Due to the availability of bovine blood and cell lines from various origins, along with the constraints of ethical approval and resources for human studies, assays were restricted to the use of these cells. Bovine blood was used to investigate the effect of honey on T cell activity (Chapter 2) cytokine mRNA expression in blood (Chapter 3) nitric oxide production by mononuclear leukocytes (Chapter 5) and neutrophil phagocytic activity (Chapter 6). While the activity of cytokines on specific cells is known to vary slightly between human and bovine systems (Collins *et al.*, 1999), it is assumed that the ability for honey to activate the inflammatory pathway would be applicable to both. The use of non-human cell-based inflammation models for human application studies is accepted as a viable model in several key wound healing studies (Goncalves *et al.*, 2001; Greenwel *et al.*, 2000; Klyubin *et al.*, 1996 and Granstein *et al.*, 1990).

The choice of assay methods described in the current study was influenced by research budget, time and the availability of resources, but were considered appropriate to address each given hypothesis. Due to the broad scope of this thesis it was not possible to examine the effect of honey on all aspects of cell signalling for each cell type studied. Therefore the primary objective of this thesis was to use key assays to screen for any effects of honey at various steps in the inflammation pathway. Assays were planned and implemented according to accepted published methods.

Immortal cell lines were used to investigate the effect of honey on; TNF-α production by human THP-1 monocytes using human WEHI macrophages as an indicator of TNF-α levels (Chapter 4), protection against tight junction disruption in a canine-derived MDCK cell line after an inflammatory challenge (Chapter 7) and proliferation in a mouse-derived 3T3-L1 fibroblast cell line (Chapter 8).

9.1.1 Limitations of the in vitro assays used in the current study

In preceding chapters of the current study, the limitations associated with use of *in vitro* experimentation for their application to the real life situation have been mentioned. While testing of honey on purified/semi-purified cell populations *in vitro* was useful to dissect responses at the cellular and molecular level, they provided minimal information about the interaction of diverse cells *in vivo*. Of particular relevance to the current study, it has been noted by Yancy *et al.* (2001) that cellular activation observed *in vitro* will be determined by the populations of cells present, as cell signalling is critically dependent on cell-cell interactions. Of additional interest here, Collins *et al.* (1999) have observed that the cellular environment *in vivo* will have profound implications for the activation response of primary cells *in vitro*. The observation that honey has immunogenic activity in cells sourced from different species and from different physiological origins (immortal cell lines and primary cultures) is encouraging because it indicates that honey has wide applicability to various cell types. Therefore, the application of the results obtained in the current study to the functioning individual are purely speculative, and further testing would be needed to establish whether the results could be realised *in vivo*.

9.1.2 Reasons for variation in cellular activation levels of primary cells in vitro

It has been noted in Section 2.3.4 of the current study, and by deJong (2003) and Gauntlett (2004), that there was variation between assays in the stimulation effect of honey on T cells obtained from primary cultures. There are a number of reports which show that cells in primary culture will vary in their cytokine production to the same stimulus according to previous exposure to cytokines *in vivo* (Collins *et al.*, 1999) such as an attenuation event (Burleson *et al.*, 2002; Kiecolt-Glaser *et al.*, 1995; Collins *et al.*, 1999; Cohen *et al.*, 1998; Herbert and Cohen, 1993; Cohen *et al.*, 1991) or psychological stress (Burleson *et al.*, 2002; Iserson, 2000). The observation that delays in blood processing led to expression of heat shock proteins (reported by Baecheler *et al.*, 2004) may offer some important clues for observed variation between assays. Induction of heat shock protein expression in cells due to an oxidative stressor

is known to interfere with the subsequent response of the cells to a further stimulus (as discussed in Section 2.4).

That different concentrations of honey were found to be optimal for inducing cellular effects in different cell types is likely to reflect differences in the physiological tolerance of cell types.

9.2 THE LIKELIHOOD THAT THESE *IN VITRO* OBSERVATIONS COULD BE REALISED *IN VIVO*

The observations in the current study that honey stimulates proliferation of lymphocytes and fibroblasts, and phagocytosis of bacteria by neutrophils are supported by what is reported anecdotally *in vivo* (Subrahmanyam, 1993;1991) and in experimental wound models (Postmes *et al.* (1997); Kumar *et al.* (1993); Oryan *et al.* (1998); Gupta *et al.* (1992) and Church (1954). While there is much evidence for the effects of honey on wounds (Cooper *et al.*, 2001; Vardi *et al.*, 1998; Subrahmanyam, 1998; Wood *et al.*, 1997; Harris, 1994; Subrahmanyam, 1994; Phuapradit and Saropola, 1992; Efem, 1988; Bergman *et al.*, 1983), and burns (Subrahmanyam, 1993; 1991), there is some limited evidence for gastroprotective activity (Ghosh and Playford, 2003; Bilsel *et al.*, 2002; Gharzouli *et al.*, 2001; Ali *et al.*, 1997; Haffejee and Moosa, 1985; Salem, 1981). The experiments described in the current study were designed to best model cellular systems as they exist *in vivo*. The method by which honey would be delivered to damaged and/or inflamed tissues was therefore of prime importance in both assay design, and interpretation of the results obtained. With regard to the stimulatory effect of honey, it is conceivable that application of honey to a non-healing wound may stimulate the activity of neutrophils to debride (Section 6.4), T cells to proliferate (Section 2.4), leukocytes to produce cytokines (Sections 3.4; 4.4) and later, fibroblasts to proliferate (Section 8.4). The ability for honey to stimulate tissue repair in non-healing recalcitrant wounds (Molan, 2004; Cooper, 2001), further lends powerful support to the demonstrated ability for honey to act as an immunostimulant in the current study.

The observations by Al-Waili (2003) that regular consumption of honey induced measurable levels of NO metabolites in saliva suggest that oral ingestion of honey could have immunogenic effects, even at low concentrations. Gross *et al.* (2004) found a direct link between honey consumption and the level of polyphenols in the blood. The regular consumption of honey by some individuals as a prophylactic, made it physiologically relevant for investigations to include prior exposure of cells to honey before a known activating agent. The four teaspoon per day dose regime reported by Al-Waili (2003), and four tablespoon per day dose reported by Gross *et al.* (2004), would have resulted in greatly diluted levels of honey, and therefore the effective concentrations of honey reaching the systemic circulation were likely to be very low. There are several reports that indicate consumption of honey has significant effects on the antioxidant profile of blood (Gross *et al.*, 2004; Schramm *et al.*, 2003) , indicating that it is feasible that honey might have anti-inflammatory activity *in vivo.* It is not known how the micromolar concentrations of antioxidants likely to be present in the systemic circulation would relate to levels hypothesised to be present *in vitro.*

Based on published evidence, it is feasible that honey could exert cellular effects at the low concentrations demonstrated to be effective *in vitro*, and that these low concentrations of honey could be achieved *in vivo.* Certainly, there is no evidence that the results obtained in this thesis would not be realised *in vivo.*

9.3 THE POSSIBLE MECHANISMS BY WHICH HONEY EXERTS IMMUNOGENIC EFFECTS

This thesis did not identify the active component in honey, but it did characterise the activity as being of a high molecular weight, and therefore achieved a prime aim of this study. In the absence of a clearly identified active compound, any discussion about the active agent is purely speculative. Here, the implications for the likely active agent are discussed in light of evidence from the current study, and from published reports.

Two key components of honey have been proposed as the most likely agents responsible for the observed stimulatory effects on cells, hydrogen peroxide and

glycated proteins. Evidence that honey contains both hydrogen peroxide and glycated proteins was discussed at length in Chapter 1. It was hypothesised that honey might stimulate cell proliferation by activating NF-κB, through the actions of hydrogen peroxide or the presence of glycated proteins (Section 2.4, 6.4). This thesis did not investigate the effect on cells of hydrogen peroxide content or protein content directly, but the observations made in the current study indicate that these agents may indeed play a role. Further, there are some indications that antioxidants may play a role in the demonstrated activity of honey (also not investigated directly in this thesis). Based on the results obtained from the investigations conducted in this thesis, it is hypothesised that hydrogen peroxide content alone is unlikely to account for all of the observed effects of honey on cells. In particular, where manuka honey has been demonstrated to have more potent activity, it is likely that proteins and antioxidants play an important role in cellular activation. This is based on the lower levels of hydrogen peroxide reported to be delivered by manuka honey compared with Otago pasture honey, and yet a higher content of proteins and antioxidants.

The most reasonable hypothesis which can be proposed based on the evidence presented in this thesis, is that a number of components present in honey may act separately or in concert to induce immunogenic effects. While such a conclusion sheds little light on the true nature of the active component(s), it fits with the inability of any studies to date to identify either UMF or the immunogenic agent in honey. Such a hypothesis is in agreement with others attempting to identify the active component(s) in honey (Gheldof *et al.*, 2002). The biphasic effect of honey, stimulatory at low concentrations and modulatory at higher concentrations suggests that there are likely to be several immunogenic agents present.

9.3.1 Evidence that honey is a cell stimulant

The ability for low concentrations of honey to stimulate proliferation was demonstrated for T cells (Section 2.3.1) and fibroblasts (Section 8.3). The results of this study mirror numerous reports that low concentrations of hydrogen peroxide directly stimulate proliferation in these same cell types (Reth, 2002; Burdon, 1995;

Los *et al.*, 1995; Chung *et al.*, 1993; Schmidt *et al.*, 1992; Rao and Berk, 1992; Murrell *et al.*, 1990).

With reference to Figure 9.1, hydrogen peroxide has been demonstrated to induce a number of anabolic cellular effects, and these are reconcilable with those seen when honey is applied topically (Molan, 1991; Postmes *et al.*, 1997; Subrahmanyam, 1993; Harris, 1994; Adesunkanmi and Oyelami, 1994; Subrahmanyam, 1993; Suguna *et al.*, 1993; Postmes *et al.*, 1993; Ndayisaba *et al.*, 1993; Efem, 1993; Gupta *et al.*, 1992; Ankra-Badu, 1992; Subrahmanyam, 1991; Zumla and Lulat, 1989; Farouk *et al.*, 1988; Efem, 1988; Dumronglert, 1983; Bergman *et al.*, 1983; Bose, 1982; Armon, 1980; Cavanagh *et al.*, 1970; Bulman, 1955). The observation that micro- and nanomolar concentrations of hydrogen peroxide were found to directly stimulate fibroblast proliferation *in vitro* (Schmidt *et al.*, 1992), mean that it is feasible that hydrogen peroxide could account for the activating effects obtained with honey. Murrell *et al.* (1990) reported that very low levels of oxidant species are needed for fibroblast proliferation to be induced (1 μM). These levels fall well within those extrapolated from the data reported by Bang *et al.* (2003) of 0.05 mmol of hydrogen peroxide obtained when 0.25% honey is added to cell suspensions. Taking into account the results obtained in this thesis and observations reported for the direct effects of hydrogen peroxide on cells, a mechanism is proposed by which hydrogen peroxide in honey might stimulate wound healing (Figure 9.1).

Evidence that honey modulates fibroblast activity means that it has potential for a topical treatment to optimise tissue repair, and especially while scar formation occurs. There is abundant anecdotal evidence that honey has effects on recovery of epithelial integrity, both *in vitro* (D. Adams, Child Health Research Institute, Adelaide) and *in vivo* (Ahmed *et al.*, 2003; Biswal *et al.*, 2003; Misirlioglu *et al.*, 2003; Aysan *et al.*, 2002; Lusby *et al.*, 2002; Topham, 2002; Agren *et al.*, 1999; Thomas, 1998; Efem, 1988). The ability of honey to increase fibroblast proliferation has been demonstrated using animal wound healing models (Gupta *et al.*, 1992).

However, the results obtained from this thesis do not entirely support the hypothesis that hydrogen peroxide alone could be responsible for stimulatory effects. While it has

been reported that progressive dilution of honey increases glucose oxidase activity because the enzyme inhibitors are progressively inactivated (Bang *et al.*, 2003; Buntting, 2001; White, 1966), activity diminishes as dilution of honey proceeds below 30% (v/v) (Bang et al., 2003; White, 1966; White *et al.*, 1963), as was the case here. Though at 0.25% concentrations the potential levels of hydrogen peroxide delivered by honey could conceivably promote fibroblast proliferation, the hundred-fold dilutions beyond this, demonstrated to activate T cell proliferation do not appear likely. Yet honey is reported to have immunogenic effects even when ingested orally (Al-Waili, 2003), and it is highly unlikely that the concentration of honey could approximate 30% (v/v) due to the dilution effects of body fluids. With these comments in mind, it is very unlikely that sufficient hydrogen peroxide would be produced by the high molecular weight fraction of honey to account for stimulation, even if the glucose oxidase enzyme had been retained. It was noted that glucose oxidase from the honeybee has a molecular weight of 16 kDa (National Honey Board USA, 2003; Eichenseer *et al.*, 1999), and the enzyme should have remained in the high molecular weight fraction after dialysis. Further, the presence of glucose in the cell culture medium would have provided the enzyme with its substrate, meaning that hydrogen peroxide could have been generated. To exclude the role of hydrogen peroxide completely, future investigations would need to use glucose-free medium, though this was not possible with the bovine-derived PBMCs used in this study.

Further evidence that discounts the probability that hydrogen peroxide could be responsible for cellular activation, relate to what is know about hydrogen peroxide content of different honey types (Section 1.3.1.5). The level of hydrogen peroxide is far greater in the pasture honey than the manuka type, and this is directly attributable to the floral source (Molan, 1992b). Components from floral sources have been demonstrated to affect the production and destruction of hydrogen peroxide, among these being the levels of catalase, ascorbic acid and iron content affect the rate of its breakdown by chemical reactions (Kerkvliet, 1996; Molan, 1992b). Therefore, it would be expected that pasture honey would have stimulatory activity at higher dilutions than manuka honey and this was not the case. While there is no dispute that honey releases hydrogen peroxide when diluted in cell suspension (Molan, 2001; Swellam *et al.*, 2003; Tonks *et al.*, 2003; Weston, 2000), it has not yet been

established what the effective concentration of hydrogen peroxide would be in the suspension when honey has been diluted a million-fold or more. Of further concern, it is unknown how the hydrogen peroxide content is affected by other factors present in the suspension.

There is evidence that the redox status of cells plays an important role in fibroblast proliferation, and the supply of bioavailable trace elements, in particular antioxidants may be expected to alter the overall redox status of the cells (Lee and Wan, 2002; Murley *et al.*, 2001; Burdon, 1995; Los *et al.*, 1995; Halliwell and Gutteridge, 1989). The higher antioxidant content of manuka honey as compared with Otago pasture honey was discussed in Section 1.3.1.5. This may help to explain why manuka honey had mitotic activity on lymphocytes beyond that of pasture honey.

Based on the established variation in chemical composition between honey types according to their floral source, it is reasonable to speculate that chemical interactions may affect the final amount of hydrogen peroxide which is released in cell suspension. The loss of activity observed for the high molecular weight fractions of manuka honey as compared with unfractionated honey is most likely due to disruption of some synergy between chemical constituents (Swellam *et al.*, 2003). Honey is a complex substance, and the interactions existing between the component molecules responsible for observed effects may have altered bioactivity after fractionation. Indeed, natural products such as honey remain elusive to chemists because they contain compounds with greater structural diversity than is possible via most synthetic chemistry routes currently in use (Harvey, 2000). However, some key differences were observed between the stimulatory action of honey on T cells and fibroblasts. Fibroblast proliferation did not occur at the same low doses which induced T cell proliferation with a minimum 0.25% concentration necessary to induce proliferation. On the other hand, it was speculated that the increased T cell proliferation which occurred with increased honey dilution might be due to the progressive removal of an inhibitor (Section 2.4). The importance of different physiological tolerances for the cell types is not presently known. The loss of stimulatory effect as honey concentrations exceeded 0.25% (v/v), and yet could not be due to osmotic effects (as established by viability

assays), may indicate the presence of an anti-inflammatory agent. Modulatory activity was only observed at higher concentrations of honey.

As blood was obtained from a different animal each time, the possibility that animals may respond differently to the stimulant can not be discounted. In a preliminary study using a similar T cell proliferation assay protocol, de Jong (2003) reported that blood obtained from various bovine differed significantly in cell response to the same honey. This needs to be investigated further. The observation that different concentrations of honey are required for measurable cellular effects to be obtained in cells from different origins may have no relevance *in vivo*, and can only be proven with *in vivo* testing.

A further difference observed for the fibroblast stimulation assays was that data obtained had less variability. This may be due in part to the observation that fibroblasts are more robust to fluctuations in culture conditions than T cells are (Deas *et al.*, 1986). However, it should be noted that the fibroblasts were obtained from a cell line, whereas T cells were present in PBMC fractions along with various numbers of other cell types. The effect of honey on the other cells present in the PBMC fraction, along with the potential for these cells to indirectly stimulate T lymphocytes after themselves being activated by honey is unknown. It was noted in Section 2.4 that bystander activation of T cells may be an important factor in perpetuating T cell proliferation after initial stimulation by honey has occurred (Los *et al.*, 1995). The role of bystander activation of fibroblasts is not known, but fibroblasts release superoxide in response to stimulants such as IL-1 and TNF (Burdon, 1995; Datta *et al.*, 1992; Larssen and Cerutti, 1989; Treisman, 1986). It was shown in Section 8.3, that honey-stimulated PBMC activated fibroblast proliferation. Drawing from the observation that honey stimulated TNF production by monocytes (Section 4.3), it is feasible that honey would have a significant effect on various cell types through indirect activation. Release of superoxide from fibroblasts is catalysed by a plasma membrane NADPH-oxidase, and superoxide dismutates spontaneously to hydrogen peroxide (Burdon, 1995; Datta *et al.*, 1992).

If honey increases the rate at which fibroblasts proliferate (and therefore fill in a wound cavity), it is likely that bystander activation would be enhanced as physical proximity of fibroblasts to each other perpetuates the stimulus (Pani *et al.*, 2000; Burdon, 1995). This suggests that once stimulation has been effected, resulting superoxide transmission between cells serves to drive further proliferation in a paracrine manner. The extent of this initial activation would then determine the rate at which subsequent mitosis will occur, so that cells stimulated with honey would rapidly outpace those in medium alone. Further investigation would need to be undertaken to examine the role of various stimulants on a paracrine stimulation system.

As discussed in Section 1.4.3, it is highly likely that the proteins in honey would be glycated (Tonks *et al.*, 2003) due to both, long-term storage of the proteins with monosaccharides and the potential for the Maillard reaction to add sugars onto proteins, along with the propensity for invertase to transfer glucose onto other functional groups (*e.g.* proteins). In Sections 1.4.3 and 6.5, the large body of evidence for glycated proteins activating a range of cell types was cited. The observation that manuka honey has a more potent activating effect on cells than Otago pasture honey may reflect its higher protein content than the Otago pasture type (Molan, 1992b). Further indications that proteins may exert cellular effects even in low honey concentrations come from reports that protein inhibitors may be removed with progressive dilution (Radwan *et al.*, 1984). Glucose oxidase is an example of a protein which does not exert effects until its inhibitor is removed with progressive dilution (Bang *et al.*, 2003; Buntting, 2001; Radwan *et al.*, 1984).

There is a large body of evidence for glycated proteins activating a diverse range of cells. Protein glycation and AGE are associated with increased free radical activity (Ahmed, 2005), induced NF-κB activation and cytokine production (Ahmed, 2005; Pullerits *et al.*, 2005; Hein and Franke, 2002), effects on intracellular signalling, gene expression, and release of pro-inflammatory molecules (Ahmed, 2005).

Sustained receptor expression of RAGE has been demonstrated to create a positive feedback loop (Pullerits *et al.*, 2005), and it has been demonstrated that AGE-RAGE

interaction leads to a more prolonged activation than NF-κB stimulation through cytokines (Hein and Franke, 2002). Cell-bound RAGE function as a counter-receptor for leukocytes, resulting in an amplification of the inflammatory response (Pullerits *et al.*, 2005. A range of AGE receptors have been identified, including RAGE, AGE-R1, AGE-R2, AGE-R3, sRAGE, and macrophages scavenger receptor) (Ahmed, 2005; Pullerits *et al.*, 2005; Hein and Franke, 2002).

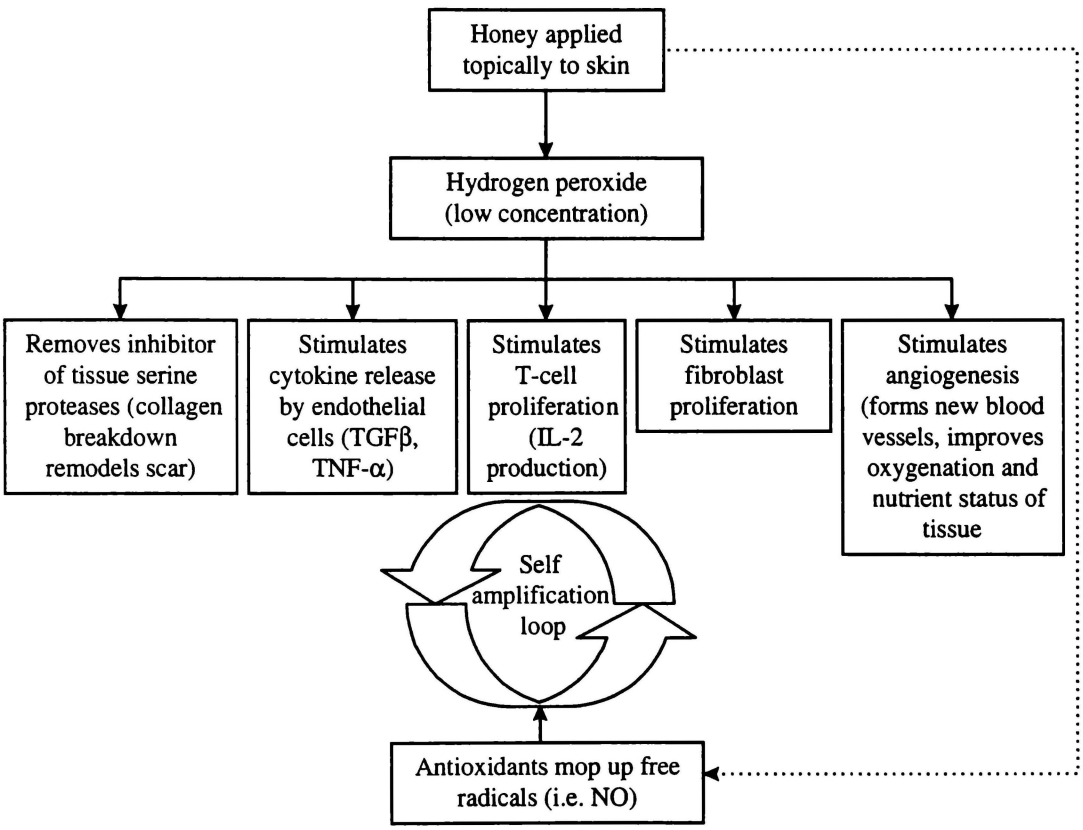


Figure 9.1: Concept diagram for the hypothesised mechanism by which hydrogen peroxide in honey may act as an immune stimulant The growing body of evidence suggests that hydrogen peroxide regulates signal transduction pathways, activates transcription factors and controls the expression of several genes concerned with cell anabolism. Observations from this study suggest that the hydrogen peroxide content of honey is a likely candidate for its effect on cell growth and immune competency. In addition, it is hypothesised that antioxidants in honey would interfere with the feedback amplification loop which exists for various inflammatory cytokines and oxidant species.

Diabetes studies have established that glycated proteins induce mitosis in fibroblasts (Huang *et al.*, 1999; Lapuz, 1997; Sharma and Ziyadeh, 1997; Guh *et al.*, 1996; Young *et al.*, 1995), through interaction with specific cell-surface acceptor molecules, ultimately activating NF-κB (Huang *et al.*, 1999; Yan *et al.*, 1994). Glycated proteins can activate the JAK/STAT pathway (Huang *et al.*, 1999; Weiss *et al.*, 1998), stimulating production of platelet-derived growth factor (PDGF) by leukocytes (Throckmorton *et al.*, 1995), which are potent activating factors for fibroblast proliferation (Burgering and Bos, 1995). These reports are reconcilable with the observed ability for honey-stimulated PBMC to activate fibroblast proliferation (Section 8.3).

A further possibility may be that tannins in honey (which would likely occur at higher concentrations in manuka honey due to its high content of polyphenols) react with proteins or sugars forming large complexes which might be antigenic (P. C Molan; personal communication). Generally, all proteins are immunogenic due to antibodies being directed against sugars present on the surface of glycoprotein moieties, and those of greater complexity induce more vigorous responses by leukocytes (Benjamini *et al.*, 1996). Binding of glycated proteins have been reported to trigger the release of cytokines by monocytes (Brownlee, 1995; Wolff *et al.*, 1991). The observation that higher doses of high molecular weight fractions of manuka honey induced greater cytokine release by resting cells may be due to increasing protein content. That activity could not be identified for the equivalent fractions of pasture honey may be evidence that synergy between several compounds in honey is vital for maximal TNF-α production by monocytes. This may explain the decrease in total activity which occurred when honey was fractionated.

However, it is unlikely that proteins alone could account for observed stimulatory effects as the protein content in full-strength honey is low (Molan, 1992b), and extremely low in Otago pasture honey, also shown to induce cell proliferation. A range of honeys were reported to have an average total protein content of 168.6 mg/100g honey (National Honey Board USA, 2003). Extrapolating from a report by

White (1975a), the average free amino acid content in an average honey, at a 0.25% concentration, would be approximately 1.12 μg/ml.

Other reports have suggested that the nutrient content of honey may account for its promotion of tissue healing (Gupta *et al.*, 1992), but this is unlikely to be of relevance in culture systems where conditions are optimal. Likewise, where glucose is available at optimal levels in culture medium, the supplementation of sugars to cells provided by honey is unlikely to promote an additional growth effect. It was observed in Section 6.3 that sugars in honey increased phagocytosis, however, the blood was not maintained in medium and this may have explained the small stimulatory effect. While others have reported that elevated glucose levels may directly stimulate various fibroblast populations, this was not observed in the current study where artificial honey had no effect on proliferation beyond culture medium (Huang *et al.*, 1999; Trevisan *et al.*, 1997; Ohgi and Johnson, 1996).

That manuka honey had a more potent stimulatory effect on T cells than Otago pasture honey is reconcilable with limited evidence that flavonoids may have a stimulatory effect on some cell types (Havsteen, 2002). Flavonoids bound to proteins may be taken up by macrophages acting as an antigen (Havsteen, 2002) and stimulate production of IFN-β. However, that Otago pasture honey would have a low flavonoid content (Molan, 1992b), and yet stimulates T cell proliferation at hundred-fold dilutions means it is very unlikely that flavonoids alone could be responsible for the observed stimulatory effects.

In Section 4.3, the ability for low concentrations of honey to induce TNF-α release by monocytes was shown. In addition to inducing the production of functional protein, it was reported that honey could induce mRNA expression for key genes involved in wound healing in blood cultures (Section 3.3). Taken together with the results from Section 4.3, and the indirect stimulation obtained with honey-stimulated PBMC on fibroblasts (Section 8.3), it is clear that honey stimulates release of various cytokines. These results confirm reports by Blair (2000) and Tonks *et al.* (2003), that honey stimulates the release of IL-1, IL-6 and TNF proteins by monocytic cell lines *in vitro*. Of particular note, the studies by Blair (2000) and Tonks *et al.* (2003), used different

monocytic cell lines and a higher concentration of honey (1%). This is likely to reflect physiological differences between the cell lines. A pilot study by de Jong (2003) demonstrated that at 0.025% and 0.25% concentrations, manuka honey induced IL-2 release from a murine T cell line. If the demonstrated ability for honey to induce cytokine mRNA expression for key genes involved in tissue repair (Section 3.3) are found to correlate with production of the functional proteins *in vivo*, this would help to explain clinical observations.

With particular relevance to wound healing, TNF-α induces macrophage activation (Leibovich *et al.*, 1987), fibroblast proliferation (Sugarman *et al.*, 1985) and angiogenesis (Leibovich *et al.*, 1987). In addition to its role in the early phase of inflammation, IL-1β stimulates the release of growth factors by macrophages and fibroblasts for keratinocyte proliferation (Clarke, 1996). The observation that honey up regulated mRNA amplicons for some of the pivotal wound healing genes (*e.g.* IL-1β) in the first 6 h is consistent with the rapid effect of honey in stimulating tissue repair as reported clinically (Tonks *et al.*, 2003; Phuapradit and Saropala, 1992; Efem, 1988). It was hypothesised in Section 3.4. that honey may augment mediator release of lymphocyte-derived factors (*e.g.* IL-2) through up regulation of IL-1 production. The production of mRNA in the early experimental phase (by 6 h) was consistent with what had been reported by Tonks *et al.* (2003) and the observation in the current study that TNF-α production by monocytes peaked at 4–6 h incubation (Section 4.3). As honey produced maximal levels of TNF-α after 4–6 h, this increases the possibility that it is interfering with NF-κB.

An additional observation in the current study which is deemed to have some significance for wound healing is that honey did not upregulate cytokine mRNA or TNF protein to the same high levels as induced by the mitogens, Con A or LPS. Further, honey did not continue to induce high levels of cytokine mRNA expression over the entire incubation period, dampening down expression relative to the mitogens by the 24 h time-point. Con A and LPS were used separately to provide models for excessive inflammation in the current study, against which to compare the magnitude of the cell response to honey. It is hypothesised that the massive, prolonged cell

response induced by the mitogens would promote inflammatory conditions such as keloid scarring. Therefore, the observation that honey elicited cytokine mRNA production at lower levels than the mitogens did was deemed a beneficial action. In the absence of measuring the production of functional protein, the feasibility of this observed regulation of cytokine mRNA expression to be realised *in vivo* cannot be known. It is recognised that wound healing takes place over a far longer period of time (up to two years in some cases, Kanzler *et al.*, 1986) and this would need to be investigated further.

In Section 2.3 it was observed that low concentrations of honey stimulate the expression of the IL-2 receptor on T cell membranes, a key activation marker as T cells need to receive IL-2 in order to undergo proliferation (Section 2.2). Taken with the report that low concentrations of honey stimulate IL-2 production in a murine T cell line (de Jong, 2003), this provides further evidence that honey stimulates cytokine production in T cells from both primary and immortal cell line origins. It is noted here that IL-2 is also known to activate fibroblast proliferation (Plaisance *et al.*, 1994). Thus, the observation that supernatants obtained from PBMCs incubated with honey had a stimulatory effect on fibroblasts (Section 6.3), is in line with the observation that low concentrations of honey induce cytokine expression by mononuclear cells.

Floral source appears to determine the extent of cytokine induction by honey. Manuka honey had a more potent inducing effect on mRNA for cytokines than Otago pasture honey. The implications of this observation for the production of functional proteins is unclear. However, it appears to be consistent with general observations made throughout this thesis for the greater potency of the manuka type in exerting immunogenic effects. While a high molecular weight fraction of manuka honey was found to activate neutrophils in the current study, an equivalent fraction of Otago pasture honey did not (Section 6.3). Evidence from studies conducted elsewhere has demonstrated that honey derived from wild flowers in Jordan stimulated phagocytic activity in neutrophils beyond that by a sugar control (Abuharfeil *et al.*, 1999). While the neutrophils studied by Abuharfeil *et al.* (1999) were from human peripheral blood, the neutrophils used in the current study were from a bovine source. Therefore, it is

not possible to conclude whether cell type or floral source of the honey could be responsible for the observed effects on phagocyte activation.

While hydrogen peroxide enters the cell by simple diffusion across cell membranes, glycated proteins must bind to receptors on the membrane (Benjamini *et al.*, 1996). As these two substances have different mechanisms by which they activate lymphocytes, it is conceivable they could both have activity on the cells. With regard to the implications of cells being stimulated by two different agents, the possibility that response to one agent may interfere with cell response to a subsequent stimulus (Terstegen *et al.*, 2000) is raised. If this were the case it would be expected that honey would not produce an additive effect on cell proliferation when present at the same time as a demonstrated mitogenic agent (*i.e.* IL-1), and this was what was observed for fibroblasts *in vitro*. Because honey is a complex mixture of chemical constituents, the specific role of each biomolecule cannot be easily separated from another in the absence of extensive isolation procedures (Swellam *et al.*, 2003).

9.3.2 Evidence that honey modulates cell response to inflammatory agents

The current study supports the hypothesis that honey modulates cell activity during inflammation. In Section 8.3, the ability for honey to modulate fibroblast proliferation in response to a mitogen was demonstrated. In Section 3.3, honey was shown to modulate production of mRNA in LPS-stimulated blood for proinflammatory cytokines. In Section 5.3, honey was demonstrated to modulate NO production by LPS-stimulated monocytes. In Section 7.3, the ability for honey to protect against disruption of tight junctions during an inflammatory challenge was shown. The observation that pre-incubation with honey prior to IL-1β-activation suppresses fibroblast proliferation suggests honey has a modulatory action on proliferation. This may be exploited to reduce the incidence of fibrotic healing disorders such as keloid scarring (Section 1.7.2). It remains to be established whether the antioxidant content of honey may interfere with bystander activation or acts on the target cells directly.

Not all of the investigations undertaken in this study gave support to the hypothesis that honey modulates cytokine production by mitogen- or endotoxin-activated cells. In Chapter 4, it was shown that honey could not modulate TNF-α production after monocytes were exposed to LPS. In Chapter 6 it was shown that honey could not modulate the response of neutrophils to an exogenous TNF-α stimulus. In Chapter 8 it was demonstrated that honey needed to be present for 2 h prior to addition of an exogenous mitogen for any suppression of proliferation to be obtained. This raises questions about how realistic it could be for any anti-inflammatory effect of honey to be gained when honey is applied topically to a wound, assuming pro-inflammatory cytokines (*e.g.* IL-1β) are already being produced. Yet the observation that honey, although in its own right stimulatory, did not augment the effect of the TNF-α stimulus on neutrophils, proliferation of IL-1β-stimulated fibroblasts, or the production of TNF-α by LPS-activated monocytes, is critically important in view of its potential role in wound healing. Once again, it is noted that it is possible that one agent may interfere with cell response to a subsequent stimulus, thereby overriding the activity of one stimulus and leading to sole activation by the other (Terstegen *et al.*, 2000). Regardless of the possibilities for the mechanisms by which honey may/may not have effects *in vitro*, the wealth of anecdotal observations, reports on experimental wound models, and clinical studies (Gharzouli *et al.*, 2002; Mahgoub *et al.*, 2002; Oryan and Zaker, 1998; Ali *et al.*, 1997; Gupta *et al.*, 1992; Haffejee and Moosa, 1985; Salem, 1981; Church, 1954), show that honey does have anti-inflammatory activity in wound healing, and mean that it is very realistic to hypothesise that these effects would be realised *in vivo*.

As this study did not examine the cellular effects of purported anti-inflammatory compounds known to be present in honey, indications to the likelihood of the modulatory compound are discussed in light of published reports. The most likely candidate for observed anti-inflammatory effects of honey is antioxidant content (Gharzouli *et al.*, 2002; Mahgoub *et al.*, 2002). As mentioned previously, human *in vivo* studies have shown that there is a direct link between consumption of honey and the level of polyphenols in blood (Gross *et al.*, 2004; Schramm *et al.*, 2003). In addition to the known antioxidant content of manuka honey (Molan 1992b), it was

observed to convey more potent modulatory effects at higher concentrations (Sections 5.3; 7.3; 8.3). That the most potent immunomodulatory effects were obtained for an ether extract of manuka honey is likely to be the result of a concentrated source of phenolic polymers (Gheldof *et al.*, 2002). High molecular weight fractions of the same honey obtained by dialysis had less immunomodulatory activity than the ether extracted fractions. While the ether extract would have contained all of the components which had dissolved in ether, including the phenolics and flavonoids, it would have no protein content (P.C Molan; personal communication). This difference in potency of the honey fractions may be explained by the established tendency for proteins to reduce the scavenging potential of phenolics (Arts *et al.*, 2002). Further, the phenolics would have been split by the dialysis process into the two fractions, leaving a less concentrated source in the retentate fraction (P.C Molan; personal communication).

Although propolis is known to contain higher concentrations of antioxidant substances than honey (Marcucci *et al.*, 2001), there is abundant evidence that this antioxidant component of honey has demonstrated bioactivity (Yao *et al.*, 2004; Gheldof *et al.*, 2002; Ali *et al.*, 1997; Hannum and Erdman, 2000). Flavonoid composition is directly correlated with the botanical source of the propolis and, therefore, it is not surprising that different honey types may vary in their ability to exert immunomodulatory effects (Section 1.3.1.5). Honey composition closely reflects the idiosyncrasies of the hive's geographical location, due to the phenolic compounds present in plants from which the honey was derived (Martos *et al.*, 1997). Therefore the antioxidant potential of different honey types will be varied, and appears to provide a likely reason for the observed differences in immunomodulatory activity of the manuka and Otago pasture types.

There is overwhelming evidence that honey has anti-inflammatory activity, when applied to the skin (Oryan and Zaker, 1998; Gupta *et al.*, 1992; Church, 1954), or to the gastric mucosa (Gharzouli *et al.*, 2002; Mahgoub *et al.*, 2002; Ali *et al.*, 1997; Haffejee and Moosa, 1985 and Salem, 1981). These studies appear to have parallels to those where antioxidants are administered directly (Ansorge *et al.*, 2003; Phan *et al.*, 2003; Havsteen, 2002; Moreno, 2000; Alliangana, 1996; Frankel *et al.*, 1993; Murrell

et al., 1990), and especially when applied to burns (Kaufman *et al.*, 1985; Tanaka *et al.*, 1995), as has been observed for honey (Subrahmanyam, 1991).

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The established antioxidant content of honey is known to include a range of antioxidants which have potent activity at micromolar concentrations (Raneva *et al.*, 2001; Middleton and Kandaswami, 1992); caffeic acid (Wahdan, 1996), ascorbic acid (White, 1975). Of relevance to this thesis, caffeic acid has been reported to reduce T cell proliferation (Ansorge *et al.*, 2003). Swellam *et al.* (2003) reported that honey at concentrations beyond 1%, had strong modulatory activity on bladder cancer cell lines, possibly due to the inhibitory effect of caffeic acid and flavonoids on tyrosine protein kinase, lipoxygenase and cycloxygenase pathway metabolites.

In general, antioxidants have been demonstrated to reduce proliferation of fibroblast cell lines in response to growth factors (Havsteen, 2002; Moreno, 2000; Frankel *et al.*, 1993), interfere with the hydrogen peroxide stimulation pathway (Phan *et al.*, 2003; Alliangana, 1996; Martinez and Moreno, 1996; Murrell *et al.*, 1990), and reduce lymphocyte proliferation in response to mitogenic substances (Ansorge *et al.*, 2003; Havsteen, 2002; Pani *et al.*, 2000; Natarajan *et al.*, 1996). It has been shown that the unoxidised flavonoids present in honey (Weston, 2000), can act as free radical scavengers (Puppo, 1992) through sequestering metal ions, so that they are unable to participate in the Fenton reaction (Dailey and Imming, 1999).

In light of the observation that prior exposure of fibroblasts to honey reduced subsequent proliferation in response to IL-1β (Section 8.3), it would be useful to investigate whether this effect was correlated with expression of heat shock proteins. The observation that a 6 h incubation of whole blood with 0.25% manuka honey upregulated mRNA expression for heat shock protein 70, may be a consequence of hydrogen peroxide activity (Section 3.3). Further indications that make it likely that hydrogen peroxide might induce heat shock protein expression come from reports that a short exposure time is all that is required for hydrogen peroxide to directly activate cells (Los *et al.*, 1995). In the current study, a two hour exposure of cells to honey was \sufficient to modulate their subsequent response to IL-1β.

However, there are a number of indicators that do not support the hypothesis that hydrogen peroxide-induced chaperonins may play a role in modulatory effects. Ether extracted fractions of manuka honey gave the most potent suppressive effects and yet would be largely devoid of the glucose oxidase enzyme needed to produce hydrogen peroxide as it is not ether soluble (P. C Molan; personal communication; Gheldof *et al.*, 2002). The known content of antioxidants in manuka honey (Schramm *et al.*, 2003; Swellam *et al.*, 2003; Weston, 2000), make it unlikely that sufficient oxidative stress could be induced for heat shock proteins to be expressed, especially by 0.25% concentrations of whole honey. Based on a report by Wang *et al.* (2001), minimum hydrogen peroxide concentrations of 0.2 mM were necessary to induce oxidative stress in fibroblasts, much higher than the likely concentration of hydrogen peroxide that could accumulate in 30 min with 0.25% honey (0.05 mM) (Bang *et al.*, 2003). However, extrapolating from the Bang *et al.* (2003) study, it is conceivable that the minimum 0.2 mM concentrations of hydrogen peroxide would be achieved with 1% concentrations of honey.

The demonstrated capacity for manuka honey to suppress NO production by LPS-stimulated monocytes (Section 5.3), along with downregulation of mRNA for iNOS in LPS-stimulated whole blood cultures (Section 3.3), raises the possibility that the honey modulates NO production through effects on iNOS. Others have shown that antioxidants can be used to regulate iNOS expression, and that this can ameliorate both NO production (Havsteen, 2002), and inflammatory damage to the intestinal mucosa (Kruidenier *et al.*, 2003). The observed ability of honey to convey protective effects to TJ integrity during an inflammatory challenge (Section 7.3), indicates that there is potential for honey to reduce infiltration of immune cells into the tissues. This agrees with the reduction of swelling which is observed when honey is applied to the skin (Oryan and Zaker, 1998; Postmes *et al.*, 1997; Gupta *et al.*, 1992; El-Banby *et al.*, 1989; Kandil *et al.*, 1987; Burlando, 1978), and reduction of vascular permeability when applied to the gastric mucosa (Ali *et al.*, 1997). This may be due to direct

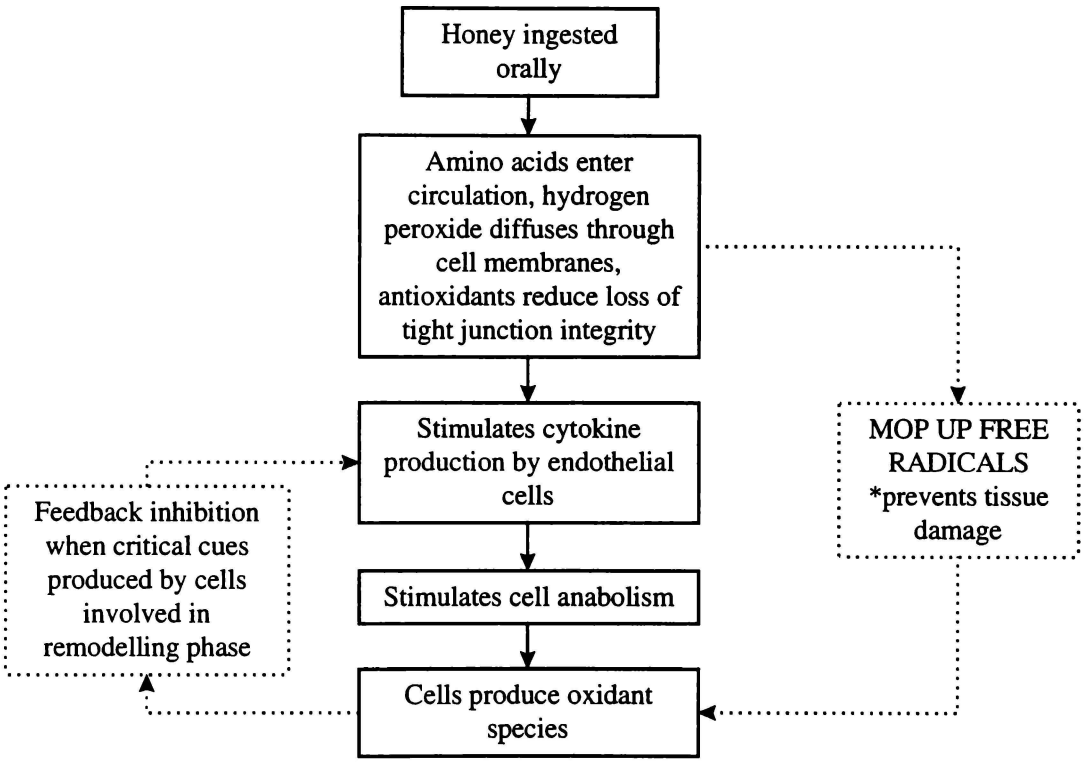

Figure 9.2: Concept diagram for the hypothesised mechanism by which honey exerts anti-inflammatory effects in inflammatory gut conditions when ingested orally Both honey types had protective effects on tight junctions against an inflammatory challenge, and reduced the proliferation response of mitogen-stimulated fibroblasts. These observations were in agreement with what is observed anecdotally. Antioxidants have been demonstrated to have a potent modulating effect on activated immune cells.

antioxidant action (Mahgoub *et al.*, 2002; Ali *et al.*, 1997), or due to enhancing epithelial growth (Molan, 2002; Efem, 1988). Subrahmanyam (1991) and Efem (1992) demonstrated that honey contains inhibin and catalase, and that these directly promote epithelial growth. It would be useful to examine whether honey causes cellular changes in the epithelium surrounding the wound, and determine if healing may be initiated through this mechanism.

Though not examined in this thesis, it has been reported that the antioxidant content of honey can downregulate superoxide production (Ali *et al.*, 1997), and this would potentially reduce superoxide-damage to gastric mucosa characteristic of irritable bowel disease (IBD) (Kruidenier *et al.*, 2003). Therefore, if honey can act at all of these levels, there is potential for it to ameliorate gastric inflammation. The hypothesised role of antioxidants in the observed anti-inflammatory effects of honey in inflammatory gut conditions is presented in Figure 9.2.

The evidence presented in the current study suggests that honey can both stimulate and modulate cell activity. The results presented in the current study show that honey interferes with a large number of regulatory steps in the inflammatory pathway, *e.g.* cell division, transcription, tight junction integrity, production of oxidant species. While the stimulatory activity was observed at lower concentrations, the modulatory effects required higher concentrations of honey. The dual ability for honey to stimulate moderate cellular activation in the absence of an immune stimulus, yet not augment mitogenic stimulation, and in some cases to modulate cell response to a mitogen, indicates that it will promote healing without setting up harmful inflammation. Figure 9.3 draws together both observations made in this thesis and a wealth of published reports to produce a hypothesised mechanism by which honey might optimise wound healing.

The observations made in this thesis demonstrate that low concentrations of honey stimulate: cytokine release, subsequent proliferation and increased phagocytosis by resting leukocytes, and dampen down the proliferative response of cells to a mitogen. Honey has been demonstrated to convey protective effects to tight junctions during an inflammatory challenge, and suppress NO release. These *in vitro* investigations are in

agreement with what is observed anecdotally. There is much evidence to suggest that honey is immunogenic due to its content of glycated proteins, complexes of tannins bound to proteins, or hydrogen peroxide (as cell stimulants), and that its antioxidant content may account for modulatory effects. Further, the role of induction of heat shock proteins in the observed modulatory effects cannot be excluded. It is proposed,

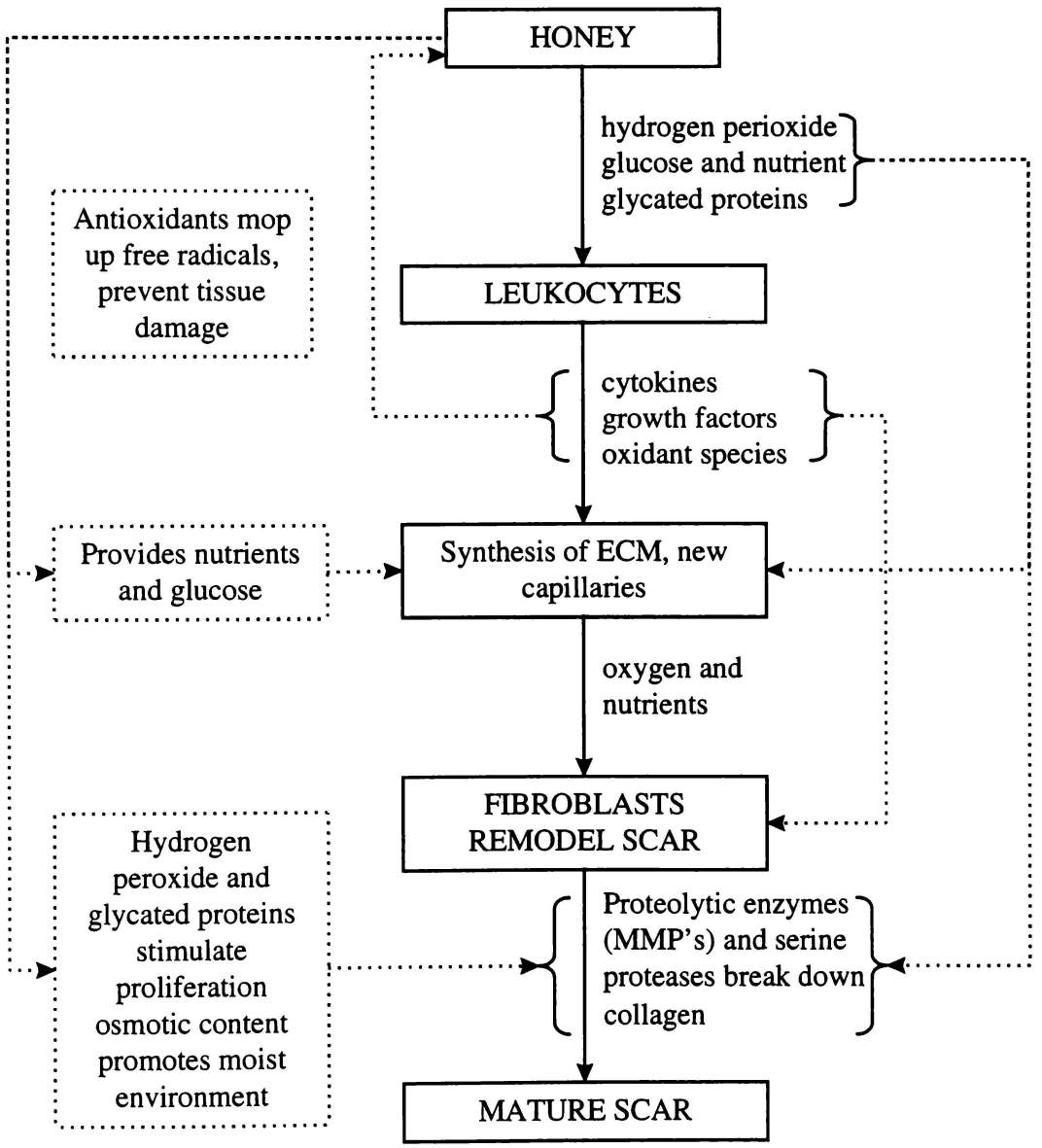

Figure 9.3: Schematic diagram summarising the hypothesised mechanisms by which honey optimises wound healing as observed in this study

on the basis of this study, that honey may provide a neatly controlled therapy for optimising tissue repair, with potential for use in inflammatory disorders. It is the central thesis of this study that the stimulatory agent in honey induces cytokine production necessary for healing to occur, but that the oxidant species produced by these cells are effectively regulated by a second agent, thereby creating a feedback-regulated delivery system. It has been demonstrated in the discussion of fibrosis in Section 1.7.2, that excessive inflammation will occur (and therefore disease) if cytokine production continues unabated, as changes in cytokine gradients are necessary for the following phases of tissue repair to begin. If further experiments confirm this to be the case, honey will have potential for therapeutic application.

9.3.3 Future experiments

The results obtained from the current study present strong justification to test the effect of honey in an animal model. Though the use of cell lines enabled dissecting out of complex signalling pathways, it would be desirable to repeat the assays on primary cells from human origins (*e.g.* skin derived fibroblasts). While cell lines have the advantage of having very defined characteristics, primary cells cultures show variation in their response to immune stimuli, and it has been noted that this occurs for cells *in vivo* as well. It is likely to be the case that the therapeutic potential of various honey types may vary among individuals and this needs to be established *in vivo*.

As there was not sufficient time to investigate all of the possible effects of honey on cell signalling for each cell type, the effect of honey on transcription factors for regulatory factors was not explored in the current study. Further, the effect of honey on cytokine antagonists was not examined as a possible source of anti-inflammatory action. It would have been valuable to examine the effect of honey on cell adhesion molecules. This information would be useful to gain a better understanding of the mechanism by which honey exerts anti-inflammatory effects.

Though the results presented in the current study show that honey has activity on cells involved in the inflammatory pathway at low concentrations *in vitro* they do not translate to dose-rates *in vivo*. Due to time constraints, plans to investigate the effect of honey on cellular adhesion molecules and selectin were abandoned. As honey had been observed to have direct effects on leukocytes and also on tight junctions, there is a strong possibility that it may manipulate the binding interactions which enable immune cells to prepare to enter the tissues. A number of compounds derived from natural products have been found to interfere with expression of leukocyte adhesion molecules (Gupta and Ghosh, 1999). It would be valuable to investigate the effect of honey on cellular adhesion molecules in future research. Due to the constraints of time and feasible scope of the current study it was not possible to pursue the identification of the stimulatory and modulatory fractions beyond the preliminary fractionation described here. Chromatographic fractionation would be required to test the activity of specific components in honey for their cellular effects, and the enormity of such an undertaking would constitute a thesis study in itself. Furthermore, identification of the stimulatory and modulatory components of honey lay outside the primary aim of the current study, which was to test the effect of honey on a range of cells functioning in the inflammatory pathway.

While this thesis has contributed much to an understanding of the mechanisms by which honey optimises wound healing, it raises many further questions which are yet to be answered. The reasons for observed variability in individual response to the stimulatory component must be further elucidated. Due to the enormity of the interactions which exist between cells in the immune system, there are many more potential investigations which may (and must) be performed. The experiments described in this thesis provide overwhelming support that honey does exert immunogenic effects as observed anecdotally. However, in the absence of *in vivo* testing, conclusions drawn will continue to be limited by the inability to demonstrate exactly how the human body may interfere with what has been observed *in vitro*. Therefore, it is vital that this research is extended to *in vivo* testing for further advances to be made in our understanding of the mechanisms by which honey has this activity.

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APPENDIX ONE

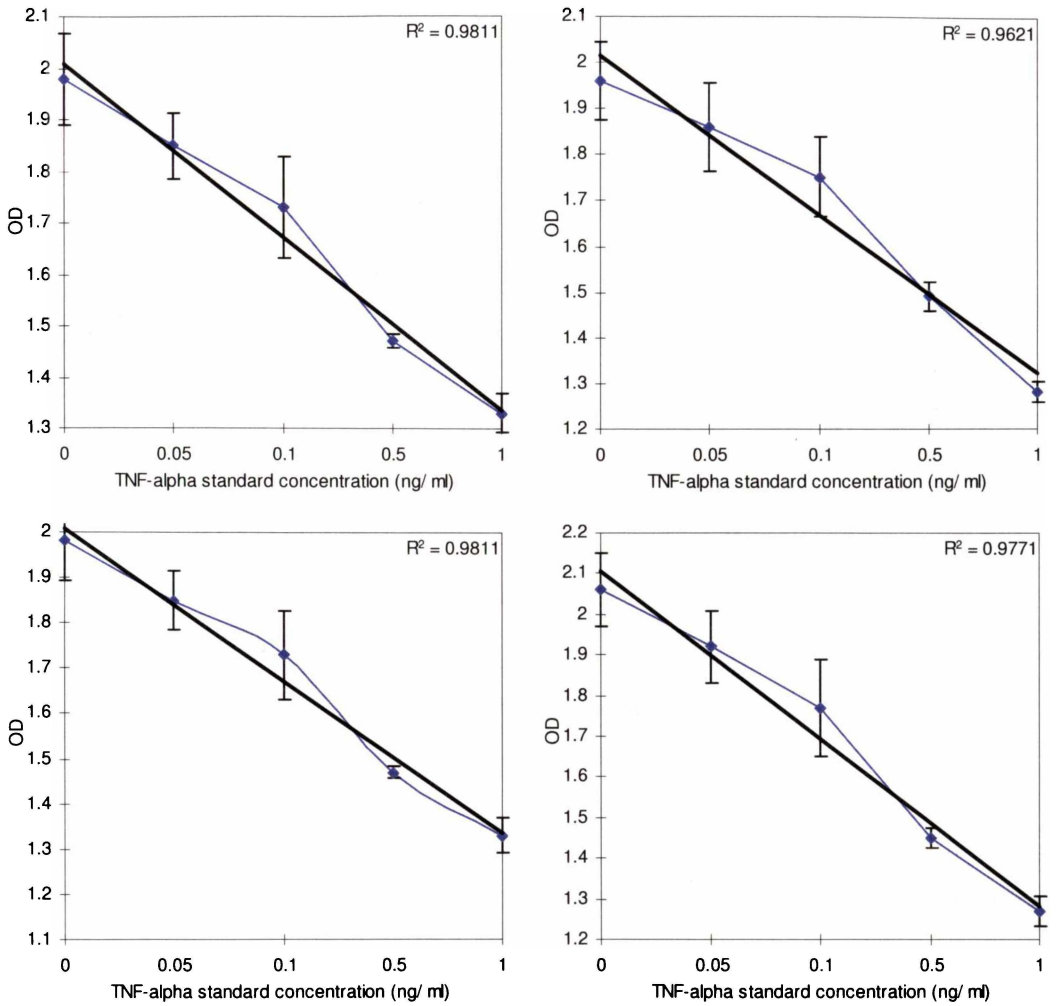

Figure A.1a: Standard curves established for MTT absorbance for WEHI cells (570 nm) against TNF-α standards. Four representative standard curves have been included for the assays presented in Chapter 4 (each assay presented was replicated in time at least twice).

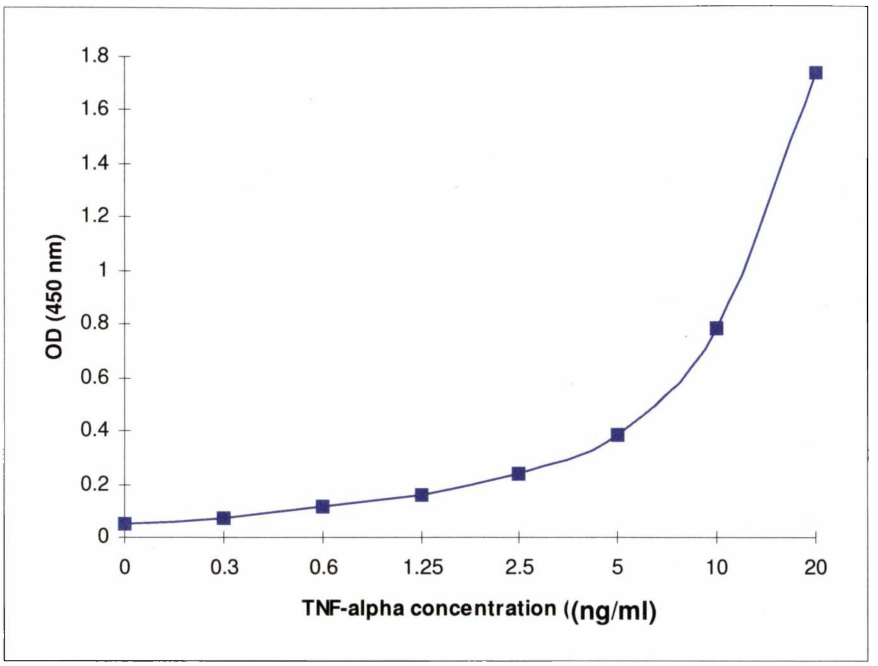

Figure A.1b: Representative standard curve for commercial TNF-α ELISA. Symbols represent the mean of three titrations.

APPENDIX TWO

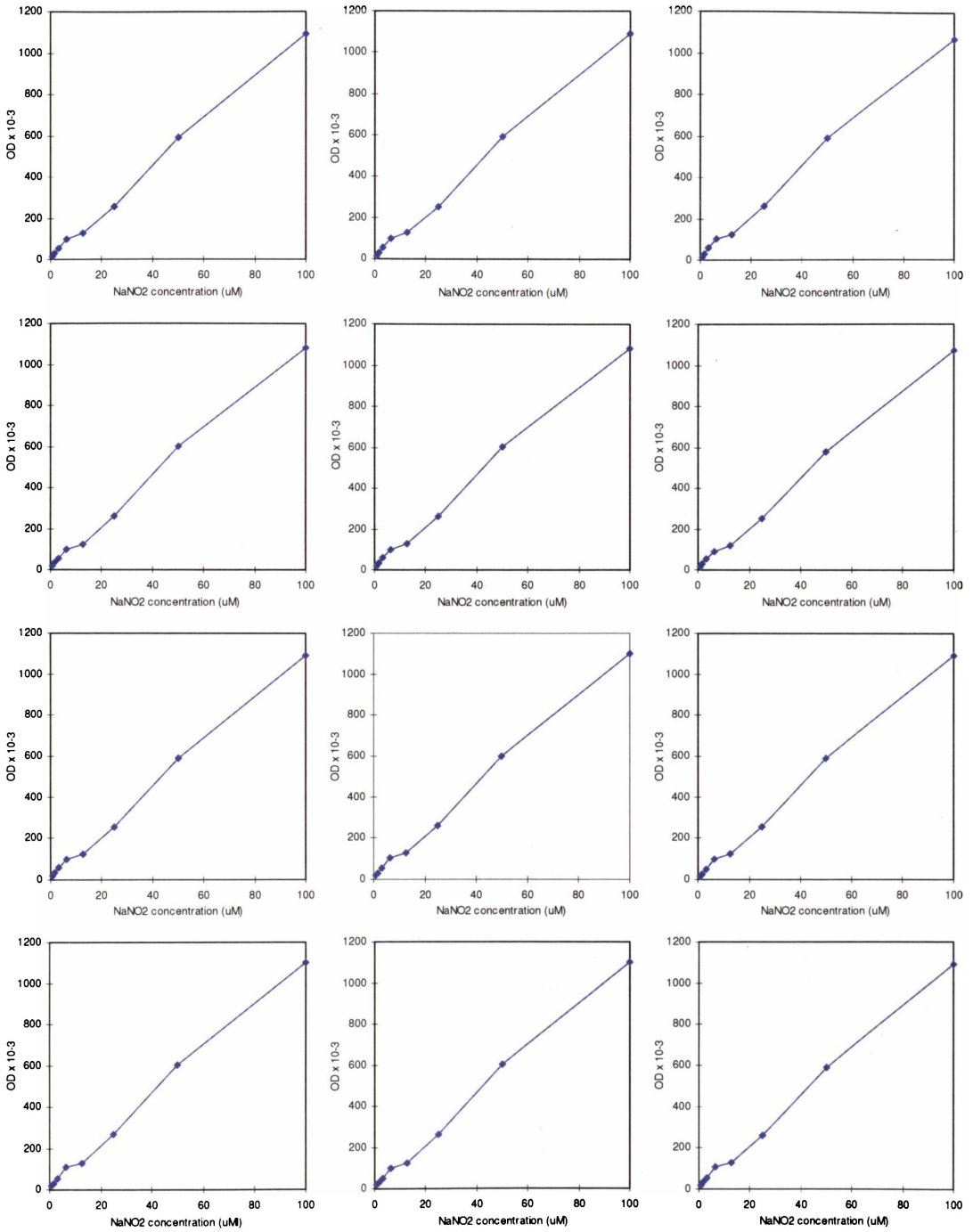

Figure A.2: Calibration curves for the NaNO2 standard using the Greiss assay. One calibration curve has been included for each assay presented in Chapter 5 (each assay presented was replicated in time at least twice).

APPENDIX THREE

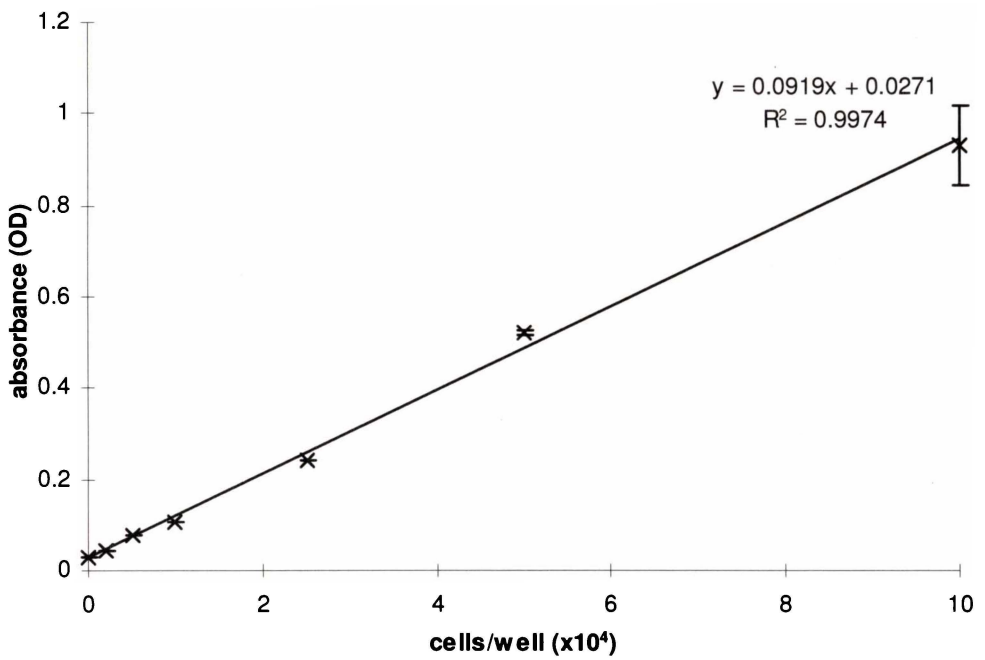

Figure A.3:Standard curve for the relationship between absorbasnce of MTT (570nm) and cell density (3T3-L1 fibroblasts).