THE ROLE OF MONONUCLEAR CELLS IN TUBERCULOSIS

by

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ABSTRACT

Sonicates derived from *Mycobacterium tuberculosis* suppressed lymphocyte proliferation. Pulsing of monocytes with mycobacterial sonicates resulted in the release of high molecular weight lipids. Both these lipids and those prepared by column fractionation of mycobacterial sonicates suppressed lymphocyte blastogenesis. This effect was due to the activation and not the proliferation of CD8+ lymphocytes by the lipid-containing mycobacterial fractions of Mr >200kDa that could be obtained *in vitro* by column fractionation. CD8+ lymphocytes in turn released suppressor molecules after being pulsed with these high molecular weight mycobacterial fractions. Untreated CD8+ or those treated with mycobacterial proteins produced no such molecules. These suppressor molecules released by CD8+ cells were identified as glycolipids of Mr 122-148kDa of which the carbohydrate moieties were responsible for immunosuppression. In addition to the inhibition of lymphocyte blastogenesis, the carbohydrates suppressed tumour necrosis factor alpha, interleukin-1ß, interleukin-2 and gamma interferon production by monocytes and lymphocytes. The production of interleukin-4 and interleukin-6, however, was increased. The decreased production of the various cytokines appeared to be related to increases in interleukin-4 and interleukin-6. Antibodies to interleukin-4 and interleukin-6 restored the production of the cytokines that were originally suppressed by CD8+ derived carbohydrates. The production of interleukin-4 and interleukin-6 appeared to be linked since raised levels of interleukin-6 led to increases in

the production of interleukin-4. Decreases in interleukin-1 and increases in interleukin-6 due to lymphocyte suppressor supernatants or carbohydrates were not related to changes in mRNA levels. This reflects interference at a post-translational level. Lymphocyte suppressor supernatants or carbohydrates also reduced HLA Class II expression by monocytes.

The results suggest that the lipid component of *Mycobacterium tuberculosis* organisms activate CD8+ lymphocytes to release carbohydrate moieties that suppress lymphocyte blastogenesis and cytokine production; and could inhibit monocyte activation. This could ensure the survival of the organism within the host.

DECLARATION

This is to certify that the thesis entitled:- "The role of mononuclear cells in tuberculosis" presented for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, is my own unaided work and has not been presented at any other university.

G.J. Sussman... 3rd. day of JUNE..., 1992

GARTH SUSSMAN

BDS (RAND)

PUBLICATIONS & PRESENTATIONS

Parts of this thesis have been published in the following journal articles:

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To my family

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LIST OF ABBREVIATIONS

α	alpha
A	Amperes
ADCC	Antibody dependent cell cytotoxicity
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
β	Beta
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
^{0}C	degrees Celsius
CB	Cyanogen bromide
CD	Cluster of differentiation
cDNA	Complementary DNA
Ci	Curie
Ci/mmole	Curie per millimole
CO_2	Carbon dioxide
Con A	Concanavalin A
CPM	Counts per minute
cRPMI	Complete RPMI containing 10% heat inactivated foetal calf serum
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
γ	Gamma
g	gravity
GZ	β galactosidase
HCl	Hydrochloric acid
HIV	Human immuno-deficiency virus
HLA	Human leukocyte antigen
Ia (antigens)	Immune associated (antigens)
i.e.	that is
IFN-gamma	Gamma-interferon
IL-1,-2,-3,-4,-6,-10	Interleukin 1,2,3,4,6,10
Ir genes	Immune response genes
IV	Intravenous
kDa	Kilodalton
Kg	Kilogram
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
ml	Millilitre
MLC	Mixed lymphocyte culture
MN cells	Mononuclear cells
Mr	Molecular mass
mRNA	Messenger RNA
MW	Molecular weight
NALs	Non-adherent lymphocytes
ng/ml	Nanogram per millilitre
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate buffered saline

LIST OF ABBREVIATIONS

α	alpha
A	Amperes
ADCC	Antibody dependent cell cytotoxicity
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
ß	Beta
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
^{o}C	degrees Celsius
CB	Cyanogen bromide
CD	Cluster of differentiation
cDNA	Complementary DNA
Ci	Curie
Ci/mmole	Curie per millimole
CO_2	Carbon dioxide
Con A	Concanavalin A
CPM	Counts per minute
cRPMI	Complete RPMI containing 10% heat inactivated foetal calf serum
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
γ	Gamma
g	gravity
GZ	ß galactosidase
HCl	Hydrochloric acid
HIV	Human immuno-deficiency virus
HLA	Human leukocyte antigen
Ia (antigens)	Immune associated (antigens)
i.e.	that is
IFN-gamma	Gamma-interferon
IL-1,-2,-3,-4,-6,-10	Interleukin 1,2,3,4,6,10
Ir genes	Immune response genes
IV	Intravenous
kDa	Kilodalton
Kg	Kilogram
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
ml	Millilitre
MLC	Mixed lymphocyte culture
MN cells	Mononuclear cells
Mr	Molecular mass
mRNA	Messenger RNA
MW	Molecular weight
NAL's	Non-adherent lymphocytes
ng/ml	Nanogram per millilitre
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate buffered saline

PGE	Prostaglandin
PG1	Phenolic glycolipid 1
PHA	Phytohaemagglutinin
pI	isoelectric point
PPD	Purified protein derivative
PMA	Phorbol myristate acetate
PMN cells/leukocytes	Polymorphonuclear cells/leukocytes
PWM	Pokeweed mitogen
RIA	Radioimmunoassay
RNA	Ribonucleic acid
S	Svedberg
SCAF	Suppressor cell activating factor
SD	Standard deviation
SE	Standard error
SKSD	Streptokinase streptodornase
SSC	Sodium chloride sodium citrate
T_H	T helper cell
TNF-alpha	Tumour necrosis factor alpha
T_S	T suppressor cell
uCi	Microcurie
ug/ml	Microgram per millilitre
U/ml	Units per millilitre
UV	Ultraviolet

LIST OF TERMS

High MW fractions	Mycobacterial fractions of Mr >200kDa obtained after fractionation of *M.tuberculosis* sonicates on a Sephacryl S-200 column.
Low MW fractions	Mycobacterial fractions of Mr <200kDa obtained after fractionation of *M.tuberculosis* sonicates on a Sephacryl S-200 column.
Monocyte control supernatants	Supernatants derived from monocytes in the presence of cRPMI
Monocyte suppressor supernatants	Supernatants derived from monocytes incubated in the presence of *M.tuberculosis* sonicates
Lymphocyte control supernatants	Supernatants derived from NAL's incubated in the presence of cRPMI
Lymphocyte suppressor supernatants	Supernatants derived from NAL's incubated with high MW fractions

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CHAPTER 1

1. SUPPRESSOR T CELLS

1.1. Introduction

The existence of a distinct subpopulation of non-cytotoxic suppressor T cells is controversial (Möller, 1988). A suggestion that a discrete subset of CD8+ suppressor cells does not exist was proposed by Möller (1988). Suppressor T cells cannot be identified by monoclonal antibodies which would separate them from the cytotoxic T cell subset (Möller, 1988). In addition the I-J gene which was postulated to control the function of suppressor T cells and suppressor factors was not found to exist in the major histocompatibility complex (MHC) site where it had been mapped. Furthermore, antigen specific suppressor clones have a 'nonsense rearrangement of genes for the T cell receptor' or lack the genes completely (Möller, 1988).

However Eichmann (1988) argued that T cells that induce suppression may not be confined to one T cell subset as determined by subset specific antigens. In addition cells with suppressor activity have been cloned *in vitro*. Furthermore suppressor cells may not utilise the alpha ß T cell receptor during antigen specific suppression and its absence or inability to function in these cells may not be necessary (Eichmann, 1988). This was confirmed by Tada (as quoted by Mitchison, 1988) who showed that I-J existed as an accessory molecule distinct from the T cell receptor which bound MHC Class II molecules at their allo-variable region. In an attempt to resolve this issue Bloom *et al.* (1992), postulated that although the evidence relating to the phenomenon of T suppressor

cell immunological suppression was valid, many of the conclusions derived from these observations were not. Although a marker separating CD8+ cytotoxic cells from non-cytotoxic clones has not been isolated, CD8+ cells can be divided into these groups based on the pattern of secretion of cytokines (Salgame *et al.*, 1991). Cytotoxic cells (CD8+) were shown to produce IL-10 and IFN-gamma consistently whereas CD8+ (non-cytotoxic) suppressor cells produced IL-4 (Salgame *et al.*, 1991).

1.2. ANTIGEN SPECIFIC NON-CYTOTOXIC SUPPRESSOR T CELLS

Swanborg (1975) showed that guinea pigs pretreated with peptide 44-89 of the myelin basic protein molecules were protected from allergic encephalomyelitis when the entire myelin basic protein in Freund's adjuvant was administered at a later stage. Other peptides, i.e. 1-20 and 114-122, derived from myelin basic protein molecules were not protective. Since the actual mechanism of protection induced by antigens 44-89 was unknown, the author postulated that suppressor lymphocytes may have been activated in an antigen specific manner. This in turn suppressed the autoimmune encephalomyelitic response (Swanborg, 1975).

Similar findings were described by Myers *et al.* (1989). Mice (DBA/1) injected with type II collagen were found to be resistant to arthritis when challenged later with native type II collagen and *Mycobacterium tuberculosis (M.tuberculosis)*.

Most of the control mice (94-100%), previously injected with type I collagen or ovalbumin (OVA), developed arthritis. In addition, mice treated with peptides 122-147 derived from type II collagen also did not develop arthritis whereas the other peptides derived from type II collagen were not protective. This included amino acids 116-137 which indicated that the suppressor determinant, i.e. the amino acid sequence or epitope able to activate suppressor cells resided in amino acids 137-147. It was also proposed that exposure to amino acids 137-147 resulted in inactivation or deletion of certain T cell clones, although the exact mechanism has not been elucidated (Myers *et al.*, 1989).

The ability of antigens to specifically induce suppression was investigated by Adorini *et al.* (1979) who demonstrated that suppressor T cells could be induced by the injection of certain purified peptide fragments derived from egg-white lysosyme. These peptides, derived from the N-terminal and C-terminal ends (i.e. amino acids 1-17, cysteine-6-cysteine 127 or amino acids 120-129) by mild acid hydrolysis, mimicked the ability of the entire antigen to induce suppressor cells. The suppressor cells accounted for suppression in B10 non-responder mice. Four weeks after administration of the peptides or intact lysosyme, parathymic spleen cells from non-responder B10 mice and responder B10.A. mice were assessed for their ability to form plaques, an indication of the presence of immunoglobulin positive cells. Numbers of plaque forming cells in B10.A. responder mice ranged from 132 - 218 x 10^6 in mice injected with peptides or the entire lysosyme antigen. The numbers of

plaque forming cells in B10 non-responder mice were less than 1×10^6. In addition when 10% of spleen cells of B10 non-responder mice were mixed with 90% of cells from B10.A. responders, suppression of plaque forming cells was observed. Suppressor cells were found to be T lymphocytes and suppression induced by these cells was abrogated when spleen cells were treated with anti-I-J serum (Adorini *et al.*, 1979). This is controversial since the existence of the I-J gene has not been shown to exist (Möller, 1988).

These findings suggested that certain peptides activated suppressor cell functions and that control of the immune response gene (Ir) was regulated by specific antigenic challenge (Adorini *et al.*, 1979).

Other evidence which elucidated the specificity of antigen specific T cells was provided by Yowell *et al.* (1979), who demonstrated that removal of amino acid residues 1-12 and 106-120 from egg white lysosyme resulted in enhanced proliferative responses to PPD *in vitro*. The T cells derived from mice injected with intact antigen could not proliferate in response to PPD. This was postulated to be due to antigen specific T suppressor cells which were activated by amino acids 1-12 and 106-120 (Yowell *et al.*, 1979).

In addition, Shivakumar *et al.* (1989) demonstrated that activation of T suppressor cells or T helper cells by an antigen with both suppressor determinants and helper determinants was

dependent on how those regulatory units were exposed or presented. A helper determinant is the epitope or amino acid sequence of an antigen which activates helper cells. This was accomplished by the use of specific peptides derived from ß-galactosidase (GZ). These peptides activated either suppressor or helper cells when injected into CBA/J ($H\text{-}Z^k$) mice as evidenced by the number of platelet forming colonies. Amino acids 60-140 (T8) derived from GZ induced only suppressor cells. However when this amino acid sequence (T8) was further cleaved into amino acids 60-92 (T8-2) and 93-140 (T8-3), only the T8-3 sequence induced suppressor cells. The T8-2 amino acid sequence induced helper cells. This indicated that the cleavage of T8 into two sequences exposed a helper determinant present in the T8-2 sequence. It was postulated that either T8 was a potent inducer of suppressor cells which suppressed helper cells or that further processing was needed to expose the helper determinant which lay in the T8-2 sequence of amino acids (Shivakumar *et al.*, 1989).

Furthermore, Krzych *et al.* (1985) showed, using amino acid sequences derived from GZ, that suppressor determinants must lie close to helper determinants for T suppressor cells to suppress target helper cells. This was demonstrated by injecting CBA/J mice with various amino acid sequences cleaved from GZ using cyanogen bromide (CB). Suppressor activity was assessed by inhibition of plaque forming colonies. Suppressor cells derived from mice injected with amino acids 3-72 (CB-2) and 98-187 (CB-3) failed to suppress T helper cells from mice injected with

amino acids 378-418 (CB-10). The CB-3 specific T suppressor cells were able to suppress T helper cells induced by the entire GZ antigen. These results indicated that not all T helper cells were suppressed by T suppressor cells. Furthermore, during antigen processing, suppressor and helper determinants must lie close together for suppressor cell activity. It was postulated that the GZ antigen itself was processed in such a way that both suppressor and helper determinants were exposed. This in turn activated a specific set of T helper cells which were suppressed (Krzych *et al.*, 1985).

Further evidence provided by Mohagheghpour *et al.* (1986) supported the postulate that antigen specific T cells existed in humans. These authors showed that suppressor T cell clones could be generated in the presence of irradiated alloantigen primed CD4+ lymphoblasts and IL-2. When these suppressor cells were added to fresh autologous CD4+ cells in a mixed lymphocyte culture, marked suppression of blastogenesis resulted. However this suppression was specific as only those suppressor cells grown in the presence of HLA DR 5+ reactive T cells were able to inhibit the response of CD4+ cells to a DR 5+ B cell line. However, a CD4+ helper cell line grown on a DRw6+ B cell line was not inhibited. In addition, suppressor donor cells only suppressed CD4+ cells from an individual with the same HLA-A and -B but not DR, suggesting suppression to be Class I restricted. It was postulated that T suppressor cells recognised the CD4+ cell that expressed an antigen from the original antigen.

However the mechanism for this non-cytolytic Class I restricted suppression still remains unknown (Mohagheghpour *et al.*, 1986).

The mechanism of cell killing was further investigated by others (Brondz *et al.*, 1987; Fedoseyeva *et al.*, 1991 as cited by Sercarz & Krzych, 1991) who demonstrated that cytotoxic determinants were different from suppressor determinants even though they overlapped on the same amino acid sequence.

With regards to the phenotype of the suppressor cell, Liew *et al.* (1982) demonstrated that Lyt 1+ 2- (helper cells) functioned as both helper and suppressor cells in mice infected with *Leishmania tropica (L.tropica).* Splenic and lymph node cells (Lyt 1+) from CBA mice resistant to *L.tropica* were shown to transfer immunity to non-immune mice when injected before administration of *L.tropica.* Resistant BALB/c mice with strong anti-leishmanial delayed type hypersensitivity reactivity were injected with (Lyt 1+) T cells from syngeneic mice that were unable to contain their *L.tropica* infections. This resulted in the disappearance of resistance to *L.tropica* in these mice. These results clearly demonstrated that Lyt 1+ (helper) cells functioned as both helper and antigen specific suppressor cells. This evidence supported the notion that antigen specific suppressor cells were not restricted to one phenotype (Liew *et al.*, 1982). This was confirmed by Jensen *et al.* (1984) who demonstrated that the failure of pork insulin to stimulate antibody responses in non-responder

H-2^b mice was due to the activation of antigen (insulin) specific T suppressor cells. These cells were found to be Lyt 2+ (suppressor) cells and their inactivation by radiation or treatment with anti-Lyt 2+ antiserum resulted in restoration of T cell help and subsequent antibody production in these mice. The findings presented here confirm other reports (Shivakumar *et al.*, 1989) that certain antigen amino acid sequences activated either helper or suppressor cells although it was not clear why these determinants are different. In addition suppressor and helper determinants must be expressed together during antigen presentation so that antigen specific suppressor cells may suppress activated helper cells. Furthermore suppressor cells are antigen specific but may belong to more than one phenotype although the overwhelming majority appear to be CD8+ cells (Sercarz & Krzych, 1991). In addition, Lyt 2+ cells were antigen specific. Mice injected with beef insulin did not develop suppressor cells which suppressed antibody responses to pork insulin. These findings showed that antigen specific suppressor cells were Lyt 2+ (suppressor) cells and that their removal restored murine antibody responses (Jensen *et al.*, 1984).

1.3. MYCOBACTERIAL DISEASE AND T SUPPRESSOR CELLS (NON-CYTOTOXIC)

Bullock *et al.* (1978) showed that T suppressor cells were generated in C3H/Anf mice following intraperitoneal injection of *Mycobacterium lepraemurium (M.lepraemurium).* These T suppressor cells appeared in the spleens at 10-11 weeks and

suppressed the platelet forming colony response of normal splenocytes by 90%. This was also accompanied by loss of delayed type hypersensitivity to *M.lepraemurium* antigens. In addition suppressor T cells were also found to be present in lymph nodes at 22-25 weeks after infection. The authors also isolated a soluble suppressor factor which was produced by splenic cells 14 weeks after infection which also induced suppression of platelet forming colonies by normal spleen cells but was not as effective. Similar findings were described by Richard *et al.* (1990) who showed that mice infected with *M.lepraemurium* produced antigen specific suppressor cells. When these cells were transferred to syngeneic mice they suppressed the expression of delayed type hypersensitivity to *M.lepraemurium* antigens but not to sheep red blood cells. In addition a soluble suppressor factor produced by mycobacterial antigen-reactive cells also inhibited the expression of delayed type hypersensitivity when administered to mice challenged with *M.lepraemurium* antigens. Furthermore the suppressor factor enhanced the growth and dissemination of bacilli. Suppressor cells responsible for these findings were shown to be Lyt 2+ (CD8+) lymphocytes. However it was not known whether this uncharacterised suppressor factor blocked T cell receptors or acted at the level of cellular proliferation.

The ability of T suppressor cells to inhibit delayed type hypersensitivity to *Mycobacterium bovis (M.bovis)* BCG in mice was also investigated by Nakamura & Tokunaga (1980). These workers showed that suppressor cells appeared in the spleens of

C3H/He mice 2-7 days after intravenous administration of BCG. However suppressor cells transferred into mice previously immunised with BCG (which already exhibited delayed type hypersensitivity) had little effect. These results indicated that suppressor cells strongly inhibited the induction of delayed type hypersensitivity to BCG but had little effect on inhibiting the expression of delayed type hypersensitivity (Nakamura & Tokunaga, 1980).

Further investigations of this phenomenon by Colizzi *et al.* (1984) found that CBA mice injected intravenously with high doses of *M.bovis* BCG failed to develop delayed type hypersensitivity. Spleen cells from these mice injected with low doses of BCG were cultured for 24 hours and the supernatants incubated in the presence of normal spleen cells stimulated with Con A. The results indicated that the supernatants derived from the non-responder mice inhibited Con A induced DNA synthesis of normal spleen cells. Supernatants from responder mice did not inhibit DNA synthesis. One of the factors present in these suppressor supernatants was found to be of Mr 50 - 70kDa as determined by gel filtration. In addition, separation of cells into T and B subsets revealed that the suppressor factor was produced by T cells. Furthermore this non-specific inhibitor of DNA present in T cell supernantants also suppressed the production of IL-2, as measured by proliferation of 4 day blasts. These findings demonstrated that T cells from BCG infected mice produced a non-specific inhibitor which resulted in anergia and loss of delayed type hypersensitivity.

Ottenhoff *et al.* (1986) cloned a CD8+ T suppressor cell line from a borderline lepromatous patient (i.e. an individual who showed a specific T cell unresponsiveness against *Mycobacterium leprae (M.leprae)*). These T suppressor cells were found to almost totally suppress the proliferation of cloned T helper cells from the same patient when stimulated with *M.leprae* or other mycobacterial antigens. However when these helper cells were stimulated with *Herpes simplex* virus or PHA, T suppressor cells had no effect. Furthermore, T suppressor cells completely suppressed the proliferation of T helper cells when stimulated with a *M.leprae* antigen of Mr 36kDa.

This indicated that a suppressor determinant was present within the 36kDa protein. T suppressor clones have been shown to be genetically restricted although this was not associated with either HLA Class I, DR or DQ. The authors postulated that although HLA-DR may be involved, genetic restriction was more complicated than that of T helper cells (Ottenhof *et al.*, 1986).

Additional findings were provided by Prasad *et al.* (1987) who showed that an antigen of *Mycobacterium leprae*, phenolic glycolipid 1, was capable of inducing suppressor cells in leprosy patients. This was demonstrated *in vitro* by the addition of phenolic glycolipid to Con A stimulated peripheral blood MN cells from leprosy patients. Similar results were presented by Kaplan *et al.* (1987) who found that *M.leprae* antigens suppressed PPD or Con A induced proliferation of MN cells derived from

leprosy patients or normal controls. In addition lipoarabinomannan B (LAM-B), derived from both *M.tuberculosis* and *M.leprae*, suppressed blastogenesis of stimulated MN cells. Depletion of T8+ (CD8+) cells did not affect LAM-B mediated suppression when cells were stimulated with Con A. However removal of CD8+ cells abrogated the suppressor effect of cells activated with PPD. These findings confirm that at least some of the suppression observed was mediated by CD8+ lymphocytes.

Other information which elucidated the role of CD8+ suppressor cells in mycobacterial diseases was provided by Ebert *et al.* (1991). These workers demonstrated that suppressor T cells were induced by *Mycobacterium paratuberculosis (M.paratuberculosis)* in normal donors and patients with inflammatory bowel disease. Suppression of blastogenesis in response to Con A was achieved when 10ug/ml of *M.paratuberculosis* was added to cultures. This effect was abrogated when CD8+ cells were removed from culture. Since CD8+ T cells in peripheral blood of these patients were not found to have increased in number, it was postulated that the heightened suppressor activity in inflammatory bowel disease was due to altered CD8+ cell function (Ebert *et al.*, 1991).

This enhanced activation but not proliferation was also noted in CD8+ cells isolated from lepromatous lesions or blood in response to antigens from *M.leprae* (Modlin *et al.*, 1986). These activated CD8+ cells in turn suppressed the proliferation of T helper cells as determined by ^{3}H-thymidine incorporation (Modlin *et al.*, 1986).

Further analysis of CD8+ clones isolated from leprosy patients demonstrated that these CD8+ (non-cytotoxic) clones produced IL-4 on stimulation with OKT3 monoclonal antibodies and *M. leprae* (Salgame *et al.*, 1991). Interleukin-4 was also noted to be of importance in suppression of CD4+ T cell clones as monoclonal antibodies to IL-4 reversed this suppression. These results suggested that although IL-4 appears to mediate suppression it is not clear as to whether this cytokine is immunosuppressive on its own (Salgame *et al.*, 1991).

1.4. MONOCYTES AS ADHERENT SUPPRESSOR CELLS

1.4.1. Introduction

In mycobacterial disease, various suppressor mechanisms have been identified. These were mediated by monocytes and macrophages amongst others (Ellner, 1978; Wadee *et al.*, 1980; Wadee & Rabson, 1981; Wadee *et al.*, 1983a,b; Tsuyuguchi, 1990; Fujiwara *et al.*, 1991; Apt, 1991).

1.4.2. Monocytes as Adherent Suppressor Cells in Mycobacterial Infections

Under certain circumstances, eg. infection with mycobacteria, monocytes may function as suppressor cells by producing a suppressor factor. Evidence for this was provided by Wadee *et al.* (1980), Wadee & Rabson (1981), Wadee *et al.* (1983a,b) and Fujiwara *et al.* (1991).

Mononuclear cell proliferation was shown to be inhibited when cells were cultured in the presence of mitogens and a heat stable suppressor factor (Wadee *et al.*, 1980). This suppressor factor, derived from incubating monocytes with *M.tuberculosis*, was found to activate suppressor T cells which in turn suppressed lymphocyte blastogenesis (Wadee *et al.*, 1980).

Similar results were described by (Fujiwara *et al.*, 1991) who noted that monocytes isolated from tuberculous patients spontaneously secreted a suppressor factor that suppressed MN cell blastogenesis to PPD. Further evidence which demonstrated the spontaneous production of a suppressor factor produced by adherent cells of *M.tuberculosis* infected guinea pig spleens was provided by Wadee & Rabson (1981). The suppressor factor suppressed the blastogenesis of normal human and guinea pig lymphocytes to PHA by the activation of suppressor lymphocytes. This suppressor cell activating factor (SCAF) was shown to be composed of mycobacterial lipids cleaved from the cell walls of *M.tuberculosis* bacilli during macrophage phagocytosis (Wadee & Rabson, 1981). Characterisation of these lipids by thin layer chromatography revealed that they were the phospholipids, phosphotidylinositol and phosphotidylethanolamine, which activated suppressor cells (Wadee *et al.*, 1983a).

In contrast to these results, Ellner (1978) demonstrated that circulating monocytes from tuberculous patients suppressed T lymphocyte blastogenesis to PPD *in vitro*. Suppression was

restored when monocytes were removed from culture. Addition of monocytes back into culture once again resulted in suppression of blastogenesis in a dose dependent manner. However, suppression was not induced by a suppressor supernatant. These results indicated that monocytes interacted directly with responding cells and suppression was not dependent on a suppressor factor (Ellner, 1978).

Another method in which monocytes suppressed T cell proliferation when infected with *M.avium-M.intracellulare* complex (MAC) was described by Tsuyuguchi *et al.* (1990). These workers demonstrated that MAC and its lipid fraction suppressed the proliferation of T cells in response to Con A, PHA and PPD. Suppression of blastogenesis was induced when macrophages were added to T cells in a dose dependent manner. This effect was not prostaglandin dependent as evidenced by the inability of indomethacin to reverse suppression. However, monocytes treated with MAC or the lipid fraction demonstrated decreased expression of CD11b, a leukocyte function-associated molecule 1 and the CD14 molecule. It was postulated that decreased expression of accessory molecules inhibited monocyte-T cell interactions which allowed the MAC organisms to evade the host response (Tsuyuguchi *et al.*, 1990).

In other experiments Apt *et al.* (1991) showed that monocytes from the lungs of mice infected with *M.tuberculosis* suppressed the blastogenesis of PPD-reactive lymphocytes.

Suppression was found to have two components and was antigen specific and non-specific. Non-specific suppression was mediated by prostaglandin E as evidenced by reversal of this phenomenon when indomethacin was added to *Staphylococcus aureus* activated cell cultures. In addition PPD-specific suppression was also mediated by macrophages since the elimination of Lyt 2+ cells and B cells did not reverse this effect. Also indomethacin failed to restore proliferation when PPD was employed in culture. These results showed that non-specific suppression mediated by prostaglandins was activated together with antigen specific suppression (Apt *et al.*, 1991).

Mycobacteria have been shown to manipulate monocytes to suppress lymphocyte proliferation in many different ways. These differed from each other possibly due to the strain of mycobacteria used in experiments, incubation periods and the varying antigens or mitogens used to stimulate responder cells. In addition differences in the action of monocytes derived from tuberculous individuals that spontaneously suppressed lymphocytes *in vitro* may have been dependent on their exposure to *M.tuberculosis in vivo.*

CHAPTER 2

2. THE CYTOKINES

2.1. Introduction

Several cytokines have been shown to play an important role in mycobacterial diseases. These include tumour necrosis factor alpha (TNF-alpha) (Valone *et al.*, 1988; Silva & Foss, 1989; Kindler *et al.*, 1989); interleukin-1ß (IL-1ß) (Chensue *et al.*, 1986; Wallis *et al.*, 1986; Denis, 1991a); interleukin-2 (IL-2) (Mohagheghpour *et al.*, 1985; Toossi *et al.*, 1986; Turcotte & Legault, 1986); interleukin-4 (IL-4) (Flesch & Kaufmann, 1990a; Denis & Gregg, 1991); interleukin-6 (IL-6) (Denis & Gregg, 1990; Denis *et al.*, 1990; Shiratsuchi *et al.*, 1991) and gamma interferon (IFN-gamma) (Flesch & Kaufmann, 1987; Wadee *et al.*, 1987; Mor *et al.*, 1989; Kaplan *et al.*, 1989).

2.2. TUMOUR NECROSIS FACTOR ALPHA (TNF ALPHA)

2.2.1. History

Observations of spontaneous tumour regression in cancer patients following bacterial infections, led to the development of a systemic cancer therapy by William Coley, at the turn of the century (Coley as cited by Old, 1988). These "Coley mixed toxins" were derived from *Streptococcus pyogenes* and *Serratia marcescens* organisms (Old, 1985). Many of the symptoms of infection could be reproduced when these "Coleys toxins" were injected into cancer patients (Beutler & Cerami, 1986). The active component in those toxins was later identified as lipopolysaccharide (LPS) or endotoxin a component

of gram negative bacterial cell walls (Shear *et al.*, 1943; Hartwell *et al.*, 1943). Carswell *et al.* (1975) showed that mice injected with endotoxin, after priming with BCG, produced a factor in their sera which caused this haemorrhagic necrosis of tumours. Exposure to LPS via the tumour necrosis factor mechanism was found to heighten the animals resistance to new bacterial infection and a lethal dose of X-rays, in addition to the necrosis of tumours (Old, 1988). This factor was called tumour necrosis factor and was found to reproduce many of the effects of BCG and LPS. Tumour necrosis factor was shown to be a protein and was finally isolated from the blood of mice and rabbits (Old, 1988). The tumour necrosis factor gene was finally cloned and expressed in *Escherichia coli* (Pennica *et al.*, 1984).

2.2.2. Structure of TNF

2.2.2.1. Cachectin and Relationship to Lymphotoxin

Although many of the *in vivo* and *in vitro* properties of TNF/cachectin and lymphotoxin were found to be very similar (Ruddle, 1987), TNF and lymphotoxin have been shown to be two structurally distinct molecules (Pennica *et al.*, 1984) produced by different cell populations (Torti *et al.*, 1985; Ruddle 1987). Tumour necrosis factor/cachectin was found to be produced by macrophages (Torti *et al.*, 1985) while lymphotoxin was shown to be produced by lymphocytes (Sung *et al.*, 1988). This distinction is not absolute since human B cell lines, tonsillar B cells (Sung *et al.*, 1988)

keratinocytes, large granular lymphocytes (Bendtzen *et al.*, 1988), basophils and mast cells have also been shown to produce TNF-alpha (Steffen *et al.*, 1989).

Cachectin, so called because of its ability to cause cachexia in experimental animals and tumour patients was found to be identical to TNF (Aderka *et al.*, 1985; Beutler & Cerami, 1986). These similarities resulted in cachectin and TNF being termed TNF-alpha and lymphotoxin, TNF-β by the Genentech group (Ruddle, 1987). Both cachectin and TNF were shown to induce cachexia by inhibiting the enzyme lipoprotein lipase as well as cause haemorrhagic necrosis of tumours in mice (Cerami & Beutler, 1988). It was confirmed that TNF and cachectin were the same cytokine, when mouse cachectin was partially sequenced and found to have a strong sequence homology with human tumour necrosis factor (Pennica *et al.*, 1984).

Both TNF-alpha and lymphotoxin genes were shown to be on the short arm of chromosome 6 of the major histocompatibility complex (MHC) (Spies *et al.*, 1986). Tumour necrosis factor-alpha was found to be produced initially as a 26kDa precursor (or pre-TNF) within the cell (Pennica *et al.*, 1984). This consisted of a 76 amino acid residue which included a hydrophobic region of 26 amino acids. The entire 76 amino acid residue could not be observed as a component of the mature 17kDa TNF molecule secreted from the cell. It was postulated that this 76 amino acid precursor was involved in secretion of TNF-alpha (Pennica *et al.*, 1984).

Tumour necrosis factor-alpha showed a 28% homology to lymphotoxin in that 44 of the 157 TNF-alpha amino acid residues were identical. Fifty percent of the amino acid residues lying between amino acid 35-66 and 110-133 were shown to be identical for both TNF-alpha and lymphotoxin. These similarities included the hydrophobic carboxyl ends of the two molecules (Pennica *et al.*, 1984).

2.2.3. Surface Receptors for TNF-alpha

Cell surface receptors for TNF-alpha and ß (lymphotoxin) on target cells were found to be shared and their expression (on cell surfaces) could be upregulated by interferon (Aggarwal *et al.*, 1985).

Cytotoxicity of target cells by TNF-alpha was shown to be mediated by interaction of the cytokine with high affinity receptors (Kull, 1988), low affinity receptors being present but outnumbered 10:1 on certain cells, particularly monocytes (Imamura *et al.*, 1987).

The correlation between TNF-alpha sensitivity and receptor number has been shown to be restricted to certain TNF-alpha sensitive tumour cell lines (Watanabe *et al.*, 1989). These included the human KYM myosarcoma, HL60 acute promyelocytic leukaemia, KM-3 acute lymphoblastic leukaemia, RPMI 4788 colon cancer cell line, the murine L-M tumourigenic fibroblast and B16 melanoma cell lines. These TNF-alpha susceptible cell lines

expressed large numbers of TNF-alpha receptors. The higher the numbers of receptors, the more susceptible the cell line was shown to be. The human KYM-R TNF resistant cell line and the murine LR TNF resistant cell line did not express TNF-alpha receptors. However evidence indicated that receptor number and affinity for the receptor did not in itself confer sensitivity to the cytokine (Watanabe *et al.*, 1989). The presence of TNF-alpha receptors on normal cells was found to result in their proliferation as in activated T cells (Scheurich *et al.*, 1987). This expression of TNF-alpha receptors was shown to peak at ~5000/cell by day 6 following activation. Tumour necrosis factor-alpha itself enhanced the proliferative effect of T cells to IL-2 (Scheurich *et al.*, 1987).

After binding to the receptor TNF-alpha was found to be rapidly internalised, and delivered to the lysosomes within 15-30 minutes (Watanabe *et al.*, 1989). Degradation and release from the cell was shown to follow. Lysosomal activity was thought to be essential for the activity of the cytokine. L-M cells (murine tumourigenic fibroblasts) known to be sensitive to TNF cytotoxicity and TNF resistant human embryonic lung (HEL) cells both expressed TNF receptors. This was determined by ^{125}I-labelled recombinant TNF. The incubation of LM cells with lysosomotropic agents or inhibitors of lysosomal activity decreased the sensitivity of the cells to the cytotoxic effects of TNF (Watanabe *et al.*, 1989). However, Hofsli & Nissen-Meyer (1989) showed that internalisation of TNF is not

required for cytotoxic activity and have suggested that membrane bound TNF-alpha binds to receptors on susceptible cells altering the levels of unknown second messengers in WEHI 164 clone 13 cells, a murine fibrosarcoma cell line.

2.2.4. Biological Properties of TNF

The biological properties of TNF-alpha have been shown to be related to the concentration of the cytokine in the tissues (Tracey *et al.*, 1989). At low concentrations these effects were most beneficial resulting in tissue remodelling whereas at higher concentrations, inflammation, cytotoxicity, cachexia, tissue injury and irreversible shock which resulted in death occurred.

Tumour necrosis factor-alpha as a growth factor stimulated fibroblast and mesenchymal cell proliferation directly and induced the biosynthesis of other growth factors (Tracey *et al.*, 1989). Epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin were shown to act synergistically with TNF-alpha (Vilcek *et al.*, 1988). This synergy with EGF was found to be due to a 2 to 5 fold increase in EGF receptor phosphorylation independent of protein kinase C (Bird & Saklatvala, 1989).

2.2.5. TNF, Infection and Endotoxic Shock

Antigen presenting cells exposed to endotoxin were found to produce large amounts of TNF, which was found to be involved in endotoxic shock (Beutler *et al.*, 1986). Symptoms of endotoxic shock were shown to include fever, metabolic acidosis, diarrhoea, hypotension and disseminated intravascular coagulation which sometimes resulted in death (Tracey *et al.*, 1989). In man these symptoms were found to begin 2 hours after IV administration of endotoxin (Tracey *et al.*, 1989). The association between endotoxic shock and TNF-alpha was further evidenced by the administration of recombinant TNF to rats which resulted in many of the described features (of endotoxic shock) (Balkwill, 1989). Beutler *et al.* (1985b) showed that endotoxin sensitive mice passively immunised against TNF, by the infusion of polyclonal rabbit anti-TNF antibodies, did not develop symptoms of endotoxic shock when challenged with *E.coli* derived endotoxin. Animals which were pre-treated with dexamethasone prior to endotoxin administration inhibited TNF production. This inhibition was shown to take place at the transcriptional and post transcriptional level, and thus protected the animal from endotoxic shock (Beutler *et al.*, 1985a). Other evidence which implicated TNF as a mediator of endotoxic shock were the circulating high levels of TNF which were found in patients who expired as a result of meningococcal disease. Patients with the same infections who were found to have lower levels of TNF-alpha survived (Waage *et al.*, 1987).

2.2.6. TNF and Fever

Dinarello *et al.* (1986) showed that recombinant TNF was capable of inducing fever in experimental animals. The intravenous injection of 1ug/Kg of TNF resulted in fever that peaked 45-55 minutes after infusion and then returned to base line levels. This fever peak was caused by direct action of TNF on the hypothalamus that resulted in an increase of hypothalamic prostaglandin E2 (PGE2) synthesis and was shown to be blocked by cyclooxygenase inhibiting drugs (Dinarello *et al.*, 1986). Cyclooxygenase was previously found to be a regulator of PGE2 (Korn *et al.*, 1989). However mice that were passively infused with anti-TNF antibodies were still shown to develop fever, even though onset of endotoxic shock was prevented (Beutler *et al.*, 1985b). This was thought to be due to IL-1, a potent endogenous pyrogen (Dinarello *et al.*, 1986). Higher concentrations of recombinant TNF (5-10ug/Kg) were shown to induce IL-1 production. This resulted in a biphasic pattern of fever, TNF found to be responsible for the first peak while IL-1 was shown to be responsible for the second (Dinarello *et al.*, 1986). In contrast to these findings, Kluger (1991) noted that 1ug/ml TNF injected into rats resulted in a decrease in body temperature. It was postulated that since minor stresses resulted in IL-6 mediated fever over a period of 10 days, the early experiments did not utilise animals accustomed to their environment at the time of experimentation. This implies that minor stresses resulted in a false raised base line level and could thus explain the discrepancy in results observed (Kluger, 1991 personal communication).

2.2.7. TNF and Cachexia

Kawakami & Cerami (1981) found that the injection of 1ug of endotoxin into endotoxin sensitive mice resulted in severe weight loss, hypertriglyceridemia and hypoglycaemia. This was shown to occur by the inhibition of the enzyme lipoprotein lipase in adipocytes isolated from epididymal fat pads 16 hours after injection. Torti *et al.* (1985) showed that RAW264 macrophages treated with 10ug/ml of endotoxin in *in vitro* produced a factor called cachectin. Cachectin (now called TNF-alpha) was found to be responsible for the observed inhibition of the adipocyte enzyme lipoprotein lipase (Torti *et al.*, 1985). Oliff *et al.* (1987) conclusively showed that TNF was responsible for cachexia by inserting the human TNF gene into a mammalian expression vector. This was transfected into Chinese hamster ovary cells which were then found to secrete low levels of TNF. The cells were injected intramuscularly into the hind limbs of nude mice. These mice, known to have no immune systems, were found to develop cachexia when compared to control animals injected with cells containing the vector but no TNF gene (Oliff *et al.*, 1987).

2.2.8. Production of TNF by Tumours

Severe weight loss has also been noted in patients with various malignant tumours. These were found to include patients with oat cell carcinoma, breast cancer and colon carcinoma (Balkwill *et al.*, 1987). High levels of TNF were shown to be detected by ELISA in the sera of many patients with such

tumours (Balkwill *et al.*, 1987). Garrett *et al.* (1987) suggested that tumours may in fact have produced TNF themselves. Their studies demonstrated that TNF-alpha and β mRNA was present in 4 human myeloma cell lines. The production of TNF mRNA in several ovarian cell lines was also reported by Spriggs (as cited by Ruddle, 1987). These findings therefore implicate TNF in severe cachexia reported in patients with malignant tumours (Balkwill *et al.*, 1987).

2.2.9. TNF and Effect on Bone and Collagen

Dayer *et al.* (1985) observed that synovial cells and dermal fibroblasts were capable of producing collagenase and PGE2 in response to stimulation with concentrations of TNF greater than 0.3nM. This effect was shown to be dose dependent. In a later study by Brenner *et al.* (1989), the production of collagenase from TNF stimulated fibroblasts was found to be due to prolonged activation of up to 6 hours of the collagenase genes. This four fold increase in collagenase gene transcription was shown to be responsible for the induction of collagenase mRNA by TNF-alpha (Brenner *et al.*, 1989). Tumour necrosis factor was further implicated in arthritic disease by Yocum *et al.* (1989). High levels of TNF could be detected by ELISA in the synovial fluids of patients with active rheumatoid arthritis, osteoarthritis and those diagnosed to have Reiter's disease; whilst TNF could not be detected in the synovial fluids of normal individuals requiring exploratory arthroscopy due to knee pain or joint trauma (Yocum *et al.*, 1989).

Another activity ascribed to TNF was the stimulation of osteoclastic bone resorption by the stimulation of osteoclasts (Bertolini *et al.*, 1986). In this study, foetal rat bone cultures treated with rTNF-alpha and ß were found to contain an increased number of multinucleated osteoclasts while untreated control bones contained few osteoclasts. Test cultures also showed significant inhibition of collagen synthesis when compared to controls. Tumour necrosis factor induced osteoclastic bone resorption could be completely inhibited by 5 units/ml of salmon calcitonin, a known inhibitor of osteoclast activity (Bertolini *et al.*, 1986).

2.2.10. TNF and the Cytotoxic Destruction of Tumours

Carswell *et al.* (1975) observed that mice injected with BCG and *E.coli* endotoxin produced a tumour necrosis factor in their sera. When 0.5ml of tumour necrosis factor positive serum was injected intravenously into BALB/c mice bearing Meth A sarcoma, haemorrhagic necrosis of these tumours became evident within 3-4 hours after injection (Carswell *et al.*, 1975). Matthews (1981) showed that monocytes exposed to 10ug/ml of *E.coli* lipopolysaccharide B *in vitro*, produced a cytotoxic factor responsible for the necrosis of some transformed cell lines at a 1:8 dilution. These susceptible cell lines tested were the Hela, HepII, HL132 AV 3 and HFF human tumour cell lines. The transformed mouse cell lines assessed were the TOL2 and L929 cell lines (Matthews, 1981). Tumour

necrosis was also postulated to be due to local effects of TNF on tumour vasculature (Gamble *et al.*, 1985). Van de Wiel *et al.* (1989) examined the effect of TNF and endotoxin on bovine endothelial cells prepared from umbilical cord arteries. Endothelial cells were shown to undergo necrosis *in vitro* in the presence of rTNF. However, the effect was extremely marked when rTNF and endotoxin were added in combination to the endothelial cells. This *in vitro* destruction of endothelial cells may have been responsible for the haemorrhagic necrosis observed *in vitro* (Van de Wiel *et al.*, 1989). Nawroth & Stern (1986) showed that bovine and human endothelial cells increased their procoagulant activity 4 hours after the addition of TNF. The increased expression of thrombomodulin on endothelial cells was also observed. This in turn was found to promote clot formation postulated to be responsible for tumour destruction if it occurred within the tumour vasculature (Nawroth & Stern 1986). Gamble *et al.* (1985) observed that TNF increased the adherence of neutrophils to human umbilical vein endothelial cells. This increased adherence of neutrophils was found to be due to the direct effect of TNF on both the endothelial cell and the neutrophil. Changes in the neutrophil were not protein synthesis dependent as they occurred in the presence of cycloheximide and actinomycin and were seen within 5 minutes. Maximal effects on the endothelial cells were protein synthesis dependent and shown to occur at 4 hours after TNF stimulation. Although the exact mechanism was not known, it was thought to be due to enhanced expression of adherence proteins including the complement receptor C3bi (CR3) (Gamble *et al.*, 1985).

No matter what the exact mechanism of TNF rejection may have been, exposure to TNF finally resulted in DNA fragmentation and lysis of susceptible cells (Balkwill, 1989). This was shown to occur by the activation of phospholipase which broke down phospholipids to arachadonic acid and resulted in the generation of leukotrienes and free oxygen radicals (Balkwill, 1989).

2.2.11. TNF and Neutrophil Activity

Apart from the TNF enhanced adherence of neutrophils to endothelial cells described by Gamble *et al.* (1985), TNF-alpha was also shown to augment phagocytosis of fluoresceinated latex beads when compared to controls (Shalaby *et al.*, 1985). Polymorphonuclear neutrophils incubated for 2 hours in the presence of 1U/ml of TNF-alpha were found to have enhanced ADCC against $Na_2{}^{51}CrO_4$ labelled chicken red blood cells (Shalaby *et al.*, 1985). Richter *et al.* (1989) showed that neutrophil degranulation was enhanced by TNF in a dose dependent manner. These doses were found to range in concentration from 10U/ml to 100U/ml and resulted in the secretion of myeloperoxidase and lactoferrin from polymorphonuclear neutrophils (Richter *et al.*, 1989).

2.2.12. Antiviral Effect of TNF

Mestan *et al.* (1986) observed that TNF protected cells from virus induced cytopathic effects. Human laryngeal carcinoma cells (HEp-2) were found to be protected from

infection with *Vesicular stomatitis* virus (VSV) *in vitro* when pre-incubated with 1ng/ml of TNF. Tumour necrosis factor was shown to only protect cells that were not infected with VSV as well as *Herpes simplex* virus (HSV) and encephalo-myocarditis virus (EMCV) (Mestan *et al.*, 1986). Vilcek *et al.* (1986) showed that TNF induced the production of Interferon-ß2 (IFN-ß2) (now referred to as IL-6) (Hirano *et al.*, 1990) in human fibroblasts. This in turn was found to be responsible for the observed antiviral effects of TNF when the infection of these fibroblasts with EMCV was attempted (Vilcek *et al.*, 1986). Anti IFN-ß2 (IL-6) antibodies were found to block these antiviral effects (Vilcek *et al.*, 1986). However, Mestan *et al.* (1986) failed to detect any IL-6 using Northern Blot techniques and IL-6 specific RNA probes. TNF was therefore implicated in having a direct antiviral effect. To this end, Wong & Goeddel (1986) explored the ability of TNF-alpha and ß to inhibit virus replication directly. These viruses included the RNA viruses, EMCV and VSV as well as the DNA viruses, adenovirus-2 (AD2) and *Herpes simplex* virus type II (HSV-2). Pretreatment with 1ng TNF-alpha or ß was shown to inhibit viral replication in a human myeloma (RPMI-8226) cell line, a glioblastoma (U87MG) cell line, C127 mouse epithelial cells and rat-1 fibroblasts. Tumour necrosis factor-alpha or ß inhibition of viral replication could be prevented using anti-TNF-alpha or ß antibodies (Wong & Goeddel, 1986).

2.2.13. Antiparasitic Effect of TNF

Scuderi *et al.* (1986) showed that patients infected with parasites had raised levels of TNF in their sera. Patients infected with kala-azar (visceral leishmaniasis) and malaria were found to have significantly higher TNF levels as determined by ELISA in their sera when compared with normal healthy controls. Serum TNF levels present in infected patients were shown to be 66% and 70% higher respectively (Scuderi *et al.*, 1986).

Silberstein & David (1986) found that TNF enhanced the cytotoxicity of eosinophils to *Schistosoma mansoni*. These eosinophils were isolated from peripheral blood of allergic rhinitis patients. Cytotoxicity was shown to occur in a dose dependent manner after 36-44 hours. Tumour necrosis factor itself was found to have no direct cytotoxic effect against *S.mansoni in vitro* but the results implicate its antiparasitic role in parasitic infections (Silberstein & David, 1986).

2.2.14. Effect of TNF on Lymphocytes

Scheurich *et al.* (1987) showed that resting T cells isolated from the peripheral blood of normal healthy volunteers were not capable of binding TNF-alpha. However, the presence of TNF-alpha was found to enhance the expression of HLA-DR antigens and IL-2 receptors on activated T cells. Tumour necrosis factor-alpha treated T cells were also shown to have enhanced proliferative responses when incubated in culture with 10ng/ml of IL-2 (Scheurich *et al.*, 1987). Malek *et al.* (1989)

showed that TNF induced a group of phosphatidylinositol anchored proteins, the Ly-6 alloantigens in T cells, thymocytes and bone marrow cells. These Ly-6 A/F antigens were found to be involved in T lymphocyte activation. Lymphocytes were shown to require a 24-48 hour period of incubation with TNF after which proliferation was measured (Malek *et al.*, 1989). Tumour necrosis factor-alpha was also found to be a potent chemoattractant for lymphocytes isolated from the peritoneal exudate of rats (Issekutz & Stoltz, 1989). Rats were injected intraperitoneally 5 days previously with vaccinia virus. Radio-labelled lymphocytes were then injected intravenously into the rats. Following the intradermal injection of 5000U of TNF into the backs of the rats, migration of radio-labelled lymphocytes was seen to peak at 6 hours. A twelve fold increase in lymphocytes were found to be recruited to the TNF puncture site when compared with other control sites injected with control medium and IL-1. These findings therefore indicate that TNF was chemotactic for lymphocytes (Issekutz & Stoltz, 1989).

2.2.15. TNF and Mycobacterial Disease

Silva & Foss (1989) reported increased levels of TNF in the sera of tuberculoid leprosy patients when compared to normal controls and lepromatous leprosy patients. The detection of TNF was determined by TNF mediated cytotoxicity against L929 mouse tumour cells. Surviving cells were fixed and stained and the absorbance of each well was determined at a wavelength of 490nm following lysis of the surviving cells. These studies showed

that between 280 and 340 units of TNF could be detected in the majority of patients with tuberculoid leprosy (Silva & Foss, 1989). Mononuclear (MN) cells and alveolar macrophages stimulated with optimal concentrations of whole *M.bovis*, BCG, PPD and *M.bovis* glycoproteins from culture filtrate were found to produce TNF-alpha after 22-24 hours *in vitro* (Valone *et al.*, 1988). Tumour necrosis factor-alpha in these supernatants was determined using a TNF sensitive L929 fibroblast cell line. Cytotoxic effects of TNF against this cell line were shown to be blocked in the presence of TNF antibodies. However, when whole *M.bovis* or mycobacterial components were added directly to the L929 fibroblast cell line, no cytotoxic effects were noted. This indicated that mycobacterial induced TNF was responsible for the cytotoxicity observed (Valone *et al.*, 1988).

Silva & Faccioli (1988) observed that when 10ug of *M.bovis* cord factor was injected into BALB/c mice, they became severely wasted within 48 hours. This cachexia, hypertriglyceridemia and hypoglycaemia was found to be mediated by TNF-alpha which was released by the host. When supernatants derived from adherent peritoneal cells incubated with cord factor were injected into mice, cachexia was also observed. However, cachexia was shown to be prevented when anti-TNF-alpha antiserum was incubated with supernatants derived from cord factor exposed adherent peritoneal cells. This implicated TNF as the central mediator of cord factor induced cachexia and explained the emaciation observed in tuberculosis patients. However, small amounts of

TNF released after stimulation with cord factor could have been of benefit to the host as this would have aided in the elimination of the invading organisms (Silva & Faccioli, 1988).

Tumour necrosis factor has also been shown to be responsible for the development of granulomas observed after intravenous injection of BCG into mice (Kindler *et al.*, 1989). Histological sections of liver, lung and spleen showed the progressive accumulation of macrophages in developing granulomas. These granulomas were found to contain bacteria surrounded by epitheloid cells, or differentiated macrophages. Granulomas reached maturity after 3 weeks. Mice injected with anti-TNF antibodies as well as BCG were shown to have fewer, smaller granulomas with the absence of epitheloid cells. In addition, these animals failed to contain the BCG infection. This implicated TNF in granuloma formation which was shown to contain the BCG infection and allow the destruction of intracellular bacteria (Kindler *et al.*, 1989).

Tumour necrosis factor was found to augment its own production by the induction of TNF mRNA in peritoneal macrophages as determined by Northern Blot analysis (Kindler *et al.*, 1989). Peritoneal macrophages cultured *in vitro* in the presence of TNF were shown to increase the production of TNF mRNA within 2 to 4 hours. Also TNF injected intraperitoneally into LPS resistant mice (C3H/HEJ) resulted in increased production of TNF mRNA in peritoneal macrophages after 2-4 hours. It was postulated that TNF released into the

microenvironment of the granuloma enhanced its own production which in turn led to further macrophage accumulation. This ultimately led to the elimination of the bacterial infection (Kindler *et al.*, 1989).

Bermudez *et al.* (1989) observed that 25ug/Kg TNF was able to induce macrophage mediated killing of *Mycobacterium avium (M.avium)* in C57 BL/6bg/+ mice. These mice were shown to be good models for disseminated *M.avium* infection. Complete clearance of the bacilli from the liver and spleen was achieved when 50ug/Kg IL-2 and 12.5ug/Kg of TNF were injected intravenously one week after the mice had been infected with *M.avium*. These findings therefore implicate TNF at non-toxic doses in the control of *M.avium* (Bermudez *et al.*, 1989).

2.3. INTERLEUKIN 1 (IL-1)

2.3.1. Introduction

Interleukin-1 was first described as pyrexin or endogenous pyrogen because of its ability to cause fevers (Dinarello *et al.*, 1974). Initial characterisation identified it to be a polypeptide unrelated to endotoxin (Atkins, 1960). Endogenous pyrogen was also shown to promote the proliferation of murine thymocytes following mitogenic or antigenic stimulation and was found to be present in culture supernatants of human peripheral adherent blood leukocytes (Gery *et al.*, 1972). Since 1972 a number of other activities have been associated with the cytokine or its degradation products (Dawson, 1991). These were

shown to include: 'leukocyte endogenous mediator' (responsible for the induction of the acute phase response by hepatocytes); 'catabolin' (destruction of cartilage); 'osteoclast activation factor' (resorption of bone); 'proteolysis inducing factor' (muscle wasting and fever); and 'B cell activation factor' (production of immunoglobulin by antigen-specific B cells) (Dawson, 1991). The term 'interleukin-1 (IL-1)' was used to describe all the above factors at the Second International Lymphokine Workshop (Aarden *et al.*, 1979).

2.3.2. Isolation of IL-1 alpha and ß

Dinarello *et al.* (1974) observed that human blood leukocytes activated with *Staphylococcus albus* produced two distinct cytokines. Both cytokines were pyrogenic when injected into rabbits. Monocyte derived IL-1 was found to have a pI of 5.1 as assessed by isoelectric focussing and had a molecular size of 38kDa. However IL-1 isolated from neutrophils appeared to be a smaller 15kDa protein and had a pI of 6.8 (Dinarello *et al.*, 1974). The acidic IL-1 with a pI value of 5.1 was later termed IL-1 alpha while the more basic IL-1 of pI 6.8 was termed IL-1ß (March *et al.*, 1985).

Subsequently IL-1 of different molecular forms and pI values from different animal sources have been reported (Goto *et al.*, 1984; 1984a). The wide range of pI values between 4.5 - 7.2 has been ascribed to a possibility of fragmentation due to endogenous protease breakdown (Dinarello, 1991) resulting in

17kDa forms of IL-1 alpha or β due to such breakdown of the 31kDa precursors. Both forms of IL-1 have been successfully cloned, sequenced and shown to be biologically active (Lomedico *et al.*, 1984). Successful cloning of the genes from both human and murine sources was accomplished (Lomedico *et al.*, 1984; Auron *et al.*, 1984). Such cloning experiments also led to the finding that human IL-1 was coded for on two gene sites on chromosome 2 (Webb *et al.*, 1986; Lafage *et al.*, 1989).

Human and murine IL-1 alpha were found to be 62% homologous (March *et al.*, 1985) while 88% homology was shown to exist between human and murine IL-1β (Dinarello, 1988). However when human IL-1 alpha was compared with human IL-1β a sequence homology of only 25% was found (Dinarello, 1991).

2.3.3. Synthesis of IL-1

Synthesis of IL-1 has been shown to occur in a wide variety of cells (Dinarello, 1988). These include monocytes, tissue macrophages, synovial cells, peritoneal macrophages, some T helper lymphocytes, B cells, NK cells, smooth muscle cells, endothelial cells, astrocytes, microglial glioma cells, keratinocytes, Langerhans cells, neutrophils, fibroblasts, chondrocytes, corneal epithelium, thymic epithelium, noradrenergic cells and gingival cells (Dinarello, 1988).

Transcription of IL-1 mRNA in blood monocytes was initiated within 15 minutes after adherence to plastic or glass (Dinarello, 1988). Other potent stimulants of IL-1 production included bacteria (Dinarello *et al.*, 1974); bacterial LPS (Lepe-Zuniga & Gery, 1984); Lyme disease spirochaetes (Habicht *et al.*, 1985); viruses (Ensoli *et al.*, 1989) and silica (Lepe-Zuniga & Gery, 1984).

Two mechanisms of secretion of IL-1 have been postulated. The first is that IL-1 may be released from damaged cells due to the perforation of the plasma membrane (Lepe-Zuniga & Gery, 1983 as cited by Oppenheim *et al.*, 1986). The second hypothesis by Matsushima *et al.* (1986) suggested that IL-1 may be released from undamaged cells following endogenous enzyme cleavage and the presence of plasminogen in culture medium.

2.3.4. IL-1 Receptors

A number of IL-1 receptor proteins have been identified (Dinarello, 1991). These range in size and include proteins of Mr 30-220kDa. Of these, only two major IL-1 receptors appear to play any significant role in immune regulation. These are proteins of Mr 80kDa and 68kDa (Dinarello, 1991).

The first receptor for IL-1 was identified to be an 80kDa protein (Sims *et al.*, 1988). The receptor, termed IL-1 RtI was initially shown to be expressed on a murine thymoma cell

line (Sims *et al.*, 1988) and on human macrophages (Uhl *et al.*, 1989). Binding of this IL-1 RtI by IL-1 was shown to enhance the monocytes phagocytic capacity and initiation of immune reactions (Uhl *et al.*, 1989).

The binding affinity of IL-1 to the IL-1 receptor is controversial since evidence has appeared indicating a single binding affinity (Qwarnstrom *et al.*, 1988) and of a double receptor binding capacity of IL-1 (Lowenthal & MacDonald, 1986). In the first type of binding, human diploid fibroblasts were shown to be capable of binding both IL-1 alpha and ß (Qwarnstrom *et al.*, 1988). Interleukin-1ß bound to these receptors was slowly internalised over a 6 hour period with an increased amount present within the cell nucleus within one hour. Once internalised, IL-1 was observed to be present in the cell cytoplasm and was not degraded as confirmed by polyacrylamide gel electrophoresis and trichloroacetic acid precipitation (Qwarnstrom *et al.*, 1988). Evidence for the second double binding affinity was provided by Lowenthal & MacDonald (1986) who showed that a murine thymoma cell line, EL 4-6.1, expressed low affinity IL-1 receptors which accounted for ~98-99% of total receptors expressed (~18 000 receptors/cell). The remaining 1-2% of IL-1 receptors expressed on this cell line were observed to be high affinity receptors (Lowenthal & MacDonald, 1986).

A second IL-1 receptor, the IL-1 RtII of Mr 68kDa was observed on Epstein Barr virus (EBV) transformed B cells (Matsushima *et al.*, 1986a). This receptor was also found to be expressed on

B cell lineages, neutrophils and bone marrow cells (Dinarello, 1991). The receptor's cytoplasmic portion was shown to be shortened when compared with IL-1 RtI and accounted for its Mr of 68kDa (Dinarello, 1991).

The IL-1 RtII expressed on EBV transformed B cells was shown to bind IL-1 alpha and ß, although IL-1ß had a greater affinity for this receptor (Matsushima *et al.*, 1986a). Binding of the receptor enhanced B lymphocyte transformation. Although small numbers of the IL-1 RtII were expressed, low occupancy of these receptors was sufficient for cell stimulation (Matsushima *et al.*, 1986a).

Horuk *et al.* (1987) showed using the RAJI cell line that once binding of IL-1 to the IL-1 RtII had occurred it was poorly internalised and remained on the cell surface for up to 1 hour at 37°C. The fate of IL-1ß once internalised was investigated by Matsushima *et al.* (1986b) using a human granular lymphocyte cell line. Interleukin-1ß, once internalised was found in a degraded form within the lysosomes 3 hours after internalisation (Matsushima *et al.*, 1986b). This was significant since it suggested different acidification mechanisms and vesicles for the two receptors (Dinarello, 1991).

2.3.5. IL-1 and Polymorphonuclear Leukocytes (PMN Cells)

Intradermal injections of IL-1 into anaesthetised rabbits indicated a chemotactic role for this cytokine (Watson *et al.*, 1989). Accumulation of PMN cells to the site of

infection was observed as early as 10 minutes following the introduction of IL-1. However it was postulated that IL-1 activity was controlled by a secondary mediator produced by connective tissue cells at the inflammatory site. This was examined by incubating human synovial cell cultures with rIL-1 alpha (Watson *et al.*, 1988). Twenty four hour fibroblast supernatants were obtained from IL-1 alpha exposed fibroblasts. Two peptides, 6kDa and 13kDa were identified in these supernatants which were produced by fibroblasts in reponse to rIL-1 alpha. These peptides were found to stimulate PMN cell locomotion as well as elevated PMN cell cytosolic Ca^{2+} production *in vitro* (Watson *et al.*, 1988). These results correlated well with those of Mason & van Epps (1989) who showed that IL-1 led to PMN cell chemotaxis in mice. Chemotaxis of PMN cells was dose dependent and was found to be greatest 6 hours after injection with 5U IL-1, when compared to chemotaxis induced by other cytokines (Mason & van Epps, 1989). An additive chemotactic response was observed when 5U IL-1 and 0.2ug/ml TNF were administered (Mason & van Epps, 1989). These findings however are somewhat inconsistent with those reported earlier by Georgilis *et al.* (1987) who reported no direct effect of IL-1 on PMN cell chemotaxis, degranulation or production of superoxide *in vitro*. Such inconsistencies may be due to the different experimental systems employed and in particular due to the finding that the *in vitro* experiments of Watson *et al.* (1988; 1989) identified a second mediator that activated PMN cells, released after the injection of IL-1. Such findings therefore suggest that IL-1 may be acting

indirectly on PMN cells. This is further substantiated by the findings of Larsen *et al.* (1989) who showed that TNF-alpha or IL-1 treated fibroblasts secreted IL-8 (neutrophil-activating-factor) *in vitro.* This cytokine was implicated in neutrophil chemotaxis *in vivo* subsequent to injection of IL-1 or TNF (Larson *et al.*, 1989).

2.3.6. Production of IL-1 by PMN Cells

The ability of PMN cells to produce IL-1 was investigated by Canning & Neill (1989) who noted that bovine PMN cells could produce IL-1. PMN cells were stimulated for 6 hours with opsonised zymosan and their ability to stimulate an IL-1 sensitive cell line (D10G4.1) assessed (Canning & Neill, 1989). A cytokine 17.8kDa in size was isolated by HPLC from PMN cell supernatants and had a pI of 4.1 as determined by isoelectric focussing (Canning & Neill, 1989). Northern Blot analysis revealed that the cytokine was IL-1 alpha.

2.3.7. The Effect of IL-1 on Vascular Endothelial Cells

Bevilacqua *et al.* (1985) showed that IL-1 increased the adhesion of PMN cells to human umbilical endothelial cells (HUVE). This effect was found to be dose and time dependent. Peak effects were noted at 4-6 hours following the administration of 10U of IL-1. Effects appeared to be protein synthesis dependent and could be inhibited by treatment of HUVE

cells with both 10ug cycloheximide or 5ug actinomycin D. These results indicated that IL-1 acted selectively on HUVE cells and was responsible for the enhanced adhesion of PMN cells (Bevilacqua *et al.*, 1985). This effect was further investigated by Pober *et al.* (1986) who found that IL-1 (and TNF) induced the synthesis and expression of a cell surface molecule on human vascular endothelial cells *in vitro*. This molecule was recognised and bound by the murine monoclonal antibody H4/18. The expression of the H4/18 protein on endothelial cells was shown to be transient and peaked at 4-6 hours following incubation with 5-10U of IL-1. Cycloheximide was found to block expression of this protein which indicated that expression was dependent on protein synthesis. The binding of the monoclonal antibody H4/18 to the protein resulted in decreased adhesion of PMN cells to endothelial cells. However as this decrease was not absolute it was suggested that other molecules or epitopes on the H4/18 binding protein may have also been involved. These findings suggested that endothelial cells expressed new functional properties in response to the presence of IL-1 at the site of local inflammation (Pober *et al.*, 1986).

However the passage of PMN cells from the blood through the endothelial cell-basal membrane barrier to the site of inflammation was known to be important. To this end, Moser *et al.* (1989) pre-incubated HUVE cells with 1U of IL-1 for 60 minutes *in vitro* and showed enhanced PMN transendothelial passage. These PMN cells were found to penetrate deep into the

subendothelium after migration through endothelial cells as assessed microscopically. Preincubation of HUVE cells with 5ng TNF produced the same results whereas HUVE cells preincubated with medium alone showed little PMN cell subendothelial infiltration. These results indicate that IL-1 (and TNF) by their action on HUVE cells thus enhanced the transendothelial passage of PMN cells by an unknown, yet IL-1 dependent, mechanism (Moser *et al.*, 1989).

2.3.8. IL-1 and T Cell Activation

Several investigators have demonstrated the importance of IL-1 in inducing the production of IL-2 by various T cell lines. Such studies have shown that the addition of exogenous IL-1 resulted in the production of IFN-gamma, IL-2 and IL-4 (Kasahara *et al.*, 1985), augmentation of the synthesis of IL-2-mRNA in leukaemic cell lines (Hagiwara *et al.*, 1987) and stimulated IL-2 production by normal murine T cells by acting on dendritic cells which in turn stimulated T cell proliferation and IL-2 release (Koide *et al.*, 1987). Other workers demonstrated that Con A activated lymphocytes expressed receptors for IL-1 in the presence of monocytes (Shirakawa *et al.*, 1987). Using different T cell clones, Lichtman *et al.* (1988) confirmed these findings and suggested that for T cell activation and proliferation to occur, antigen presenting cells were necessary for cytokine production whilst IL-1 enhanced Class II antigen mediated production of cytokines and T cell proliferation.

2.3.9. IL-1 and Antibacterial Resistance

Czuprinski & Brown (1987) observed that 0.17ug of IL-1 alpha enhanced the antimicrobial resistance of mice challenged with *Listeria monocytogenes (L.monocytogenes)*. It was postulated that the enhanced antimicrobial resistance was due to activation of cellular immunity rather that a direct effect of rIL-1 alpha on *L.monocytogenes* since rIL-1 alpha incubated with *L.monocytogenes* had no effect (Czuprinski & Brown, 1987).

A similar study by van der Meer *et al.* (1988) showed that a low dose of rIL-1β protected granulocytopenic mice from lethal *Pseudomonas aeruginosa (P.aeruginosa)* infection. Administration of IL-1β was not protective unless administered at least 24 hours before *P.aeruginosa* infusion (van der Meer *et al.*, 1988). Interleukin-1β itself was not observed to have any direct anti-pseudomonas activity. It was postulated that IL-1β either counteracted the toxicity of pseudomonas exotoxins or stimulated acute phase proteins which in turn bound exotoxin (van der Meer *et al.*, 1988). These findings correlated with those of Czuprinski & Brown (1987) which indicated that IL-1 enhanced non-specific resistance to bacteria.

2.3.10. IL-1 and Septic Shock

In contrast to the beneficial effects of IL-1 observed in antibacterial resistance, the excess production of either IL-1 alpha or IL-1β was found to result in septic shock and possibly

death of both human and animal subjects (Butler *et al.*, 1989; Waage *et al.*, 1989). This apparent correlation between IL-1 and shock was further investigated by Okusawa *et al.* (1988) who noted that the intravenous injection of 5ug/Kg of human rIL-1ß into rabbits resulted in the development of a shock-like state including hypotension. The hypotension observed in IL-1 induced shock appeared to be mediated by cyclooxygenase products (Okusawa *et al.*, 1988). Similar findings were reported by Butler *et al.* (1989) in a murine model using IL-1 alpha and ß. In contrast to these findings, pretreatment of mice with 5ug of IL-1ß completely inhibited the development of shock when a lethal dose of IL-1 alpha or ß was administered. These results indicated that IL-1 alpha and ß are at least one of a number of cytokines responsible for the development of endotoxic shock although the tolerance induced by pretreatment with sublethal doses of IL-1ß could not be explained (Butler *et al.*, 1989).

The role of IL-1 in shock was further examined by Waage *et al.* (1989) who found that extremely high levels of IL-1 (and IL-6) could be detected in the serum of patients with meningococcal septic shock. These high levels of IL-1 (and IL-6) were associated with death of three individuals whereas IL-1 could not be detected in the sera of survivors (Waage *et al.*, 1989). In addition Cannon *et al.* (1990) showed that patients with septic shock had high plasma levels of IL-1ß (120 ± 17pg/ml) compared to normal healthy individuals (<70pg/ml) as determined by RIA. However in contrast to

findings by Waage *et al.* (1989) patients with higher levels of IL-1ß tended to survive while those septic shock individuals with lower levels of IL-1 did not (Cannon *et al.*, 1990). It was therefore postulated that the failure to produce enough IL-1 during endotoxic shock resulted in death (Cannon *et al.*, 1990) whilst in contrast to these findings excess production of IL-1 also resulted in death (Waage *et al.*, 1989). Furthermore, evidence presented by Fong *et al.* (1989b) using baboons demonstrated a relationship between TNF, IL-1ß and septic shock. Infusion of anti-TNF antibodies 2 hours before the intravenous injection of live *E.coli* prevented the production of IL-1ß (and IL-6) and the subsequent induction of endotoxic shock. Baboons infused with saline 1-2 hours before *E.coli* infusion developed the symptoms of shock and died. High levels of IL-1ß were found in the serum of those animals 3 hours after *E.coli* administration. This data suggested that TNF was essential for the production of IL-1 during lethal gram negative infection (Fong *et al.*, 1989b).

2.3.11. IL-1 and Fever

Interleukin-1 or 'endogenous pyrogen', isolated from acute granulomatous exudate fluid, was initially shown to induce fever when injected into animals or humans (Atkins, 1960). Further investigations by Dinarello *et al.* (1984) demonstrated that IL-1 isolated from the plasma of febrile patients induced a rise in temperature when injected into mice. The active component of IL-1 that induced fever was found to be a 4.2kDa peptide that

could be cleaved by trypsinisation of the original 15kDa molecule. Subsequently these authors injected rIL-1 into mice and rabbits and observed a monophasic fever peak which was thought to be due to an increase in hypothalamic prostaglandin synthesis in response to the IL-1 injected (Dinarello *et al.*, 1986a). Their studies also implicated the arachadonic acid pathway in the induction of fever.

Other workers (Cannon *et al.*, 1990) measured serum IL-1β levels in human volunteers challenged with endotoxin. Their studies showed a rise in temperature corresponding with a rise in IL-1ß levels at 180 minutes following such challenge. However, even though the rise in IL-1ß levels may have corresponded to a rise in temperature as a function of time, the possibility that raised plasma levels may have caused an increase in fever was excluded by the findings of Fontana *et al.* (1984), who suggested that the monokine did not cross the blood-brain barrier. Studies in mice have demonstrated that IL-1 was produced within the hypothalamus following the intraperitoneal injection of LPS into these animals (Fontana *et al.*, 1984).

2.3.12. The Effects of IL-1 on Muscle Proteolysis, Acute Phase Protein Production, Bone and Cartilage Resorption

The 4.2kDa fragment of IL-1 isolated by Dinarello *et al* (1984) was also found to induce proteolysis of rat muscle *in vitro* (Clowes *et al.*, 1983). The mechanism by which IL-1

induced muscle proteolysis has been suggested to be due to an increase in the production of PGE2 by the 4.2kDa fragment (Baracos *et al.*, 1983). Raised levels of PGE2, in turn, induced intralysosomal proteolysis ultimately resulting in protein breakdown.

Interleukin-1 has also been shown to have marked effects on the production of acute phase proteins (Gauldie *et al.*, 1987). These included the production of serum amyloid A; alpha 2 macroglobulin; alpha 1-acid gylcoprotein; fibrinogen and alpha 1-acute phase globulin by mouse hepatocytes injected with purified human IL-1. The production of albumin, however, appeared to be decreased in response to the injection of the cytokine. It has been suggested that this effect on acute phase protein production was due to a direct action of IL-1 on hepatocytes (Gauldie *et al.*, 1987).

The effect of IL-1 on bone resorption was effectively demonstrated by Gowen *et al.* (1983) in experiments whereby IL-1 was responsible for bone resorption of mouse calvaria. Later, Eastgate *et al.* (1988) suggested a casual relationship between IL-1 and rheumatoid arthritis by demonstrating raised IL-1β levels in such patients.

In addition, IL-1 appeared to play an important role in cartilage degradation by inducing the release of glycosaminoglycan and inhibiting the further synthesis of the disaccharide (Smith *et al.*, 1989). Erosion of cartilage by

IL-1 however appeared to be dependent on the presence of viable chondrocytes for active cartilage erosion.

2.3.13. IL-1, Granuloma Formation and Mycobacterial Disease

Since granulomatous inflammation was known to be associated with many human diseases including tuberculosis, Kasahara *et al.* (1988) investigated the role of IL-1 in granuloma formation. Sephadex G-50 beads injected intratracheally in mice produced small granulomas primarily composed of macrophages (Kasahara *et al.*, 1988). Two peaks of IL-1β, 12-25kDa and 25-67kDa, were isolated from extracts of such granulomas after application to a Sephacryl S-200 column. Granuloma formation was observed when mice were injected intratracheally with Sepharose 4B beads coupled with either IL-1 fraction or rIL-1β. Sepharose 4B beads alone or those coupled with rIL-2 produced considerably smaller granulomas (Kasahara *et al.*, 1988).

These findings correlated well with a previous study by Kobayashi *et al.* (1985) who showed that the intratracheal injection of BCG into sensitised C57 BL/6 and BALB/c mice resulted in granuloma formation. Granulomas were found to consist of large macrophages, mature lymphocytes and a small number of neutrophils (Kobayashi *et al.*, 1985). Interleukin-1 activity was detected in supernatants from extracts of such granulomas (Kobayashi *et al.*, 1985). However intratracheal injection of BCG into CBA/J mice did not

result in significant granuloma formation. In addition, very low levels of IL-1 were isolated from these granulomas (Kobayashi *et al.*, 1985). MN cells isolated from the lymph nodes of BALB/c or C57 BL/6J mice which developed large granulomas associated with high levels of IL-1 exhibited marked suppression of lymphocyte blastogenesis. MN cells were isolated from lymph nodes and placed in culture with antigens or mitogens. Mononuclear cells isolated from the lymph nodes of CBA/J mice which developed granulomas associated with low levels of IL-1 were only mildly suppressed when placed in culture with antigens or mitogens. These results indicated that granuloma formation associated with high IL-1 levels was dependent on a genetic ability to mount a granulomatous response to BCG. Furthermore, since IL-2 was not detected in granuloma extracts, it was postulated that BCG activated cells within the granuloma to produce an unknown inhibitor which resulted in suppression of lymphocyte blastogenesis in granuloma bearing mice. This 'inhibitor' would in turn suppress the production of IL-1 and result in smaller granuloma formation (Kobayashi *et al.*, 1985).

The role of IL-1 in mycobacterial disease was also investigated by (Chensue *et al.*, 1986). Monocytes isolated from tuberculous individuals were found to spontaneously secrete significantly larger amounts of IL-1β than healthy controls. These findings supported the postulate that IL-1 was an important mediator in chronic infectious diseases like tuberculosis (Chensue *et al.*, 1986). Further evidence of

the role of IL-1 in mycobacterial diseases was provided by Dunlap & Tilden (1991) who showed that CD4+ cells prestimulated with 1ug/ml of PPD and added to untreated monocytes induced the secretion of IL-1ß in a dose dependent manner. However monocytes failed to secrete IL-1ß as determined by RIA unless cell-to-cell contact between macrophages and PPD-stimulated T cells had occurred (Dunlap & Tilden, 1991). This data suggested that sufficiently activated T cells could induce the secretion of IL-1ß by monocytes which may have been important in the normal cellular immune response (Dunlap & Tilden, 1991).

Mycobacterial components themselves have also been shown to stimulate IL-1 release (Wallis *et al.*, 1986). MN cells isolated from PPD-non-reactive individuals stimulated with PPD were observed to release IL-1. This release occurred in the absence of CD3+ lymphocytes. In addition, antigen 5, a partially purified cytoplasmic antigen derived from *M.tuberculosis*, was also found to induce the secretion of IL-1 from MN cells. This indicated that *M.tuberculosis* components could directly activate IL-1 release by monocytes. This resulted in the increased production and secretion of IL-1 (Wallis *et al.*, 1986).

Whatever the method of activation of IL-1ß release may have been, IL-1ß did not appear to benefit *M.avium* infected individuals (Denis, 1991a). Monocytes isolated from the blood of healthy individuals were infected with *M.avium in vitro*. Mycobacterial growth was significantly enhanced intracellularly

when IL-1ß was added to culture medium. This was compared with mycobacterial growth in untreated macrophages or monocytes treated with IFN-gamma. Interleukin-1ß added to *M.avium* grown in cell free medium also exhibited enhanced mycobacterial cell growth when compared with *M.avium* grown in medium alone. The enhanced growth of *M.avium* was blocked when anti-IL-1ß (and anti-IL-6) antibodies were added to the culture. Thus at the site of local infection, *M.avium* could bind IL-1 produced. This would enhance the growth of *M.avium* while at the same time remove IL-1 necessary for the host to mount an immune response (Denis, 1991a).

Shiratsuchi *et al.* (1991) showed that monocytes infected with one of two strains of *M.avium* did not demonstrate enhanced growth in the presence of rIL-1ß. Recombinant IL-1 alpha however significantly augmented the intracellular and extracellular growth of *M.avium* when added to monocyte cultures as compared to monocyte cultures in medium alone. This effect was neutralised with anti-IL-1 alpha, suggesting that IL-1 favoured the growth of *M.avium* and influenced the outcome of *M.avium* infection (Shiratsuchi *et al.*, 1991).

2.4. INTERLEUKIN 2 (IL-2)

2.4.1. Introduction

In 1976, Morgan *et al.* demonstrated that normal human T cells could be cultured indefinitely in "conditioned" media, derived from PHA stimulated MN cells. Later T cells were identified as the source of the mitogenic lymphokine with the

result that it was termed T cell growth factor (TCGF) (Gillis *et al.*, 1978) and that was subsequently changed to "Interleukin 2" (IL-2) (Aarden *et al.*, 1979).

In addition to its role as a T and B cell growth factor (Meuer *et al.*, 1984; Tsudo *et al.*, 1984), IL-2 has also been found to enhance the destruction of tumours (Grimm *et al.*, 1982). Incubation of lymphocytes in the presence of IL-2 for 48 hours resulted in the development of lymphokine activated killer (LAK) cell formation. These cells displayed enhanced cytotoxic abilities (Grimm *et al.*, 1982).

2.4.2. Cellular Sources of IL-2

Interleukin-2 has been shown to be produced by all T cells (Dawson 1991) although T helper cells appeared to provide the major source of IL-2 (Pfizenmaier *et al.*, 1984). This was evidenced by the observation that 90% of murine Lyt 2- T cell clones were found to produce IL-2 when stimulated with Con A while only 8-10% of the Lyt 2+ cells produced the cytokine under the same conditions. Interleukin-2 appeared to be produced by lymphocytes in response to Con A (Mosmann *et al.*, 1986) and PHA (Morgan *et al.*, 1976) stimulation. Human PPD- specific T helper cell clones have also been found to produce the same cytokine in response to stimulation with PPD (Del Prete *et al.* as cited by Romagnani 1991).

2.4.3. Structure of IL-2 and Gene Location

The IL-2 gene was located on the long arm of chromosome 4 in humans (Seigal *et al.*, 1984). Human IL-2, a glycoprotein, was shown to be secreted as a single polypeptide chain of 133 amino acids (Robb & Smith, 1981). A hydrophobic 20 amino acid leader sequence necessary for secretion from the cell was cleaved from a 153 amino acid precursor. The 133 amino acid sequence was found to contain a single disulphide bond between amino acids 58 and 105 necessary for structure and hence bioactivity of the cytokine (Robb, 1984). Previous analysis of the cytokine indicated that it was of Mr 15.5kDa with a single isoelectric point of 8.2 (Robb & Smith, 1981).

2.4.4. The IL-2 Receptors

Interleukin-2 has been shown to exert its effects by binding to IL-2 receptors on target cell surfaces (Cantrell & Smith, 1983). The IL-2 receptor was found to consist of two polypeptide chains which may be expressed individually or in combination on the cell surface (Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987). The first of these, the Tac receptor of Mr 55kDa (p55) consisted of 272 amino acids (Greene & Robb, 1985 as cited by Dawson, 1991). The second IL-2 receptor, a binding protein of Mr 75kDa (p75) expressed on a T cell acute lymphoblastic leukaemia cell line was described by Tsudo *et al.* (1986). This p75 binding protein has also been isolated on normal stimulated T cells and bound IL-2 with intermediate affinity (Teshigawara *et al.*, 1987).

Interleukin-2 may therefore bind to a low affinity receptor, the p55 receptor, or to the p75 receptor of intermediate affinity (Wang & Smith, 1987). However when both receptors are expressed together, intact IL-2 may bind both receptors at once with high affinity (Tsudo *et al.*, 1986). Under these conditions the p75 receptor has been termed the alpha-chain whilst the p55 protein has been termed the ß-chain of the high affinity receptor (Teshigawara *et al.*, 1987). Of these, only the p75 (ß-chain) stimulated T cell proliferation whilst the p55 (alpha-chain) served only as helper binding sites (Wang & Smith, 1987).

Approximately 2 000 high affinity receptors and ~11 000 low affinity were expressed on normal human T cells (Wang & Smith, 1987). Intermediate binding receptors numbered ~2 000 and could not be distinguished from high affinity receptors (Wang & Smith, 1987).

2.4.5. IL-2 and T Cell Proliferation

Meuer *et al.* (1984) demonstrated that IL-2 promoted the proliferation of human T cells *in vitro* following activation with anti-Ti monoclonal antibodies. Once activated, T cells increased the expression of surface IL-2 receptors by ~6 fold. Interleukin-2 secreted by activated T cells was bound by IL-2 receptors resulting in T cells moving from the activated (G1) phase into the proliferative (S) phase, an effect which was blocked by either anti-IL-2 antibodies or anti-IL-2 receptor

antibodies. However once T cells were exposed to either lectin or antigen, a critical number of receptors were expressed before cells would progress into the proliferative phase due to IL-2 bound (Cantrell & Smith, 1983). Tac receptors on PHA activated blasts reached maximum expression between 3 and 5 days after stimulation. Removal of this stimulus resulted in decreased receptor numbers with a concomitant decrease in proliferation regardless of the concentration of IL-2 present. These results indicated that antigen or lectin-induced proliferation was regulated by endogenous IL-2 in an autocrine manner, which once secreted, bound to IL-2 receptors triggering proliferation (Cantrell & Smith, 1983).

2.4.6. IL-2 and B Cells

Tsudo *et al.* (1984) showed that Tac receptors were expressed on human B cells activated with *Staphylococcus aureus* Cowan I stain. Addition of purified IL-2 to these activated B cells *in vitro*, resulted in proliferation which was completely abrogated in the presence of an anti-Tac antibody (Tsudo *et al.*, 1984). Similar findings were reported by Mittler *et al.* (1985) who found that 20U/ml of rIL-2, in addition to inducing proliferation in ~25-65% of activated human B cells, also enhanced the secretion of immunoglobulins. This effect was blocked by ~90% when anti-IL-2 receptor antibodies were employed in cultures (Mittler *et al.*, 1985).

These findings suggested that once T cells have been activated to produce IL-2, the cytokine supported the growth of activated B cells.

2.4.7. IL-2 and Mycobacterial Diseases

Mycobacterial infections have been associated with decreased levels of IL-2 and/or decreased IL-2 receptor expression by a number of workers (Mohagheghpour *et al.*, 1985; Toossi *et al.*, 1986; Turcotte & Legault, 1986).

MN cells isolated from lepromatous leprosy patients, stimulated with *M. leprae* antigens *in vitro* failed to produce IL-2 or express IL-2 receptors (Mohagheghpour *et al.*, 1985). However patients with borderline tuberculoid leprosy produced IL-2 and expressed IL-2 receptors after 6 days in culture with 10ug/ml of *M. leprae*. No proliferative responses could be induced in cells derived from patients with lepromatous leprosy even when incubated in the presence of PPD and exogenous IL-2. This was shown to be due to failure to express IL-2 receptors (Mohagheghpour *et al.*, 1985).

Similar findings were reported by Toossi *et al.* (1986) who showed that peripheral blood MN cells isolated from newly diagnosed tuberculous individuals produced ~81% less IL-2, detected in 48 hour supernatants, than normal controls in response to PPD. In addition IL-2 receptor expression was also decreased. However defects in IL-2 production and proliferation appeared to be restricted to stimulation with the antigen PPD when employed in culture since the MN cells of both patients and controls proliferated in response to streptococcal antigen. The authors were able to separate the patients into two groups. The

first of these were patients with identifiable adherent suppressor cells which suppressed PPD induced IL-2 production and lymphocyte blastogenesis. Removal of these suppressor cells resulted in the production of normal amounts of IL-2 by lymphocytes *in vitro*. The second group with more extensive pulmonary disease had no identifiable adherent suppressor cells and the cause of their low IL-2 levels could not be established (Toossi *et al.*, 1986).

Interleukin-2 production may also be suppressed due to a defect in synthesis as demonstrated by Turcotte & Legault (1986). Mice (C57BL/6) were injected with *M.bovis* BCG. Two to three weeks later spleen cells or cells derived from lymph nodes were stimulated with Con A in culture and the concentration of IL-2 assessed 20 hours thereafter. Interleukin-2 production by spleen cells was completely suppressed whilst some IL-2 was secreted by lymph node cultures. In addition, spleen cells from BCG infected mice suppressed the production of IL-2 by non-infected murine spleen cells activated with Con A when added to cultures. This was postulated to be due to a defect in IL-2 synthesis by IL-2 producing cells. Lymph node cultures from BCG infected mice also produced low levels of IL-2. Further identification of cell types present in lymph nodes indicated that IL-2 producing cells had been replaced by Ig+ (B) cells. This accounted for the decreased production of IL-2 detected in these cultures (Turcotte & Legault, 1986).

These investigations were continued by Turcotte (1987) who stimulated spleen cells from normal C57BL/6 mice with Con A *in vitro*. Interleukin-2 detected in supernatants peaked at 24 hours and then rapidly declined for the next 24 hours as it was bound and internalised by IL-2 receptor positive cells. Spleen cells incubated with Con A from mice also produced IL-2 after 24 hours in culture, although these levels were not compared with normal IL-2 production. However IL-2 persisted in these supernatants for up to 72 hours remaining unbound due to suppression of IL-2 receptor expression. To elucidate the mechanism for this suppression, spleen cells from infected mice were added to spleen cells from normal mice in increasing numbers and activated with Con A. Significant suppression of IL-2 production was evident when compared with IL-2 in supernatants derived from normal Con A activated spleen cells. Suppressor cells responsible for this effect belonged to the B cell lineage and IL-2 production was restored when they were removed from culture. These findings intimated that suppressor B cells acted by either preventing binding of IL-2 to its receptor or by down regulation of IL-2 receptor expression on T cells (Turcotte, 1987).

Addition of exogenous IL-2 proved to be beneficial in *M.lepraemurium* or *M.bovis* BCG infections such as evidenced by Jeevan & Asherson (1988). Administration of rIL-2 60 days after infection of BALB/c mice with the above mycobacteria resulted in a ~50-85% decrease in *M.lepraemurium* organisms present in the liver, foot pads and lymph nodes after

6 months when compared with mice infected but not treated with rIL-2. In addition, mice infected with *M.bovis* BCG and treated with 100U of IL-2 significantly reduced total splenic bacterial counts when compared to controls (without rIL-2). It was postulated that IL-2 had a direct effect on macrophages which enhanced killing of mycobacteria or by increasing IFN-gamma production which in turn activated macrophage intracellular killing of mycobacteria (Jeevan & Asherson, 1988).

In summary, these results demonstrated that mycobacterial diseases suppressed IL-2 production and/or IL-2 receptor expression resulting in a decreased proliferative response. Conflicting evidence has been presented with regards to restoration of the proliferative response by the addition of exogenous IL-2. This may be due to the type of mycobacterial disease examined and the possibility that lymphocytes from patients with some mycobacterial diseases had become refractory to exogenous IL-2.

2.5. INTERLEUKIN 4 (IL-4)

2.5.1. Introduction

Interleukin 4 was initially identified by Howard *et al.* (1982) as a B cell growth factor (BCGF) and by Isaksen *et al.* (1982) as an IgG1 enhancing factor (BCDF-gamma). Later it was called B cell stimulatory factor 1 (BSF-1). When the DNA was finally cloned in 1986, in both the human and murine models, it was termed IL-4 (Yokoto *et al.*, 1986; Noma *et al.*, 1986).

Interleukin-4 has been shown to act on a variety of cells including macrophages (Stuart *et al.*, 1988), B cells (DeFrance *et al.*, 1987) and T cells (Spits *et al.*, 1987). In addition IL-4 was termed mast cell growth factor II (MCGF-II) because it promoted the growth of mast cells (Nabel *et al.*, 1981).

2.5.2. Cellular Sources of IL-4

It has been shown that the major producers of IL-4 were T cells (Nabel *et al.*, 1981), some B cell lymphomas (O'Garra *et al.*, 1989), mast cells (Brown *et al.*, 1987) and bone marrow stromal cell lines (King *et al.*, 1988). The stimuli used to produce IL-4 were mitogens (Chretien *et al.*, 1989) or antigens, eg. influenza virus (Horohov *et al.*, 1988). In the murine model the major producers of IL-4 were T_{H2} cells (T helper cell clone 2) which secreted IL-4 in response to Con A stimulation (Mosmann *et al.*, 1986). However in the human model the existence of T_{H1} and T_{H2} cells is controversial.

Parronchi *et al.* (1991 as cited by Romagnani) has shown that a clone of human T cells stimulated with *Toxocara canis* (TES) antigens secrete IL-4. It is not clear whether T_{H1} and T_{H2} cells exist in humans or whether T helper cells secrete different patterns of cytokine in response to different infectious agents. What is clear however is that human T helper cells secrete IL-4 in response to PHA, Con A and PMA (Chretien *et al.*, 1989).

2.5.3. Structure of IL-4

The IL-4 gene was located on the long arm of human chromosome 5 (Le Beau *et al.*, 1988). Interleukin-4 was initially produced as a 153 amino acid sequence within the cell (Yokoto *et al.*, 1986). However the secreted glycoprotein of Mr 15-19kDa which consisted of 129 amino acids was obtained following cleavage of the first 24 amino acids. This hydrophobic 'leader sequence' was postulated to facilitate secretion of the cytokine from the cell (Yokoto *et al.*, 1986). Human and murine IL-4 amino acid sequences shared extensive homology with the exception of ~40 amino acids and are thus closely related (Yokoto *et al.*, 1986).

2.5.4. The IL-4 Receptor

Interleukin-4 has been shown to initiate its effect through binding to cell surface receptors (Keegan *et al.*, 1991). These receptors have been found to be present on a wide range of haematopoietic cells including T, thymoma, T stem, pre-B, B and myeloid cell lines (O'Hara *et al.*, 1987). Receptor numbers on the surface of murine cells ranged from 100-5 000 sites per cell with one class of binding affinity, i.e. high affinity, identified (Idzerda *et al.*, 1990). However, receptors with both high and low binding affinity have been detected on human blood lymphocytes (Foxwell *et al.*, 1989).

Experiments utilising murine cells that expressed IL-4 receptors in large numbers, demonstrated each receptor to comprise of 3 polypeptide binding chains (Keegan *et al.*, 1991). These were of Mr 40, 70, 120-140kDa. Of these the intact 140kDa chain appeared to be neccessary for binding of IL-4 to the cell surface. Following binding, the 140kDa polypeptide was found to be more susceptible to breakdown into a 70kDa component. The significance of the 40kDa chain is as yet unknown (Keegan *et al.*, 1991).

2.5.5. IL-4 and B Cells

The expression of Ia on normal resting murine B cells appeared to be significantly increased in the presence of IL-4 (Roehm *et al.*, 1984; Callard, 1991). Expression of Ia on these cells appeared to be dependent on the dose of IL-4 and the time for which these cells were incubated with the cytokine. Other experiments demonstrated that rIL-4 enhanced the proliferative response of pre-activated B lymphocytes (DeFrance *et al.*, 1987). This was true of B cells activated with *Staphylococcus aureus* or insolubilised anti-IgM and suggested a role for IL-4 in the proliferative response to B cell mitogens and antigens.

Recently IL-4 has been implicated in playing a role in B cell migration (Clinchy *et al.*, 1991). These authors effectively demonstrated the chemotactic potential of IL-4 for B cells *in vitro*. Their findings suggest a role for IL-4 in enhancing T and B cell contact by increasing the motility of B lymphocytes.

2.5.6. IL-4 and T Cells

Spits *et al.* (1987) showed that rIL-4 promoted the growth of human T cells *in vitro*. Interleukin-4 augmented the proliferation of cells prestimulated with PHA. To exclude the effects of IL-2, T cells were activated with PHA, the IL-2 receptors blocked with an anti-Tac antibody and the cells placed in culture with IL-4. This resulted in marginal inhibition of the IL-4 mediated proliferation. In addition IL-4 stimulated the proliferation of CD4+ and CD8+ cell lines which peaked at 2-3 days. These results indicated that IL-4 acted as a T cell growth factor independent of IL-2 (Spits *et al.*, 1987).

2.5.7. IL-4 and Macrophages

Stuart *et al.* (1988) demonstrated that bone marrow derived murine macrophages expressed both Class I and Class II MHC gene products when exposed to rIL-4 or IL-4 containing supernatants. Surface expression of Class I and Class II antigens increased by 1.5-4 fold, 72 hours after incubation with IL-4. In addition, induction of Class I and Class II expression occurred at the level of mRNA. However IL-4 failed to induce the expression of Class I and Class II by WEHI-3 cells (a myelomonocytic cell line) or thioglycolate elicited peritoneal macrophages, probably due to the presence of unknown inhibitors. Another posibility is that the latter cells could be refactory to IL-4 due to long term culture or exposure *in vivo* to the same cytokine (Stuart *et al.*, 1988).

2.5.8. IL-4 and Mycobacterial Disease

Giant multinucleated cells have been observed as a feature of tuberculous granulomas (Langhans, 1868, as cited by Most *et al.*, 1990). To investigate the relationship between IL-4 and giant cell formation further, McInnes & Rennick (1988) incubated alveolar macrophages and bone marrow cells from the femurs of CBA/J mice in the presence of purified mouse rIL-4 and IL-3. The presence of IL-3 was required because these cells were shown to be dependent on IL-3. Multinucleated giant cell formation was noted only in cultures containing only IL-4 and IL-3. Interleukin-3 on its own failed to induce such giant cell formation but was necessary for culture maintenance. Giant cell formation was blocked by anti-IL-4 antibodies which indicated that IL-4 directly stimulated multinucleated cell formation (McInnes & Rennick, 1988).

Other workers have demonstrated that the growth of *M.bovis* within macrophages could be inhibited by IL-4 (Flesch & Kaufmann, 1990a). Macrophages infected with 1×10^6 live mycobacteria after 24 hours in culture in the presence of IL-4 were not found to inhibit the numbers of viable organisms determined by the growth of cell forming units. However, macrophages first infected with *M.bovis* for 18 hours and then placed in culture with IL-4 showed significant inhibition of mycobacterial growth. This occured in a dose dependent manner with 500U/ml of IL-4 found to inhibit 44% of mycobacterial growth. These findings indicated that rIL-4

activated tuberculostatic functions by an unknown mechanism in murine macrophages already infected with mycobacteria (Flesch & Kaufmann, 1990a).

Further evidence in support of the bacteriostatic role played by IL-4 was provided by Denis & Gregg (1991) who showed that rIL-4 could inhibit the growth of a virulent strain of *M.avium* in the organs of BALB/c mice. Peritoneal macrophages isolated from mice infected with *M.avium* were grown in culture in the presence of 40-500U/ml of IL-4 or in combination with 500U of interferon gamma. Interleukin-4, at these doses, significantly reduced the growth of *M.avium* between 2 and 4 days when compared to the control which contained medium (cRPMI) alone. This effect was further significantly enhanced when IL-4 and IFN-gamma were added to the culture. Although the exact mechanism of IL-4 induced bacteriostasis has remained unknown, it was proposed that IL-4 acted by enhancing phagosome-lysosome fusion in infected macrophages (Denis & Gregg, 1991). This theory was proposed since *M.avium* was known to inhibit phagosome-lysosome fusion.

2.5.9. IL-4 and Other Cytokines

Essner *et al.* (1989) examined the effect of human rIL-4 on IL-1 and TNF gene expression in human peripheral blood monocytes. Monocytes preincubated with IL-4 for between 24 and 96 hours were activated with 10ug/ml of LPS in the final 24 hours of culture. Messenger RNA levels for IL-1 and TNF from

these cells were then assessed. Interleukin-4 suppressed mRNA production of both IL-1 and TNF (Essner *et al.*, 1989). These results correlated with those of te Velde *et al.* (1990) who also showed that human rIL-4 inhibited the production and secretion of IL-1β and TNF-alpha from LPS activated human monocytes by 90-100% *in vitro*. Reduced levels of IL-1β and TNF-alpha were found to persist for 40 hours after IL-4 was added to cultures. In addition, IL-4 also inhibited the production and secretion of IL-6 by 70-85% from human monocytes under the same experimental conditions (te Velde *et al.*, 1990). The mechanism whereby IL-4 inhibits IL-1 and TNF production is unclear. However, since IL-1 has been known to induce the production of IL-6, it has been postulated that this reduced production of IL-1 subsequently resulted in decreased production of IL-6 (te Velde *et al.*, 1990). Furthermore, suppression of IL-6 production by LPS-stimulated monocytes was also shown to take place at the mRNA level when 2.5U/ml of IL-4 was added to cultures *in vitro* (Cheung *et al.*, 1990). Contrasting results have been presented by Smeland *et al.* (1989) who showed that human rIL-4 induced the production of IL-6 from purified, normal, resting B lymphocytes. One hundred units/ml of IL-4 incubated with resting B cells for 48 hours significantly enhanced production of IL-6. Since different cell populations were used in the last two studies the contrasting findings of IL-4 on IL-6 production may be an effect of the cell type used. In the former studies te Velde *et al.* (1990) examined the role of IL-4 on monocyte cytokine production

whereas the studies of Smeland *et al.* (1989) examined the effects of IL-6 on B lymphocytes. It is therefore difficult to evaluate the findings of the two studies.

Interleukin-4 has also been shown to inhibit the production of IFN-gamma (Peleman *et al.*, 1989). This effect was observed only in MN cell cultures in which IL-4 was present at the initiation of culture. Inhibition was found to be at the level of mRNA synthesis. Such findings suggested that the initial production of IL-4 resulted in inhibition of IFN-gamma release. However, if IFN-gamma production had already been initiated, addition or production of IL-4 had no effect on IFN-gamma secretion (Peleman *et al.*, 1989).

Gaya *et al.* (1991) showed that IL-4 inhibited the synthesis of IL-2 produced by human CD4+ lymphocytes stimulated with Con A. The suppression of IL-2 appeared to be directly dependent on the amount of IL-4 present in cultures. Inhibition of IL-2 occured at the level of mRNA. These results indicated that IL-4 directly inhibited IL-2 production (Gaya *et al.*, 1991).

2.6. INTERLEUKIN-6 (IL-6)

2.6.1. Introduction

Interleukin-6 was first described as interferon-ß2 (IFN-ß2) produced by fibroblasts infected with vesicular stomatitis virus (Weissenbach *et al.*, 1980). Subsequently IL-6 was termed B cell stimulatory factor 2 (BSF 2); 26kDa protein hybridoma or

plasmacytoma growth factor (HPGF or IL-HP1); hepatocyte stimulating factor (HSF); monocyte granulocyte inducer type 2 (MGI-2); and T cell activation factor (TAF) (as cited by Dawson, 1991). However, molecular analysis of the structure of these factors determined that they were all identical to IL-6 (Sehgal *et al.*, 1987).

The activities of IL-6 were found to be widespread and included: differentiation of B cells into antibody producing cells (Hirano *et al.*, 1986), augmented IgG, IgA and IgM production by B cells (Muraguchi *et al.*, 1988), co-stimulation of T cell proliferation (Habetswallner *et al.*, 1988), production of acute phase proteins (Nijsten *et al.*, 1987), and induction of fever (Kluger, 1991). Interleukin-6 was also found to augment the growth of *M.avium* (Denis & Gregg, 1990).

2.6.2. IL-6 Structure

The human IL-6 gene was found to be on chromosome 7 of the MHC (Sehgal *et al.*, 1986). Interleukin-6 was produced as a 212 amino acid polypeptide chain of which 28 amino acids formed a hydrophobic signal sequence which was lost during secretion (Hirano *et al.*, 1986). The secreted 26kDa cytokine consisted of 184 amino acids with two potential N-glycosylation sites and four cysteine residues (Hirano *et al.*, 1986). The four cysteine residues, as well as amino acids 56-65, were found to be conserved when comparison between murine and human IL-6 amino acid sequences were made (Tanabe *et al.*, 1988). This indicated conservation of ~42% between human and murine IL-6 (Tanabe *et al.*, 1988).

2.6.3. Production and Stimulation of IL-6

Many different cell populations have been shown to produce IL-6 (Kishimoto, 1989). These included T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells and tumour lines such as the myelomas and plasmacytomas (Kishimoto, 1989). The production of IL-6 in these various cells can be induced by a number of stimuli (Kishimoto, 1989). Interleukin-6 mRNA production was shown to be induced in macrophages after 5 hours in culture in the apparent absence of a stimulus, whilst B and T lymphocytes expressed IL-6 mRNA which peaked at 48 hours after stimulation with PHA or *Staphylococcus aureus* Cowan strain 1 (SAC) (Horii *et al.*, 1988). Lipopolysaccharide was also found to strongly enhance IL-6 mRNA production in macrophages (Horii *et al.*, 1988). Viruses, for example the HIV, was also observed to stimulate IL-6 mRNA and production of the cytokine in MN cells isolated from HIV positive patients, while IL-6 levels in their sera were also elevated (Breen *et al.*, 1990). In addition, IL-1 and TNF also rapidly stimulated the production of IL-6 in mice (Shalaby *et al.*, 1989).

2.6.4. The IL-6 Receptor

The effects of IL-6 on target cells was shown to be mediated by interaction of the cytokine with IL-6 receptors (Yamasaki *et al.*, 1988). These receptors of Mr 80kDa were expressed by a number of cells which included B cells, T cells, myeloma and hepatoma cell lines (Kishimoto, 1989). Receptors expressed

varied in number and binding affinity on cell types examined (Coulie *et al.*, 1989). IL-6 receptors were found to be both high and low affinity receptors, although low affinity receptors outnumbered high affinity receptors by ~50:1 (Yamasaki *et al.*, 1988). Further examination of the receptor structure indicated that it was produced as a 468 amino acid sequence which included a signal sequence of ~19 amino acids and a cytoplasmic sequence of ~82 amino acids (Yamasaki *et al.*, 1988). Murine T cells were found to express high affinity receptors only, while plasmacytomas and hybridomas expressed both high and low affinity receptors (Coulie *et al.*, 1989). These results correlated with those of Taga *et al.* (1987) who examined activated human T and B cells which were only found to express high affinity receptors. Normal resting T cells expressed ~100-1 000 receptors per cell while IL-6 receptors could not be detected on the surface of resting B cells (Taga *et al.*, 1987).

2.6.5. The Effect of IL-6 on T Cells

Habetswallner *et al.* (1988) demonstrated that 5-10ng of human rIL-6 enhanced the proliferation of purified T cells in response to PHA and 2U/ml of IL-2. This effect was compared with T cells incubated in the presence of PHA and IL-2 alone. PHA activated T cells incubated in the presence of IL-6 demonstrated enhanced proliferation compared to control systems (Habetswallner *et al.*, 1988). These findings indicated that IL-6 markedly potentiated the proliferative effect of suboptimal amounts of IL-2 on PHA stimulated T lymphocytes (Habetswallner *et al.*, 1988).

Houssiau *et al.* (1989) extended these studies using small tonsillar human T cells and showed that rIL-6 induced a significant increase in cell size and protein synthesis. Once this increase in cell size had occurred T cells were observed to become more responsive to small amounts of IL-2. In addition these lymphocytes were found to produce their own IL-2 in response to activation with IL-1. Cell enlargement due to IL-6 was accompanied by a 5-20 fold increase in protein synthesis and cell area increased from $94\pm20u^2$ to $131\pm42u^2$. Thus it appeared that IL-6 was able to induce T cells to move from G_o to the G_i phase of the cell activation cycle. This primed cells for entry into the S-phase or proliferative phase of the cell cycle which was induced by IL-2 (Houssiau *et al.*, 1989).

2.6.6. IL-6 and B Cells

Muraguchi *et al.* (1988) examined the effect of rIL-6 on PWM stimulated B cell differentiation. B cells were derived from tonsillar and peripheral blood. Recombinant IL-6 at a concentration of 1-10ng/ml augmented the *in vitro* production of IgG, IgA and IgM in a dose dependent manner by ~3-10 fold. This effect was blocked when anti-IL-6 antibodies were employed in the system. Interleukin-6 was found to act on antibody production, a late phase of the B cell response. Addition of anti-IL-6 antibodies 4 days after initiation of culture also resulted in abrogation of antibody production. Interleukin-6, however, had no proliferative effect or antibody inducing

effects on unstimulated B cells. These results indicated that IL-6 acted on the late phase of the activated B cell response and augmented antibody production (Muraguchi *et al.*, 1988).

Similar findings were described by Lue *et al.* (1991) who used peripheral blood B cells from human volunteers immunised with polyvalent pneumococcal vaccine or diptheria toxoid. Approximately one week later antigen specific antibody secreting B cells were detected in peripheral blood and incubated in the presence of rIL-6. Recombinant IL-6 increased the frequency of antigen secreting cells as well as the amount of antibody produced in a dose dependent manner. This effect was blocked by anti-IL-6 polyclonal antiserum. These results demonstrated that locally produced IL-6 promoted the terminal differentiation of antigen activated B cells and resulted in antibody production (Lue *et al.*, 1991).

2.6.7. IL-6 and Bacterial Infection

Fong *et al.* (1989a) examined the ability of 20U/kg of LPS injected intravenously into human volunteers to induce the production of IL-6. Serum levels of IL-6 peaked at 2-4 hours after endotoxin challenge as determined by the production of alpha 1-antichymotrypsin by a hepatoma cell line (Hep 3B clone 2) in response to IL-6. Twenty hours after LPS administration c-reactive protein (CRP) levels of 1,7±0,3mg/dl were detected in the serum of all 6 volunteers. These findings indicated that challenge with LPS resulted in a rapid IL-6 response which, in

turn, may have been responsible for the clinical and immunologic response observed in bacterial infection (Fong *et al.*, 1989a). Furthermore, Helfgott *et al.* (1989) noted that raised IL-6 levels were found in the CSF and serum of patients with acute bacterial meningitis as well as the ankle 'fluid' of a patient with *Streptococcus canus* arthritis. These results suggested that IL-6 participated in the host response to bacterial infections (Helfgott *et al.*, 1989). However extremely high levels of serum IL-6 in meningococcal septic shock was associated with poor prognosis (Waage *et al.*, 1989). Interleukin-6 could be detected in 69 of 79 patients with meningococcal meningitis. Patients with septic shock had serum IL-6 levels of 189ng/ml as compared with 0.2ng/ml in patients with meningitis and 0.05ng/ml in healthy controls. All patients with IL-6 serum levels ≤3.0ng/ml survived while 11 out of 21 patients with >3ng/ml died. In addition, all 4 patients with IL-6 levels >750ng/ml died. It thus appeared that high levels of IL-6 in meningococcal shock were associated with fatal outcome whilst those with low levels of IL-6 survived (Waage *et al.*, 1989).

2.6.8. IL-6 and Acute Phase Proteins

Nijsten *et al.* (1987) investigated the involvement of IL-6 in the acute phase response of patients with severe burns. Interleukin-6 and the acute phase proteins, alpha-2-macroglobulin, c-reactive protein (CRP) and alpha-1-antitrypsin (AAT) in serum were closely monitored on admission, at 24 hours

and 2 months later. Interleukin-6, as determined by ^{3}H-thymidine incorporation into IL-6 dependent cell lines on admission was found to be 2-100 fold greater than normal levels and began to decline by 24 hours. However, AAT and CRP rose slowly after admission and increased serum levels were detected at 24 hours. It was therefore postulated that raised IL-6 levels were responsible for the increase in acute phase proteins found in the serum (Nijsten *et al.*, 1987). These findings were confirmed by Ramadori *et al.* (1988) who showed that purified human IL-6 placed in culture with human hepatocytes for 20 hours increased the production of C3 and ceruloplasmin while albumin production decreased. This IL-6 effect was found to be dose dependent and could be blocked with anti-IL-6 antiserum. In addition, IL-6 injected into endotoxin resistant C3H/HeJ mice resulted in an increase in serum amyloid A (SAA) in a dose dependent manner, 2 hours after injection. These findings indicated that IL-6 directly induced the synthesis of specific acute phase proteins in hepatocytes (Ramadori *et al.*, 1988).

2.6.9. IL-6 and Fever

Helle *et al.* (1988) observed that when 5ug/Kg of human rIL-6 was injected intravenously into rabbits it induced a monophasic fever peak. Rabbits injected with IL-6 were shown to have raised temperatures of ~1.2^{o}C which peaked at ~1 hour when compared with rabbits injected with phosphate buffered saline containing bovine serum albumin. Since rIL-6 has been

shown to induce the production of IL-6 in human thymocytes, IL-6 may have been responsible for the induction of fever previously attributed to IL-1 (Helle *et al.*, 1988).

Further evidence supporting the role of IL-6 in fever was provided by Frei *et al.* (1988) who showed that mice innoculated intracerebrally with lymphocytic choriomeningitis virus (LCMV) produced BSF-β2 (IL-6) in both sera and CSF. Interleukin-6 levels in CSF were found to be ~60 fold more concentrated in CSF than in sera of mice at 4-6 days after infection. These findings indicated that IL-6 is produced within the CNS during viral meningitis infection (Frei *et al.*, 1988). In addition, Kluger (1991) has shown that pre-treatment of rats with small amounts of anti-IL-6 antiserum resulted in significant reduction of LPS induced fever. These findings suggested a role for IL-6 in the induction of fever (Kluger, 1991).

2.6.10. IL-6 and Mycobacterial Disease

The role of rIL-6 in mycobacterial diseases was investigated by Flesch & Kaufmann (1990b) who showed that rIL-6 activated antimycobacterial function in murine bone marrow-derived macrophages previously infected with *M.bovis in vitro*. This occurred in a dose dependent manner and inhibited the intracellular growth of *M.bovis* by 55% at 100U/ml of rIL-6. However pre-incubation of macrophages with IL-6 followed by subsequent infection with *M.bovis* failed to inhibit

mycobacterial growth. It was postulated that within the granuloma IL-6 preferentially affected macrophages which harboured mycobacteria (Flesch & Kaufmann, 1990b).

In contrast to these results, Denis & Gregg (1990) observed that rIL-6 resulted in enhanced growth of *M.avium*, an effect which was abrogated by anti IL-6 antiserum. Human monocytes were treated with 100U of IL-6, 24 hours before infection with *M.avium*. Growth of *M.avium* was determined by cell forming units after incubation on *M.avium* agar for 21 days. In addition, rIL-6 directly enhanced the growth of *M.avium* organisms in the absence of macrophages or other cells. This effect was reversed with anti-IL-6 antiserum. These findings therefore indicated that *M.avium* utilised IL-6 as a growth factor. Thus the production of IL-6 would have been detrimental to the host (Denis & Gregg, 1990). It has been suggested by Huygen *et al.* (1991) that conflicting results obtained by Flesch & Kaufmann (1990b) and Denis & Gregg (1990) could have been due to differences in the cell populations and time kinetics studied by the two groups.

However the findings of Denis & Gregg (1990) correlated well with those of Shiratsuchi *et al.* (1991) who showed that rIL-6 enhanced the growth of 2 different strains of *M.avium* in human monocyte cultures. Monocytes precultured with *M.avium* were incubated in the presence of rIL-6 which enhanced the intracellular growth of *M.avium* in a dose dependent manner. Furthermore IL-6 significantly enhanced the

growth of *M.avium* in cell free culture after 7 days when compared to *M.avium* cultured in cRPMI or the cytokines IL-1 alpha, IL-1ß, IL-2, IL-3, IL-4 and IFN-gamma. Such findings lend credibility to the theory that IL-6 enhanced the growth of *M.avium* to the detriment of the host (Shiratsuchi *et al.*, 1991). Similar findings were described by Denis *et al.* (1991a) who proposed that human monocytes infected with *M.avium* secreted IL-6 (and IL-1ß). This in turn enhanced the growth of *M.avium.* Mycobacterial components themselves have also been shown to stimulate IL-6 release (Huygen *et al.*, 1991). Spleen cells isolated from BALB/c mice previously injected with 10-100ug/ml of *M.bovis* BCG produced higher levels of IL-6 when stimulated with PPD bacterial extract or culture filtrate antigens. This was compared to IL-6 levels produced by spleen cells isolated from untreated BALB/c mice and stimulated with the same mycobacterial antigens. Unstimulated spleen cells incubated in medium alone produced low levels of IL-6 *in vitro.* These results indicated that *M.bovis* components potently stimulated IL-6 production in both BCG treated and untreated mice (Huygen *et al.*, 1991).

2.6.11. The Effect of IL-6 on IL-1 and TNF Production

Schindler *et al.* (1990) showed that rIL-6 suppressed the production of IL-1ß and TNF production in human mononuclear cells stimulated with LPS or PHA *in vitro.* IL-6 induced suppression was found to be dose dependent and maximal suppression of IL-1ß and TNF was achieved with 100ng/ml of

IL-6. Furthermore IL-6 suppressed LPS and PHA induced production of IL-1 and TNF mRNA which indicated that suppression occurred at the level of transcription (Schindler *et al.*, 1990). It is possible that IL-6 may have provided a negative feedback signal which in turn could have inhibited IL-1ß and TNF production.

These results were supported by Aderka *et al.* (1989) who also showed that rIL-6 inhibited LPS-induced production of TNF by cultured monocytes. Preincubation of monocytes with IL-6, 24 hours before LPS stimulation suppressed TNF mRNA production more effectively than if IL-6 and LPS were added simultaneously to monocytes. This simultaneous addition of IL-6 and LPS to monocyte cultures also resulted in a 45-50% inhibition of TNF. In addition, mice injected with IL-6, 4 hours prior to injection with LPS showed significant inhibition of TNF production (42±20pg) whereas control mice injected with LPS only were found to have TNF levels of 163.5±58pg/ml. These results indicated that IL-6 inhibited TNF production *in vitro* and *in vivo*. Furthermore the IL-6-induced inhibition of TNF production reflected an anti-inflammatory function of IL-6 (Aderka *et al.*, 1989).

Based on these findings, once high levels of IL-6 were secreted it then acted on monocytes or macrophages to inhibit the production of TNF alpha and IL-1.

2.7. GAMMA INTERFERON (IFN-GAMMA)

2.7.1. Introduction

Wheelok (1965) first described IFN-gamma as an antiviral agent present in supernatants derived from PHA stimulated human leukocyte cultures. Biological properties of the cytokine included the induction of Class II antigens on macrophages (Cao *et al.*, 1989); the killing of intracellular parasites by macrophages activated with IFN-gamma (Nathan *et al.*, 1983); production of selective antibodies (IgG2a) by B cells (Finkleman *et al.*, 1988) as well as the inhibition of growth of the HeLa cell line and osteosarcoma tumours in culture (Rubin & Gupta, 1980).

2.7.2. Cellular Sources of IFN-gamma

Human T lymphocytes were found to produce IFN-gamma following stimulation with OKT3 antibodies *in vitro* (Anderson *et al.*, 1986). These included both T4+ (CD4+) and T8+ (CD8+) lymphocytes. Other producers of this cytokine were natural killer cells (Leu 7+ or B73.1+) (Andersson *et al.*, 1986). At the same time Mosmann *et al.* (1986) demonstrated the production of IFN-gamma and IL-2 by murine (T_{H1}) cell clones. More recently T cell clones producing IFN-gamma and IL-2 have been isolated from human subjects (Del Prete *et al.* as cited by Romagnani, 1991).

Stimuli used to induce the secretion of IFN-gamma *in vitro* were mitogens (PHA), viral antigens (Epstein Barr Virus)

(Sandvig *et al.*, 1987), bacterial antigens (*Listeria monocytogenes*) (Havell *et al.*, 1982) and PPD (Del Prete *et al.* as cited by Romagnani, 1991). In addition, IL-2 has been shown to stimulate the production of IFN-gamma from resting or Con A activated human T4+ and T8+ cells *in vitro* (Kasahara *et al.*, 1983).

2.7.3. Structure of IFN-gamma and Gene Location

The human IFN-gamma gene has been located on chromosome 12 of the MHC (Trent *et al.*, 1982). Gamma interferon of Mr 20kDa and 25kDa was shown to consist of 146 amino acids after secretion from the cell (Rinderknecht *et al.*, 1984). Secretion is believed to be facilitated by a hydrophobic 'leader sequence' found to consist of 20 amino acids which is cleaved from the cytokine during secretion. The protein was shown to be glycosylated in 2 sites which accounted for the different sizes of IFN-gamma. However the significance of such glycosylation has remained unknown (Rinderknecht *et al.*, 1984).

2.7.4. The IFN-gamma Receptor

Gamma interferon has been shown to exert its effects on target cells by binding to specific high affinity receptors (Dawson, 1991). These receptors were found to be present on many human cells including peripheral blood MN cells (Orchansky *et al.*, 1986) and fibroblasts (Anderson *et al.*, 1983). Receptor

numbers varied from ~2 400 on human fibroblasts to ~20 000 present on WISH amnion cell lines (Orchansky *et al.*, 1986).

Two forms of the IFN-gamma receptor have been found to exist (Orchansky *et al.*, 1986). Although receptors on both WISH cells and macrophages bound ^{125}IFN-gamma, receptors expressed by macrophages appeared to bind IFN-gamma with different affinities as determined by Scatchard analysis. In addition, acid treated IFN-gamma was shown to block the receptors on WISH cells but not on macrophages. This acid treated IFN-gamma was found to induce the expression of HLA DR on WISH cells only but not on monocytes. Based on these findings, the authors concluded that two types of receptors existed, one on monocytes and the other on non-haematopoietic cells (Orchansky *et al.*, 1986).

2.7.5. IFN-gamma and Macrophages

The effect of IFN-gamma as a macrophage activating factor was initially described by Nathan *et al.* (1983). Partially purified IFN-gamma, rIFN-gamma and IFN-gamma present in Con A stimulated lymphoid supernatants enhanced the peroxide releasing capacity of human monocytes by ~9 fold *in vitro*. This effect was blocked by antibodies to IFN-gamma. In addition, incubation of macrophages in the presence of IFN-gamma for 3 days resulted in the enhanced ability to kill *Toxoplasma gondii (T.gondii) in vitro*. Replication of *T.gondii* within macrophages was also totally inhibited when macrophages

were pre-incubated with IFN-gamma (Nathan *et al.*, 1983). Furthermore, 100U/ml of murine rIFN-gamma stimulated murine peritoneal macrophages to kill greater numbers of tumour cells *in vitro* (Svedersky *et al.*, 1984). Anti-tumour activity was prevented when anti-IFN-gamma antibodies were added to cultures. This implicated IFN-gamma as an activator of macrophage tumouricidal activity in addition to its ability to increase the macrophage antibactericidal ability (Svedersky *et al.*, 1984).

Murine rIFN-gamma has also been shown to induce the expression of Class II MHC (Ia) antigens on murine peritoneal macrophages (Cao *et al.*, 1989). Sixty to eighty percent of IFN-gamma activated macrophages expressed Ia which peaked at 48 hours and remained at this level for 6 days.

Sihvola & Hurme (1989) showed that IFN-gamma potentiated the production of IL-1 from human macrophages stimulated with suboptimal doses of LPS *in vitro*. Gamma interferon had to be present at the beginning of culture. However, in contrast to these findings, IFN-gamma inhibited the production of IL-1 in macrophages stimulated with silica dust. Such results suggested that IFN-gamma either enhanced or decreased IL-1 production depending on the stimulus. This implied that the effects of IFN-gamma on macrophage IL-1 production is dependent on the nature of the invading organism as evidenced by these results (Sihvola & Hurme, 1989).

2.7.6. IFN-gamma and B Cells

Gamma interferon has been shown to induce the secretion of antibodies by normal resting murine splenic B cells and the B cell tumour line WEHI-2791 (Sidman *et al.*, 1984). This effect was blocked by antibodies to IFN-gamma and indicated that IFN-gamma may mature resting B cells into antibody secreting plasma cells (Sidman *et al.*, 1984). Later Finkelman *et al.* (1988) showed that BALB/c mice injected with goat-anti-mouse IgD (GaMD) significantly increased serum levels of IgG1 and IgE. Gamma interferon injected 2-5 days after administration of GaMD inhibited the production of IgG1 and IgE whilst at the same time stimulated the production of IgG2a. Furthermore mice injected with the bacterium *Brucella abortus* resulted in IFN-gamma production and subsequent IgG2a production. This IgG2a production was suppressed by the injection of anti-IFN-gamma antibodies while at the same time IgG1 antibody production was enhanced. These results implicated IFN-gamma as an inhibitor of IgG1 and IgE production and a possible potentiator of IgG2a production (Finkelman *et al.*, 1988).

2.7.7. IFN-gamma and Mycobacterial Components

Mycobacterial components themselves blocked the ability of IFN-gamma to activate macrophages. Sibley *et al.* (1988) showed that lipoarabinomannan (LAM) derived from the walls of *M.leprae* and *M.tuberculosis* inhibited IFN-gamma mediated activation of murine macrophages *in vitro.* Macrophages incubated in the presence of LAM for 24 hours and then activated with IFN-gamma failed to restrict the growth of *T.gondii*

when compared with the control which consisted of macrophages which were not treated with LAM. However this inhibition was only observed when sufficient LAM had accumulated within the cytoplasmic vacuoles of the macrophages. These results indicated that LAM inhibited IFN-gamma mediated activation of macrophages which was the mechanism responsible for defective macrophage activation observed in macrophages isolated from mycobacterial granulomas (Sibley *et al.*, 1988).

2.7.8. IFN-gamma and Mycobacterial Diseases

Flesch & Kaufmann (1987) demonstrated that when murine derived bone marrow macrophages were first activated with rIFN-gamma for 24 hours and later infected with either *M.bovis* BCG Phipps strain or *M.tuberculosis* strain H37RV, growth of these organisms was reduced by 96%. This effect was blocked when anti-IFN-gamma antibodies were added to cultures. Since the growth of *M.bovis* was almost totally inhibited by hydrogen peroxide (H_2O_2), it was postulated that IFN-gamma activated H_2O_2 production, which resulted in reduced *M.bovis* growth. However the addition of catalase, an H_2O_2 scavenger, during culture did not reverse the inhibition of *M.bovis* growth. This indicated that IFN-gamma suppressed the growth of *M.bovis* by an H_2O_2 independent mechanism (Flesch & Kaufmann, 1987).

When poly-D-glutamic acid, a polybasic anion which inhibited phagosome-lysosome fusion, was added to mycobacterial infected

macrophages, inhibition of mycobacterial growth remained intact. Chloroquine, a tertiary amine which enhanced phagosome-lysosome fusion but did not affect the viability of macrophages or *M.bovis* at concentrations used (5ug/ml), significantly inhibited the growth of *M.bovis* in unstimulated macrophages. These findings suggested that IFN-gamma inhibited mycobacterial growth by enhancing phagosome-lysosome fusion rather than due to stimulation of the oxidative burst (Flesch & Kaufmann, 1988). Findings presented by Wadee *et al.* (1987) showed that IFN-gamma reversed the inhibition of phagosome-lysosome fusion in human PMN cells and monocytes induced by a 25kDa glycolipoprotein.

Another activity ascribed to IFN-gamma was described by Denis (1991) who showed that mice continuously infused with murine rIFN-gamma showed increased resistance to a lethal dose of *M.tuberculosis*. This was associated with decreased microbial growth in the spleen and liver, an effect which was abrogated by the infusion of anti-IFN-gamma antibodies. To investigate the role of IFN-gamma in the development of acquired resistance, mice were infected with *M.tuberculosis* which was later cleared by isoniazid. Approximately 3 weeks after resolution of the infection mice were reinfected with *M.tuberculosis* in the presence or absence of anti-IFN-gamma antibodies. Acquired resistance was decreased by anti-IFN-gamma antibodies and mice were unable to limit bacterial multiplication. These results suggested that IFN-gamma was an important mediator in acquired resistance to *M.tuberculosis*. The mechanism of the role of IFN-gamma in acquired resistance remains to be elucidated.

Gamma interferon, injected intradermally into skin lesions of lepromatous patients, reduced the numbers of *M. leprae* bacilli by 5-10 000 fold within 21 days (Kaplan *et al.*, 1989). Gamma interferon administration was accompanied by an infiltrate of monocytes and T cells into the skin which remained elevated for 3 weeks. Of these, CD4+ cells were found to predominate in contrast to untreated lesions where CD8+ cells predominated. In addition keratinocytes overlying the injection site synthesised and expressed large amounts of Class II MHC antigens. It was postulated that IFN-gamma enhanced the cellular response, an effect which persisted long after the cytokine had been cleared from the site (Kaplan *et al.*, 1989).

These results were in contrast to a study by Mor *et al.* (1989) who showed that IFN-gamma enhanced the growth of *M. lepraemurium* in murine peritoneal macrophages *in vitro*. Gamma interferon decreased macrophage phagocytosis when compared with macrophages which were not treated with IFN-gamma. *M. lepraemurium* growth increased by ~6 fold in the first 12 days when incubated with control macrophages. However when IFN-gamma was added to the culture system in a dose dependent manner, *M. lepraemurium* grew by ~18-24 fold. In addition, this cytokine had no direct effect on the growth of *M. lepraemurium in vitro*. Since it was observed that IFN-gamma stimulated macrophages it was postulated that the cytokine enhanced endocytosis of nutrients from the medium which

were delivered to the phago-lysosomal compartments. This in turn promoted the growth of *M.lepraemurium* in macrophages (Mor *et al.*, 1989).

Similar results were reported by Toba *et al.* (1989) who showed that IFN-gamma enhanced the replication of *M.avium* in human peripheral blood macrophages *in vitro*. This was accompanied by a decreased capacity for phagocytosis (Toba *et al.*, 1989). Although conflicting results have been presented, differences may be explained by the different strains of mycobacterial organisms used in the various studies. In addition, detrimental findings induced by IFN-gamma were all reported in *in vitro* experiments whilst the beneficial effects of IFN-gamma therapy were reported *in vivo*.

2.8. AIMS OF THIS PROJECT

It has previously been demonstrated that when cultured human peripheral blood adherent cells ingest mycobacteria, they release suppressor cell activating factors (SCAF) which induce CD8+ cells to inhibit lymphocyte proliferation and lymphokine production (Wadee *et al.*, 1980; 1983a,b). Subsequent studies demonstrated that these SCAF molecules were lipids of mycobacterial origin (Wadee *et al.*, 1980).

The present study was thus undertaken to examine the effects of factors derived from *M.tuberculosis* on CD8+ cell functions as well as factors derived from CD8+ cells, their effects on cytokine production and HLA-DR expression.

CHAPTER 3
MATERIALS & METHODS

3.1. PREPARATION OF MYCOBACTERIAL SONICATES

M.tuberculosis organisms were cultured and grown on Lowenstein-Jensen slopes from human sputum positive individuals for 2-3 weeks at 37^0C. Colonies were scraped off into physiological saline, pooled and centrifuged at 3 000 x g for 30 minutes, then washed three times for 15 minutes at 3 000 x g. After autoclaving, colonies were homogenised, sonicated in a MSE Soniprep 150 Ultrasonic disintegrator (MSE Scientific Instruments, Sussex, UK), centrifuged for 15 minutes, and the supernatant containing soluble mycobacterial components collected. A protein estimation of these was performed using the Biorad protein estimation kit (Biorad Laboratories, Richmond, California, USA) and the concentration adjusted to 1mg/ml, aliquoted and stored at -20^0C until used.

3.2. BACTERIAL PREPARATION

Listeria monocytogenes, Escherichia coli, Escherichia coli enterobacteriaceae, Staphylococcus aureus and *Nocardia asteroides* were cultured by the Microbiology Department, SAIMR. Colonies were scraped off into physiological saline, autoclaved and washed three times in physiological saline. This was followed by centrifugation for 15 minutes at 3 000 x g. Bacterial sonicates were prepared in the same manner as described above.

3.3. PREPARATION OF *SACCHAROMYCES CEREVISIAE* (BAKER'S YEAST)

Commercially available Baker's yeast was prepared by suspending a small pellet in physiological saline, washing 3 times by centrifuging 50ml suspensions at 400g and resuspending in saline each time. The yeast was killed by heating at 80°C for 1 hour. Organisms were then washed with saline, resuspended to a concentration of 6 x 10^7/ml in saline and stored at -20°C until used.

3.4. MONONUCLEAR (MN) CELL SEPARATION

Peripheral venous blood from normal, healthy volunteers (who had given their consent previously) was collected into heparinised bottles (10U/ml Heparin Sodium - Evans, Sigma, St Louis, USA). The whole blood was separated by sedimentation on Hypaque-Ficoll gradients (Sigma, St Louis, USA), the mononuclear fraction at the plasma-Ficoll interphase was collected, the cells washed three times in physiological saline and resuspended in RPMI 1640 (Highveld Biologicals, Johannesburg, RSA) containing 10% heat inactivated (56°C for 30 minutes) foetal calf serum (cRPMI) (Highveld Biologicals, Johannesburg, RSA). MN cells were adjusted to 1 x 10^6/ml and stored on ice until used.

3.5. PREPARATION OF HUMAN ADHERENT CELLS

MN cell suspensions were separated into adherent and non-adherent cell populations as follows:

MN cells were suspended at 10 x 10^6/ml in cRPMI and allowed to

adhere for one hour onto a plastic petri dish in a 37°C incubator containing 5% CO_2. Non-adherent cells were then removed using physiological saline at 37°C. Adherent cells were resuspended using a sterile rubber tipped syringe plunger and cRPMI at 4°C, after which cells were dispensed into a 50ml tube, washed twice in cRPMI at 4°C and the pellet resuspended in cRPMI and stored on ice until used.

3.6. PRODUCTION OF SUPPRESSOR CELL ACTIVATING FACTOR (SCAF)

Adherent cells at a concentration of 4 x 10^6/ml were incubated in sterile tubes (Sterilin, Hounslow, UK) for 2 hours at 37°C with and without *M.tuberculosis* sonicates at a predetermined concentration of 10ug/ml. At the end of this period the medium was discarded and the cells washed twice in cRPMI at 4°C and reconstituted to their original volume. Cultures were incubated for 24 hours and the supernatants collected and stored at -20°C until assessed for suppressor activity. Supernatants derived from monocytes pulsed with *M.tuberculosis* sonicates were termed "monocyte suppressor supernatants" while those derived from monocytes incubated in the presence of cRPMI were termed "monocyte control supernatants".

3.7. LYMPHOCYTE TRANSFORMATION

MN cells or adherent cell depleted lymphocytes (NAL's) were cultured in 96 well round bottom microtitre plates (Sterilin, Hounslow, UK) containing 1 x 10^6 cells/well. Cells were

stimulated with the mitogens phytohaemagglutinin (PHA - Wellcome Diagnostics, Dartford, UK), or concanavalin A (Con A - Pharmacia, Upsalla, Sweden), or with the antigens *Candida albicans* (Hollister-Stier Laboratories, Elkhart, USA), purified protein derivative of *M.tuberculosis* (PPD - Connaught Laboratories, Toronto, Canada) or SKSD (Lederle Laboratories, New York, USA). All experiments were performed in triplicate. Plates were incubated at 37°C in 5% CO_2 in humidified air for 72 hours (mitogens) or 5 days (antigens). Tritiated thymidine (methyl- ^{3}H-thymidine, specific activity 24Ci/mmole - Amersham, Buckinghamshire, UK) at 1uCi/well was added for the final 18 hours of culture, after which they were harvested onto glass fibre filters (Flow Laboratories, Lier, Norway) using an automated cell harvester 550 (Flow Laboratories, Irvine, California), washed with distilled water and allowed to dry. Five millilitres of Instafluor (Packard, Illinois, USA) was added and radioactivity measured using a LKB 1217 RackBeta liquid scintillation spectrometer (Bromma, Sweden). Counts were expressed as counts per minute (CPM) and compared with control radioactive counts obtained from cells in culture with medium alone.

3.8. ASSESSMENT OF THE EFFECTS OF BACTERIAL SONICATES ON LYMPHOCYTE TRANSFORMATION

Bacterial sonicates at a concentration of 10ug/ml were added to mitogen or antigen stimulated MN cells or lymphocytes at the initiation of culture. Tritiated thymidine was added for the final 18 hours of culture and cells were harvested as described

above. Counts per minute (CPM) of radioactivity incorporated were compared with the control radioactive counts and suppression expressed as a percentage of control systems.

In all experiments where suppressor effects of lymphocyte blastogenesis were assessed, suppression was calculated according to the formula:

$$\text{Percent suppression} = \frac{\text{CPM of control cultures} - \text{CPM of test cultures}}{\text{CPM of control cultures}} \times 100$$

3.9. ACTIVATION OF SUPPRESSOR LYMPHOCYTES BY MONOCYTES PULSED WITH *M. TUBERCULOSIS*

Adherent cells were incubated with *M.tuberculosis* sonicates at a concentration of 10ug/ml for 2 hours. Control systems contained adherent cells in medium alone. The supernatants were discarded and the adherent cells washed twice and reconstituted to 1 x 10^6/ ml in cRPMI. The adherent cells were then added to NAL's from the same donor in the ratio of 1 adherent cell to 3 NAL's. The cultures were incubated in microtitre well plates for the assessment of lymphocyte blastogenesis.

3.10. INCUBATION OF MN CELLS IN THE PRESENCE OF *M. TUBERCULOSIS* SONICATES AND INDOMETHACIN

MN cells were incubated in the presence of PHA, Con A and PPD. At the initiation of culture *M.tuberculosis* sonicates were added to cultures in the presence or absence of a

predetermined 1ug/ml of indomethacin. Cells were harvested after the addition of tritiated thymidine and radioactive counts assessed as previously described (Section 3.7).

3.11. ETHER TREATMENT OF *M.TUBERCULOSIS* SONICATES

Ten millilitres of *M.tuberculosis* sonicates (1mg/ml) were mixed with 2.5 volumes of anaesthetic ether (Natal Cane By-Products Ltd., Merebank, RSA). The mixture was frozen at -70°C, thawed and the interface containing the lipid removed. This was repeated until lipid was no longer observed at the interface. Lipid samples were pooled and the ether removed by evaporation over nitrogen. The lipid was dissolved in 1ml of ethanol (BDH, Poole, UK) and the suspension reconstituted to the original starting volume in physiological saline. This was followed by dialysis against three changes of distilled water and three changes of PBS. The remaining delipidated fractions were reconstituted in saline to their starting volume. Both delipidated and lipid fractions were assessed for their ability to suppress lymphocyte blastogenesis.

3.12. CHEMICAL TREATMENT OF *M.TUBERCULOSIS* SONICATES

Five millilitres of *M.tuberculosis* sonicates (1mg/ml) were individually incubated at room temperature for 1 hour with 1ug/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), 1ug/ml aprotinin (Sigma, Illinois, USA), 1ug/ml chymotrysinogen (Boehringer Mannheim, Mannheim, Germany) or 150U/ml

neuraminidase (Sigma, Illinois, USA). At the end of the incubation period, all solutions were mixed vigorously after which they were dialysed against distilled water (3 x 2l each at 4^0C) and PBS (3 x 2l each at 4^0C) for 48 hours. They were then reconstituted to their starting volumes and assessed for their ability to suppress lymphocyte blastogenesis. Carbohydrate moieties were degraded according to the method of Owhashi *et al.* (1983). Nine millilitres of *M.tuberculosis* sonicates (1mg/ml) were incubated with 1ml of sodium metaperiodate (0.05M - Merck, Darmstadt, Germany) in the dark for 72 hours at 4^0C. In later experiments, lymphocyte derived supernatants were chemically treated in the same way as described here. Supernatants were produced as described in Section 3.19.

3.13. THE EFFECT OF HEAT TREATMENT ON *M.TUBERCULOSIS* SONICATES

To assess the effects of heat on *M.tuberculosis* sonicates, 1ml aliquots of *M.tuberculosis* sonicates (1mg/ml) in McCartney bottles were heated at 37^0C for 24 hours or 42^0C, 65^0C, 100^0C or 121^0C for 30 minutes, after which they were cooled and stored at 4^0C until used.

3.14. FRACTIONATION OF *M.TUBERCULOSIS* SONICATES

Mycobacterial sonicates were separated into a number of fractions according to the method of Wadee *et al.* (1987). Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden)

was packed in a 96 x 1.5cm (Pharmacia) column. The column was equilibrated with PBS (pH 7.4) at a flow rate of 1ml/minute for 3 days at 4°C. Cytochrome C (from horse heart - Sigma Chemical Co., St Louis, Missouri, USA) was passed through the column to ensure that even packing of the column had occurred. Blue dextran T 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for the determination of the void volume. Proteins greater than 200kDa were excluded by the Sephacryl S-200 column and passed through in the void volume at 100ml.

Proteins of known molecular weight were used to standardise the column. These included: ribonuclease (14kDa), chymotrypsin (25kDa), ovalbumin (43kDa), bovine serum albumin (BSA) (67kDa), aldolase (158kDa), catalase (232kDa), ferritin (440kDa) and thyroglobulin (669kDa) (all from Pharmacia Fine Chemicals, Uppsala, Sweden). Molecular weight was determined by plotting the log of the molecular weight of the proteins against the elution volume according to the method described by Andrews *et al.* (1964) (Figure 2). Proteins were dissolved in PBS to a final concentration of 5mg/ml, 1ml was layered onto the column and the fractions were collected with a LKB fraction collector (LKB Bromma 7000, Stockholm, Sweden). Once the protein solution was judged to have entered the column, fraction collection was initiated. The effluent volume which corresponded to the maximum concentration of the protein was estimated to the nearest 1ml for an elution diagram by extrapolating both sides of the solute peak to the apex.

One millilitre of *M.tuberculosis* sonicates was applied to the column. Fractions were collected in 1ml aliquots and a protein elution profile plotted by measuring the protein concentrations present in each fraction. This resulted in ten protein peaks labelled A-J which ranged in Mr size from 669 to 14kDa (Figure 3).

M.tuberculosis fractions present in the void volume which corresponded to peaks A-E and those fractions which corresponded to peaks F-J were pooled and placed in spectrapor membrane tubing (Spectrum Medical Industries Inc, USA). Dialysis bags were then placed in Aquacide II (Calbiochem Corp., La Jolla, USA) at 4^{0}C until the contents were concentrated ten-fold. A protein estimation was performed on the concentrated fractions after which they were stored at -20^{0}C.

3.15. ASSESSMENT OF SUPPRESSOR ACTIVITY OF MYCOBACTERIAL FRACTIONS OF Mr >200kDa OR <200kDa

The effects of the two major mycobacterial fractions on lymphocyte proliferation was assessed by incubating 10ug/ml of each fraction with mitogen stimulated NAL's. The proliferation assay was essentially as described in Section 3.7.

3.16. ETHER TREATMENT OF FRACTIONATED MYCOBACTERIAL SONICATES

Ten millilitres of Mr >200kDa mycobacterial fractions in the void volume (10ug/ml) were delipidated as described in Section

3.12. Both delipidated and lipid fractions were assessed for their ability to affect lymphocyte blastogenesis by incubating 10ug/ml lipid or delipidated fractions with mitogen stimulated NAL's.

3.17. ASSESSMENT OF HIGH MW (OF Mr>200kDa) FRACTION CYTOTOXICITY TO MN CELLS

MN cells were incubated in the presence of high molecular weight fractions or cRPMI alone (control) for various intervals. At the end of 2, 24, 48 and 72 hour intervals cytotoxicity was assessed by Trypan blue exclusion (Gürr, London, UK) and compared with the control.

3.18. PREPARATION OF ENRICHED LYMPHOCYTE SUBPOPULATIONS USING MONOCLONAL ANTIBODIES

Enriched lymphocyte populations were obtained by incubating 5ul of reconstituted anti-CD3, anti-CD4, anti-CD8 or anti-B7 monoclonal antibodies (Orthoclone, Ortho Pharmaceutical Corp, NJ, USA) per 10^6 cells at room temperature for 45 minutes. Fresh rabbit complement (Reagent Services, SAIMR, Johannesburg, RSA) was added to the tubes for 1 hour at room temperature and gently mixed. Cell death to determine the efficacy of such treatment, was determined by Trypan blue exclusion. The cells were reconstituted to 5ml with RPMI (4^oC), the contents layered onto 5ml of Hypaque-Ficoll and centrifuged at 1 300g for 15 minutes at 4^oC. The Hypaque-Ficoll-RPMI bilayer containing enriched viable CD3+, CD4+, CD8+ or B7 cells was removed and

washed twice in saline. Cells were then adjusted to 1 x 10^6/ml in cRPMI and incubated alone or in the presence of high MW fractions. In addition, enriched cells were incubated with the mitogens PHA, Con A and PWM in the absence (control) and the presence of high MW fractions as previously described (Section 3.7). Counts per minute were compared with control counts and expressed as a percentage of the control.

3.19. GENERATION OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS

NAL's at 2 x 10^6/ml were incubated with cRPMI (control) and with:

a) 10ug/ml unfractionated mycobacterial sonicates
b) the fraction present in the void volume Sephacryl S-200 column (10ug/ml final concentration)
c) the delipidated fraction derived from *M.tuberculosis* sonicates (10ug/ml final concentration)
d) the lipid fraction derived from *M.tuberculosis* sonicates (10ug/ml final concentration)

for 2 hours at 37°C in polystyrene tissue culture flasks.

After the 2 hour pulse, the cells were washed 3 times in physiological saline and resuspended to their original volumes in cRPMI. The cultures were incubated at 37°C in 5% CO_2 in humidified air for various periods of time. Cell free supernatants were collected at 2, 4, 24 and 48 hours and 3, 4 and 5 days, centrifuged at 4 000g for 10 minutes, filter

sterilised through a 0.22um filter (Millipore Corp., Massachusetts, USA) and stored at -20°C until assessed for suppressor activity.

3.20. ASSESSMENT OF THE SUPPRESSOR ABILITY OF VARIOUS SUPERNATANTS

NAL's at 2 x 10^6/ml were cultured with medium (RPMI), or the mitogen PHA in the presence of an equal volume of each of the above supernatants (Section 3.19) in round-bottomed microtitre plates. All experiments were performed in triplicate. Plates were incubated for 72 hours in 5% CO_2 in humidified air. In the final 18 hours, 1uCi tritiated thymidine was added and cells were harvested and radioactivity assessed in a liquid scintillation counter.

3.21. OPTIMISATION OF SUPPRESSOR SUPERNATANT PRODUCTION

Various concentrations of NAL's were pulsed for 2 hours with cRPMI or mycobacterial fractions of Mr >200kDa. The NAL's were washed 3 times in physiological saline and reconstituted to their original volume. Cell free supernatants were collected at 24 hours (Section 3.19) and used in lymphocyte transformation tests as previously described (Section 3.7).

3.22. THE EFFECT OF SUPPRESSOR SUPERNATANTS ON CD4+ CELLS

An enriched population of CD4+ cells at 1 x 10^6/ml was isolated as previously described (Section 3.18). The cells

were stimulated with PHA alone or in the presence of cRPMI, lymphocyte control supernatants or suppressor supernatants in a lymphocyte transformation test (Section 3.7).

3.23. IDENTIFICATION OF THE LYMPHOCYTE SUBSET RESPONSIBLE FOR SECRETION OF SUPPRESSOR MOLECULES

Enriched lymphocyte populations were obtained by incubating 5ul of reconstituted anti-CD3, anti-CD4, anti-CD8 or anti-B7 monoclonal antibodies per 10^6 NAL's followed by the addition of rabbit complement as previously described (Section 3.18). Viable CD3+, CD4+ and CD8+ enriched cells at 2 x 10^6/ml were incubated in plastic round-bottomed tubes (Sterilin, Middlesex, UK) at 37°C in 5% CO_2 in humidified air in cRPMI alone or in the presence of low or high MW mycobacterial fractions at a concentration of 10ug/ml for 2 hours. After this period the cells were washed twice in physiological saline, reconstituted to their original volume in cRPMI and cell free supernatants collected after 24 hours. Equal volumes of cells and such supernatants were used in proliferation assays.

3.24. SALT TREATMENT OF SUPPRESSOR SUPERNATANTS

Twenty four hour supernatants from NAL's incubated with high MW fractions were treated with ammonium sulphate as follows:- A saturated solution of ammonium sulphate (Holpro Analytics, Johannesburg, RSA) was slowly added to the supernatants to produce a 60% solution which was gently mixed for 2 hours at

room temperature, after which additional ammonium sulphate was added to produce a 75% saturated mixture. The mixture was gently mixed for a further 1 hour at room temperature. Control supernatants were treated in the same way.

The mixtures were centrifuged at 10 000g for 30 minutes and supernatants placed in spectrapor membrane tubing (Spectrum Medical Industries Inc., USA). The dialysis bags were then dialysed against three changes of distilled water and two changes of PBS at 4°C until no $(NH_4)_2SO_4$ could be detected.

The pellets were resuspended in distilled water to 20% of their starting volume, placed in spectrapor membrane tubing and dialysed in the same way. Dialysed pellets and supernatants were filter sterilised through a 0.45um filter (Millipore Corp., Bedford, Massachusetts, USA), aliquoted, a protein estimation performed and stored at -20°C until used.

The ability of pellets and supernatants to affect lymphocyte blastogenesis was assessed using standard proliferation assays in response to the mitogen PHA.

3.25. SDS-PAGE AND WESTERN BLOT IMMUNOASSAY

SDS-PAGE analysis of NAL derived suppressor molecules was performed by loading 150ug of 10x concentrated supernatants onto gels of 10% acrylamide under non-reducing conditions (Hudson &

Hay, 1980). Samples were electrophoresed in a Tris-glycine SDS buffer (0.2M Tris-HCl; 1.92M glycine; 1% SDS) system using a Biorad Protean II slab gel apparatus under conditions of constant current (25mA/gel). Protein markers were identified on gels by silver staining (Quick Silver silver staining kit, Amersham, Buckinghamshire, UK), whereas glycoprotein-containing bands were stained with Schiffs reagent (Clinical Sciences Diagnostics, Booysens, Johannesburg, RSA) according to the methods of Zacharius *et al.* (1987).

Electroblotting of unstained gels was performed under conditions of constant current and voltage (0,3 amps; 70 volts) for 3 hours as described by Towbin *et al.* (1979). The Biorad TM Transblot cell apparatus (Biorad Laboratories) was used for electroblotting.

Upon completion of electroblotting, the nitrocellulose sheet (Hybond C, 0.45um; Amersham, International, Buckinghamshire, UK) was rinsed and both lymphocyte control and suppressor molecule lanes were cut into 2mm^2 pieces according to the method of Abou-Zied *et al.* (1987). These were sterilised and dissolved in 250ul of dimethyl sulphoxide (DMSO - BDH Laboratory Reagents, Poole, UK). A precipitate was obtained by the addition of an equal volume of a sterile aqueous solution of 0.05M carbonate/ bicarbonate buffer (Merck, Darmstadt, Germany) and left overnight. The precipitated particles were collected by high speed centrifugation (10 000g for 10 minutes) and washed three times in RPMI. These suspensions were used in lymphocyte proliferation assays. Unused nitrocellulose membranes were employed as controls.

3.26. PURIFICATION OF CARBOHYDRATES FROM LYMPHOCYTE SUPPRESSOR SUPERNATANTS

After a 2 hour pulse with high MW mycobacterial fractions, CD8+ lymphocytes were washed 3 times in saline and resuspended to their original volume in saline for 24 hours at 37°C. Cell free supernatants were delipidated as described in Section 3.12. The lipid was reconstituted to one tenth of its original volume and dialysed against 3 changes of distilled water to remove any possible sodium chloride. The lipid was then hydrolysed for 3 hours in 2M solution of hydrochloric acid (HCl - BDH, Poole, UK) according to the method of Lowenstein (1969). Two millilitres of distilled water were added to the hydrolysate and remaining HCl blown off under a jet of air. The hydrolysate was treated with an equal volume of ether until lipid was no longer present at the interface. Ether present in aqueous phase was evaporated under a jet of nitrogen gas. The remaining aqueous layer was lyophilised overnight in an Edwards Freeze Drier (Sussex, UK).

3.27. THE EFFECT OF SUPPRESSOR CARBOHYDRATES ON LYMPHOCYTE BLASTOGENESIS

Various concentrations of suppressor carbohydrates were dissolved in cRPMI and added at the initiation of culture to either resting MN cells or those activated with mitogens or antigen. Since optimal suppression of blastogenesis was attained with 100ng/ml of suppressor carbohydrate, this concentration was used in all subsequent experiments. To investigate the effect of pulsing MN cells with the

carbohydrate, experiments were undertaken whereby MN cells were incubated with 100ng/ml of suppressor carbohydrate for 2 and 4 hours in round bottomed tubes at 37°C. After the pulse, cells were washed 3 times and stimulated with PHA or PPD in standard proliferation assays.

3.28. THE EFFECT OF SUPPRESSOR SUPERNATANTS AND SUPPRESSOR CARBOHYDRATES ON A MIXED LYMPHOCYTE CULTURE (MLC)

MN cells at a concentration of 1 x 10^6 isolated from the peripheral blood of normal donors, were irradiated at 3 000 rads. An equal volume of irradiated MN cells from donor (1) was incubated with an equal volume of non-irradiated MN cells from donor (2) and vice versa. In addition, 100ul of control supernatants, suppressor supernatants or 100ng/ml of suppressor carbohydrates were added to cultures. Cultures were incubated for 5 days. During the last 18 hours of culture, tritiated thymidine was added and cells were harvested as previously described. Counts were compared with control counts and suppression expressed as a percentage of the control.

3.29. ASSAYS FOR CYTOKINE PRODUCTION

Fresh MN cells, adherent cells and NAL's at a concentration of 1 x 10^6/ml were incubated with an equal volume of lymphocyte control supernatants, suppressor supernatants or 100ng/ml suppressor carbohydrates for 4 hours at 37°C. The cells were subsequently washed, resuspended to their original volume in

cRPMI and incubated for a further 24, 48 and 72 hours. Cells were washed and resuspended for further culture at the end of each incubation period. Cell free supernatants from these cultures were collected at the termination of each incubation period, and stored at -20°C until used.

To produce cytokines, cell cultures (MN cells, adherent monocytes and NAL's) were incubated with an equal volume (1ml) of lymphocyte control or suppressor supernatants.

Cultures also included 10ug PHA (NAL cultures) or 20ug/ml LPS (*E.coli* endotoxin, Difco Labs, Detroit, Michigan, USA) (MN cells and adherent monocytes).

At the end of the incubation periods, cell free supernatants were again collected and stored at -20°C for use in assays of cytokine detection.

The cytokines IL-1β, IL-2, IFN-gamma and TNF-alpha were assayed using commercially available radioimmunoassay (RIA) kits, while IL-4 and IL-6 were detected using ELISA kits according to the manufacturers instructions (IL-4 kits fom R & D Systems, Minneapolis, USA and IL-6 kits from Medgenix Diagnostics, Brussels, Belgium). TNF-alpha, IL-1β and IFN-gamma were assayed by incubating 200ul of the respective supernatant with specific antibody to these cytokines bound to tubes. The amount of cytokine captured was determined by a further overnight incubation with a second ^{125}I labelled antibody. The amount

of radioactivity bound was counted in an auto-gamma 500C counter (Packard Instruments, Illinois, USA) and the amount of cytokine in supernatants determined by comparison with a standard curve as suggested by the supplier. Interleukin-2 was determined by using an IL-2 competitive binding RIA. The amounts of IL-4 and IL-6 in the various experiments were assessed by ELISA. In all the assays the amount of cytokine produced in culture was determined by comparison with a standard curve, drawn from standards provided in the respective kits. In addition, control samples provided in each kit were used to assess the efficacy of each assay by comparison with the standard curve drawn. In each case these controls were within the expected range as suggested by the manufacturer. All samples were assayed in duplicate and the results were expressed as means of duplicate assessments per sample assayed.

To ascertain the percent suppression or the percent increase in cytokine production the following formulae were employed:

$$\text{Percent suppression of cytokine production} = \frac{\text{CPM/OD of control supernatants} - \text{CPM/OD of test supernatants}}{\text{CPM/OD values of control supernatants}} \times 100$$

$$\text{Percent increase of cytokine production} = \frac{\text{CPM/OD of test supernatants} - \text{CPM/OD of control supernatants}}{\text{CPM/OD values of test supernatants}} \times 100$$

3.30. THE EFFECT OF IL-6 ON THE PRODUCTION OF IL-1ß, TNF-ALPHA, IL-2, IL-4, IL-6 AND IFN-GAMMA

Initial studies undertook to evaluate the effect of different concentrations of rIL-6 (Boehringer Mannheim, Mannheim, Germany) on the production IL-1ß and IL-4 by resting MN cells or those

stimulated with LPS (20ug/ml). MN cells (1×10^6) were cultured for 4 or 24 hours with rIL-6 and cell free supernatants collected after each incubation period. Control systems contained cultures without rIL-6. Such studies indicated that optimal suppression of IL-1β was attained with 50U/ml of rIL-6 (Figure 13). This concentration of rIL-6 was subsequently used in experiments to detect the production of TNF-alpha, IFN-gamma and IL-2 by MN cells.

Further experiments examined the effects of rIL-6 on purified monocytes and assessed the production of IL-1β and TNF-alpha in supernatants at 4 or 24 hours respectively. Because of the known macrophage stimulatory effect of IFN-gamma, this cytokine (rIFN-gamma- Amgen Biologicals, Amersham, UK) was added to monocyte cultures containing rIL-6. Such cultures therefore contained monocytes stimulated with LPS together with rIL-6 and IFN-gamma. The rationale for this experiment was to explore the possibility of reversing the suppressor effects of rIL-6 on TNF-alpha and IL-1β production.

In order to determine the effects of exogenous rIL-6 on the production of this cytokine by MN cells, LPS stimulated cells were pulsed with rIL-6 for 4 hours, washed and cultured for a further 24 hours, after which the levels of IL-6 in these supernatants were determined.

stimulated with LPS (20ug/ml). MN cells (1 x 10^6) were cultured for 4 or 24 hours with rIL-6 and cell free supernatants collected after each incubation period. Control systems contained cultures without rIL-6. Such studies indicated that optimal suppression of IL-1ß was attained with 50U/ml of rIL-6 (Figure 13). This concentration of rIL-6 was subsequently used in experiments to detect the production TNF-alpha, IFN-gamma and IL-2 by MN cells.

Futher experiments examined the effects of rIL-6 on purified monocytes and assessed the production of IL-1ß and TNF-alpha in supernatants at 4 or 24 hours respectively. Because of the known macrophage stimulatory effect of IFN-gamma, this cytokine (rIFN-gamma- Amgen Biologicals, Amersham, UK) was added to monocyte cultures containing rIL-6. Such cultures therefore contained monocytes stimulated with LPS together with rIL-6 and IFN-gamma. The rationale for this experiment was to explore the possibility of reversing the suppressor effects of rIL-6 on TNF-alpha and IL-1ß production.

In order to determine the effects of exogenous rIL-6 on the production of this cytokine by MN cells, LPS stimulated cells were pulsed with rIL-6 for 4 hours, washed and cultured for a further 24 hours, after which the levels of IL-6 in these supernatants were determined.

3.31. THE EFFECT OF OF rIL-4 ON THE PRODUCTION OF IL-1ß, TNF-ALPHA, IL-2, IL-6 AND IFN-GAMMA

Similar to the experiments utilising IL-6, rIL-4 (Biogenesis, Bournemouth, UK) was added to MN cells alone or those stimulated with LPS in various concentrations. At the end of the incubation period (4 hours for IL-1ß or 24 hours for other cytokines) the amount of each cytokine present in cell free supernatants was determined.

3.32. THE EFFECT OF OF rIL-4 AND rIL-6 ON THE PRODUCTION OF IL-1ß, TNF-ALPHA, IL-2, IL-6 AND IFN-GAMMA

At the time when these experiments were undertaken the optimal dose of IL-4 required for inhibiting the various cytokines had not been determined. For this reason LPS stimulated MN cells were incubated with various concentrations of rIL-4 and 50U/ml of rIL-6. Cell free supernatants were collected at appropriate times for assessment of the production of the various cytokines. Control systems contained LPS stimulated MN cells alone.

3.33. THE EFFECT OF ANTI-IL-6 ON CYTOKINE PRODUCTION

Different concentrations of anti-IL-6 antibodies (Boehringer Mannheim, Mannheim, Germany) were added to MN cells activated with LPS or those activated with LPS together with lymphocyte control supernatants, suppressor supernatants or suppressor carbohydrates. MN cells stimulated with LPS only served as

control systems. After 4 hours cell free supernatants were assessed for IL-1ß production. Restoration of IL-1ß production in the presence of lymphocyte suppressor supernatants to those of control levels was evident when concentrations of 100U/ml, and higher, of anti-IL-6 antibodies were added to cultures. As a result 100U/ml of anti-IL-6 antibodies were used in all further experiments.

To detect the production of the cytokines TNF-alpha, IL-2, IL-4, IL-6 and IFN-gamma, 100U/ml of these antibodies were added to similar cultures with the exception that cell free supernatants were collected at 24 hours.

3.34. THE EFFECT OF ANTI-IL-4 ANTIBODIES ON THE PRODUCTION OF IL-1ß, IL-2, TNF-ALPHA, IL-6 AND IFN-GAMMA

In preliminary experiments the use of anti-IL-4 antibodies (Amersham, Buckinghamshire, UK) demonstrated that optimal restoration of cytokine production was obtained when 1ug/ml of the antibody was employed. In the current studies this concentration of anti-IL-4 was added to LPS stimulated MN cells that were incubated with lymphocyte suppressor carbohydrates or supernatants or control supernatants. The antibody was present in cultures throughout the duration of the experiment and supernatants were collected at the appropriate interval of 4 or 24 hours. The amounts of various cytokines produced were determined in these supernatants.

3.35. THE EFFECT OF BOTH ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES ON RESTORATION OF CYTOKINE PRODUCTION

To assess the effects of the antibodies to the two cytokines, predetermined doses of 100U/ml anti-IL-6 and 1ug/ml anti-IL-4 were employed in these experiments. These concentrations of anti-IL-4 or anti-IL-6 antibodies were added to LPS stimulated MN cells incubated in the presence of lymphocyte suppressor supernatants or suppressor carbohydrates. As a control, cells were stimulated with LPS alone or those incubated with LPS and lymphocyte control supernatants. At the end of the incubation period (4 hours for IL-1β or 24 hours for TNF-alpha, IL-2, IL-4, IL-6 and IFN-gamma) and the levels of the various cytokines were assayed.

3.36. ASSESSMENT OF MESSENGER RNA (mRNA)

3.36.1. Preparation of Monocytes for mRNA Extraction

Monocytes were isolated as previously described (Section 3.5). The cells were incubated at 37°C for 3½ hours with LPS in cRPMI or with lymphocyte control or suppressor supernatants. At the end of the incubation period supernatants were aspirated. Total cellular RNA for cytokine analysis was isolated according to the method of Chomczynski & Sacchi (1987).

3.36.2. Isolation of Total Cellular mRNA

Half a millilitre of the denaturing solution was added to monocytes. To reduce RNAse contamination all solutions were mixed in diethyl pyrocarbonate (DEPC) (Sigma, Illinois, USA)

treated water (200ul/l of distilled water), allowed to stand overnight, followed by autoclaving to remove DEPC. The denaturing solution (Solution D) contained 4M guanidinium thiocyanate (Sigma, St Louis, USA); 25mM sodium citrate, pH7 (Merck, Darmstadt, Germany); 0.5% sarcosyl (Sigma, St Louis, USA) and 0.1M 2-mercaptoethanol (Sigma, St Louis, USA). In addition 50ul of 2M sodium acetate, pH4 (Merck, Darmstadt, Germany); 0.5ml of water saturated phenol (Sigma, St Louis, USA) and 150ul chloroform:isoamylalcohol (49:1) (BDH, Poole, UK) were added to the pellet. The pellet was sequentially vortexed thoroughly for 10 seconds and cooled on ice for 15 minutes. The samples were centrifuged at 10 000g in a microfuge (Eppendorf, Hamburg, Germany) for 20 minutes at 4°C. The RNA in the aqueous phase was removed and precipitated with an excess volume of isopropanol (BDH, Poole, UK) at -20°C overnight. RNA was pelleted at 10 000g twice for 20 minutes at 4°C, resuspended in 0.3ml of Solution D and reprecipitated with an equal volume of isopropanol for 1 hour at -20°C. The pellet was briefly washed in 75% ethanol, recentrifuged as above and the RNA pellet air dried. The pellet was resolubilised in 0.5% sodium dodecyl sulphate (SDS - Merck, Darmstadt, Germany) for 10 minutes at 65°C.

Total RNA concentration was determined spectrophotometrically (at lambda = 260nm) in a Beckman Du 65 spectrophotometer (Fullerton, California, USA). The concentration was determined using the following formula:

A_{260} (absorbance) x 40 x 400 (Dilution factor) and expressed in ug/ml.

3.36.3. Gel Fractionation of Cellular mRNA

The integrity of the RNA was assessed according to the method of Maniatis *et al.* (1984). A 1% agarose gel was prepared by mixing the following reagents:

0.5g agarose (Sigma, St Louis, USA)

5ml 10x morpholino-propane-sulphonic acid (MOPS - Sigma, St Louis, USA)

42.3ml DEPC treated distilled water.

The mixture was melted by boiling, cooled to 42^0C and 2.7ml formaldehyde (BDH, Poole, UK) as well as 0.5ug/ml of ethidium bromide (Boehringer Mannheim, Mannheim, Germany) from a stock solution at 10mg/ml was added for visualisation.

The gel was poured and allowed to set for ~20 minutes.

3.36.4. Preparation of Sample Buffer

The buffer was prepared by mixing the following reagents according to the method of Maniatis *et al.* (1984):

360ul formamide (Sigma, St Louis, USA)

80ul 10x MOPS

130ul formaldehyde

140ul DEPC treated distilled water

40ul glycerol (BDH, Poole, UK)

5ul bromophenol blue (Boehringer Mannheim, Mannheim, Germany)

Five microlitres of RNA was added to 15ul of sample buffer and boiled for 3 minutes to denature the sample. The sample was

then loaded onto the gel and electrophoresed at 60A constant current (Hoefer Scientific Instruments, San Francisco, USA). The running buffer consisted of 1 x MOPS and 50ul of a 10mg/ml stock of ethidium bromide.

Following electrophoresis the gel was visualised on a UV illuminator (Transilluminator, California, USA) for detection of 28S, 18S and 5S RNA.

3.36.5. Northern Blot of Total Cellular RNA

Electrophoresis was performed according to the method of Maniatis *et al.* (1984) as described above excluding the use of ethidium bromide. Following electrophoresis the gel was washed in 10x SSC for 10 minutes (3M sodium chloride - Associated Chemicals, Alrode, RSA and 0.3M sodium citrate). RNA was transferred onto a nylon membrane (Amersham, Buckinghamshire, UK) as follows. The gel was placed on a flat surface, RNA side facing up. A nylon membrane of equal size was placed on the gel and air bubbles removed. Two pieces of blotting paper (3M Whatman International, Maidstone, UK) were placed on top of the nylon membrane. The edges of the gel were sealed with parafilm (American National Can., Greenwich, USA) and several layers of absorbent tissues (KimDri, Kimberley-Clark, Johannesburg, RSA) were placed on top. This was left overnight under constant weight. Following this procedure the nylon membrane was washed in 2x SSC for 10 minutes, air dried and the RNA crosslinked by UV illumination (5 minutes).

3.36.6. Prehybridisation of the Northern Blot

Blocking of non-specific sites was achieved by the use of herring sperm DNA (Boehringer Mannheim, Mannheim, Germany) according to the method of Maniatis *et al.* (1984). The herring sperm DNA stock solution was prepared in the following way. A stock solution of 10mg/ml was aspirated through an 18 gauge needle repeatedly to solubilise the DNA. To reduce the viscosity of the solubilised material, it was then sonicated. One millilitre of herring sperm DNA was denatured by boiling for 5 minutes and placed on ice.

The prehybridisation solution was prepared as follows:

- 6.25ml of 20x SSPE which consisted of NaCl (Merck, Darmstadt, Germany); 27.6g NaH_2PO_4 (Merck, Darmstadt, Germany); 7.44g EDTA (Merck, Darmstadt, Germany) pH 7.7. The volume was made to 1l with DEPC treated distilled water.
- Formamide (12.5ml)
- 100x Denhardt's (1.25ml) which consisted of 10g of Ficoll (Sigma, St Louis, USA); 10g of polyvinyl pyrollidine (Sigma, St Louis, USA); 10g BSA. DEPC treated distilled water was used to make up the volume to 500ml.
- DEPC treated distilled water (3.75ml)
- 10% SDS 0.25ml
- herring sperm DNA (1ml)

This solution was added to the blot and incubated at 42°C for 18 hours.

3.36.7. Preparation of Labelled Probes

Oligonucleotide probes (IL-1ß - Oncogene Science, New York, USA; TNF-alpha and IL-6 - British Biotechnology, Oxford, UK) were 5'-end labelled using the suppliers' instructions. One microlitre (2.5pmol) of probe was added to 4ul 5x kinase buffer (0.5M Tris HCl, pH 9.5, 50mM $MgCl_2$; 50mM DTT (dithiothreitol); 4ul of gamma-^{32}P (ATP) (40uCi; >7 000Ci/mmol - NEN, Dreieich, Germany). The labelling reaction was initiated by the addition of the T_4 polynucleotide kinase (5-10 units - Amersham, Buckinghamshire, UK) and allowed to incubate at 37°C for 1 hour. The reaction was stopped by the addition of 40ul of 0.1M EDTA.

This solution was added to the hybridisation solution which consisted of the following:

- 20x SSPE (6.25ml)
- Formamide (12.5ml)
- 100x Denhardt's (1.25ml)
- DEPC treated distilled water (2ml)
- Herring sperm DNA (1ml) as prepared previously
- 40% PEG (polyethylene glycol - Sigma, St Louise, USA) (2.5ml)

The prehybridisation solution was removed from Northern blots and replaced by the hybridisation solution which contained the labelled probe. This was incubated overnight at 42°C (Maniatis *et al.*, 1984).

3.36.8. Stringency Washes of Northern Blots

The hybridisation solution was aspirated from the Northern Blots and a regimen of stringency washes performed. Blots were washed twice in a solution of 2x SSPE and 0.1% SDS for 15 minutes at 42°C. This was followed by washing in a solution of 1x SSPE and 0.1% SDS for 30 minutes at 42°C, followed by immersion in a solution of 0.1% SSPE and 0.1% SDS for 15 minutes at room temperature. Excess liquid was removed from blots by placing them on blotting paper after which they were wrapped in cling wrap and exposed to radiograph (X-ray) film at -70°C overnight.

3.36.9. Development of Radiographs

Radiographs were developed using Ilford developer and fixer (Ilford, Isando, RSA).

3.37. ASSESSMENT OF DR EXPRESSION

3.37.1. Induction of DR Expression

MN cells at a concentration of 3 x 10^6/ml were allowed to adhere to 96-well flat-bottomed microtitre plates (200ul/well) (Sterilin Ltd, Hounslow, UK) at 37°C. NAL's were removed by gentle agitation using cRPMI at 37°C. Remaining NAL's were removed the next day by vigorous washing with warm (37°C) cRPMI. The monocytes were incubated for a further 2 days in cRPMI at 37°C. DR expression was induced using 20ug/ml of LPS or 6 x 10^6/ml of Baker's yeast. In addition 100ul of lymphocyte control supernatant, suppressor supernatant or suppressor carbohydrate were added to the cultures and incubated for a further 5 or 7 days at 37°C.

3.37.2. Cellular Enzyme-Linked Immunosorbent Assay (ELISA) for DR Expression

After 5 or 7 days the monocytes were fixed with 0.5% paraformaldehyde for 1 hour. All incubation periods were performed at room temperature. Plates were washed three times with PBS-Tween and treated with 0.5% BSA in a carbonate buffer, pH 9.6 (Appendix 2) for 1 hour to prevent non-specific binding of the monoclonal antibody. Wells were washed three times as before and 100ul of 1:100 dilution of monoclonal antibody to DR (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) was added to each well and incubated for 2 hours. This was followed by washing as before. One hundred microlitres of peroxidase labelled anti-mouse IgG (Amersham International, Buckinghamshire, UK) was added to each well and incubated for 1 hour. The washing step was repeated to remove unbound conjugated antibody. One hundred microlitres of peroxidase substrate was added to each well and incubated in the dark. The reaction was stopped after 30 minutes with 50ul of 2.5M sulphuric acid (BDH Chemicals Ltd., Poole, UK). Optical densities (OD) were measured at 492nm on a Titretek Multiscan MC spectrophotometer (Flow Laboratories, Bioggio, Switzerland).

To examine the effects of IFN-gamma (a macrophage stimulator) and rIL-6 (shown in this study to inhibit cytokine production) on DR expression 100U/ml of IFN-gamma was added at the initiation of culture to monocytes stimulated with LPS alone or those stimulated with LPS and lymphocyte control supernatants, suppressor supernatants or suppressor carbohydrates. In addition 50U/ml of rIL-6 or 100U/ml of anti-IL-6 antibodies were added to monocyte cultures and DR expression assessed.

CHAPTER 4
RESULTS

4.1. THE EFFECT OF *M.TUBERCULOSIS* SONICATES ON LYMPHOCYTE BLASTOGENESIS

When *M.tuberculosis* sonicates were added to normal MN cells stimulated with PHA, Con A or the antigens *Candida albicans*, SKSD or PPD, blastogenesis of these cells as measured by ^{3}H-thymidine uptake was suppressed (Table 1). Optimal suppression was observed when a final concentration of 10ug/ml *M.tuberculosis* sonicates were employed in these cultures.

As optimal stimulation was obtained with a 1:50 dilution of *C.albicans*, a 1:50 dilution of SKSD, 20ug/ml of PPD, 20ug/ml of PHA or 10ug/ml of Con A, these concentrations were used in all further experiments.

4.2. THE EFFECT OF OTHER ORGANISMS ON MONONUCLEAR CELL BLASTOGENESIS

When organisms other than *M.tuberculosis* were added to cultures of MN cells stimulated with PHA, no inhibition of MN cell proliferation was observed (Figure 1). These included sonicates of *Staphylococcus aureus, Escherichia coli, Escherichia coli enterobacteriaceae, Nocardia asteroides* and *Listeria monocytogenes.*

TABLE 1. The effect of *M.tuberculosis* sonicates on the proliferative response of normal (MN) cells to mitogens and antigens.

Cell Systems	Mean CPM ± SD of 3 individual experiments Medium	*M.tuberculosis* sonicates	% Suppression
MN cells alone	1 940 ± 849	1 200 ± 162	0
MN cells + PHA (20ug/ml)	68 382 ± 4 420	41 690 ± 5 412**	39
MN cells + Con A (10ug/ml)	54 161 ± 3 046	30 188 ± 4 976**	44.3
MN cells + *C.albicans* 1:50 dilution of stock	12 200 ± 1 300	3 300 ± 560*	73
MN cells + SKSD 1:50 dilution of stock	7 800 ± 1 026	2 808 ± 463*	64
PPD (20 ug/ml)	18 642 ± 3 016	10 056 ± 2 167**	46

* - $P < .001$

** - $P < .01$

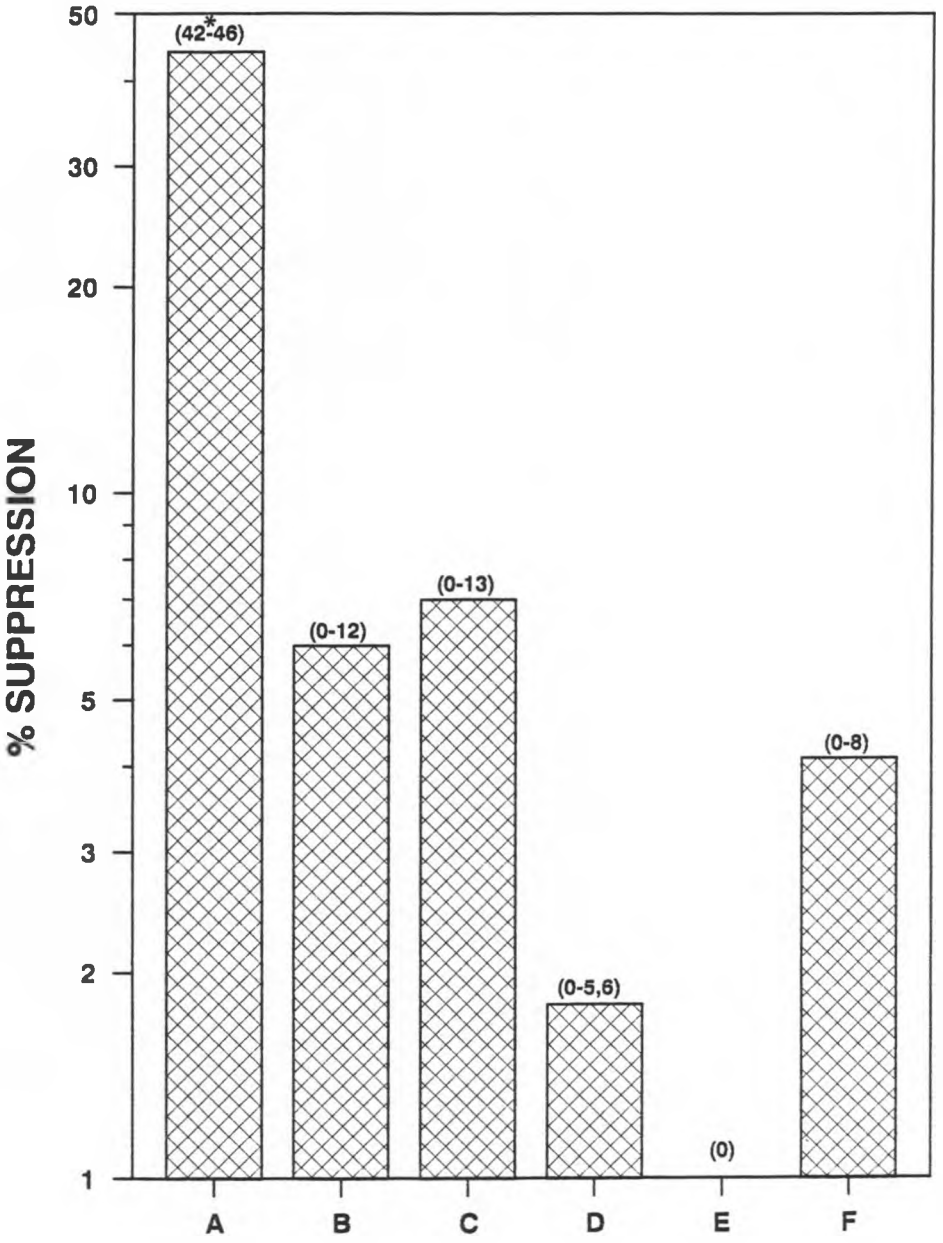


FIGURE 1. Percent suppression of PHA-induced MN cell blastogenesis

MN cells + PHA incubated with:
A. *M.tuberculosis* sonicates
B. *S.aureus* sonicates
C. *E.coli* sonicates
D. *E.coli enterobacteriaceae* sonicates
E. *Nocardia asteroides* sonicates
F. *L.monocytogenes* sonicates
* - P < .005

4.3. THE EFFECT OF *M.TUBERCULOSIS* SONICATES ON ^{3}H-THYMIDINE INCORPORATION BY MN CELLS DEPLETED OF ADHERENT CELLS (NALS)

Mononuclear cells, depleted of adherent cells, were unable to suppress the incorporation of ^{3}H-thymidine when treated with *M.tuberculosis* sonicates (Table 2). To elucidate the role of adherent cells in suppression of MN cell proliferation, adherent cells were pulsed with *M.tuberculosis* sonicates for 2 hours then extensively washed and added back to adherent cell depleted lymphocytes at a ratio of 1 adherent cell to 3 lymphocytes. The cultures were then activated with 20ug/ml of PHA or 10ug/ml of Con A. Significant suppression of ^{3}H-thymidine incorporation could only be observed when adherent cells previously treated with *M.tuberculosis* extracts were added to lymphocyte cultures (Table 3). Control adherent cells which were incubated with cRPMI alone demonstrated no significant suppressor activity (Table 3).

To determine whether the suppressor effect was due to a soluble factor released from monocytes, cells were pretreated with *M.tuberculosis* sonicates for a pre-determined 2 hour period after which they were washed and resuspended to their original volume in cRPMI. Supernatants derived from adherent cells pretreated for less than 2 hours failed to have any suppressor effect on lymphocyte blastogenesis. Supernatants collected 24 hours following the 2 hour pulse were termed monocyte suppressor supernatants and suppressed lymphocyte blastogenesis to mitogens. Monocyte control supernatants (ie. 24 hour supernatants derived from monocytes incubated in cRPMI) added to such cultures did not result in suppression of blastogenesis (Table 4).

TABLE 2. ^{3}H-thymidine incorporation of mitogen activated monocyte depleted lymphocytes incubated in the presence of *M. tuberculosis* sonicates.

Culture Systems	Mean CPM ± SD of 3 experiments
Enriched lymphocytes incubated with:	
Medium alone	2 774 ± 1 764
Medium + mycobacterial sonicates	4 104 ± 636
Medium + PHA	52 945 ± 4 453
Medium + PHA + *M. tuberculosis* sonicates	47 768 ± 8 666
Medium + Con A	43 867 ± 1 121
Medium + Con A + *M. tuberculosis* sonicates	46 423 ± 7 808

TABLE 3. ^{3}H-thymidine incorporation by mitogen activated monocyte depleted lymphocytes incubated with adherent cells previously treated with *M.tuberculosis* sonicates or medium.

Culture Systems	Mean CPM ± SD of 5 experiments	% Suppression
Enriched lymphocytes incubated with:		
Medium alone	1 522 ± 800	
Medium + PHA	52 945 ± 4 453	
Medium + PHA + *M.tuberculosis* sonicates	47 768 ± 8 666	10
Medium + PHA + cRPMI treated adherent cells	53 242 ± 4 394	0
Medium + PHA + adherent cells treated with *M.tuberculosis* sonicates	39 410 ± 3 687*	26
Enriched lymphocytes incubated with:		
Medium + Con A	49 046 ± 3 010	
Medium + Con A + cRPMI treated adherent cells	46 323 ± 4 260	6
Medium + Con A + adherent cells treated with *M.tuberculosis* sonicates	27 014 ± 5 012**	45

* - $P < .005$
** - $P < .001$

TABLE 4. The effect of supernatants derived from monocytes treated with *M.tuberculosis* sonicates and cRPMI on lymphocyte blastogenesis. (% Suppression in parenthesis)

Culture Systems	Mean CPM ± SD of 3 experiments PHA	Con A
Lymphocytes + medium	48 869 ± 6 861	27 190 ± 2 180
Lymphocytes + monocyte control supernatants	44 995 ± 4 567 (8)	26 241 ± 1 283 (3,5)
Lymphocytes + monocyte suppressor supernatants	24 161 ± 1 755* (50)	16 155 ± 2 788* (41)
CPM of lymphocytes alone:	2 108 ± 338	

* - P < .005

4.4. THE EFFECT OF INDOMETHACIN ON INHIBITION OF LYMPHOCYTE BLASTOGENESIS DUE TO MYCOBACTERIAL SONICATES

To exclude the possibility that suppression of lymphocyte blastogenesis was mediated by prostaglandin release from monocytes, cultures were incubated in the presence of indomethacin. Inhibition of lymphocyte blastogenesis by *M.tuberculosis* sonicates was unaffected by indomethacin treatment (Table 5). This applied to cultures stimulated with the mitogens PHA and Con A and to the proliferative response of MN cells to PPD. Suppression in systems containing *M.tuberculosis* sonicates ranged from between 34-49% whereas the suppression of blastogenesis in cultures containing both indomethacin and *M.tuberculosis* sonicates ranged between 32-42% (Table 5).

4.5. CHARACTERISATION OF THE COMPONENT IN SONICATES FROM *M.TUBERCULOSIS* RESPONSIBLE FOR SUPPRESSION OF LYMPHOCYTE BLASTOGENESIS

To identify the component(s) present in mycobacterial sonicates responsible for the suppression of lymphocyte blastogenesis, *M.tuberculosis* sonicates were treated by various means to remove lipid and to destroy protein or carbohydrate moieties. The results indicate that when the carbohydrate or protein moieties were destroyed, suppression by such mycobacterial sonicates was still apparent (Table 6). Prior to the use of chemically treated *M.tuberculosis* sonicates in culture, the sonicates were extensively dialysed as indicated in Section 3.11, 3.12 to avoid any direct effects of chemicals/enzymes on the tissue culture systems.

TABLE 5. The effect of indomethacin on the inhibition of lymphocyte blastogenesis due to mycobacterial sonicates.

Culture Systems	Mean CPM ± SD of 3 experiments	% Suppression
MN cells incubated with:		
Medium	2 107 ± 422	
indomethacin	1 692 ± 310	
PHA	49 816 ± 5 102	
PHA + mycobacterial sonicates	31 699 ± 4 523*	36.4
PHA + mycobacterial sonicates + indomethacin	33 786 ± 3 970	32.2
Con A	45 626 ± 4 217	
Con A + mycobacterial sonicates	29 967 ± 3 085*	34.4
Con A + mycobacterial sonicates + indomethacin	31 237 ± 3 762	32
PPD	17 170 ± 1 287	
PPD + mycobacterial sonicates	8 677 ± 1 280**	49,5
PPD + mycobacterial sonicates + indomethacin	9 895 ± 958	42,4

* - $P < .01$
** - $P < .005$

A convenient control in these systems was to assess the effect of dialysis or such treatment of sonicates by using neuraminidase treated, followed by extensively dialysed sonicates in these cultures. Such results therefore indicate that as expected, sialic acid residues (if any present) were not responsible for the observed suppression. Of greater importance however is the fact that various treatments to remove salts and enzymes did not have any significant effect on lymphocyte blastogenesis. In other experiments, mycobacterial sonicates were dialysed as were chemically treated sonicates. These untreated but dialysed sonicates still retained their suppressor activity (results not shown).

When *M.tuberculosis* extracts were treated with ether and the lipid layer and remaining ether removed, the delipidated extract failed to suppress mitogen stimulated MN cell blastogenesis (Table 6). The ether in the lipid fraction was evaporated over nitrogen and the lipid fraction reconstituted with 1:100 (ethanol:RPMI). When the remaining lipid fraction was added to mitogen activated MN cell cultures, suppression of blastogenesis was once more observed indicating that suppressor activity may reside in the lipid fraction of *M.tuberculosis* (Table 6).

TABLE 6. The effect of chemically modified *M. tuberculosis* sonicates on the proliferation of MN cells activated with PHA and Con A (% suppression in parenthesis)

Cell Systems	Mean CPM ± SD of 5 experiments MN cells in the presence of PHA	Con A
MN cells	51 180 ± 4 667	45 453 ± 3 790
MN cells + *M. tuberculosis* sonicates	32 784 ± 5 423 (36)	27 210 ± 3 096 (40)
MN cells + *M. tuberculosis* treated with:		
Sodium metaperiodate	33 779 ± 4 073 (34)	30 613 ± 2 740 (33)
Chymotrypsin	34 291 ± 5 063 (33)	25 627 ± 3 098 (44)
Proteinase K	38 385 ± 6 179 (25)	29 203 ± 5 924 (36)
Aprotinin	36 338 ± 3 124 (29)	21 668 ± 4 260 (52)
Neuraminidase	35 830 ± 3 216 (30)	32 378 ± 3 354 (29)
Ether	44 460 ± 3 745 (13)	41 808 ± 5 069 (8)
Extracted lipid from *M. tuberculosis* sonicates	35 850 ± 6 390* (30)	31 857 ± 2 190* (30)
CPM ± SD of MN cells in medium alone : 1 177 ± 223		

* - P < .01

4.6. THE EFFECT OF HEAT TREATMENT ON THE SUPPRESSOR ACTIVITY OF *M. TUBERCULOSIS* SONICATES

When mycobacterial sonicates previously heat treated at different temperatures were incubated in culture with PHA, they retained their ability to suppress lymphocyte blastogenesis (Table 7). This was true of sonicates treated at 37°C, 42°, 65°, 100° and 121° for various intervals (30 minutes and 24 hours).

4.7. MOLECULAR WEIGHT ESTIMATION OF STANDARDS OFF A SEPHACRYL S-200 COLUMN

Figure 2 demonstrates a plot of the elution volume of known molecular weight standards off a Sephacryl S-200 column against the log molecular weight for Ribonuclease (14kDa), Chymotrypsin (25kDa), Ovalbumin (43kDa), Bovine serum albumin (67kDa), Aldolase (158kDa), Catalase (232kDa), Ferritin (440kDa) and Thyroglobulin (669kDa). The experimental points lie close to a straight line. The molecular weight estimation of the active fractions were read off this standard curve by extrapolating the fractions eluted against the log molecular weight.

TABLE 7. The effect of heat treated *M.tuberculosis* sonicates on the blastogenic response of normal human peripheral blood MN cells to PHA.

Cell Systems	Mean CPM ± SD of 3 experiments	% Suppression
MN cells alone	601 ± 112	
MN cells + PHA	50 566 ± 3 138	
MN cells + PHA + untreated *M.tuberculosis* sonicates	33 518 ± 3 794*	34
MN cells + PHA + *M.tuberculosis* sonicates heated at:-		
37°C/24 hours	34 669 ± 3 834*	31
42°C/30 minutes	34 109 ± 2 448*	33
65°C/30 minutes	30 525 ± 2 320*	40
100°C/30 minutes	35 939 ± 4 432*	29
121°C/30 minutes	32 520 ± 2 501*	36

* - $P < .01$

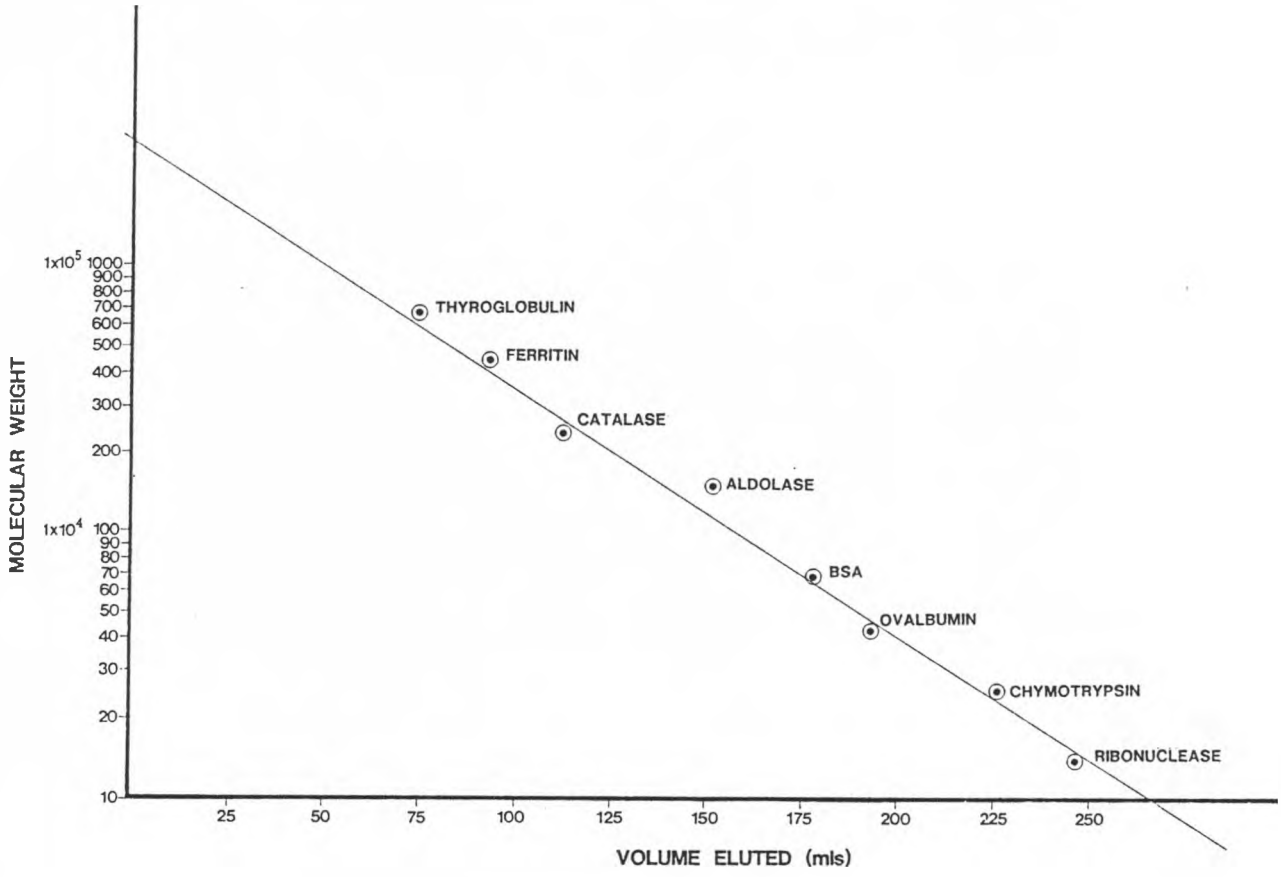


FIGURE 2. The relationship of proteins with known molecular weights and elution volumes off a Sephacryl S-200 column

This figure demonstrates a plot of the elution volume of known molecular weight standards off a Sephacryl S-200 column against the log molecular weight. (Mean of 3 column runs).

4.8. PARTIAL PURIFICATION OF *M.TUBERCULOSIS* SONICATES ON SEPHACRYL S-200 COLUMNS

Fractionation of *M.tuberculosis* extracts yielded 10 major protein peaks arbitrarily designated A-J, as determined by Biorad protein estimation (Figure 3). When eluates of Mr >200kDa present in the void volumes corresponding to peaks A-E were incubated with mitogen activated MN cells or MN cells activated with PPD, suppression of blastogenesis was observed (Table 8). Optimal suppression of lymphocyte proliferation was observed with a final concentration of 1ug/ml of these high MW fractions (i.e. >200kDa) (Table 8). Eluates present in peaks F-J (i.e. <200kDa) failed to suppress mitogen or PPD stimulated MN cell blastogenesis (Table 8). Eluates present in the void volume of the Sephacryl S-200 column applied to a Sepharose 2B column could not be further separated (results not shown).

4.9. THE EFFECT OF HIGH MOLECULAR WEIGHT FRACTIONS ON ^{3}H-THYMIDINE INCORPORATION BY MN CELLS DEPLETED OF ADHERENT CELLS (NAL's)

When mitogen activated NALs were incubated with high MW fractions (>200kDa) lymphocyte blastogenesis was significantly suppressed (Table 9). Fractions <200kDa failed to suppress mitogen activated NAL blastogenesis (Table 9). Similarly, suppression of lymphocyte blastogenesis was not observed in the presence of unfractionated mycobacterial sonicates (Table 9).

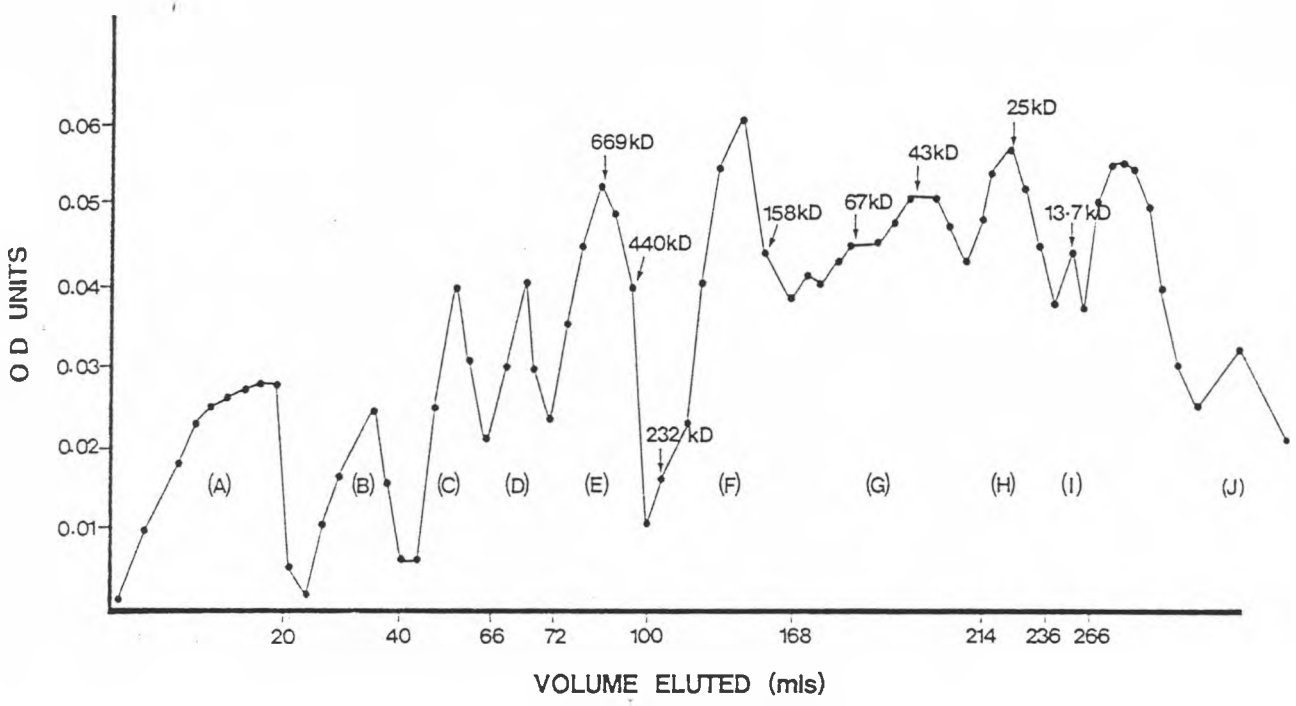


FIGURE 3. Protein elution profile of *M. tuberculosis* sonicates off a Sephacryl S-200 column (mean of 3 column runs)

TABLE 8. The effect of mycobacterial fractions on MN cell blastogenesis to PHA, Con A and PPD.

Culture Systems	Mean CPM ± SD of 5 experiments	% Suppression
MN cells incubated with:		
Medium	1 055 ± 207	
PHA	45 886 ± 4 589	
Con A	51 687 ± 3 325	
PPD	17 048 ± 437	
MN cells incubated with unfractionated mycobacterial sonicates +		
Medium	1 872 ± 421	
PHA	30 560 ± 3 672*	33,5
Con A	28 897 ± 3 069**	44
PPD	10 010 ± 2 167**	41
Fractions of Mr <200kDa +		
Medium	2 338 ± 612	
PHA	50 232 ± 5 698	0
Con A	48 162 ± 5 210	7
PPD	16 827 ± 1 070	1
Fractions of Mr >200kDa +		
Medium	2 510 ± 667	
PHA	32 028 ± 4 542*	30
Con A	28 294 ± 4 326**	45
PPD	9 214 ± 1 495**	46

* - P < .005
** - P < .001

TABLE 9. The effects of mycobacterial fractions on NAL's activated with PHA and Con A.

Culture Systems	Mean CPM ± SD of 5 experiments	% Suppression
NAL's alone with:		
Medium	1 277 ± 223	
PHA	51 180 ± 4 667	
Con A	51 285 ± 4 135	
NAL's incubated with unfractionated mycobacterial sonicates +		
Medium	1 273 ± 321	
PHA	50 981 ± 3 762	0,4
Con A	54 105 ± 2 140	0
Fractions of Mr <200kDa +		
Medium	2 306 ± 882	
PHA	49 802 ± 3 160	3
Con A	50 612 ± 2 917	1
Fractions of Mr >200kDa +		
Medium	1 973 ± 832	
PHA	33 621 ± 2 972*	34
Con A	35 367 ± 3 981*	31

* $P < .005$

4.10. THE EFFECT OF ETHER ON THE SUPPRESSOR ACTIVITY OF HIGH MOLECULAR WEIGHT FRACTIONS

When high molecular weight fractions were treated with ether and the lipid layer removed, the delipidated fraction failed to suppress the blastogenesis of MN cells stimulated with PHA, Con A or PPD in culture (Table 10). However, suppression of blastogenesis was evident when the lipid component was added to cultures (Table 10). Untreated fractions of Mr >200kDa also suppressed MN cell blastogenesis when incubated with PHA, Con A and PPD (Table 10).

4.11. ASSESSMENT OF THE CYTOTOXIC EFFECT OF THE HIGH MW MYCOBACTERIAL FRACTION ON MN CELLS

To determine whether the decreased incorporation of ^{3}H-Thymidine into lymphocyte DNA was due to cytotoxicity by the high MW fraction, MN cells were incubated in the presence of the high MW fraction for 2, 24, 48 and 72 hours. After each interval, cytotoxicity was assessed by Trypan Blue exclusion and compared to the control (RPMI only). As can be seen (Table 11) the high MW fraction was not cytotoxic to MN cells at all time intervals assessed.

TABLE 10. The effect of chemically modified high molecular weight fractions on the blastogenesis of MN cells activated with PHA, Con A and PPD.

Culture Systems	Mean CPM ± SD of 3 experiments	% Suppression
MN cells alone with:		
Medium	1 029 ± 197	
PHA	52 712 ± 5 655	
Con A	45 424 ± 4 758	
PPD	17 048 ± 437	
Fractions of Mr >200kDa +		
Medium	1 330 ± 351	
PHA	36 012 ± 7 142*	32
Con A	33 966 ± 5 233*	25
PPD	9 214 ± 1 495**	46
Ether treated fractions of Mr >200kDa +		
Medium	2 245 ± 762	
PHA	50 169 ± 4 989	5
Con A	47 627 ± 5 089	0
PPD	17 296 ± 1 895	0
Extracted lipids from fractions of Mr >200kDa +		
Medium	2 721 ± 997	
PHA	36 898 ± 4 126*	30
Con A	29 980 ± 3 690*	34
PPD	10 107 ± 1 207**	41

* - $P < .01$
** - $P < .001$

TABLE 11. The effects of mycobacterial fractions of Mr >200kDa on MN cells: Assessment of cytotoxicity.

Culture Systems	Mean % Cytotoxicity and Range of 3 experiments	
2 HOURS:		
MN cells + medium	3	0 - 6
MN cells + >200kDa mycobacterial fractions	3	1 - 5
24 HOURS:		
MN cells + medium	4	1 - 7
MN cells + >200kDa mycobacterial fractions	6	2 - 10
48 HOURS:		
MN cells + medium	4	3 - 7
MN cells + >200kDa mycobacterial fractions	7	1 - 13
72 HOURS:		
MN cells + medium	9	7 - 12
MN cells + >200kDa mycobacterial fractions	7	5 - 9

4.12. EFFECT OF HIGH MOLECULAR WEIGHT FRACTIONS ON ENRICHED CELL POPULATIONS

High molecular weight fractions were assessed for their ability to induce proliferation of separated lymphocyte populations alone, or in the presence of the mitogens PHA, Con A, or PWM. The high MW fractions suppressed the blastogenesis of unseparated NAL's as well as the blastogenesis of CD3+ lymphocytes when placed in culture (Table 12). In addition, resting or mitogen stimulated CD4+, CD8+ or B lymphocytes did not proliferate when high MW fractions were added to such cell cultures (Table 12).

4.13. THE EFFECT OF SUPERNATANTS FROM NAL's PULSED WITH MYCOBACTERIAL COMPONENTS ON LYMPHOCYTE BLASTOGENESIS

Since high MW fractions appeared to activate CD8+ suppressor lymphocytes rather than increase their numbers, experiments were undertaken in which cell free supernatants were collected at various intervals after pulsing NAL's with high MW fractions, or the lipid or delipidated components derived from these fractions. As can be seen (Figure 4), supernatants from NALs incubated with high MW fractions or fractions containing mycobacterial lipids suppressed the blastogenesis of fresh untreated lymphocytes stimulated with PHA. Significant suppression was observed when these supernatants were added to fresh NAL's stimulated with PHA (Figure 4). Maximal suppression was observed when 24 hour supernatants were used in these assays and gradually declined until it was totally lost at 72 hours (Figure 4).

TABLE 12. The effect of mycobacterial fractions of Mr >200kDa on the proliferation of lymphocyte subsets.
(% suppression in parenthesis)

Culture Systems	Mean CPM ± SD of 3 experiments Medium	PHA	Con A	PWM
NAL's alone	1 841 ± 553	36 400 ± 1 566	42 905 ± 4 020	14 159 ± 2 690
+ >200kDa fraction	2 750 ± 734	19 500 ± 3 635 (46)*	25 432 ± 3 609 (41)*	7 796 ± 2 701 (45)*
Enriched CD3+ lymphocytes	1 472 ± 812	58 212 ± 1 807	43 963 ± 3 279	20 782 ± 3 189
+ >200kDa fraction	2 998 ± 944	38 141 ± 4 680 (34,5)**	31 249 ± 5 021 (29)**	14 143 ± 3 190 (32)**
Enriched CD4+ lymphocytes	1 812 ± 946	37 836 ± 3 950	27 845 ± 2 580	15 897 ± 2 846
+ >200kDa fraction	2 867 ± 1 932	41 012 ± 7 783 (0)	30 831 ± 3 649 (0)	14 817 ± 3 003 (7)
Enriched CD8+ lymphocytes	1 032 ± 174	33 028 ± 1 764	29 559 ± 5 074	4 822 ± 1 342
+ >200kDa fraction	3 676 ± 1 474	32 101 ± 1 668 (0)	32 158 ± 5 335 (0)	4 376 ± 722 (9,3)
Enriched B lymphocytes	2 752 ± 1 226	9 235 ± 1 060	9 328 ± 3 546	3 924 ± 1 410
+ >200kDa fraction	3 207 ± 1 611	9 084 ± 2 320 (2)	9 962 ± 4 538 (0)	3 685 ± 1 378 (6)

* - $P < .005$
** - $P < .01$

Supernatants from NALs pulsed with high MW fractions (of Mr >200kDa) were termed lymphocyte suppressor supernatants while those treated with low MW fractions (of Mr <200kDa) were referred to as lymphocyte control supernatants. Supernatants derived from NALs pulsed with unfractionated mycobacterial sonicates or those pulsed with delipidated high MW fractions did not induce suppression (Figure 4).

4.14. THE EFFECT OF SUPERNATANTS DERIVED FROM DIFFERENT CONCENTRATIONS OF NALs ON LYMPHOCYTE BLASTOGENESIS

To assess the number of activated lymphocytes required for the optimal production of lymphocyte suppressor supernatants, various concentrations of NALs were pulsed with mycobacterial fractions of Mr >200kDa or cRPMI. Twenty-four hour supernatants derived from these NALs were placed in culture with fresh untreated NALs activated with PHA. As can be seen (Figure 5) optimal suppression of PHA induced lymphocyte blastogenesis was achieved when equal volumes of supernatants derived from 2 x 10^6 NALs pulsed with fractions of Mr >200kDa were added in culture to 2 x 10^6 NALs/ml in a 1:1 ratio (Figure 5). Supernatants derived from 2 x 10^6 NALs pulsed with cRPMI did not suppress blastogenesis of fresh NALs (Figure 5). Since optimal suppression of blastogenesis was achieved using supernatants from 2 x 10^6 NALs/ml, this concentration of cells was used to generate lymphocyte suppressor supernatants in all further experiments. Supernatants derived from lower cell concentrations did not demonstrate such suppressor activity. Although supernatants from higher cell concentrations (greater than 2 x 10^6) were immunosuppressive, the effects were no greater than those obtained with the optimal cell number used.

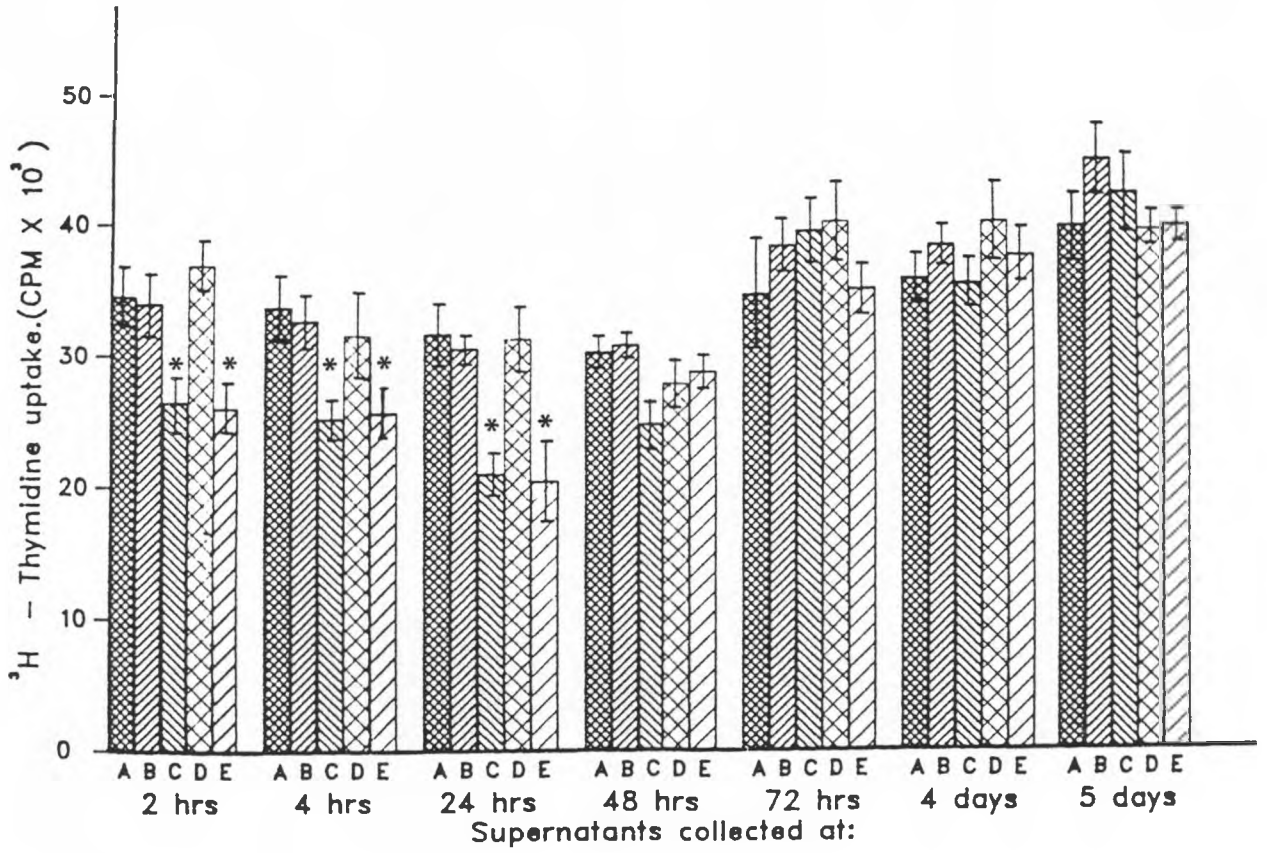


FIGURE 4. The effects of various supernatants on lymphocyte blastogenesis to PHA (mean ± SD of 5 experiments).

Supernatants were collected from cultures pulsed with:
medium (A); unfractionated mycobacterial sonicates (B); fractions of mycobacterial sonicates in the void volume of Sephacryl S-200 columns (C) delipidated (D) lipid components (E) of these fractions; and incubated with fresh lymphocytes in the presence of PHA.

Significant suppression (* - P <.005) of lymphocyte blastogenesis was observed in the presence of: (C) mycobacterial sonicates of Mr >200kDa and (E) fractions containing mycobacterial lipids at 2, 4 and 24 hours.

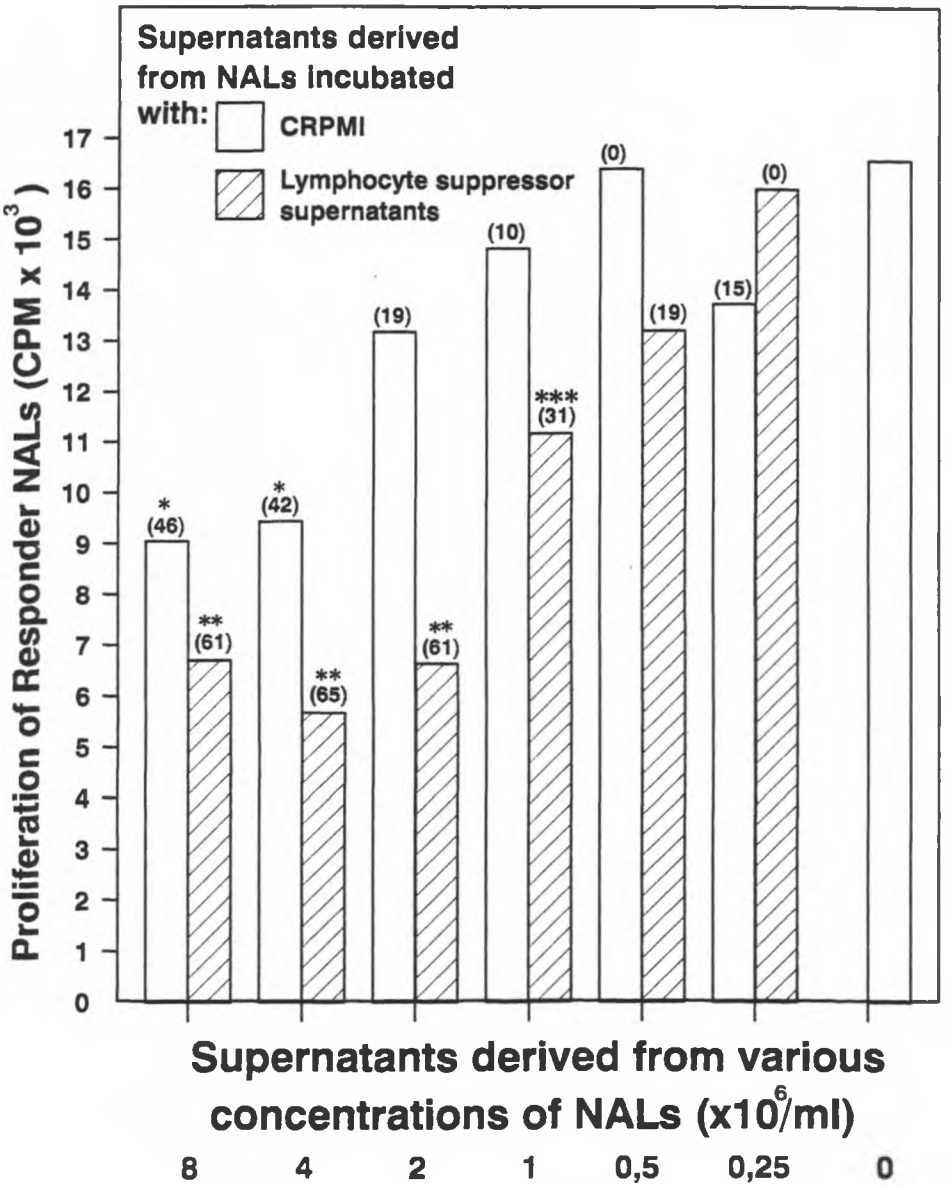


FIGURE 5. The effect of lymphocyte suppressor supernatants on blastogenesis:- Kinetic studies of suppressor supernatant generation.

Supernatants were generated by using various concentrations of NAL's. The % suppression indicated in parenthesis was calculated by comparison of CPM's of experimental systems with those obtained by incubating NAL's with PHA alone (control).

* - $P < .005$

** - $P < .001$

*** - $P < .01$

4.15. IDENTIFICATION OF THE LYMPHOCYTE SUBSET RESPONSIBLE FOR SECRETING SUPPRESSOR MOLECULES

To assess which lymphocyte subset was responsible for the release of suppressor molecules, NAL's and enriched CD4+ or CD8+ lymphocytes were pulsed with high and low MW fractions for 2 hours. Once the cells were washed, 24 hour cell free supernatants from respective cultures were incubated with fresh untreated lymphocytes stimulated with PHA. Supernatants from enriched CD8+ lymphocytes pulsed with high MW fractions suppressed lymphocyte blastogenesis significantly (Table 13) but was not cytotoxic to these cells (results not shown). No such suppressor activity was observed in supernatants derived from CD4+ lymphocytes, from untreated CD8+ cells or CD8+ cells treated with mycobacterial fractions with molecular masses below 200kDa, incubated with PHA stimulated lymphocytes (Table 13).

4.16. THE EFFECT OF SUPPRESSOR SUPERNATANTS ON CD4+ CELLS

To confirm that lymphocyte suppressor supernatants inhibited the proliferation of any enriched population of CD4+ cells, these cells were placed in culture with lymphocyte control or suppressor supernatants in the presence of PHA (Table 14). Lymphocyte suppressor supernatants inhibited the blastogenesis of CD4+ cells by ~41% compared to mitogen stimulated cell proliferation of CD4+ cells in medium alone (Table 14). Lymphocyte control supernatants had no such suppressor effect (Table 14). Similar effects were observed in control systems that contained unfractionated NAL's (Table 14).

TABLE 13. The effects of supernatants from CD4+ and CD8+ enriched populations pulsed with mycobacterial fractions on lymphocyte blastogenesis. Mean CPM ± SD of 3 experiments (% suppression in parenthesis)

Culture Systems	Response of NAL's in the presence of PHA Supernatants derived from CD4+ lymphocytes	 CD8+ lymphocytes
Enriched populations incubated with:-		
Medium	49 653 ± 2 290	50 329 ± 2 543
Fractions of Mr <200kDa	49 521 ± 2 311 (0)	49 982 ± 2 943 (0)
Fractions of Mr >200kDa	48 503 ± 1 981 (1)	37 470 ± 1 846* (23)
The mean CPM ± SD of NAL's incubated with PHA alone in these experiments was	48 892 ± 2 183	

*(P < .01)

TABLE 14. The effect of suppressor supernatants on NAL's and enriched CD4+ lymphocyte populations. (% suppression in parenthesis)

	Mean CPM ± SD of 3 experiments	
Culture Systems	NAL's	CD4+ lymphocytes
Medium	3 602 ± 1 171	4 390 ± 1 769
PHA	47 621 ± 5 109	51 619 ± 6 709
PHA + lymphocyte control supernatants	44 221 ± 5 217 (7)	48 916 ± 4 562 (5)
PHA + lymphocyte suppressor supernatants	28 309 ± 3 760* (40)	30 602 ± 4 252* (41)

* - $P < .005$

4.17. SALT FRACTIONATION OF LYMPHOCYTE SUPERNATANTS

Salt fractionation with 60 and 75% ammonium sulphate resulted in a pellet and supernatant. When the various lymphocyte derived supernatants were treated with ammonium sulphate, the resultant components were separated and dialysed extensively. On analysis reconstituted pellets from any of the test systems had no effect on lymphocyte blastogenesis (Table 15). Furthermore the unprecipitated components of ammonium sulphate treated supernatants of NAL's alone or those treated with delipidated high MW fractions were also without effect. Suppressor activity was only retained in the unprecipitated components of supernatants from NAL's treated with high MW fractions or the lipid components of these fractions (Table 15).

4.18. CHARACTERISATION OF SUPPRESSOR MOLECULES IN CD8+ CULTURE SUPERNATANTS

To characterise the molecules responsible for inducing suppression, 24 hour CD8+ suppressor supernatants were subjected to various treatments that removed lipid, or degraded carbohydrate or polypeptide portions and sialic acid residues as described in Section 3.11, 3.12. As can be seen destruction of sialic acid residues had no effect on the suppressor activity of these supernatants (Table 16). When supernatants containing lipid and carbohydrates, in which protein moieties

TABLE 15. Separation of supernatants pulsed with various mycobacterial fractions by ammonium sulphate precipitation.
(Mean CPM ± SD of 3 experiments)

Culture Systems	Response of NAL's in the presence of PHA	% Suppression
Source of 24 hour supernatants:		
NAL's +		
Medium	56 123 ± 4 082	0
Delipidated >200kDa fractions	55 074 ± 3 106	6
Lipid component of >200kDa fraction	41 598 ± 4 010*	29
>200kDa mycobacterial fractions	40 576 ± 3 790*	31
Source of 24 hour pellet:		
NAL's +		
Medium	60 273 ± 2 972	0
Delipidated >200kDa fractions	55 290 ± 5 138	6
Lipid component of >200kDa fraction	60 014 ± 6 585	0
>200kDa mycobacterial fractions	57 066 ± 4 242	2,6
The mean CPM ± SD of NAL's incubated with PHA alone in these experiments was	58 589 ± 4 586	

* - $P < .01$

were destroyed were used, suppression of blastogenesis remained unaffected (Table 16). However, suppressor activity was no longer evident in supernatants treated with sodium metaperiodate (Table 16). Supernatants containing only lipid, in which protein and carbohydrates moieties were destroyed, no longer induced the suppression of lymphocyte blastogenesis (Table 16).

4.19. RESOLUTION OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS BY SDS-PAGE

When lymphocyte suppressor supernatants were resolved by SDS-PAGE, protein staining demonstrated major bands of ~67 and 150kDa (Figure 6i). Staining for carbohydrate moieties demonstrated bands in the region of ~122 to 148kDa, ~90kDa, ~55kDa and ~50kDa (Figure 6ii, lane C). Lymphocyte control cultures or those that were not immunosuppressive contained bands in the region of ~90kDa, ~55kDa and ~50kDa. These included supernatants from CD4+ cultures incubated with high MW mycobacterial fractions (Figure 6ii, lane A), low MW mycobacterial fractions (Figure 6ii, lane B) and supernatants from unstimulated NAL's (Figure 6ii, lane D). Other controls such as mycobacterial sonicates <200kDa (Figure 6i, lane E) or >200kDa (Figure 6ii, lane F) or medium control containing cRPMI only (Figure 6i and ii, lane G) did not demonstrate bands in the same areas as test systems when stained for protein and carbohydrate (Figure 6i and ii).

TABLE 16. The effects of chemically modified suppressor supernatants on lymphocyte blastogenesis.

Culture Systems	Mean CPM ± SD of 3 experiments % suppression in parenthesis NAL's (PHA)	MN Cells (PPD)
NAL's (PHA) / MN cells (PPD) incubated with:		
Medium	41 326 ± 1 642	17 048 ± 437
Lymphocyte control supernatants	49 521 ± 2 311 (0)	15 101 ± 1 456 (11,5)
Lymphocyte suppressor supernatants	20 530 ± 1 556 (50)*	8 843 ± 669 (48)*
Lymphocyte suppressor supernatants chemically modified with:-		
Neuraminidase	24 882 ± 1 431 (40)*	9 501 ± 1 364 (44)*
Proteinase k	21 179 ± 1 263 (49)*	8 216 ± 1 509 (52)*
Sodium metaperiodate	41 746 ± 1 378 (0)	17 693 ± 1 329 (0)
Proteinase k + sodium metaperiodate	38 433 ± 1 991 (7)	16 195 ± 798 (5)

* - $P < .001$

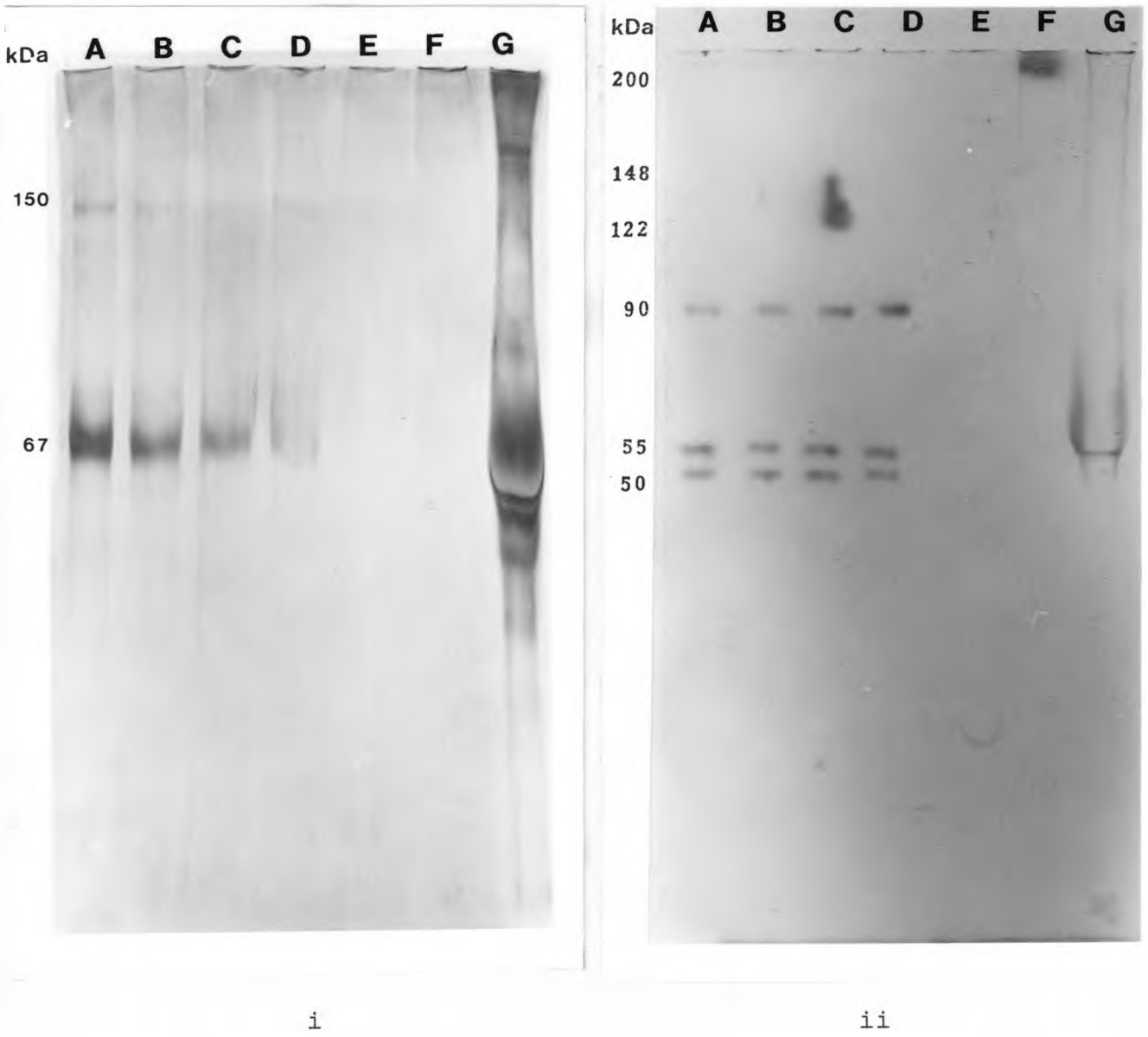


FIGURE 6. SDS-PAGE of 24 hour supernatants from CD4+, CD8+ and NAL's.

(A) Supernatants from CD4+ cultures incubated with mycobacterial fractions of Mr >200kDa
(B) Supernatants from CD8+ cultures incubated with mycobacterial fractions of Mr <200kDa
(C) Supernatants from CD8+ cultures incubated with mycobacterial fractions of Mr >200kDa
(D) Supernatants from unstimulated NAL cultures
(E) *M.tuberculosis* sonicates of Mr <200kDa
(F) *M.tuberculosis* sonicates of Mr >200kDa
(G) cRPMI (medium control)
The arrows indicate the approximate Mr of protein staining bands (silver stain) (6i) and carbohydrate staining bands (Schiffs) (6ii).

4.20. THE EFFECT OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS ON MN CELL PROLIFERATION FOLLOWING TRANSFER ONTO NITROCELLULOSE MEMBRANES

After transfer onto nitrocellulose membranes, areas corresponding to identifiable carbohydrates or protein bands were excised and used in proliferation assays. Suppression of PHA induced MN cell blastogenesis was observed with molecules present in ~122-148kDa region derived from lymphocyte suppressor supernatants (Figure 7). Suppressor activity was not observed in regions above or below this range (Figure 7). Corresponding nitrocellulose strips from lymphocyte control culture supernatants also did not suppress MN cell blastogenesis (Figure 7). These studies were extended to exclude the possibility of the presence of immunosuppressive molecules in protein or carbohydrate bands other than those in the 122-148kDa region. In these experiments, supernatants derived from CD4+ cells pulsed with mycobacterial fractions of Mr >200kDa or CD8+ cells pulsed with mycobacterial fractions of Mr <200kDa>, suppressor effects on MN cell blastogenesis were assessed (Table 17). When nitrocellulose strips from areas corresponding to molecules other than those seen at Mr of 122-148kDa were assessed for their ability to influence lymphocyte blastogenesis, none of the transferred components were immunosuppressive (Table 17). The results are extended in Table 18 and indicate that the immunosuppressive properties of suppressor supernatants reside only in the 122-148kDa fraction.

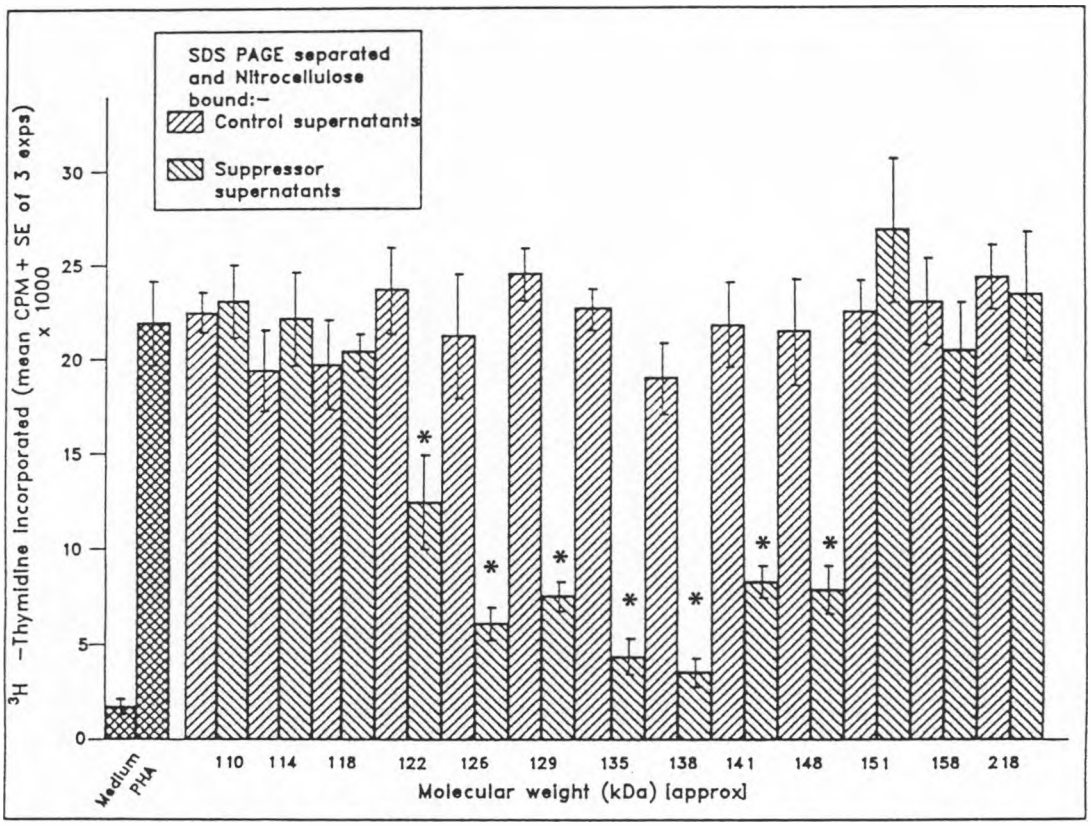


FIGURE 7. MN cell proliferation to nitrocellulose-bound supernatants separated on SDS-PAGE.

Significant suppression of MN cell blastogenesis was only observed when fractions of Mr 122-148kDa from lymphocyte suppressor supernatants were employed.
* P < .001

TABLE 17. The effects of SDS-PAGE separated supernatants on MN cell blastogenesis

(a) The effects of components other than those of Mr 122-148kDa.

Protein/carbohydrate fractions obtained by SDS-PAGE	Source of supernatants (% Suppression and range)		
	CD4+ cells + >200kDa mycobacterial fractions	CD8+ cells + <200kDa mycobacterial fractions	CD8+ cells + >200kDa mycobacterial fractions
50kDa	2 (1 - 3)	0	0
55kDa	0	0	4 (2 - 6)
67kDa	0	3 (0 - 6)	0
90kDa	0	0	0
150kDa	0	0	0

The results shown in Table 18 reflect the effects of nitrocellulose strips corresponding to regions of Mr 122-148kDa. These were areas of lanes containing supernatants from CD4+ or CD8+ cells in medium or those stimulated with mycobacterial fractions of Mr >200kDa (CD4+ cells) or of Mr <200kDa (CD8+ cells). None of these areas were immunosuppressive. Table 18 also demonstrates control systems which contained MN cells stimulated with PHA alone (CPM = 22 446 ± 2 092) or those to which mycobacterial fractions were added. The latter control served to confirm that the system still recognised mycobacterial fractions of Mr >200kDa which activated immunosuppressive mechanisms (60%).

4.21. THE EFFECT OF SUPPRESSOR CARBOHYDRATES ON LYMPHOCYTE BLASTOGENESIS

Figure 8 demonstrates that carbohydrates cleaved from the glycolipid component of lymphocyte suppressor supernatants suppressed MN cell blastogenesis. This effect was dose dependent with maximal suppression observed at a final concentration of 100-250ng/ml which is optimal for cell systems activated with mitogens. When MN cells were incubated with PPD however, 500ng/ml of the carbohydrate was required for optimal suppression.

TABLE 18. The effects of SDS-PAGE separated supernatants on MN cell blastogenesis (Mean CPM ± SD of 3 experiments)

Source of 24 hour supernatants	Response of MN cells in the presence of PHA to nitrocellulose bound regions of Mr 122-148kDa (% suppression in parenthesis)	
Supernatants derived from:		
CD4+ cells + medium	25 158 ± 3 138	
CD8+ cells + medium	23 806 ± 2 593	
CD4+ cells + >200kDa fractions	24 655 ± 2 631	(0)
CD8+ cells + <200kDa fractions	25 716 ± 2 484	(0)
Mycobacterial fractions >200kDa	8 960 ± 1 080*	(60)
Mycobacterial fractions <200kDa	24 191 ± 1 595	(0)
The mean CPM ± SD of MN cells incubated with PHA alone in these experiments was	22 446 ± 2 092	

* - P < .001

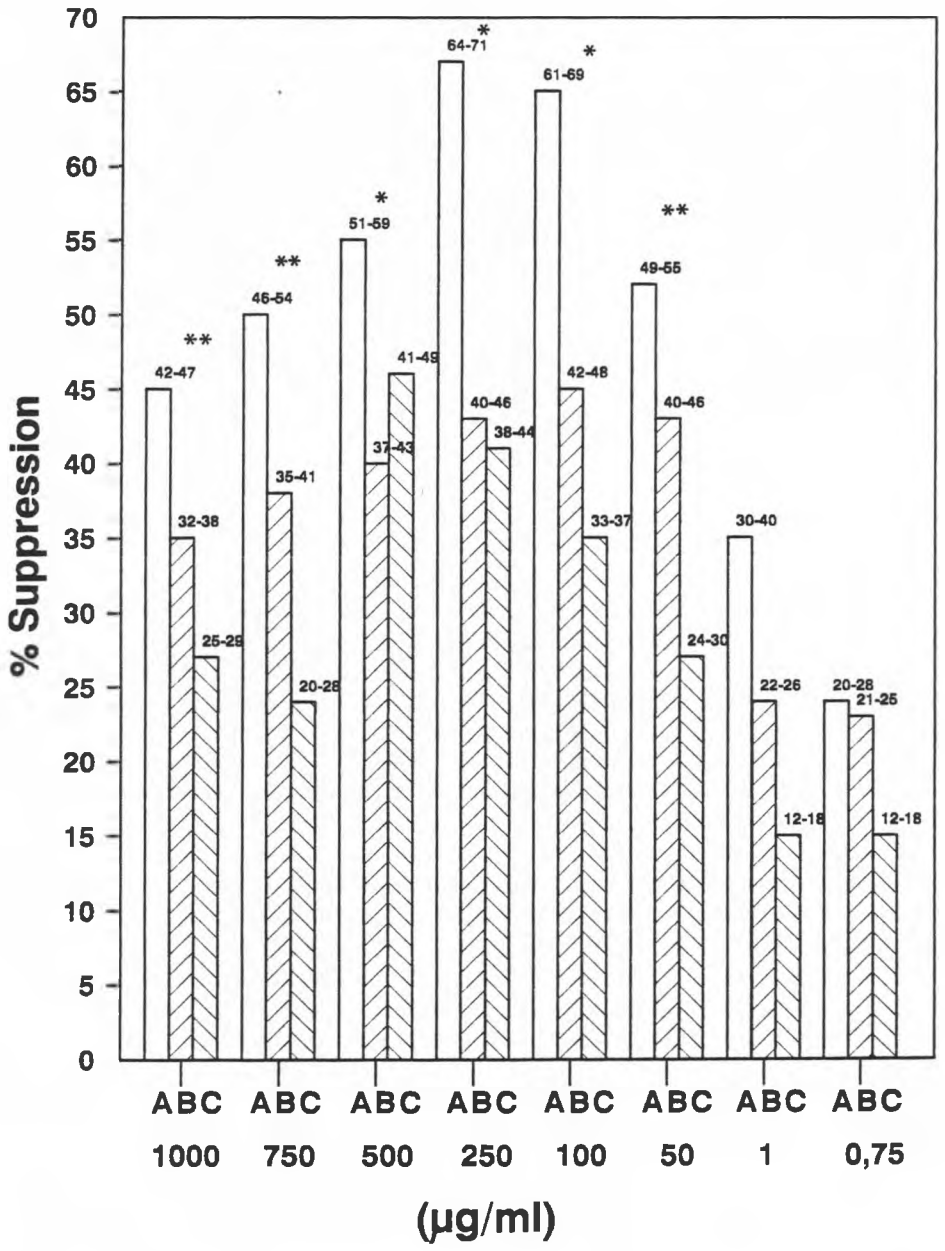


FIGURE 8. The effect of suppressor carbohydrates on lymphocyte blastogenesis.

MN cells + suppressor supernatants at different concentrations (ng/ml) incubated with:-
(A) PHA
(B) Con A
(C) PPD

The bars reflect the % inhibition of blastogenesis when compared to the proliferation of MN cells stimulated with mitogens (A & B) or antigens (C).

* - $P < .001$

** - $P < .01$

4.22. THE EFFECT OF PRE-INCUBATION OF MN CELLS WITH SUPPRESSOR CARBOHYDRATES

Since suppressor carbohydrates were able to inhibit lymphocyte blastogenesis in culture, experiments were undertaken whereby MN cells were pulsed for different time intervals with optimal concentrations of suppressor carbohydrates, washed and incubated with PHA, Con A and PPD (Table 19). The results indicate that pulsing of MN cells for 4 hours with suppressor carbohydrates inhibited lymphocyte blastogenesis when stimulated in culture. This effect was not observed when pulsing was performed for 2 hours only. A moderate though not significant increase in lymphocyte blastogenesis was seen when suppressor carbohydrate pulsed MN cells were placed in culture with medium alone (Table 19).

4.23. THE EFFECT OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS AND CARBOHYDRATES ON MIXED LYMPHOCYTE CULTURE (MLC)

When MN cells derived from peripheral blood of one individual (MN ^{1R}, R = responder) were incubated in culture with irradiated MN cells (MN ^{2S}, S = stimulator) of a second individual, responder MN cells (MN ^{1R}) proliferated to the stimulator MN ^{2S} cells (Table 20). Similar effects were seen when the stimulator and responder cells were reversed (Table 20). The addition of lymphocyte suppressor supernatants or 100ng/ml of suppressor carbohydrate significantly suppressed the proliferation of MN ^{R} cells when added to culture (Table 20). This effect was not seen when control supernatants were added to cultures (Table 20).

TABLE 19. The effect of pulsing MN cells with suppressor carbohydrates on the blastogenic response of MN cells. (% suppression in parenthesis)

Culture Systems	Mean CPM ± SD of 3 experiments cRPMI (medium)	PHA	PPD
MN cells alone	3 217 ± 1 706	52 559 ± 6 172	17 982 ± 2 160
MN cells + suppressor carbohydrates 2 hours*	3 692 ± 1 276	49 786 ± 5 967 (5)	18 612 ± 1 665 (0)
MN cells + suppressor carbohydrates 4 hours[1]	3 809 ± 1 529	39 466 ± 4 127 (25)*	12 899 ± 1 062 (28)*

[1] see text for details.

* - P < .01

TABLE 20. The effect of lymphocyte suppressor supernatants and carbohydrates on mixed lymphocyte culture (MLC) (% suppression in parenthesis)

Cell Systems	Mean CPM ± SD of 3 experiments	
MN ^{1R} cells alone	5 710 ± 662	
MN ^{2S}* cells alone	1 069 ± 212	
MN ^{1R} cells + MN ^{2S}* cells	17 457 ± 1 500	
MN ^{1R} cells + MN ^{2S}* cells control supernatants	16 496 ± 1 217	(6)
MN ^{1R} cells + MN ^{2S}* cells suppressor supernatants	9 299 ± 1 062	(47)**
MN ^{1R} cells + MN ^{2S}* cells suppressor carbohydrate	11 358 ± 980	(35)***
MN ^{2R} cells alone	6 794 ± 547	
MN ^{1S}* cells alone	1 182 ± 306	
MN ^{2R} cells + MN ^{1S}* cells	14 960 ± 1 319	
MN ^{2R} cells + MN ^{1S}* cells control supernatants	13 383 ± 1 415	(10,6)
MN ^{2R} cells + MN ^{1S}* cells suppressor supernatants	9 514 ± 876	(36)***
MN ^{2R} cells + MN ^{1S}* cells suppressor carbohydrate	9 874 ± 1 045	(34)***

* - MN cells irradiated at 3 000 rads.
R - responder cells
S - stimulator cells

** - $P < .001$
*** - $P < .01$

4.24. THE EFFECTS OF SUPPRESSOR SUPERNATANTS ON CYTOKINE PRODUCTION BY MN CELLS

Lymphocyte suppressor supernatants increased the production of IL-4 and IL-6 by MN cells incubated with LPS (Figure 9 II,VI - mean ± SD of 3 experiments). Lymphocyte control supernatants demonstrated no such effect. Increases in the production of these cytokines were evident as early as 4 hours, with maximal levels observed at 24 hours. Interleukin-4 and IL-6 activity was still observed at 48 hours of culture, but could not be detected at 72 hours (Figure 9 II,VI). An increased level of IL-4 could be detected at 48 hours of culture but no differences in IL-6 levels could be detected at this time.

Lymphocyte suppressor supernatants appeared to inhibit the production of IL-1β, TNF-alpha, IL-2 and IFN-gamma (Figure 9 I,III,IV,V). Inhibition of IL-1β, TNF-alpha and IL-2 production was apparent within 4 hours of incubation. Inhibition of TNF-alpha and IL-2 remained for a further 24 hours. The inhibitory effects of lymphocyte suppressor supernatants on IFN-gamma production was only observed in 24 hour cultures (Figure 9 V). These effects were not evident in cultures maintained for longer periods. Lymphocyte control supernatants did not demonstrate any inhibition of these cytokines at any time (Figure 9 I,III,IV,V).

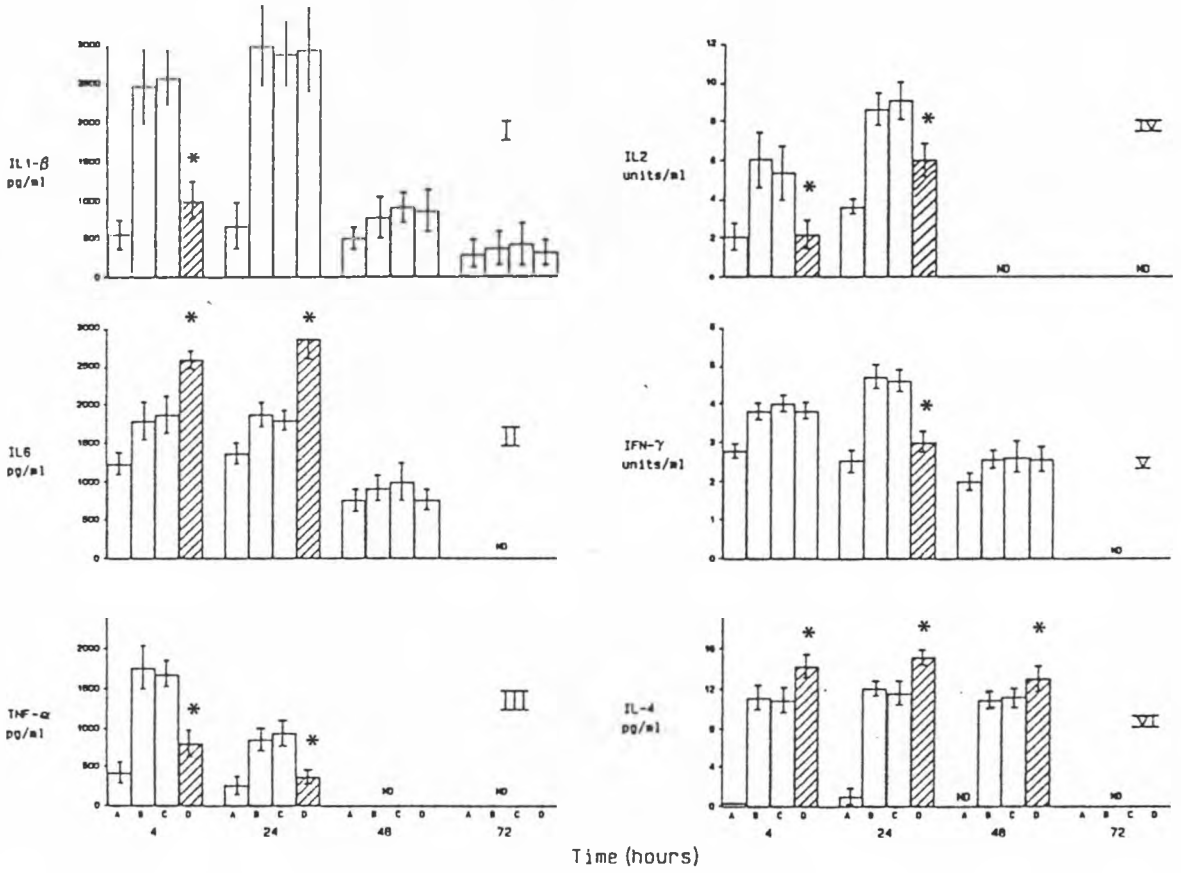


FIGURE 9. Cytokine production by MN cells

MN cells incubated with:
(A) Medium alone
(B) LPS
(C) LPS + lymphocyte control supernatants
(D) LPS + lymphocyte suppressor supernatants

Hatched bars indicate cytokine levels significantly different from control levels (* $P < .005$).
(Mean ± SD of 3 experiments)
ND = NOT DETECTED.

4.25. THE EFFECTS OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS ON CYTOKINE PRODUCTION BY PURIFIED PERIPHERAL BLOOD MONOCYTES

In order to assess the effects of lymphocyte suppressor supernatants on monocytes, experiments were undertaken whereby purified peripheral blood monocytes were utilised in studies of IL-1ß, IL-6 and TNF-alpha production (Figure 10 I,II,III). The presence of suppressor molecules significantly increased the production of IL-6 within 4 hours reaching maximal production at 24 hours of culture (Figure 10 II - mean ± SD of 3 experiments). The production of IL-1ß and TNF-alpha, however, was significantly suppressed (Figure 10 I,III). Suppression of IL-1ß was only apparent early (4 hours) during culture, whereas the production of TNF-alpha was inhibited for as long as 24 hours (Figure 10 I,III). Lymphocyte control supernatants demonstrated no such effect.

4.26. THE EFFECTS OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS ON LYMPHOKINE PRODUCTION

The production of IFN-gamma and IL-2 by purified NAL's stimulated with PHA was significantly reduced by lymphocyte suppressor but not control supernatants (Figure 11 I,II - mean ± SD of 3 experiments). Suppression of lymphokine production was observed at 4 and 24 hours (IL-2) and at 48 hours (IFN-gamma) (Figure 11 I,II). The production of IL-4 and IL-6 by purified NAL's, however, was significantly elevated at 4, 24 and 48 hours (Figure 11 III, IV). The production of these four lymphokines was too low to determine differences in 72 hour culture supernatants with the assays used.

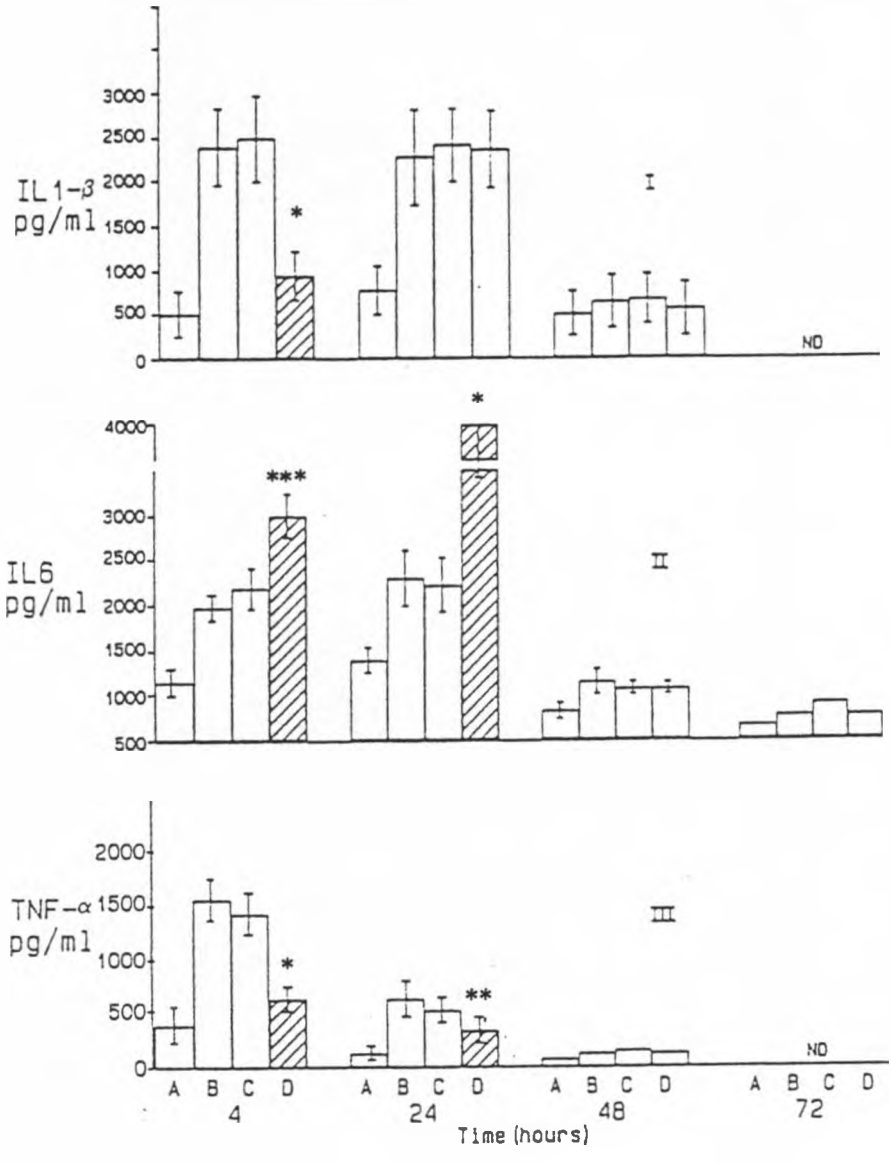


FIGURE 10. The production of IL-1ß, TNF-alpha and IL-6 by purified monocytes.

Monocytes incubated with:
(A) Medium alone
(B) LPS
(C) LPS + lymphocyte control supernatants
(D) LPS + lymphocyte suppressor supernatants

Supernatants were collected for cytokine assessment as for MN cells.
Hatched bars indicate values significantly different from control levels
(Mean ± SD of 3 experiments)
ND = NOT DETECTED
* $P < .005$
** $P < .05$
*** $P < .01$

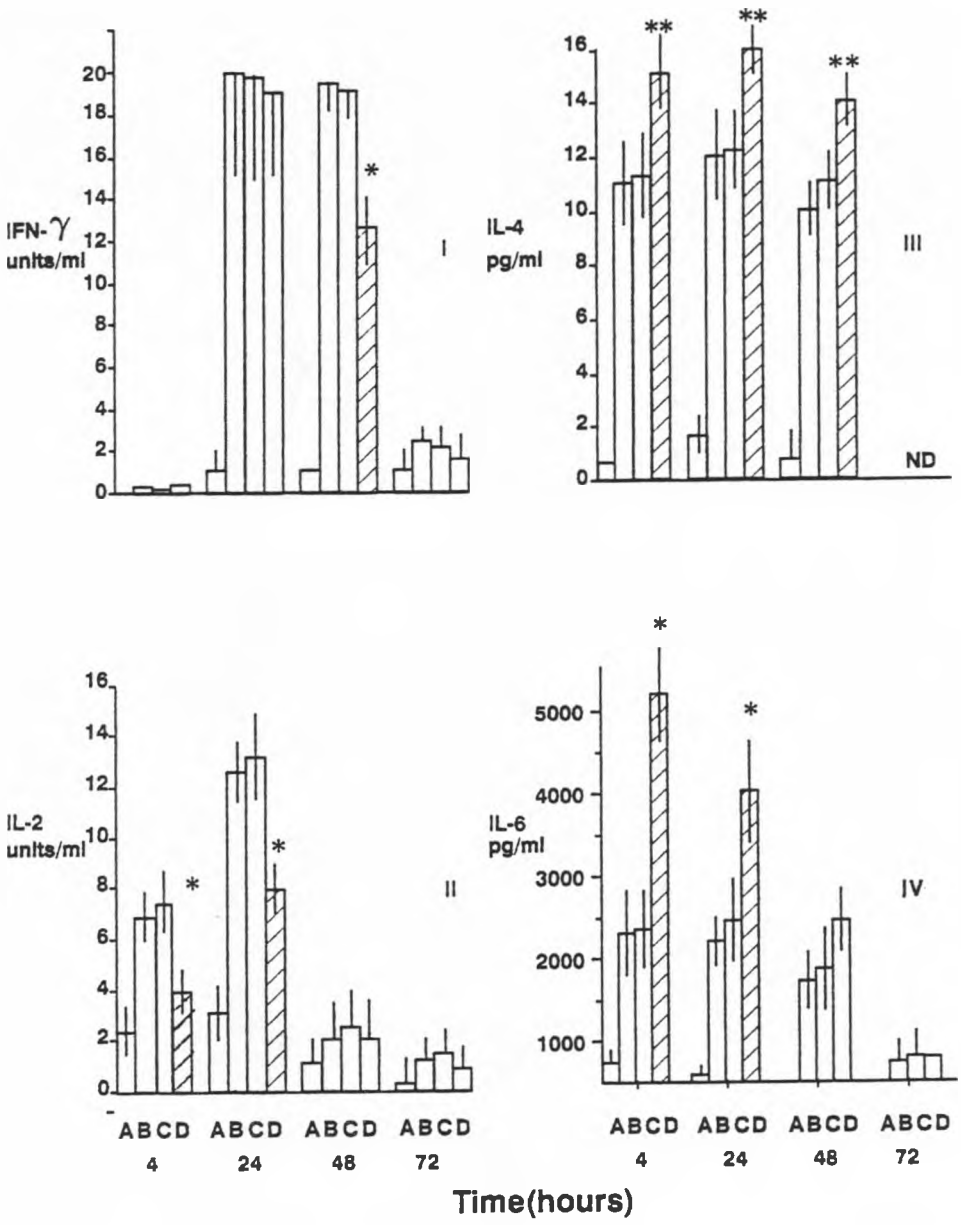


FIGURE 11. The production of IL-2, IFN-gamma, IL-6 and IL-4 by NAL's

NAL's incubated with:
(A) Medium alone
(B) PHA
(C) PHA + lymphocyte control supernatants
(D) PHA + lymphocyte suppressor supernatants

Supernatants were collected at various time intervals as indicated.
Hatched bars indicate cytokine levels significantly different from control levels
(Mean ± SD of 3 experiments)
ND = NOT DETECTED
* $P < .005$
** $P < .01$

4.27. THE EFFECTS OF SUPPRESSOR CARBOHYDRATES ON CYTOKINE PRODUCTION BY MN CELLS

When suppressor carbohydrates cleaved from suppressor glycolipids (Section 3.26) were incubated with LPS activated MN cells, the production of IL-4 and IL-6 increased (Figure 12VI,II - mean ± SD of 3 experiments). The same effects were observed when supernatants containing suppressor molecules were used in culture (Figure 12VI, II). Supernatants from control cultures did not increase the production of IL-4 and IL-6.

Supernatants containing suppressor molecules and purified carbohydrates inhibited the production of IL-1ß, TNF-alpha, IL-2 and IFN-gamma (Figure 12I, III, IV, V). Inhibition of IL-1ß was observed after 4 hours in culture whilst TNF-alpha, IL-2 and IFN-gamma levels could be seen in 24 hour cultures. Lymphocyte control supernatants did not inhibit the production of any of these cytokines (Figure 12).

4.28. MEDIATION OF CYTOKINE PRODUCTION BY rIL-6:- EFFECTS ON IL-1ß PRODUCTION

To assess the direct effects of IL-6 on monokine production, different concentrations of rIL-6 were added to cultures containing MN cells alone or those stimulated with LPS. The results indicate that the presence of 25U/ml of IL-6 was capable of inhibiting the production of IL-1ß by LPS stimulated MN cells (4 hour cultures) (Figure 13 - mean ± SD of 3 experiments).

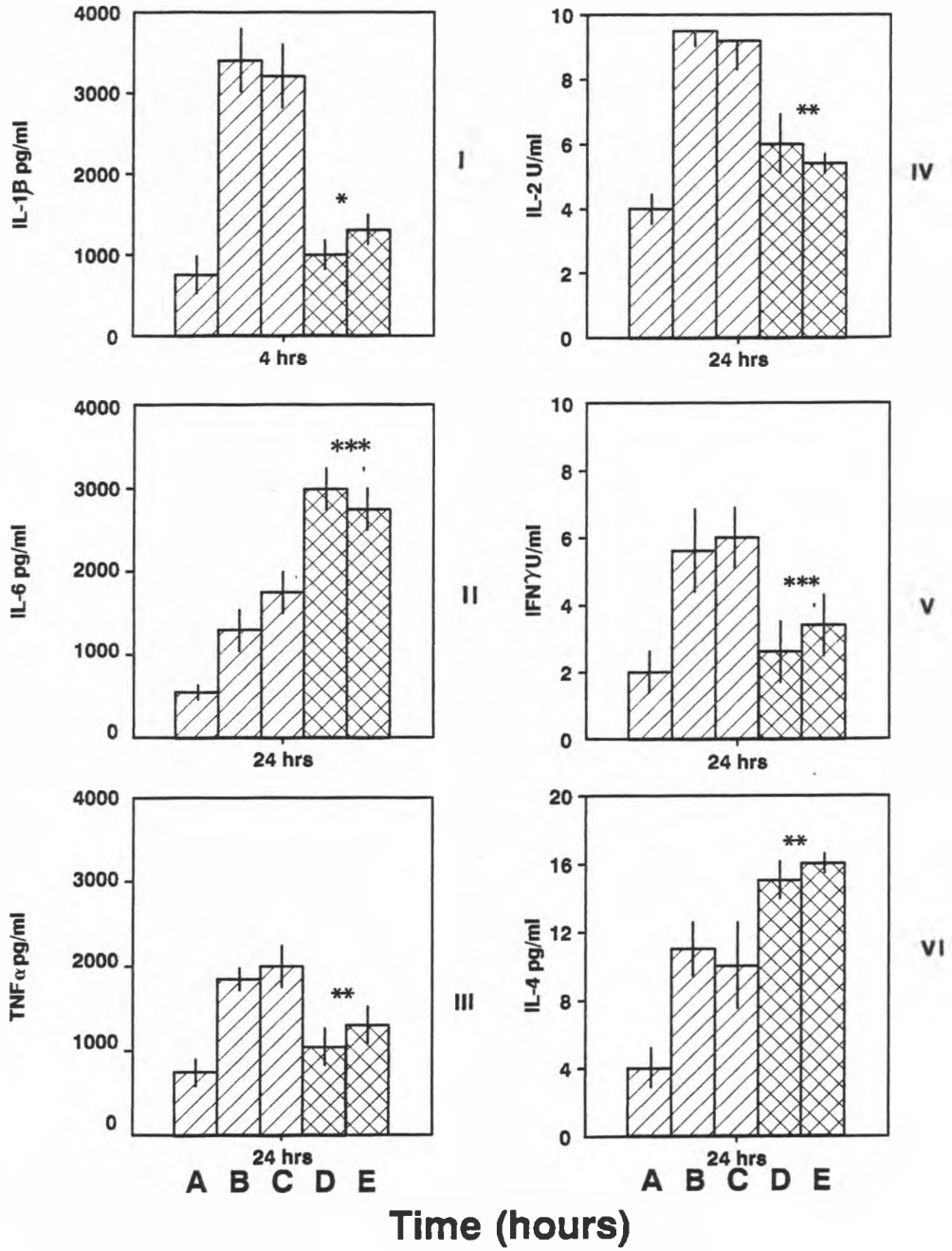


FIGURE 12. Cytokine production by MN cells:- Effect of suppressor carbohydrate

MN cells incubated with:
(A) cRPMI alone
(B) LPS
(C) LPS + lymphocyte control supernatants
(D) LPS + lymphocyte suppressor supernatants
(E) LPS + suppressor carbohydrate

Hatched bars indicate values significantly different from control levels
(Mean ± SD of 3 experiments)
* $P < .001$
** $P < .01$
*** $P < .005$

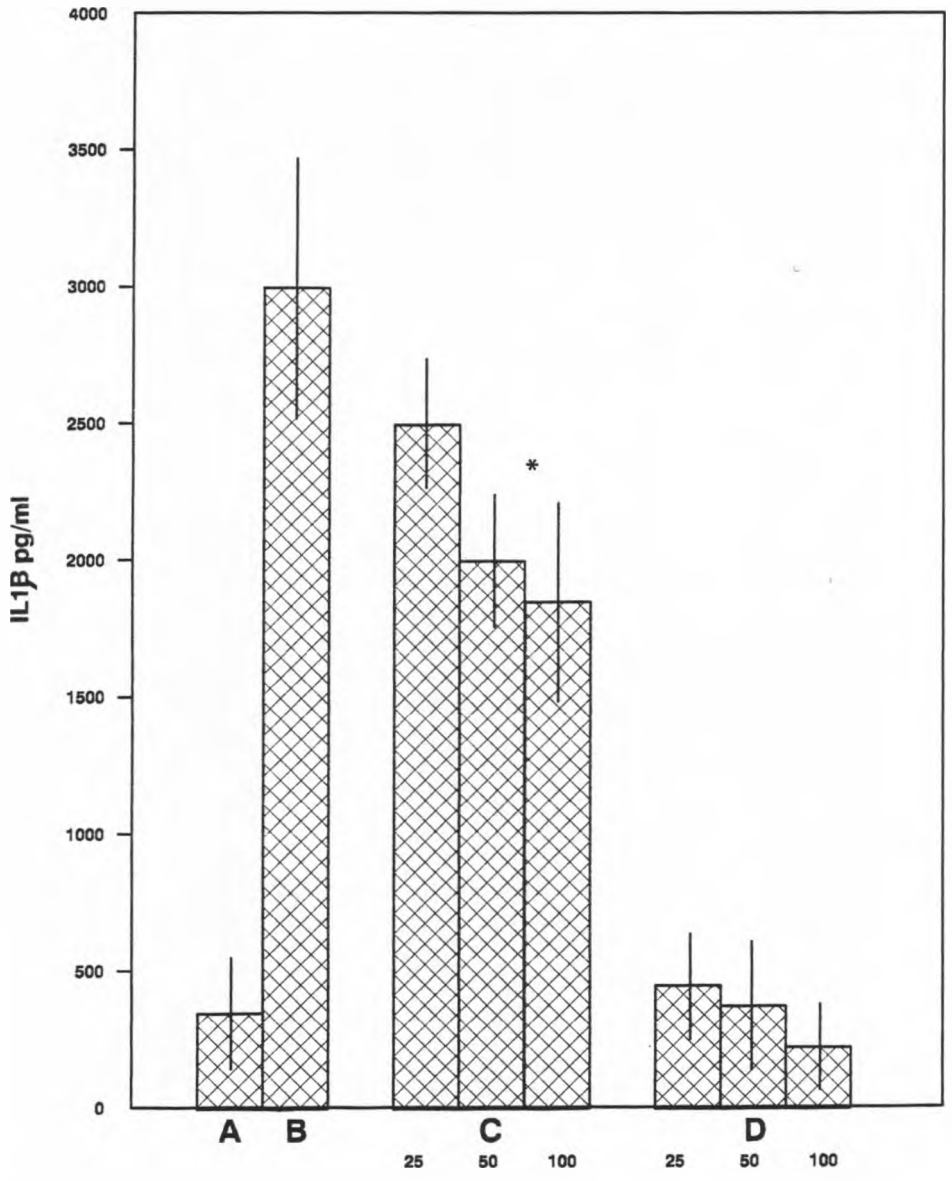


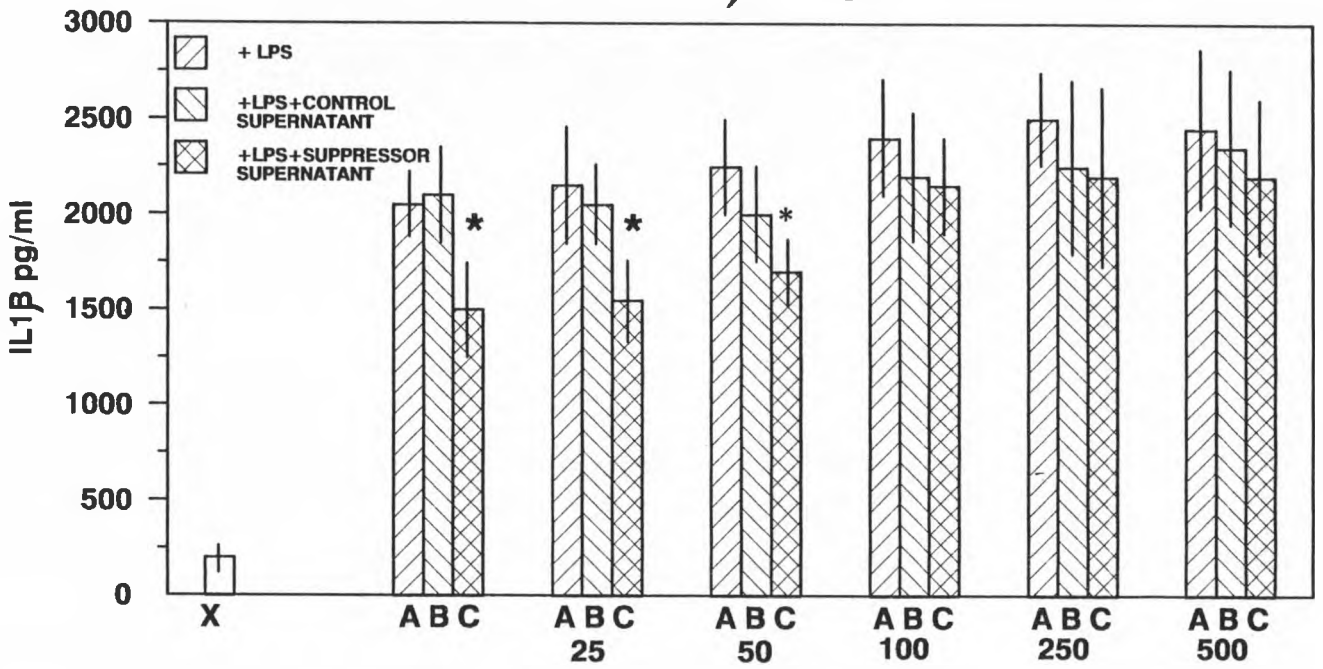
FIGURE 13. IL-1ß production by MN cells:- Effects of rIL-6

MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + different doses (U/ml) of IL-6
(D) Different doses (U/ml) of IL-6
(Mean ± SD of 3 experiments)
* P < .05

Significant suppression of IL-1ß production was obtained when 50U/ml of IL-6 was added to cultures. This suppression slightly improved with 100U/ml of IL-6 (Figure 13) but remained constant when higher concentrations were used (results not shown). Recombinant IL-6, at any of the concentrations used, had no effect on the production of IL-1ß by resting MN cells.

In other experiments lymphocyte suppressor supernatants were added to LPS-stimulated MN cell cultures in the presence of different concentrations of anti-IL-6 antibodies. The results of such studies (mean ± SD of 3 experiments) demonstrate that anti-IL-6 (100U/ml) restores the production of IL-1ß in 4 hour cultures treated with lymphocyte suppressor supernatants (Figure 14). Concentrations of anti-IL-6 below 100U/ml (25 and 50U/ml) failed to reverse inhibition of IL-1ß (Figure 14C). The production of IL-1ß by control systems which contained LPS-stimulated MN cells or these cells in the presence of lymphocyte control supernatants were not affected by any concentration of anti-IL-6 in culture (Figure 14 A, B). Anti-IL-6 added to resting cultures of MN cells also had no effect on IL-1ß production (results not shown).

Production of IL1β by MN cells.



Mn cells incubated with

X) CRPMI
A) CRPMI
B) CONTROL SUPERNATANT
C) SUPPRESSOR SUPERNATANT

} + DIFFERENT CONCENTRATIONS (U/ml of anti - IL6 antibodies)

(Mean - SD of 3 experiments)
* (p< 0,01)

4.29. MEDIATION OF CYTOKINE PRODUCTION BY rIL-4 AND rIL-6:- (a) EFFECTS ON IL-1ß PRODUCTION

In addition to a rise in the level of IL-6, previous studies (Figure 9) have demonstrated that IL-4 was concomitantly increased. It was therefore of interest to assess the effects of IL-4 on IL-1ß production. To this end, different concentrations of rIL-4 were added to MN cell cultures stimulated with LPS. The results (Figure 15C) demonstrate that 100, 200 and 400U/ml of IL-4 inhibits the production of IL-1ß in 4 hour supernatants of MN cell cultures when compared to control systems (Figure 15B). Inhibition of IL-1ß production was further augmented with the addition of 50U/ml of IL-6 (Figure 15E). However the inhibition of IL-1ß production was not significantly increased in the presence of IL-6. To compare the inhibition of IL-1ß production by rIL-4 with inhibition due to rIL-6, additional cultures were included in this series of experiments. Cultures containing 50U/ml of rIL-6 alone (previously optimised) demonstrated that MN cell IL-1ß production was significantly reduced (Figure 15D). Further experiments utilised this concentration of rIL-6 in combination with several doses of rIL-4. Such studies showed that a combination rIL-6 and rIL-4 minimally increased the inhibition of IL-1ß production when compared to cultures containing rIL-4 alone (Figure 15E vs Figure 15C). To ensure that IL-1ß suppression could also be due to the presence of rIL-4 in the culture system, anti-IL-4 antibodies were added to LPS stimulated MN cells. The results (Figure 15F) demonstrate that the production of IL-1ß was restored by 1ug/ml anti-IL-4

antibodies (Figure 15F). Concentrations above 1ug/ml of anti-IL-4 antibodies had no increased effect whilst those below this concentration did not significantly restore IL-1ß production by MN cells. Recombinant IL-4 added to LPS-stimulated MN cells in the presence of lymphocyte suppressor supernatants or carbohydrates did not inhibit the production of IL-1ß (Figure 15d, e) any further when compared to control systems (Figure 15b, c). However the production of IL-1ß by all of these cultures (Figure 15b, c, d, e) was suppressed when compared to IL-1ß produced by LPS stimulated MN cells alone (Figure 15B) or those incubated in the presence of lymphocyte control supernatants (Figure 15a).

4.30. RESTORATION OF IL-1ß PRODUCTION BY ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES

To confirm that IL-1ß produced by LPS-stimulated MN cells was inhibited by IL-4 and IL-6, experiments were undertaken whereby lymphocyte suppressor supernatants or suppressor carbohydrates were added to LPS-stimulated MN cells in the presence of optimal concentrations of anti-IL-4 and anti-IL-6 antibodies. The results indicate that 1ug/ml of anti-IL-4 (Figure 16a) or 100U/ml of anti-IL-6 (Figure 16b) or the addition of both antibodies (Figure 16c) completely restores the production of IL-1ß when compared to control systems. These were MN cells stimulated with LPS alone (Figure 16A) or those stimulated with LPS in the presence of lymphocyte control supernatants (Figure 16B).

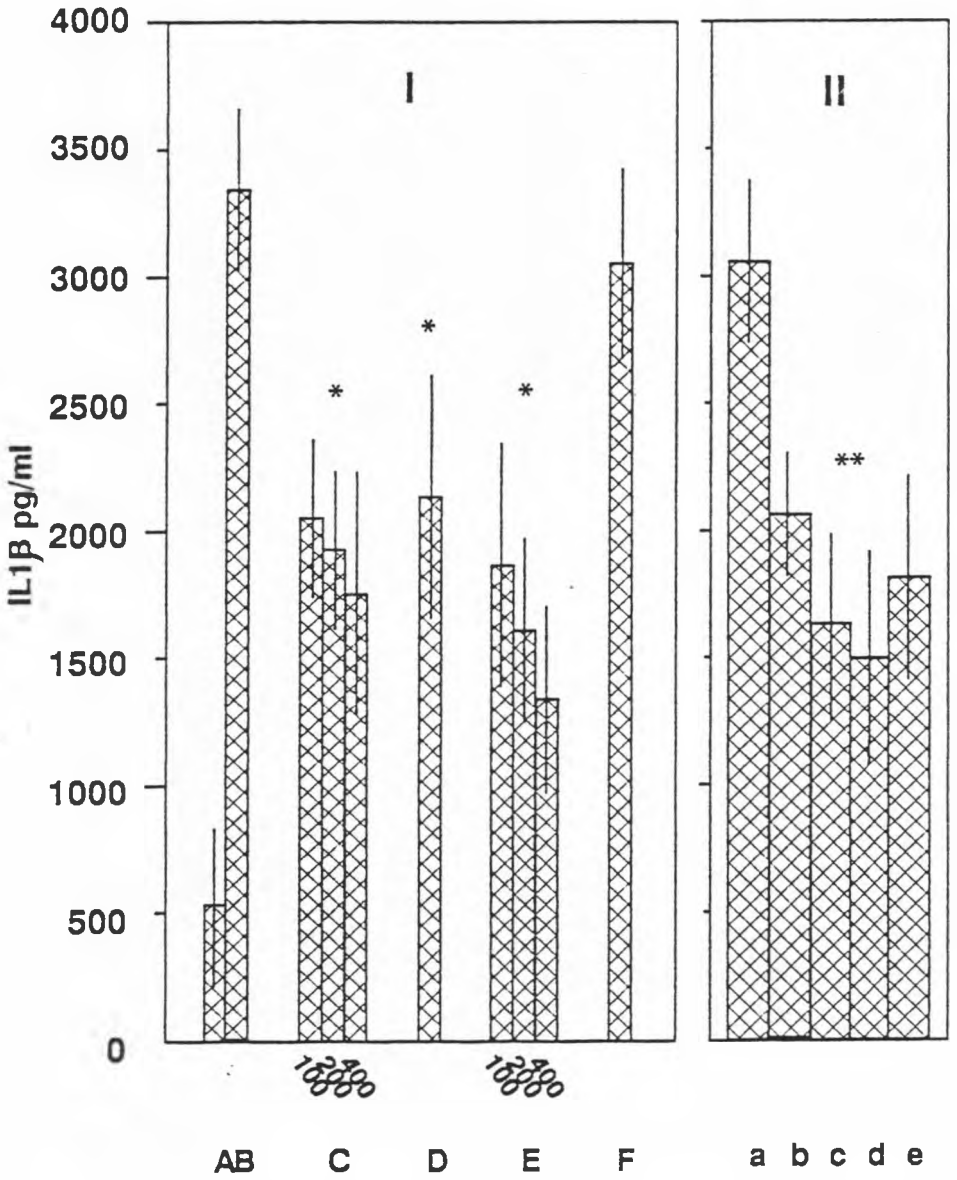


FIGURE 15. Production of IL-1ß by MN cells:- Effects of IL-6 and IL-4

(I)MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + different concentrations (U/ml) of IL-4
(D) LPS + IL-6 (50U/ml)
(E) LPS + different concentrations (U/ml) of IL-4 + IL-6 (50U/ml)
(F) LPS + IL-4 (200U/ml) + anti-IL-4 antibodies (1ug/ml)

(II)MN cells + LPS incubated with:
(a) Lymphocyte control supernatants
(b) Lymphocyte suppressor supernatants
(c) Suppressor carbohydrates
(d) Lymphocyte suppressor supernatants + IL-4 (200U/ml)
(e) Suppressor carbohydrates + IL-4 (200U/ml)
Mean ± SD of 3 experiments
* - $P < .05$
** - $P < .01$

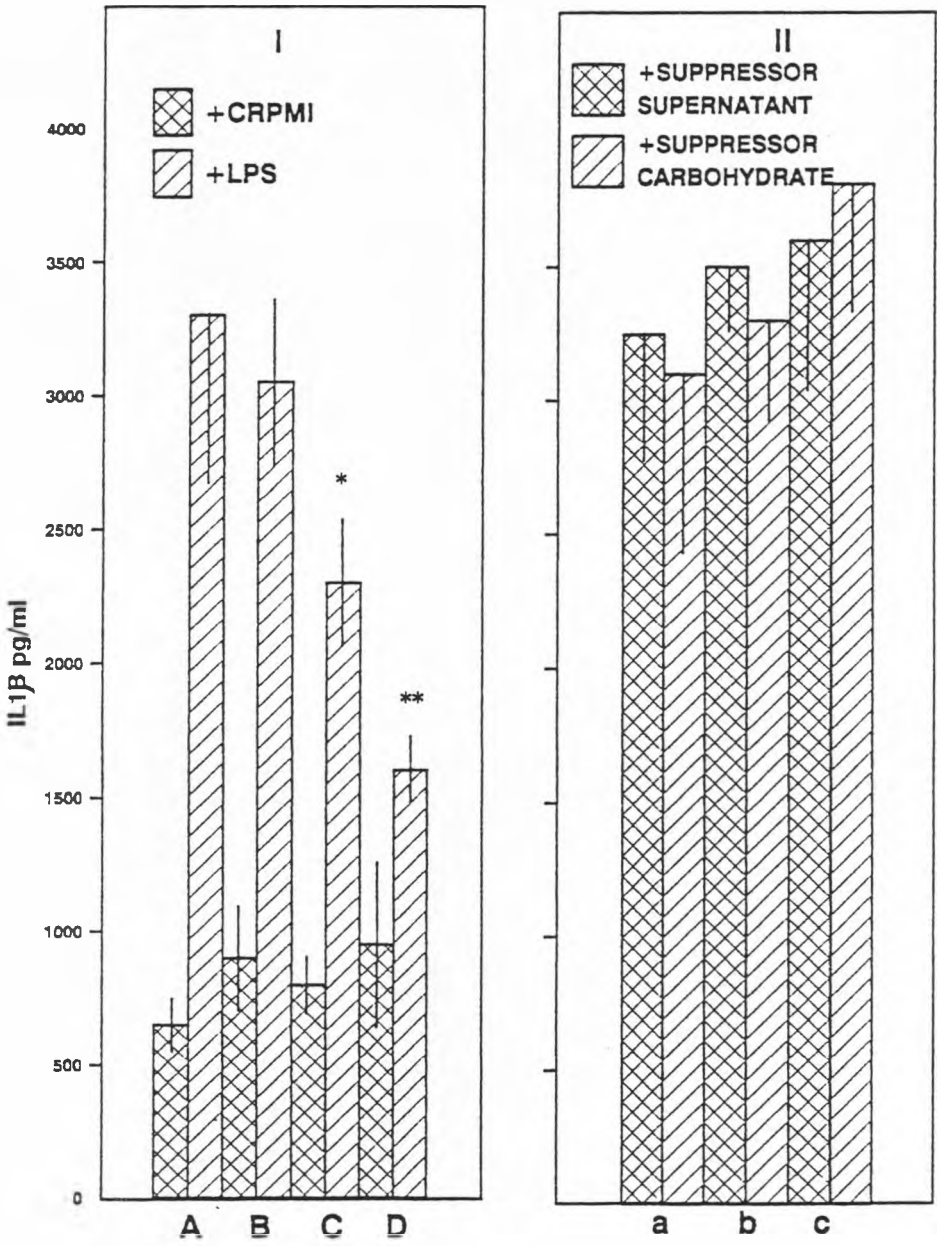


FIGURE 16. Production of IL-1ß by MN cells:- Effects of anti-IL-4 and anti-IL-6 antibodies

(I)MN cells incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrates

(II)MN cells incubated with LPS +:
(a) Anti-IL-4 antibodies (1ug/ml)
(b) Anti-IL-6 antibodies (100U/ml)
(c) Anti-IL-4 (1ug/ml) + anti-IL-6 (100U/ml) antibodies
Mean ± SD of 3 experiments
* - $P < .01$
** - $P < .001$

To ensure that lymphocyte suppressor supernatants and carbohydrates could indeed suppress IL-1ß production, suppressor supernatants or carbohydrates (Figure 16C, D) were added to MN cells stimulated with LPS. Lymphocyte control, suppressor supernatants (Figure 16 B, C) or suppressor carbohydrates (Figure 16D) when added to resting MN cells did not affect IL-1ß production above resting levels (Figure 16A).

4.31. IFN-GAMMA DOES NOT REVERSE IL-6 MEDIATED IL-1ß SUPPRESSION

IFN-gamma has been shown to be a potent stimulator of monocyte functions. It was therefore of interest to assess if this lymphokine could reverse the inhibition of IL-1ß production by exogenous rIL-6. The results indicate that the addition of IFN-gamma to cultures containing rIL-6 did not affect the production of IL-1ß by LPS-stimulated monocytes (Figure 17D). IFN-gamma did not affect IL-1ß production by LPS-stimulated monocytes (Figure 17C). Lipopolysaccharide stimulated control systems which contained IL-6 alone still demonstrated marked suppression of IL-1ß production (Figure 17B) whereas addition of anti-IL-6 antibodies completely restored the production of IL-1ß (Figure 17E) that was inhibited by exogenous rIL-6 (Figure 17B).

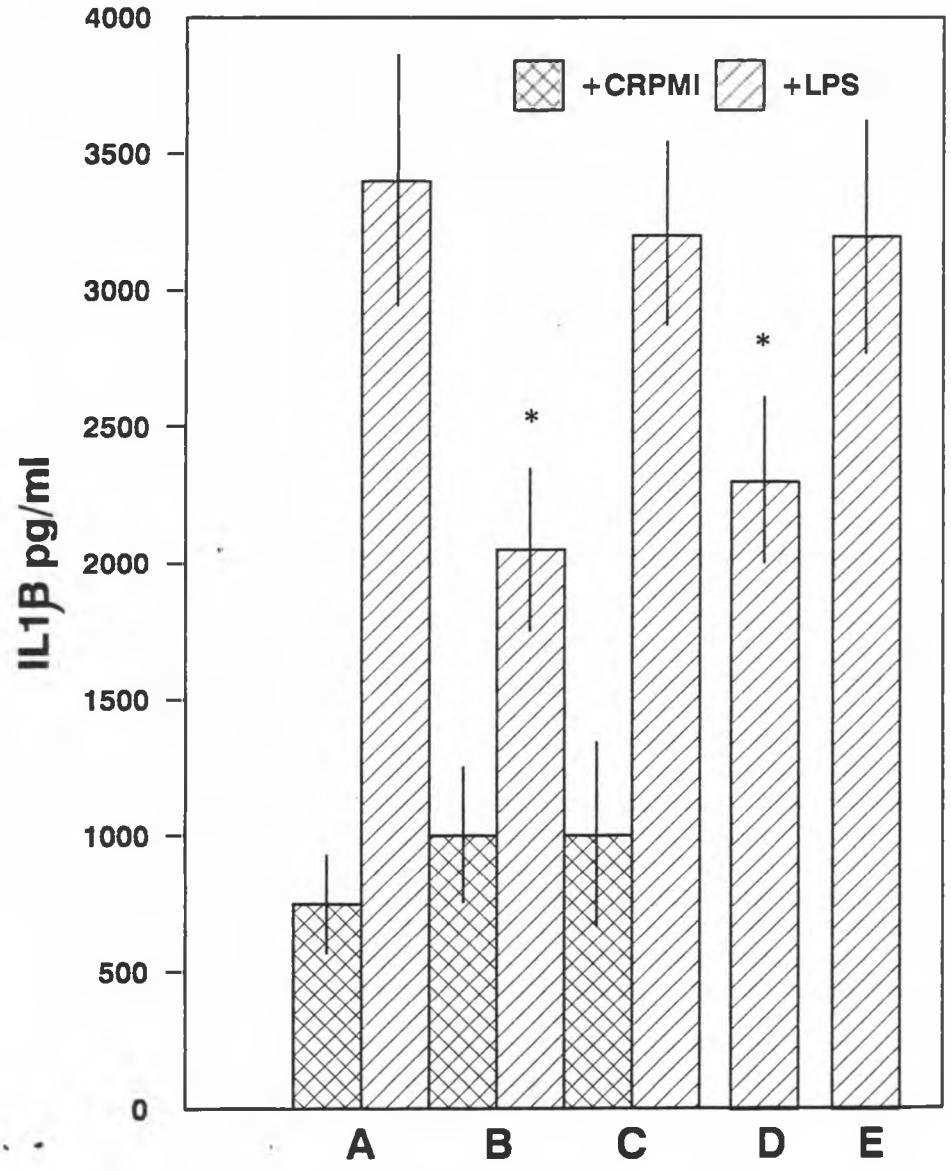


FIGURE 17. IL-1β production by monocytes: Effect of IFN-gamma

Monocytes incubated with:
(A) cRPMI
(B) IL-6 (50U/ml)
(C) IFN-gamma (100U/ml)
(D) IL-6 (50U/ml) + IFN-gamma (100U/ml)
(E) IL-6 (50U/ml) + IFN-gamma (100U/ml) + anti-IL-6 antibodies (100U/ml)
(Mean ± SD of 3 experiments)
* P < .001

4.32. MEDIATION OF CYTOKINE PRODUCTION BY rIL-4 AND rIL-6:- (b) EFFECT ON TNF-ALPHA PRODUCTION

When varying concentrations of rIL-4 were added to cultures containing MN cells stimulated with LPS, significant suppression of TNF-alpha production was observed (Figure 18C - mean ± SD of 3 experiments). Interleukin-4 at concentrations of 100, 200 or 400U/ml could inhibit TNF-alpha production by ~21-26% when compared to the control (LPS alone) (Figure 18B). Similarly, 50U/ml of IL-6 was capable of suppressing the production of TNF-alpha by MN cells (Figure 18D). However, the simultaneous addition of both IL-4 and IL-6 to LPS stimulated cells did not suppress TNF-alpha production any further (Figure 18E). To determine that rIL-4 was responsible for suppression of TNF-alpha by LPS stimulated MN cells, anti-IL-4 antibodies were added to cultures containing rIL-4. This restored the production of TNF-alpha (Figure 18F). Recombinant IL-4 added to LPS stimulated MN cells in the presence of lymphocyte suppressor supernatants or carbohydrates did not inhibit the production of TNF-alpha any further (Figure 18d, e) when compared to control systems (Figure 18 b, c). However the production of TNF-alpha by all of these cultures (Figure 18b, c, d, e) was suppressed when compared to TNF-alpha produced by LPS stimulated MN cells alone (Figure 18B) or those incubated with lymphocyte control supernatants (Figure 18a).

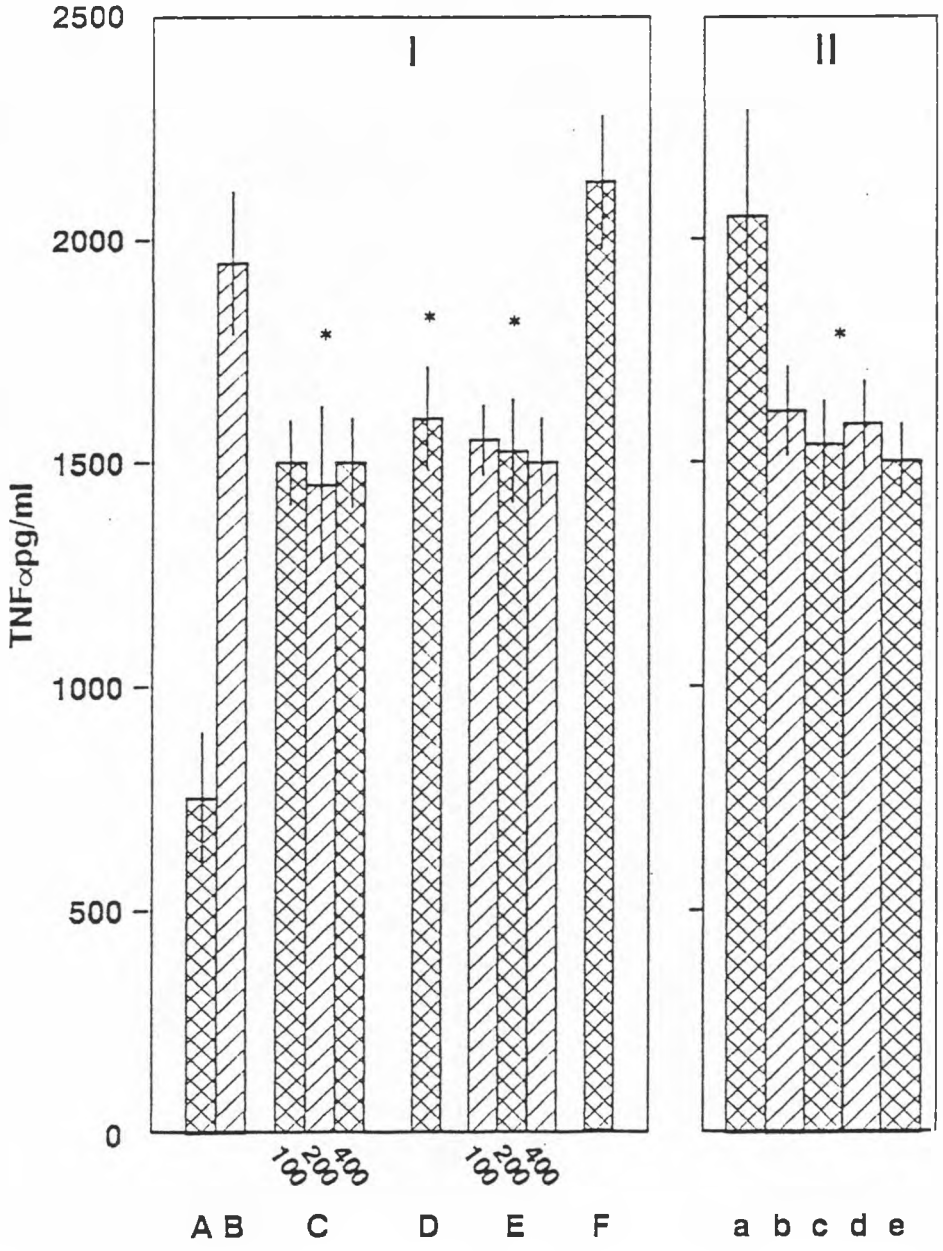


FIGURE 18. TNF-alpha production by MN cells:- Effect of rIL-6 and rIL-4

(I)MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + various concentrations (U/ml) of IL-4
(D) LPS + IL-6 (50U/ml)
(E) LPS + various concentrations (U/ml) of IL-4 + IL-6 (50U/ml)
(F) LPS + IL-4 (200U/ml) + anti-IL-4 antibodies (1ug/ml)

(II)MN cells + LPS incubated with:
(a) Lymphocyte control supernatants
(b) Lymphocyte suppressor supernatants
(c) Suppressor carbohydrates
(d) Lymphocyte suppressor supernatants + IL-4 (200U/ml)
(e) Suppressor carbohydrates + IL-4 (200U/ml)
Mean ± SD of 3 experiments
* - P < .01

4.33. RESTORATION OF TNF-ALPHA PRODUCTION BY ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES

Lymphocyte suppressor supernatants and carbohydrates were found to suppress TNF-alpha production when compared to MN cells incubated with LPS alone (Figure 19A vs Figure 19C, D) or LPS and lymphocyte control supernatants (Figure 19B). When anti-IL-4 and/or anti-IL-6 antibodies or both were added to cultures containing lymphocyte suppressor supernatants or carbohydrates, the production of TNF-alpha was completely restored (Figure 19a, b, c).

4.34. IFN-GAMMA DOES NOT REVERSE IL-6 MEDIATED TNF-ALPHA SUPPRESSION

Figure 20B confirms the previous finding that addition of rIL-6 significantly inhibits TNF-alpha production by LPS stimulated monocytes. Since IFN-gamma has been known to potentiate monocyte functions, this lymphokine was added to cultures containing rIL-6. The results demonstrate that IFN-gamma could not restore the inhibition of TNF-alpha production caused by rIL-6 (Figure 20D). Control systems which contained IFN-gamma only, did not alter TNF-alpha production (Figure 20C). However, the second control containing anti-IL-6 antibodies showed reversal of inhibition due to rIL-6 (Figure 20E). This reversal however was not enhanced by the addition of IFN-gamma over and above that seen with IFN-gamma alone (Figure 20C) or LPS alone (Figure 20A).

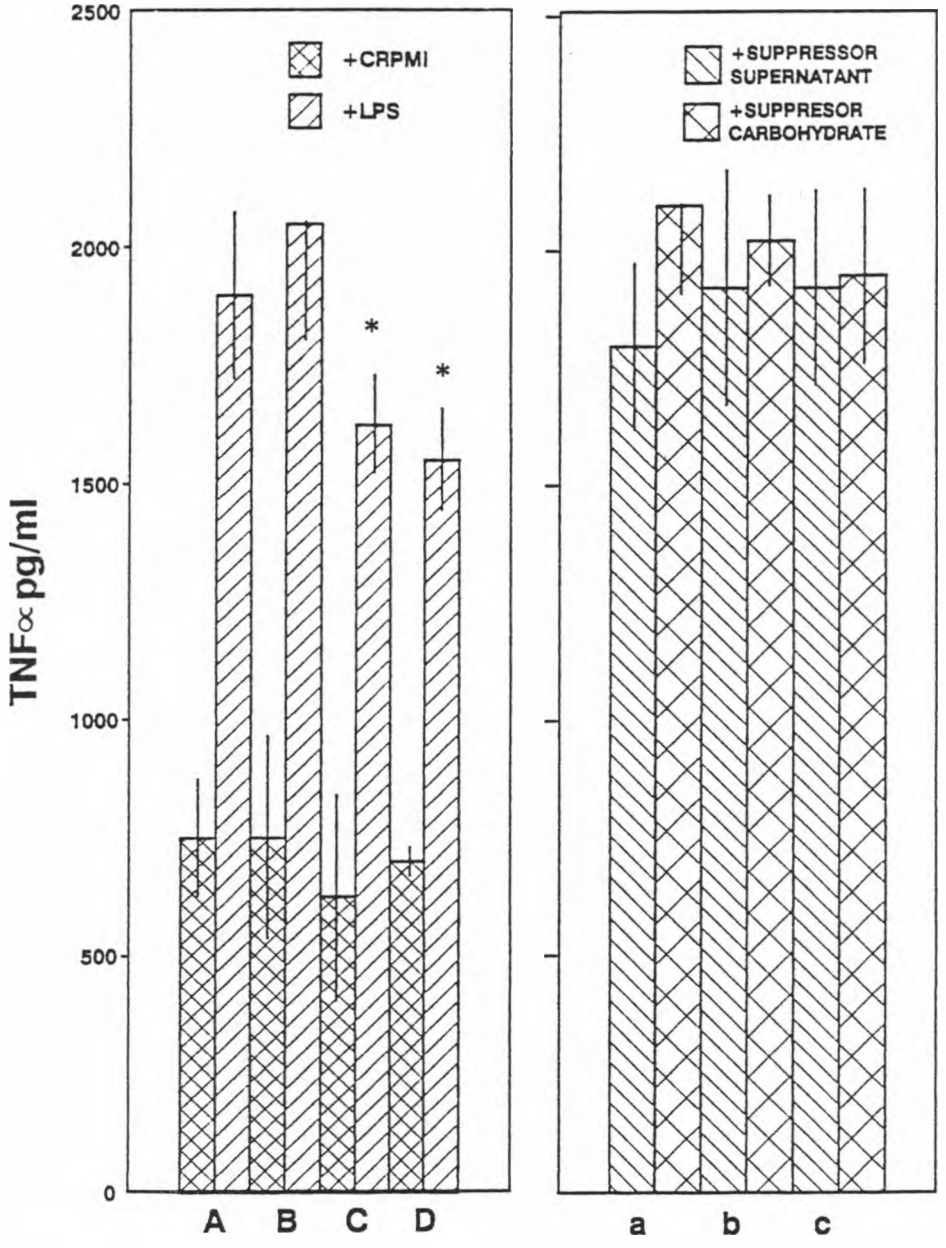


FIGURE 19. TNF-alpha production by MN cells:- Effect of anti-IL-4 and anti-IL-6 antibodies

(I)MN cells incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrates

(II)MN cells + LPS incubated with:
(a) Anti-IL-4 antibodies (1ug/ml)
(b) Anti-IL-6 antibodies (100U/ml)
(c) Anti-IL-4 (1ug/ml) + anti-IL-6 (100U/ml) antibodies
Mean ± SD of 3 experiments
* - $P < .05$

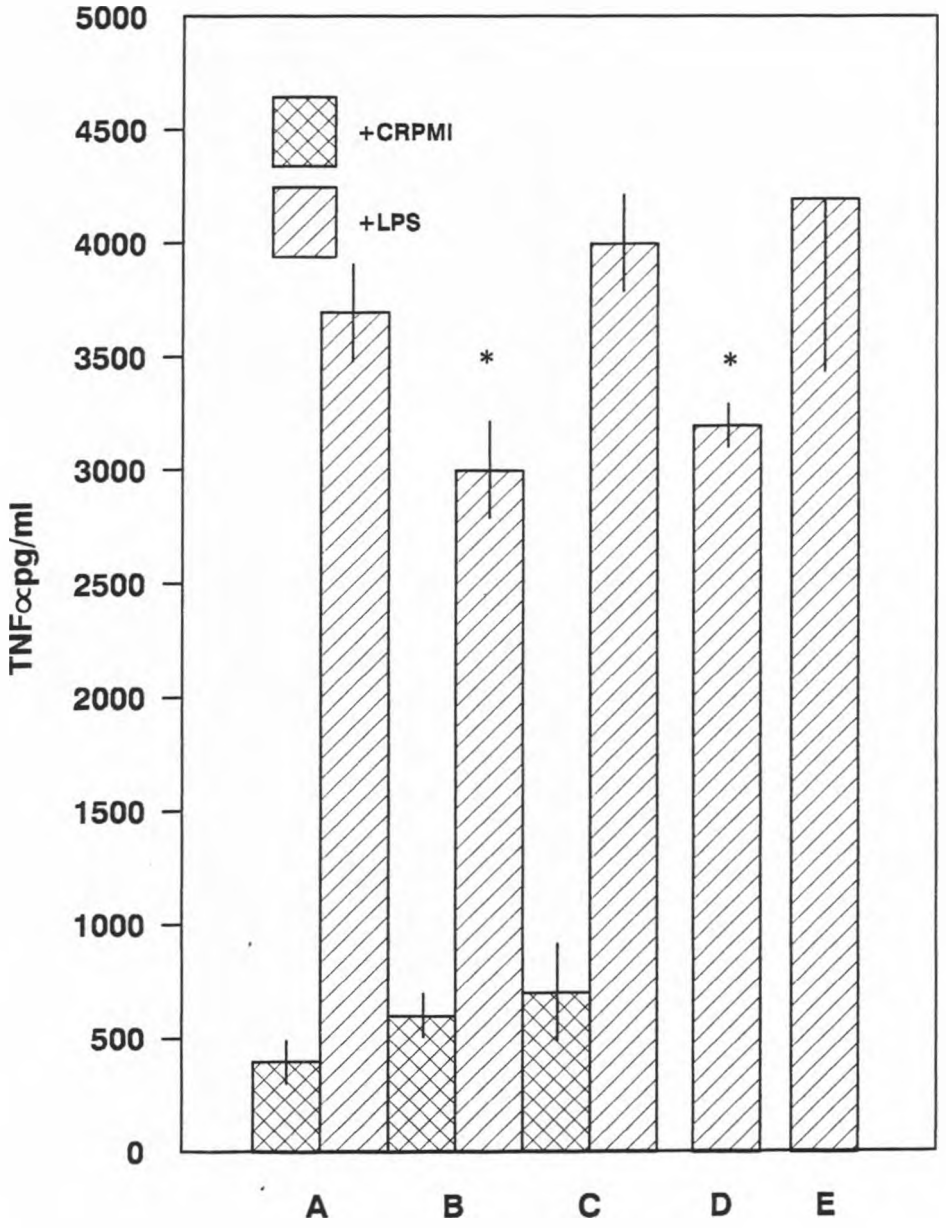


FIGURE 20. TNF-alpha production by MN cells: Effect of IFN-gamma

Monocytes incubated with:
(A) cRPMI
(B) IL-6 (50U/ml)
(C) IFN-gamma (100U/ml)
(D) IL-6 (50U/ml) + IFN-gamma (100U/ml)
(E) IL-6 (50U/ml) + IFN-gamma (100U/ml) + anti-IL-6 antibodies (100U/ml)
(Mean ± SD of 3 experiments)
* $P < .05$

4.35. MEDIATION OF SUPPRESSOR EFFECTS BY IL-6

To assess the direct effects of IL-6 on monokine production, rIL-6 was added to cultures containing purified monocytes alone or those stimulated with LPS. The results indicate that the presence of IL-6 inhibits the production of IL-1ß and TNF-alpha by monocytes stimulated with LPS (in 4 hour (IL-1ß) and 24 hour (TNF-alpha) cultures) (Figure 21, mean ± SD of 3 experiments). Interleukin-6 had no effect on the production of IL-1ß and TNF-alpha by resting monocytes.

4.36. INHIBITION OF IL-6 PRODUCTION BY IL-4

Since IL-6 levels in LPS activated MN cell supernatants returned to control levels 48 hours after treatment with lymphocyte suppressor supernatants, whilst IL-4 remained raised at this time (Figure 9 II, VI), experiments were undertaken to assess the effect of IL-4 on IL-6 production. When exogenous IL-4, at different concentrations, was added to LPS stimulated MN cells, significant inhibition of IL-6 production was observed (Figure 22C). Addition of rIL-6 to MN cells incubated with LPS did not enhance or suppress IL-6 production above that of the control (Figure 22D). When rIL-4 and rIL-6 were added to LPS stimulated MN cells, production of IL-6 was still significantly suppressed (Figure 22E). The suppression of IL-6 by IL-4 could be restored by the addition of anti-IL-4 antibodies (Figure 22F). Recombinant IL-4 added to LPS stimulated MN cells in the presence of lymphocyte suppressor

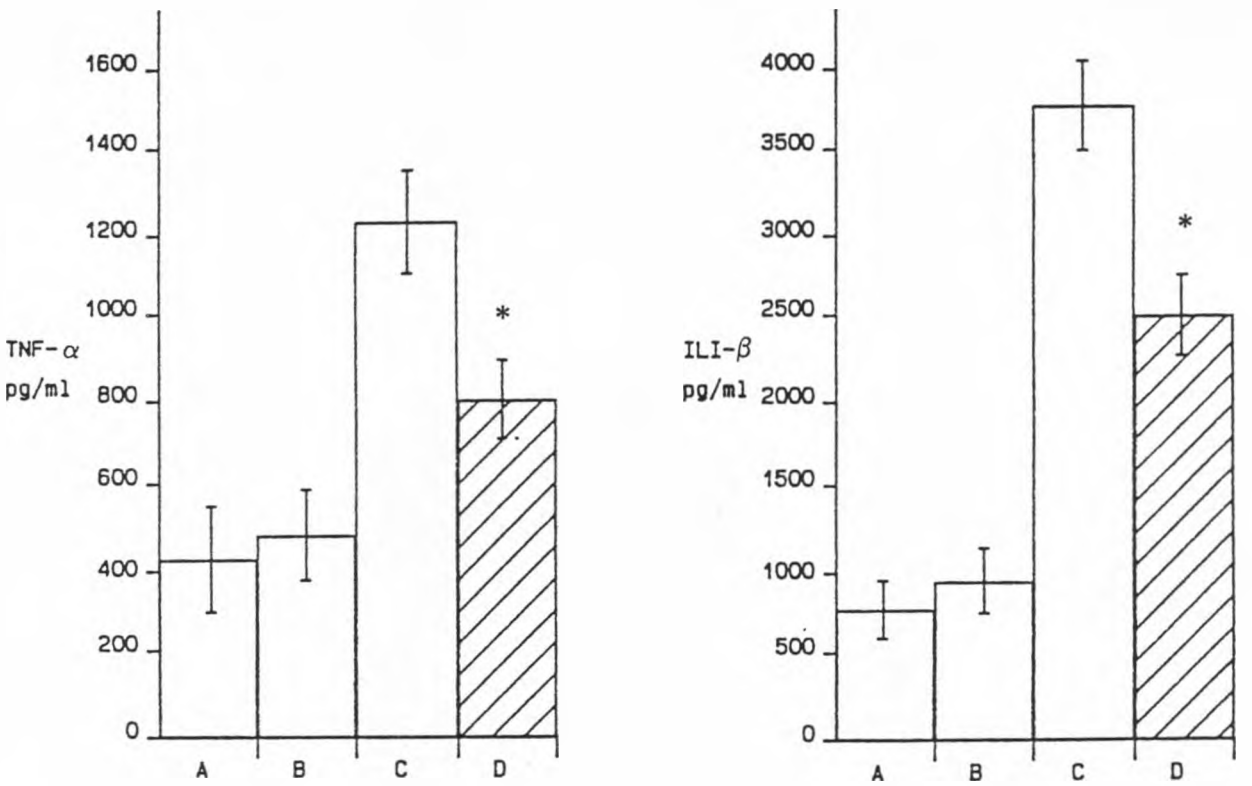


FIGURE 21. The effect of rIL-6 on IL-1ß and TNF-alpha production by purified monocytes in 4 hour (IL-1ß) and 24 hour (TNF-alpha) cultures.

MN cells incubated with:
(A) Medium alone
(B) rIL-6
(C) LPS
(D) LPS + rIL-6

Hatched bars indicate cytokine levels significantly different from control levels
(Mean ± SD of 3 experiments)
* $P < .005$

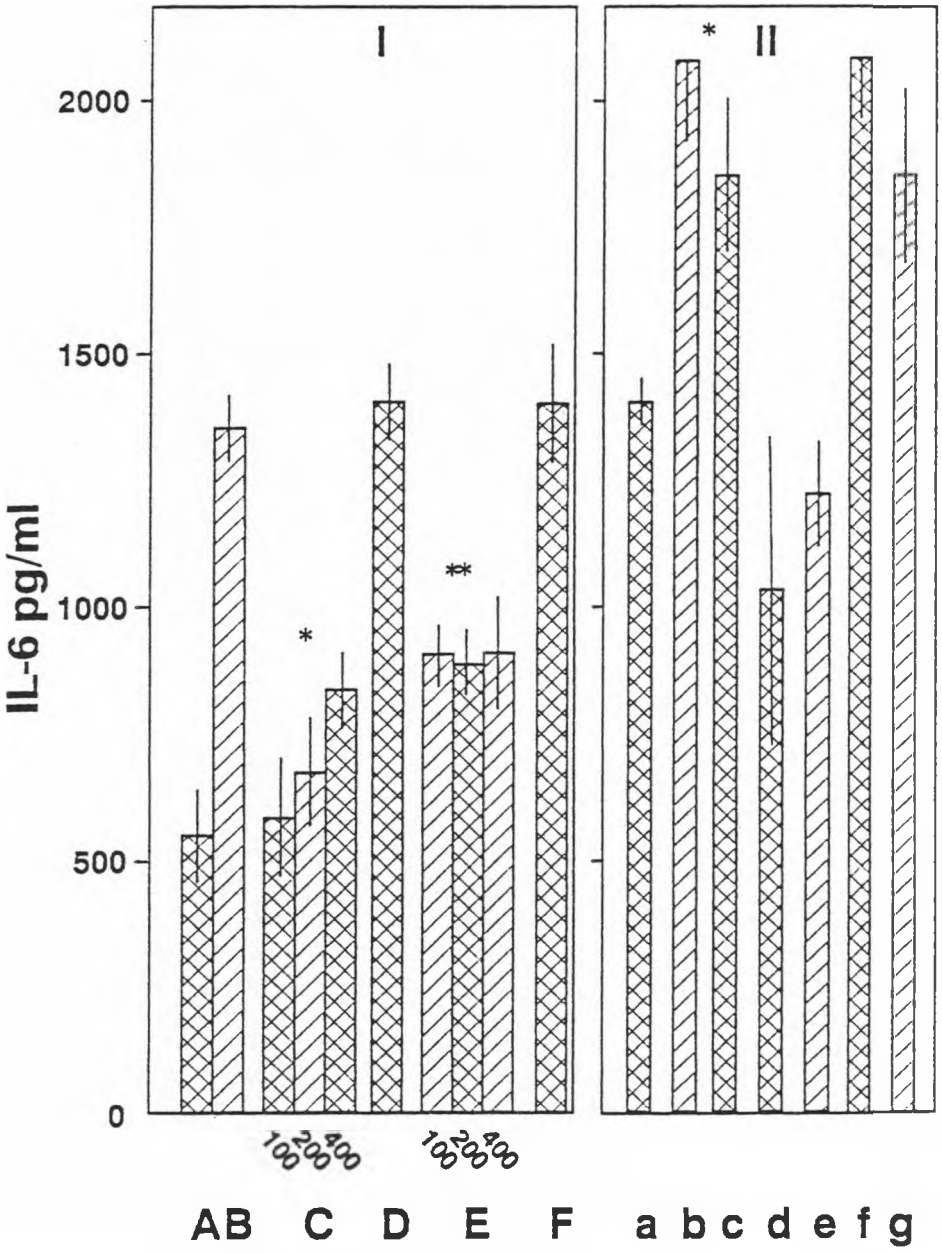


FIGURE 22. IL-6 production by MN cells:- The effect of rIL-4

(I)MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + different concentrations (U/ml) of IL-4
(D) LPS + IL-6 (50U/ml)
(E) LPS + various concentrations (U/ml) of IL-4 + IL-6 (50U/ml)
(F) LPS + IL-4 (200U/ml) + anti-IL-4 antibodies (1ug/ml)

(II)MN cells + LPS incubated with:
(a) Lymphocyte control supernatants
(b) Lymphocyte suppressor supernatants
(c) Suppressor carbohydrates
(d) Lymphocyte suppressor supernatants + IL-4 (200U/ml)
(e) Suppressor carbohydrate + IL-4 (200U/ml)
(f) Lymphocyte suppressor supernatants + anti-IL-4 antibodies (1ug/ml)
(g) Suppressor carbohydrates + anti-IL-4 antibodies (1ug/ml)
(Mean ± SD of 3 experiments)
* - $P < .001$
** - $P < .005$

supernatants or carbohydrates inhibited the production of IL-6 (Figure 22d, e) when compared to control systems (Figure 22b, c). The addition of anti-IL-4 antibodies to cultures containing lymphocyte suppressor supernatants or carbohydrates which already contained raised levels of IL-6 were without effect (Figure 22f, g). Interleukin-6 produced by LPS-stimulated MN cells incubated in the presence of lymphocyte control supernatants (Figure 22a) was unchanged when compared to IL-6 produced by LPS-stimulated MN cells alone (Figure 22B).

4.37. THE EFFECT OF IL-4 ON IL-2 PRODUCTION

To assess the effect of rIL-4 on IL-2 production, different concentrations of rIL-4 were added to LPS stimulated MN cells (Figure 23C - Mean ± SD of 3 experiments). Suppression of IL-2 ranged from 11% with 10U/ml of IL-4 to ~53% in the presence of 400U/ml (Figure 23C). The simultaneous addition of both IL-4 and IL-6 to LPS stimulated cells did not suppress IL-2 production any further (Figure 23E). The addition of anti-IL-4 antibodies completely reversed the inhibition of IL-2 production in cultures containing rIL-4 (Figure 23D). Since maximal suppression was achieved with 200U/ml of IL-4, this concentration was used in all subsequent experiments.

4.38. MEDIATION OF CYTOKINE PRODUCTION BY rIL-4 AND rIL-6:- EFFECT ON IL-2 PRODUCTION

Production of IL-2 by LPS stimulated MN cells was significantly decreased when these cells were pulsed with lymphocyte suppressor supernatants or carbohydrates (Figure 24F, I) compared to

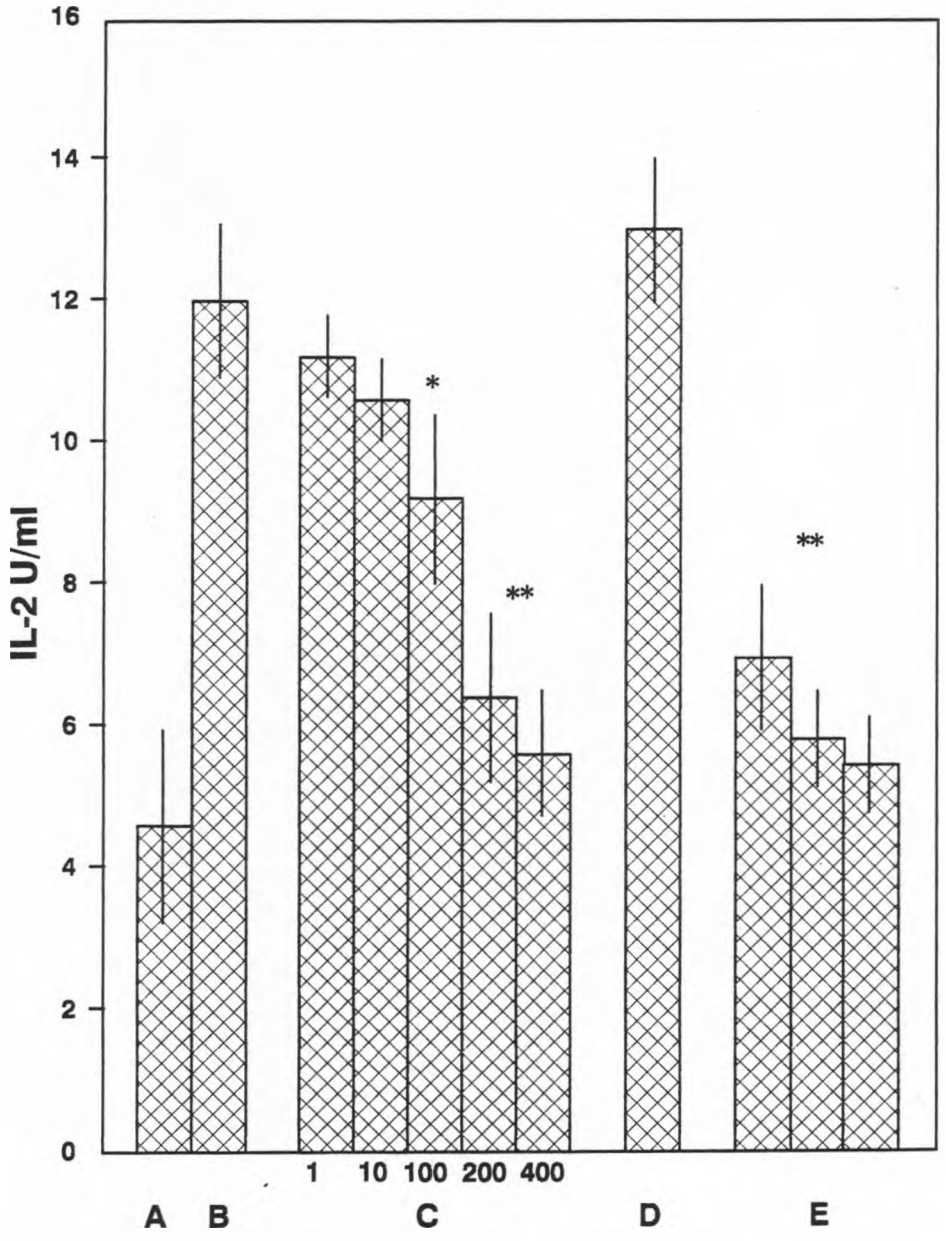


FIGURE 23. IL-2 production by MN cells: Effect of rIL-4

MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + different concentrations (U/ml) of IL-4
(D) LPS + rIL-4 (200U/ml) + anti-IL-4 antibodies (1ug/ml)
(E) LPS + different doses (U/ml) of rIL-4 + IL-6 (50U/ml)
(Mean ± SD of 3 experiments)
* - P < .01
** - P < .001

control systems containing LPS alone (Figure 24B) or those pulsed with lymphocyte control supernatants (Figure 24E). When anti-IL-6 antibodies were added to cultures together with lymphocyte suppressor supernatants the inhibition of IL-2 production was completely reversed (Figure 24G).

When 50U/ml of rIL-6 was added to LPS stimulated MN cell cultures the production of IL-2 was inhibited (Figure 24C). The addition of anti-IL-6 antibodies to such cultures resulted in the complete restoration of IL-2 production (Figure 24D). The addition of rIL-4 to LPS stimulated MN cells incubated with lymphocyte suppressor supernatants or carbohydrates did not suppress IL-2 production any further (Figure 24H, J).

4.39. PRODUCTION OF IL-2 BY MN CELLS INCUBATED WITH LYMPHOCYTE SUPPRESSOR SUPERNATANTS:- THE EFFECT OF ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES

Lymphocyte suppressor supernatants or carbohydrates inhibited the production of IL-2 by LPS stimulated MN cells (Figure 25C, D) when compared to control cultures of LPS stimulated MN cells alone (Figure 25A) or those incubated with lymphocyte control supernatants (Figure 25B - mean ± SD of 3 experiments). When anti-IL-4 (Figure 25a) or anti-IL-6 (Figure 25b) or both (Figure 25c) were added to cell cultures containing suppressor supernatants or carbohydrates, IL-2 production was restored.

Lymphocyte suppressor supernatants or carbohydrates (Figure 25C, D) or control supernatants (Figure 25B) added to resting MN cells had no effect on IL-2 production when compared with resting MN cell production of IL-2 (Figure 25A).

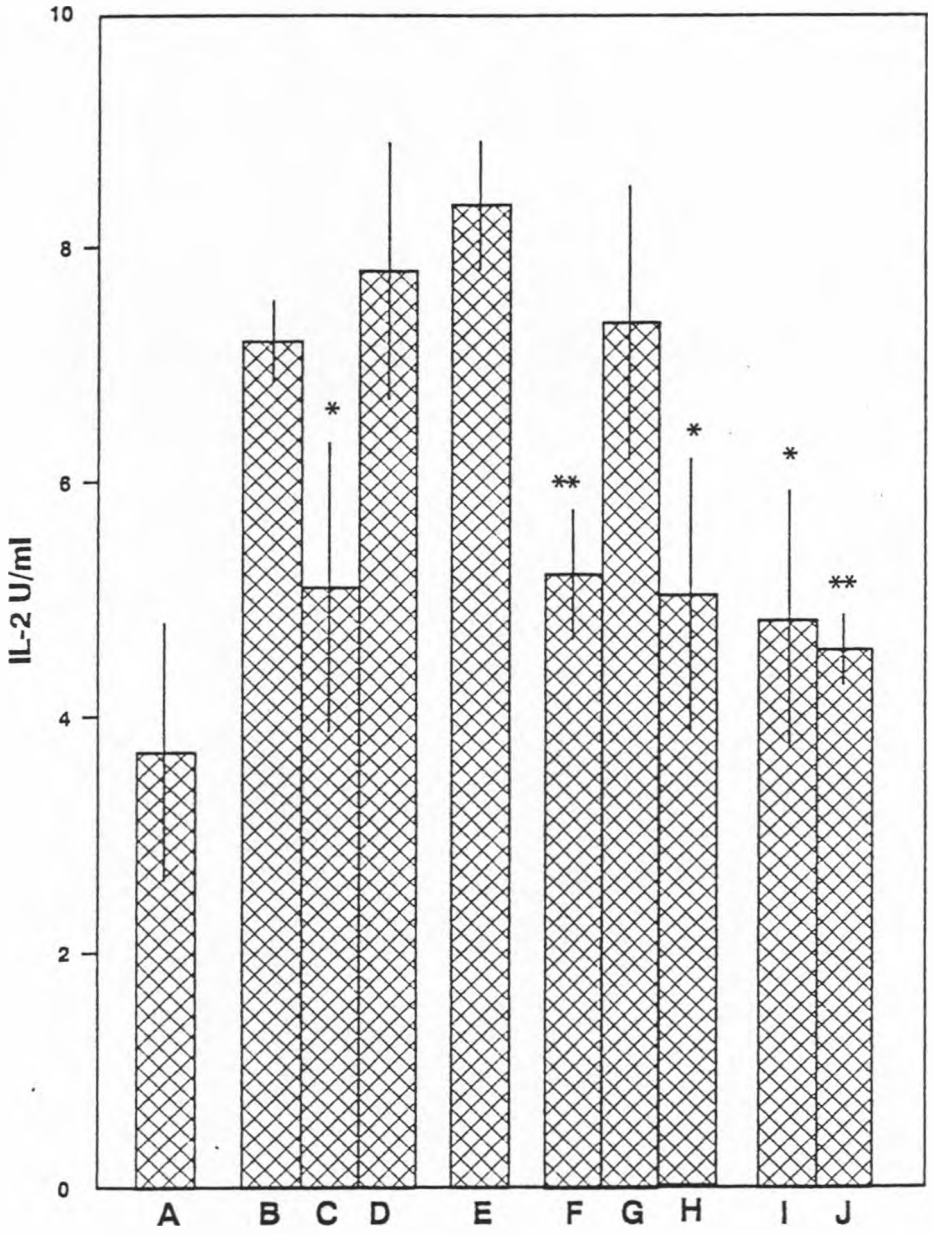


FIGURE 24. IL-2 production by MN cells:- Effect of rIL-6 and rIL-4

MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + rIL-6 (50U/ml)
(D) LPS + rIL-6 (50U/ml) + anti-IL-6 antibodies (100U/ml)
(E) LPS + lymphocyte control supernatants
(F) LPS + lymphocyte suppressor supernatants
(G) LPS + lymphocyte suppressor supernatants + anti-IL-6 antibodies (100U/ml)
(H) LPS + lymphocyte suppressor supernatants + rIL-4 (200U/ml)
(I) LPS + lymphocyte carbohydrates
(J) LPS + lymphocyte carbohydrates + rIL-4 (200U/ml)
Mean ± SD of 3 experiments
* - $P < .05$
** - $P < .01$

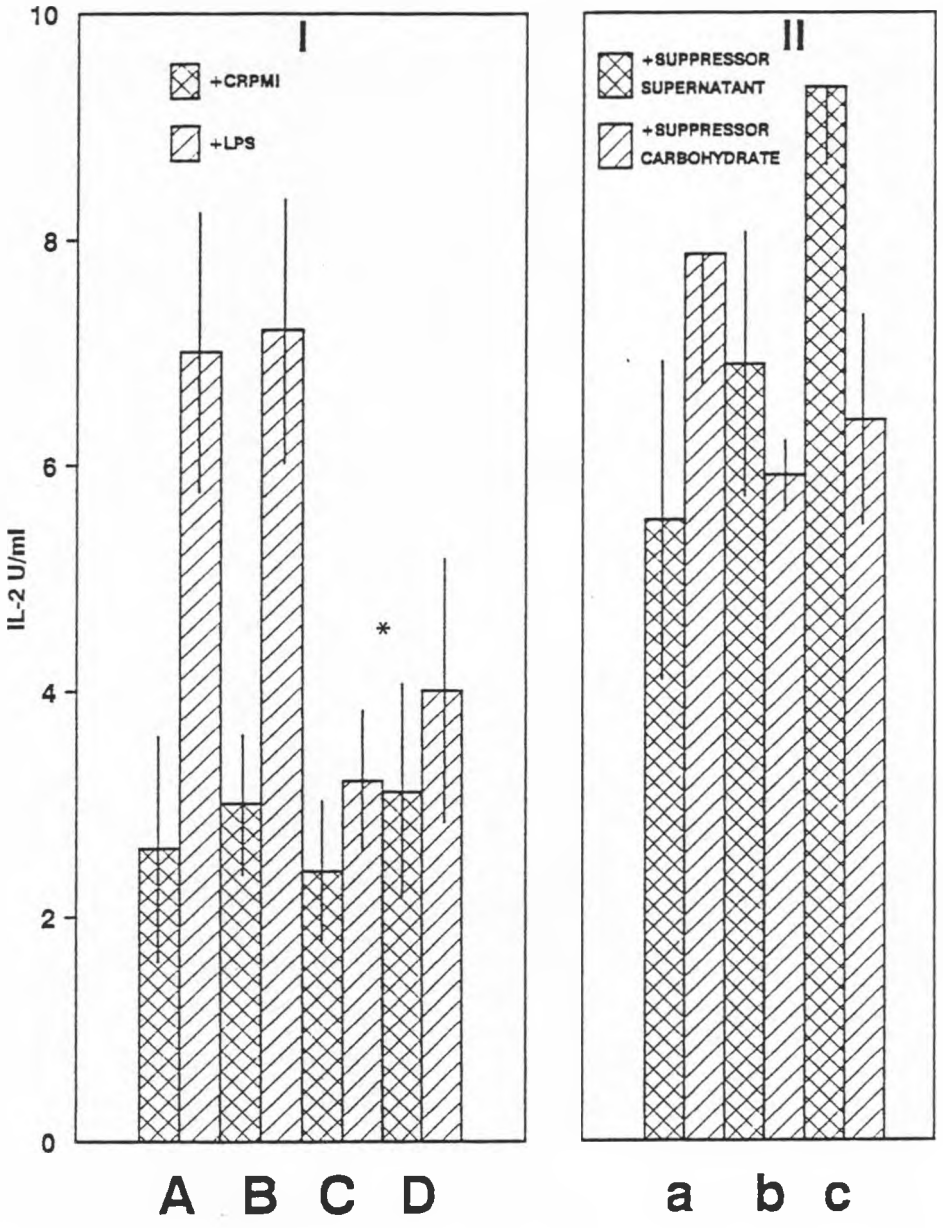


FIGURE 25. IL-2 production by MN cells: Effect of anti-IL-4 and anti-IL-6

(I)MN cells incubated in the presence of:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Lymphocyte suppressor carbohydrate

(II)MN cells incubated in the presence of LPS +:
(a) Anti-IL-4 antibodies (1ug/ml)
(b) Anti-IL-6 antibodies (100U/ml)
(c) Anti-IL-4 (1ug/ml) + anti-IL-6 (100U/ml) antibodies
Mean ± SD of 3 experiments
* - $P < .001$

4.40. MEDIATION OF CYTOKINE PRODUCTION BY rIL-4 AND rIL-6:- (d) EFFECT ON IFN-GAMMA PRODUCTION

The addition of various concentrations of IL-6 to LPS stimulated MN cells resulted in suppression of IFN-gamma production. Suppression was observed with as little as 100U/ml with significant inhibition of IFN-gamma production observed when 200U/ml or greater of IL-4 was added to cultures (Figure 26C - mean ± SD of 3 experiments) Similarly, the addition of IL-6 also resulted in the inhibition of IFN-gamma production by MN cells activated with LPS (Figure 26D). Furthermore the addition of both IL-4 and IL-6 also resulted in significant suppression of IFN-gamma production by activated MN cells (Figure 26F). The addition of either anti-IL-4 or anti-IL-6 antibodies abrogated the inhibitory effects of the two cytokines (Figure 26E, G). Recombinant IL-4 added to LPS stimulated MN cells in the presence of lymphocyte suppressor supernatants or carbohydrates did not inhibit the production of IFN-gamma any further (Figure 26d, e) when compared to control systems (Figure 26b, c). However the production of IFN-gamma by all of these cultures (Figure 26b, c, d, e) was suppressed when compared to IFN-gamma produced by LPS stimulated MN cells alone (Figure 26B) or those incubated with lymphocyte control supernatants (Figure 26a).

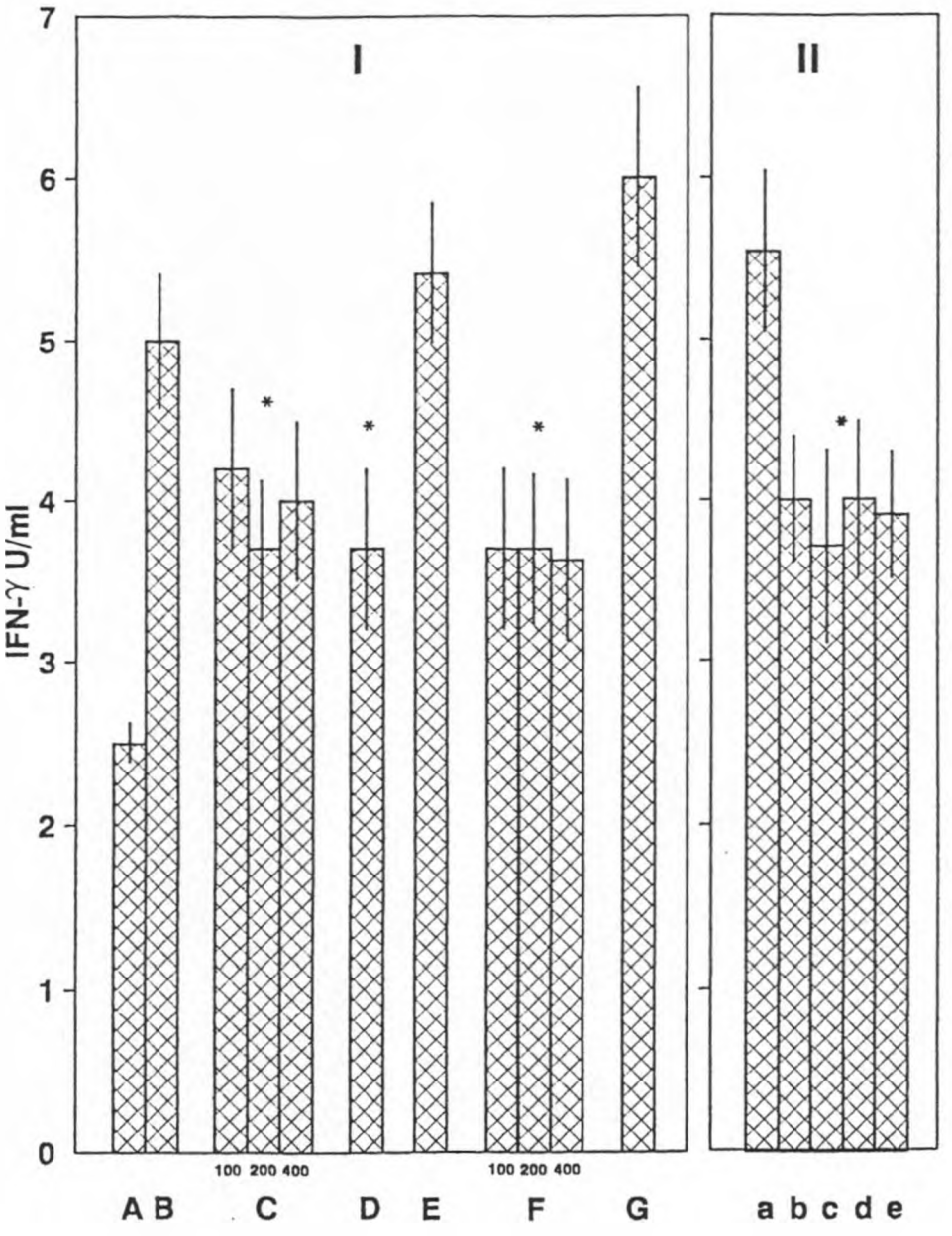


FIGURE 26. IFN-gamma production by MN cells:- Effect of rIL-4 and rIL-6

(I)MN cells incubated with:
(A) cRPMI
(B) LPS
(C) LPS + different concentrations (U/ml) of IL-4
(D) LPS + IL-6 (50U/ml)
(E) LPS + IL-6 (50U/ml) + anti-IL-6 antibodies (100U/ml)
(F) LPS + different concentrations (U/ml) of IL-4 + IL-6 (50U/ml)
(G) LPS + IL-4 (200U/ml) + anti-IL-4 antibodies (1ug/ml)

(II)MN cells + LPS incubated with:
(a) Lymphocyte control supernatants
(b) Lymphocyte suppressor supernatants
(c) Suppressor carbohydrates
(d) Lymphocyte suppressor supernatants + rIL-4 (200U/ml)
(e) Suppressor carbohydrates + rIL-4 (200U/ml)
Mean ± SD of 3 experiments
* - $P < .05$

4.41. IFN-GAMMA PRODUCTION BY MN CELLS INCUBATED WITH LYMPHOCYTE SUPPRESSOR SUPERNATANTS:- EFFECT OF ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES

Figure 27(C, D) demonstrates suppression of IFN-gamma by lymphocyte suppressor supernatants or carbohydrates. This effect parallels the inhibitory activity of IL-4 and IL-6 as seen in Figure 26 C and D.

To explore if the production of IFN-gamma could be restored, anti-IL-4 and anti-IL-6 antibodies or a combination of the two were incubated with LPS stimulated MN cells in the presence of suppressor supernatants or carbohydrates. The results (Figure 27) indicate that the addition of specific antisera to cultures containing suppressor molecules completely restores the suppression of IFN-gamma (Figure 27a, b). This was confirmed in experiments indicating that the incubation of both anti-IL-4 and anti-IL-6 antibodies also restored the production of IFN-gamma (Figure 27c).

4.42. THE EFFECT OF ANTI-IL-4 AND ANTI-IL-6 ANTIBODIES ON IL-4 PRODUCTION

Although previous experiments (Figure 9, 11) have shown that both LPS and PHA were capable of initiating the production of IL-4 by lymphocytes, previous studies (Hashimoto *et al.*, 1991) and current studies (not presented here) have indicated that PMA and ionomycin could induce the secretion of greater amounts of IL-4. For these reasons, the production of IL-4 was

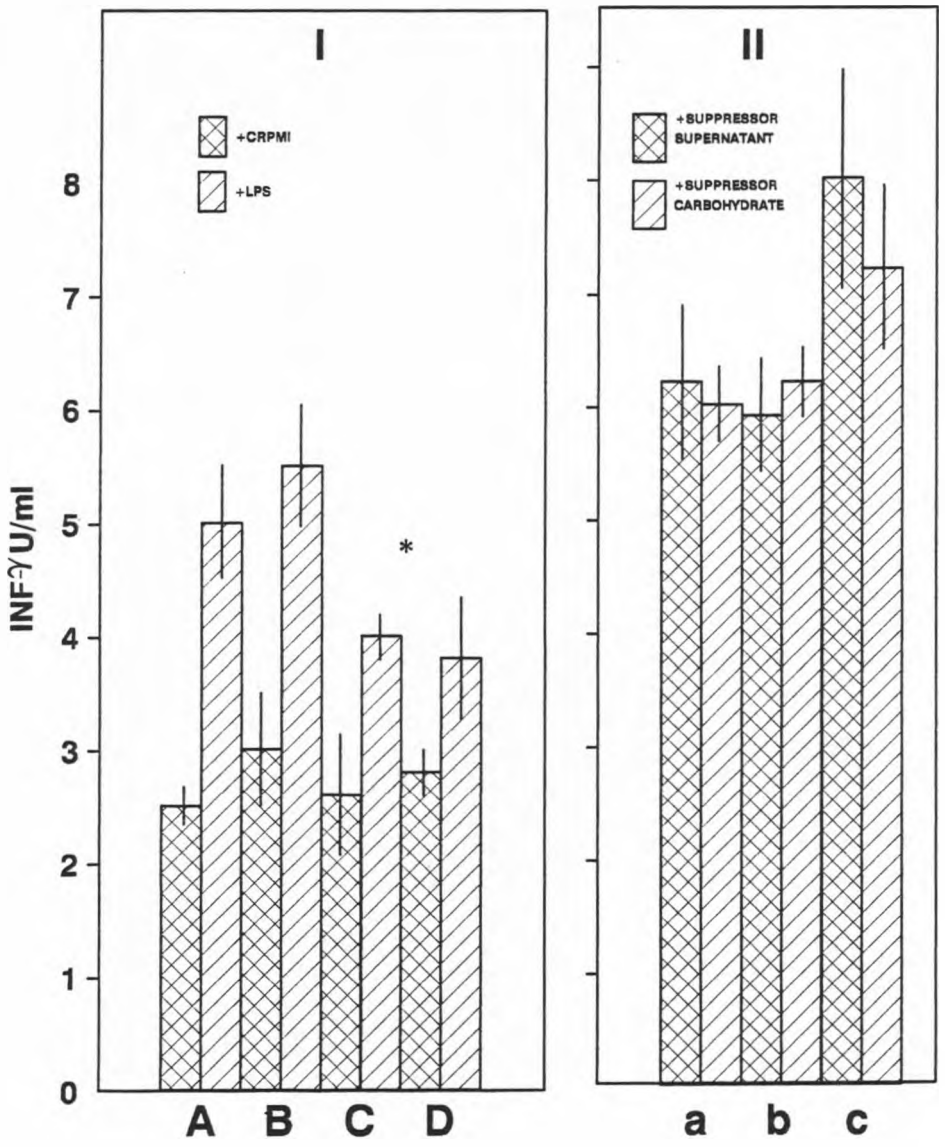


FIGURE 27. IFN-gamma production by MN cells:- Effect of anti-IL-4 and anti-IL-6 antibodies

(I)MN cells incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrate

(II)MN cells + LPS incubated with:
(a) Anti-IL-4 antibodies (1ug/ml)
(b) Anti-IL-6 antibodies (100U/ml)
(c) Anti-IL-4 (1ug/ml) + anti-IL-6 (100U/ml) antibodies
(Mean ± SD of 3 experiments(
* $P < .05$

assessed using a previously determined suboptimal dose of a combination of PMA (1ng/ml) and ionomycin (2,5ug/ml). The addition of lymphocyte suppressor supernatants or carbohydrates to MN cells incubated with these agents resulted in an increase of IL-4 (Figure 28C, D). This increase in IL-4 production was significantly greater than the amount of IL-4 produced by PMA and ionomycin (Figure 28A) alone or with lymphocyte control supernatants (Figure 28B).

The addition of anti-IL-4 antibodies to cultures containing lymphocyte suppressor supernatants (at the initiation of culture) reduced IL-4 production to resting control levels (Figure 28b - IL-4 secreted by unstimulated cells).

Similarly when anti-IL-6 antibodies were added to cultures containing lymphocyte suppressor supernatants (Figure 28a), IL-4 production was reduced to levels obtained with PMA and ionomycin (Figure 28A, B). The addition of anti-IL-4 and anti-IL-6 antibodies resulted in a complete abrogation of IL-4 production as seen in Figure 28c.

4.43. THE EFFECT OF VARYING CONCENTRATIONS OF rIL-6 ON IL-4 PRODUCTION

Since the presence of anti-IL-6 antibodies appeared to reduce IL-4 production (Figure 28a), experiments were undertaken to examine the direct effects of rIL-6 on the production of IL-4 by activated MN cells. Figure 29C demonstrates a dose related increase of IL-4 production due to the presence of rIL-6.

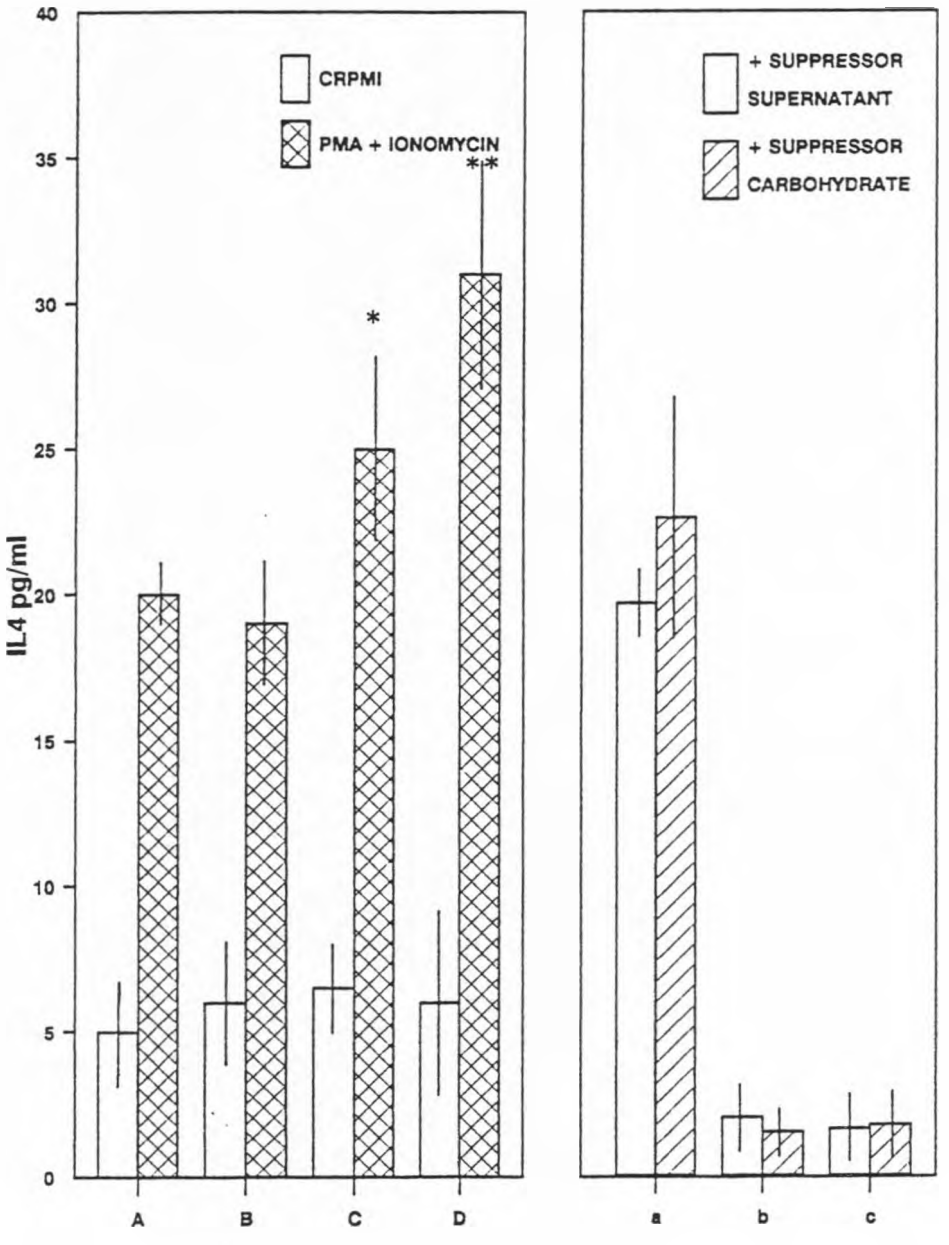


FIGURE 28. The effect of anti-IL-4 and anti-IL-6 antibodies on IL-4 production

(I)MN cells incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrate

(II)MN cells + PMA (1ng/ml) + ionomycin (2,5ug/ml) incubated with:
(a) Anti-IL-6 antibodies (100U/ml)
(b) Anti-IL-4 antibodies (1ug/ml)
(c) Anti-IL-4 (1ug/ml) + anti-IL-6 (100U/ml) antibodies
Mean ± SD of 3 experiments
* - $P < .05$
** - $P < .01$

activated MN cells. Figure 29 (C) demonstrates a dose related increase of IL-4 production due to the presence of rIL-6. This was significantly higher than the control systems which contained MN cells stimulated with ionomycin and PMA (Figure 29B). As little as 25U/ml enhanced IL-4 production with peak activity observed at 50U/ml (Figure 29C) whilst higher concentrations did not improve this enhanced secretion of IL-4. The addition of anti-IL-6 to these latter cultures, containing rIL-6, effectively demonstrates the neutralising effect of anti-IL-6 antibodies. Figure 29D shows that the level of IL-4 detected in such cultures is reduced to control values that utilised PMA and ionomycin only.

4.44. INTEGRITY OF mRNA

To assess the varied effects of lymphocyte suppressor supernatants on cytokine production, it was of interest to assess their effects on specific mRNA production by monocytes. Figure 30 demonstrates the integrity of total cellular RNA as evidenced by the presence of the 28S, 18S and 5S bands. In addition an equal amount of RNA has been loaded in each lane.

4.45. DETECTION OF IL-1ß mRNA

The presence of IL-1ß mRNA was detected in resting monocytes (Schindler *et al.*, 1990). In the present study the addition of LPS enhanced the expression of IL-1ß mRNA 3½ hours after stimulation (Figure 31B). The addition of lymphocyte control and suppressor supernatants to MN cells did not affect IL-1ß mRNA expression by these cells at 3½ hours (Figure 31C, D).

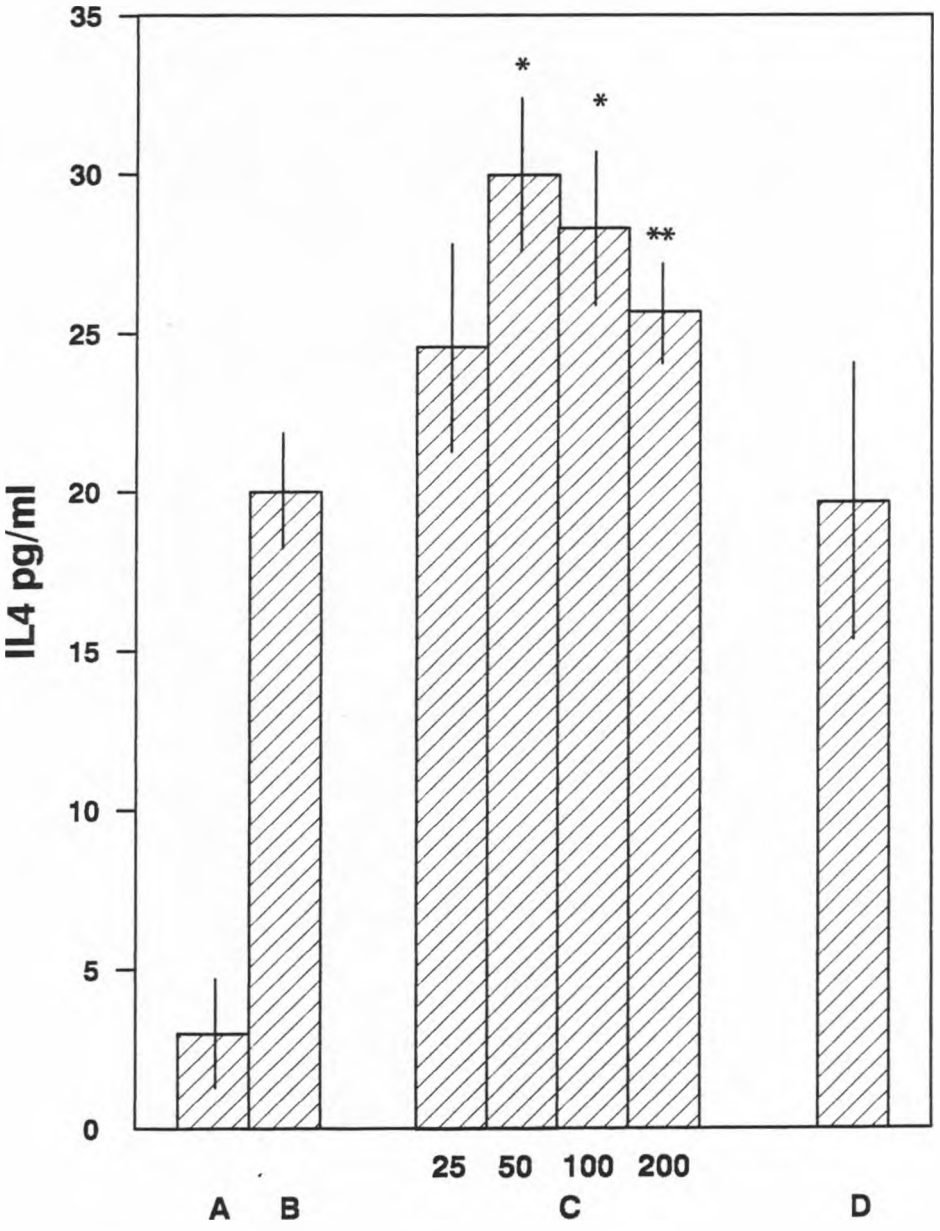


FIGURE 29. The effect of varying concentrations of rIL-6 on IL-4 production

MN cells incubated with:
(A) cRPMI

MN cells + PMA (1ng/ml) + ionomycin (2,5ug/ml) incubated with:
(B) cRPMI
(C) Varying concentrations of IL-6 (U/ml)
(D) IL-6 (50U/ml) + anti-IL-6 antibodies (100U/ml)
(Mean ± SD of 3 experiments(
* P < .005
** P < .01

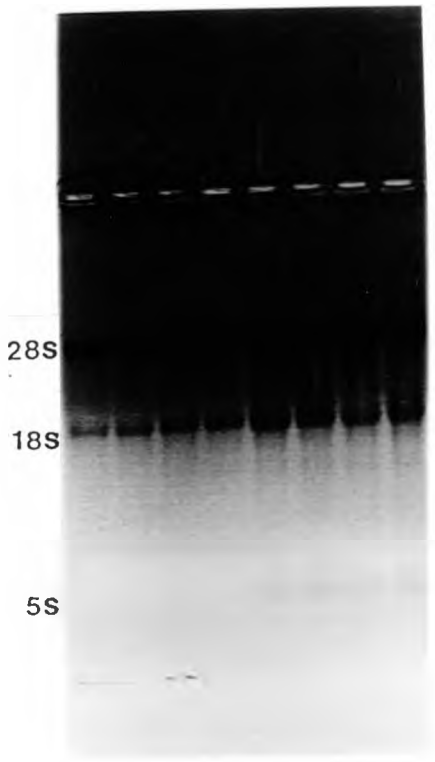


FIGURE 30. Agarose gel electrophoresis of mRNA

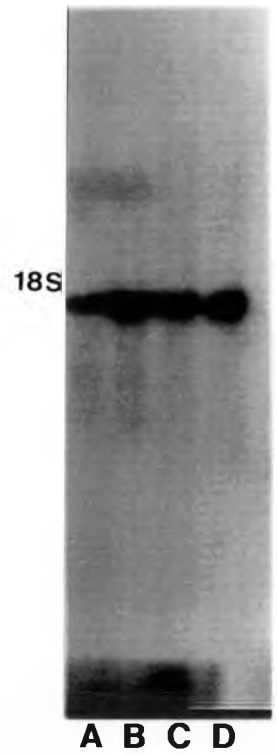


FIGURE 31. Detection of IL-1ß mRNA by agarose gel electrophoresis

IL-1ß mRNA isolated from monocytes incubated with:
(A) cRPMI
(B) LPS
(C) LPS + lymphocyte control supernatants
(D) LPS + lymphocyte suppressor supernatants

4.46. DETECTION OF IL-6 mRNA

Figure 32B demonstrates the expression of IL-6 mRNA in resting monocytes. The addition of LPS to these monocytes resulted in the enhanced expression of IL-6 mRNA (Figure 32A). The presence of lymphocyte control (Figure 32C) or suppressor supernatants (Figure 32D) in these cultures did not affect the expression of IL-6 mRNA in any way.

4.47. THE EFFECT OF LYMPHOCYTE SUPPRESSOR SUPERNATANTS AND CARBOHYDRATES ON THE EXPRESSION OF HLA CLASS II ANTIGENS BY MONOCYTES

The expression of HLA-DR antigens was determined following stimulation of monocytes with LPS or yeast in the presence of lymphocyte suppressor supernatants (Figure 33C) or carbohydrates (Figure 33D). These were compared with the expression of such antigens by monocytes incubated with yeast or LPS alone (Figure 33A) or those incubated with lymphocyte control supernatants (Figure 33B). The results indicate a significant suppression of HLA-DR expression 5 or 7 days after initiation of culture (Mean ± SD of 3 experiments). Suppression of HLA-DR expression ranged from ~30-40% at both 5 and 7 days. Lymphocyte control supernatants, suppressor supernatants or carbohydrates (Figure 33B, C, D) when added to unstimulated monocytes did not have any effect on HLA Class II expression (Figure 33B, C, D compared with Figure 33A).

Additional experiments utilised IFN-gamma as a monocyte activator to confirm the effects seen when LPS or Baker's yeast were used.

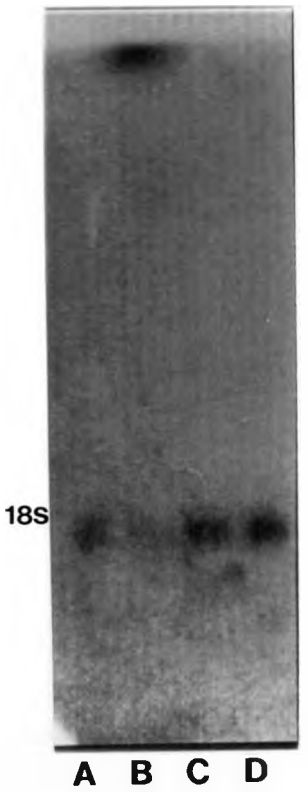


FIGURE 32. Detection of IL-6 mRNA by agarose gel electrophoresis

IL-6 mRNA isolated from monocytes incubated with:
(A) LPS
(B) cRPMI
(C) LPS + lymphocyte control supernatants
(D) LPS + lymphocyte suppressor supernatants

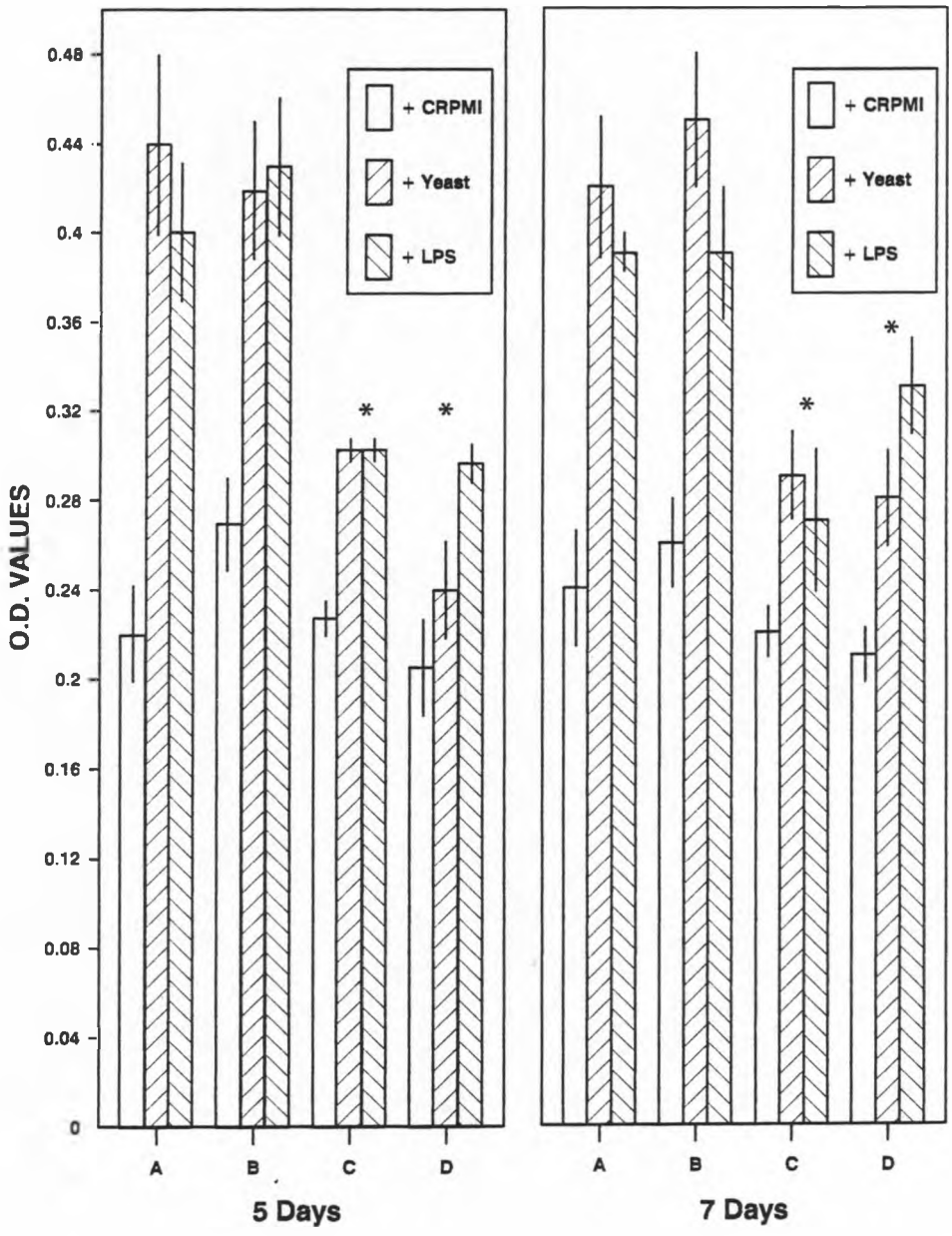


FIGURE 33. HLA Class II (DR) expression by monocytes

Monocytes incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrates
(Mean ± SD of 3 experiments)
* P < .001

Figure 34 demonstrates the expression of HLA-DR antigens as assessed after cells had been in culture for 7 days. The addition of LPS at the initiation of culture resulted in a significant increase in the expression of these antigens compared to monocytes cultured in medium alone. Gamma interferon added to LPS stimulated cultures further increased the expression of these antigens (Figure 34A). The addition of lymphocyte control or suppressor supernatants or suppressor carbohydrates had no effect on the expression of DR antigens by such resting monocytes (Figure 34A-D - unhatched bars). When suppressor supernatants or carbohydrates were added to LPS, or LPS and IFN-gamma stimulated cultures, a significant reduction in the expression of DR antigens was observed (Figure 34C, D compared with Figure 34A, B). No such effect was seen with lymphocyte control supernatants (Figure 34B).

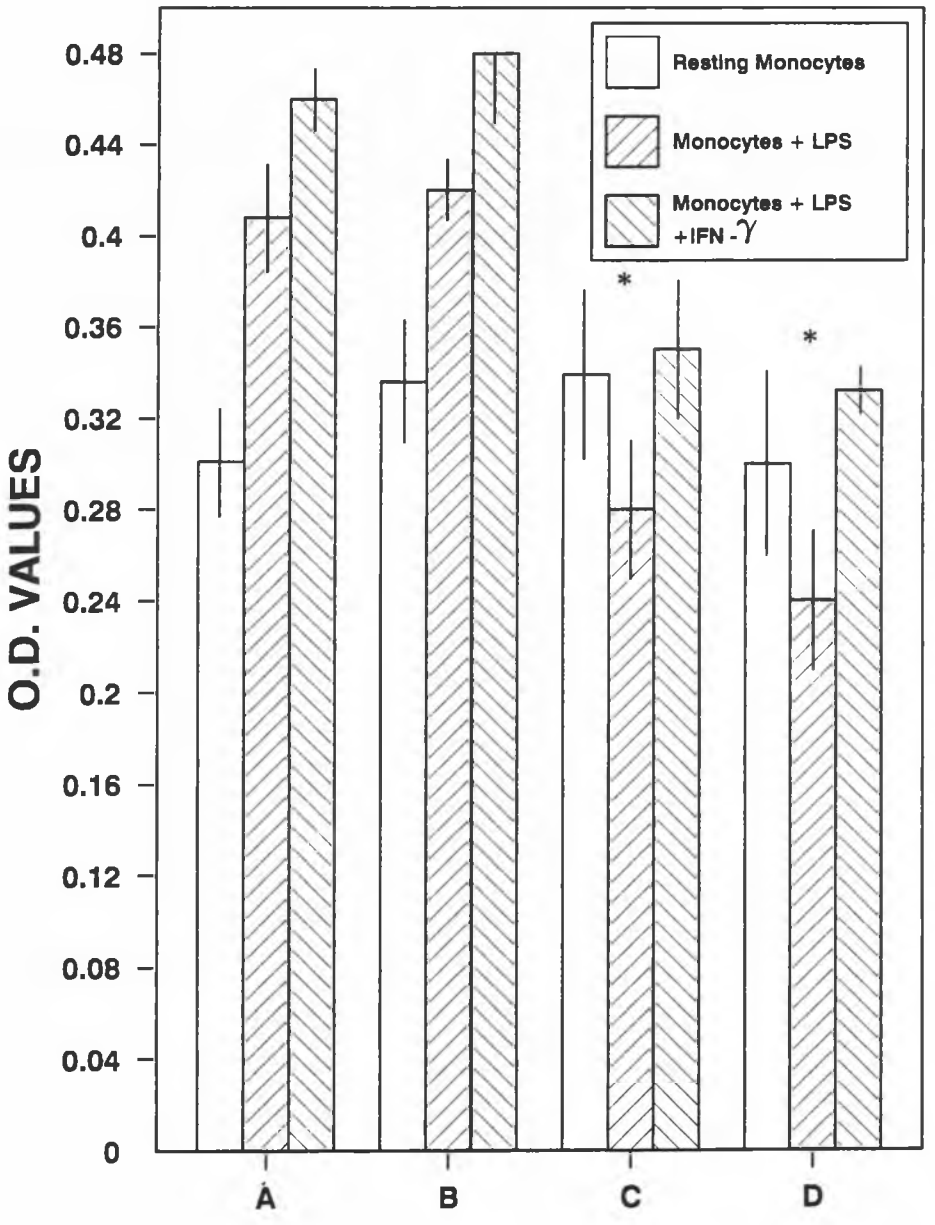


FIGURE 34. HLA Class II (DR) expression by monocytes

Monocytes incubated with:
(A) cRPMI
(B) Lymphocyte control supernatants
(C) Lymphocyte suppressor supernatants
(D) Suppressor carbohydrate
(Mean ± SD of 3 experiments)
* $P < .05$

CHAPTER 5
DISCUSSION

The results of the initial studies undertaken in this project demonstrate that *M.tuberculosis* sonicates, when added to normal mononuclear cells, suppress the proliferation responses of these cells to both antigens and mitogens (Table 1). In particular suppression of blastogenesis was observed when MN cells were activated with PHA, Con A or with the antigens PPD, SKSD and *C.albicans*. These findings confirm the results of Wadee *et al.* (1980) who showed that whole heat killed mycobacteria inhibited the mitogen induced proliferation of MN cells. In the present study bacterial sonicates other than *M.tuberculosis* organisms did not induce such suppression of MN cell blastogenesis (Figure 1).

These initial results suggest that mycobacterial sonicates possess a component that is immunosuppressive. It has been suggested that mycobacteria require processing by monocytes to release suppressor cell activating factors (SCAF) that inhibit lymphocyte proliferation (Wadee *et al.*, 1980; 1983b). To confirm that such processing was necessary the present study undertook to examine the role (if any) of unfractionated *M.tuberculosis* sonicates on the blastogenic response of normal peripheral blood lymphocytes. In these experiments, crude mycobacterial sonicates were added to monocyte depleted NAL cultures. The results (Table 2) indicate that when unfractionated sonicates derived from *M.tuberculosis* were added to NAL cultures, the blastogenesis of such cells to mitogens was not affected (Table 2). This is in keeping with the suggestion that in order for mycobacteria to suppress

immmune responses some processing of these organisms is necessary (Wadee *et al.*, 1980). Such previous reports have gone further to demonstrate the release of phospholipids (Wadee *et al.*, 1983a) or lipids of mycobacterial origin by macrophages (Tsuyuguchi *et al.*, 1990) that appeared to be activating suppressor cell functions (Wadee *et al.*, 1980; 1983b; Wadee & Rabson, 1983). To explore and confirm this crucial role played by monocytes in the suppressor activity exhibited by mycobacteria, experiments were undertaken whereby *M.tuberculosis* sonicate pretreated monocytes were shown to inhibit the blastogenic response of purified NAL's (Table 3). This finding is not novel since previous workers have demonstrated a direct suppressor effect of mycobacteria treated monocytes (Ellner, 1978) or their supernatants (Wadee *et al.*, 1980) on lymphocyte blastogenesis.

Further experiments evaluated the effects of monocyte derived supernatants on the proliferative responses of NAL's to the mitogens PHA and Con A. In brief, monocyte control supernatants were those derived from monocyte cultures not treated with *M.tuberculosis*. Monocyte suppressor supernatants were those obtained from monocyte cultures pulsed with mycobacterial sonicates for 2 hours. Table 4 demonstrates the effects of such monocyte derived supernatants on lymphocyte blastogenesis and indicates that only monocyte suppressor supernatants were able to significantly inhibit lymphocyte proliferation. Such monocyte suppressor supernatants have previously been termed suppressor cell activating factor(s) (SCAF) by Wadee *et al.* (1983a).

The technique used in the current study suggests that a similar or identical component is responsible for the observed immunosuppressive properties of the monocyte suppressor supernatant. Other workers have demonstrated the presence of a soluble suppressor factor that was produced by monocytes (Fujiwara *et al.*, 1991). Even though this factor has not been purified or characterised, these authors have demonstrated the ability of this factor to activate suppressor lymphocytes.

Wadee *et al.* (1983a) identified the active component in their mycobacteria treated monocytes as being a phospholipid of mycobacterial origin. It was therefore of interest to attempt to determine if monocytes treated with mycobacterial sonicates would release prostaglandins or whether the lipid components present in the mycobacteria were indeed responsible for the observed suppressor activity. The results (Table 5) clearly demonstrate that the inhibition of lymphocyte proliferation was not due to an increased production of prostaglandins by monocytes that had ingested mycobacterial sonicates. In these experiments, MN cells were incubated with mitogens or the antigen PPD and mycobacterial sonicates in the presence or absence of indomethacin. In all cases suppression of blastogenesis was unaffected by indomethacin treatment. These results confirm the findings of Wadee *et al.* (1980) who suggested that mycobacterial mediated suppression is not due to an increase in the production of prostaglandins. Recent studies have indicated that suppression of blastogenesis could be a

direct effect of prostaglandins produced by monocytes (Apt *et al.*, 1991). The results of the present study do not necessarily contradict these findings, but merely suggest another mechanism of immune suppression. This is especially true since cultures containing sonicates of other bacteria (Figure 1) were not able to induce suppression of lymphocyte blastogenesis. If prostaglandin release by monocytes was the only mechanism responsible for suppression, then monocytes that had been incubated with sonicates derived from bacteria other than *M.tuberculosis* would also have caused significant suppression of lymphocyte blastogenesis. Together these findings therefore suggest another mechanism of immune suppression. Since increased prostaglandin release was excluded as a means of immune suppression, preliminary studies undertook to characterise the mycobacterial sonicates that were employed in the current studies. This characterisation is similar to the initial work done in this laboratory in that the mycobacterial sonicates used were treated using different regimens. The results indicate a heat stable lipid (Table 6; 7) that was primarily responsible for the suppression observed. This is in keeping with the findings of Wadee *et al.* (1980) who demonstrated that delipidated whole heat killed mycobacteria lost their suppressor ability.

Previous reports have also suggested that the suppressor effect of mycobacterial lipids was due to processing and presentation of these mycobacterial components by monocytes (Wadee *et al.*, 1980). To explore the possibility of bypassing such

monocyte processing, mycobacterial sonicates were fractionated on Sephacryl S-200 columns and resulted in a protein elution profile of 10 major protein peaks arbitrarily labelled A-J (Figure 3). On assessment, only those fractions present in the void volume, i.e. of Mr >200kDa, were shown to suppress mitogen or PPD stimulated MN cell blastogenesis. Fractions of Mr <200kDa (i.e. F-J) did not demonstrate such suppressor effects (Table 8). When fractions of Mr >200kDa were used in proliferation assays containing purified NAL's, significant suppression of lymphocyte blastogenesis was observed (Table 9). Such suppression was not evident when NAL's were incubated with unfractionated crude mycobacterial sonicates. Similarly, fractions of Mr<200kDa failed to suppress lymphocyte blastogenesis. These results suggest that *M.tuberculosis* bacilli had been sufficiently fractionated to release components that could act directly on the lymphocyte population. The *in vitro* fractionation of *M.tuberculosis* antigens as described herein therefore appears to result in the exposure of mycobacterial components that do not require processing by monocytes for T cell recognition. To determine if these components contained similar lipid fractions described by Wadee *et al.* (1983a) experiments undertook to delipidate the immunosuppressive high MW mycobactial fractions. When the isolated lipids derived from such treatment were added to mitogen or PPD stimulated MN cell cultures, MN cell blastogenesis was suppressed significantly (Table 10). In contrast the remaining control systems containing delipidated high MW fractions was without effect (Table 10). Untreated high

MW fractions of Mr >200kDa also induced suppression of blastogenesis as shown previously (Table 9).

Even though the present studies did not undertake to reproduce the previous findings of Wadee *et al.* (1983a), which demonstrated that phosphatidylinositol and phosphatidyl-ethanolamine were the active components of mycobacteria that resulted in immunosuppression; the results of the fractionation and delipidation studies suggest that the lipids present in the high MW mycobacterial fractions could be the same as those mentioned above.

Other studies have implicated high MW mycobacterial antigens in suppression of immune responses (Bloom & Mehra, 1984; Ottenhoff *et al.*, 1989). Some evidence, although controversial, appears to favour the role of CD8+ suppressor lymphocytes as playing a central role in the mediation of anergy observed in lepromatous leprosy (Bloom & Mehra, 1984). In addition, Ottenhoff *et al.* (1989) has suggested that mycobacterial antigens of Mr >150kDa selectively activated T lymphocytes from anergic individuals with lepromatous leprosy but not tuberculoid leprosy. Furthermore, suppression due to high MW bacterial components other than mycobacteria has been demonstrated by Lehner *et al.* (1985). These workers found that a 185kDa streptococcal cell wall antigen was responsible for the activation of CD8+ lymphocytes.

A plausible mechanism of immune suppression could be cell death due to the presence of the lipids present in the high MW fractions. To exclude this possiblity experiments examined the cytotoxic effect of these components on MN cells for various time periods. The results (Table 11) clearly demonstrate that the mycobacterial fractions of Mr >200kDa were not cytotoxic to MN cells, even when cultured for up to 72 hours.

Since suppression was not mediated by cytotoxicity, it was of interest to explore the effects of the high MW mycobacterial fractions on enriched lymphocyte populations and T lymphocyte subsets. Using negative selection criteria, selected populations were lysed with monoclonal antibodies and rabbit complement, and the remaining purified population isolated on Hypaque-Ficoll density gradients. The results (Table 12) indicate that the proliferative responses of NAL's and CD3+ lymphocytes stimulated with mitogens were suppressed by the high MW fraction. When B lymphocytes or purified T cell subsets (CD4+ or CD8+) were incubated with the mitogens PHA and Con A, they still retained their proliferative ability. The enriched B cell population showed low responses to the various mitogens, probably due to the presence of residual T lymphocytes providing the necessary helper factors. However when this mycobacterial fraction was added to any of these latter three cultures, no additional effects were seen. Furthermore, resting or mitogen stimulated subsets were also unaffected by the addition of the high MW fraction. Of importance to note is that the high MW mycobacterial fraction did not selectively increase the

proliferation of any of the subsets assessed. These results therefore clearly demonstrate that suppression of lymphocyte blastogenesis was not due to an increase in proliferative responses of CD8+ cells or a direct inhibition of CD4+ cell proliferation. Similar findings have been reported by Wadee *et al.* (1980) and Ebert *et al.* (1991). The latter authors demonstrated that *M.paratuberculosis* activated the function of CD8+ suppressor T cells from normal individuals and those with ulcerative colitis. This heightened suppressor activity was also not due to increased CD8+ cell numbers.

The results thus far have indicated that the high MW lipid components derived from mycobacterial sonicates inhibit lymphocyte proliferation (Table 10). This effect was not due to cytotoxicity since MN cells incubated in culture with high MW fractions of mycobacterial sonicates still remained viable (Table 11). Furthermore, the lipid containing high MW fractions did not appear to have any proliferative effect on either resting or mitogen stimulated lymphocyte subsets (Table 12).

To examine the mechanism(s) of suppression further, experiments were undertaken to explore the possibility that mediators were released by lymphocytes upon contact with the mycobacterial fractions of Mr >200kDa. In these experiments NAL's were initially pulsed with high MW mycobacterial fractions for 2 hours after which the cells were washed and cultured in cRPMI for varying periods of time. After each incubation period, cell free supernatants were collected and added to cultures of fresh

NAL's stimulated with PHA. Supernatants derived from high MW pulsed NAL's were capable of suppressing lymphocyte blastogenesis (Figure 4). These 'second suppressor molecules' appeared in the supernatants of mycobacterial pulsed NAL cultures as early as 2 hours. Maximal suppressor activity was noted when supernatants collected 24 hours after pulsing with high MW mycobacterial fractions were added to PHA stimulated NAL's (Figure 4C). Non-adherent lymphocytes pulsed with the lipid components of these fractions suppressed lymphocyte blastogenesis following a similar time course (Figure 4E). These 'second messengers' in lymphocyte suppressor supernatants were not cytotoxic to MN cells as evidenced by Trypan blue exclusion tests which demonstrated viable cells even after 5 days in culture with the latter molecules (results not shown). Supernatants derived from NAL's incubated with unfractionated mycobacterial sonicates (Figure 4B) or proteins (Figure 4D) derived from the high MW fractions did not contain suppressor molecules and were unable to suppress PHA stimulated blastogenesis. The release of suppressor molecules declined at 48 hours and was absent at 72 hours of culture as evidenced by the results (Figure 4) showing that NAL's incubated with these supernatants retained their proliferative responses to PHA when compared with control systems (Figure 4A) containing medium alone. The results from these experiments therefore suggest that once NAL's have been incubated with high MW mycobacterial components or their lipids, the lymphocytes in turn release a second set of molecules with suppressor activity. This does not occur when NAL's are incubated with low MW mycobacterial

fractions (results not shown) or the proteins from mycobacteria. The results also suggest that the release of this second set of suppressor molecules is short lived. Suppression of PHA stimulated lymphocyte blastogenesis is observed when 2, 4 or 24 hour supernatants from high MW mycobacterial fraction or lipid treated NAL's were employed in culture.

To avoid confusion, these supernatants have been referred to as lymphocyte suppressor supernatants and indicate the release of suppressor molecules into the surrounding medium by NAL's pulsed with high MW mycobacterial fractions or their lipids, washed extensively and now cultured without any further stimulus for a further 2, 4 or 24 hours (page viii). Similarly lymphocyte control supernatants were produced by incubating NAL's with cRPMI for 2 hours, washed, reconstituted and incubated for 2, 4 and 24 hours. In experiments not reported here supernatants collected from NAL's pulsed with low MW proteins had the same effects as did supernatants from NAL's cultured in medium alone. Furthermore, pulsing NAL's with proteins from mycobacteria had the same effects. For these reasons the production of lymphocyte control supernatants was performed using cells incubated with medium alone.

In order to optimise the production of lymphocyte suppressor supernatants, cultures containing different concentrations of NAL's pulsed with high MW mycobacterial fractions were incubated for 24 hours and the resulting supernatants assessed for suppressor activity. The results clearly indicate that the

optimal concentration of NAL's required to produce suppressor supernatants is 2 x 10^6/ml (Figure 5). In these experiments lymphocyte control supernatants from concentrations of NAL's ≥4 x 10^6 were also found to be immunosuppressive. It is possible that this suppression could be due to the release of proteins or a variety of factors, due to overcrowding or cell death. For this reason and because control supernatants derived from cultures containing 2 x 10^6NAL's/ml did not demonstrate such suppression (Figure 5), this concentration was used in all further experiments to produce lymphocyte suppressor supernatants.

Since the culture system employed utilised pulsed, washed cells after each incubation period, the results favour suppression being due to the release of 'second messengers' by lymphocytes pulsed with particular mycobacterial antigens. The results also suggest that the lipid component present in the high MW fraction of mycobacteria selectively activates NAL's to release these 'second messengers' with suppressor activity. In this regard it is of interest to note that unfractionated mycobacterial sonicates do not induce the release of suppressor molecules (Figure 4B) and that these unfractionated mycobacterial sonicates are also unable to suppress lymphocyte blastogenesis (Table 9). These results therefore confirm the previously described activation of suppressor cell function by lipids of mycobacterial origin (Wadee *et al.*, 1983a; Wadee & Rabson, 1983). It is also possible that such unfractionated mycobacterial sonicates were not sufficiently processed to

reveal hidden suppressor determinants which would then induce suppressor cells.

Adorini *et al.* (1979) showed that certain sequences from egg white lysosyme activated suppressor cells while others stimulated helper cells. Similar results were described by Shivakumar *et al.* (1989) using peptides derived from ß-galactosidase (GZ). To identify the cell type that is active in mediating suppression, 24 hour supernatants from enriched populations of CD4+ and CD8+ lymphocytes pulsed with high MW mycobacterial fractions were incubated with fresh NAL's in the presence of PHA. The results indicate that only supernatants derived from enriched CD8+ cultures were capable of suppressing lymphocyte blastogenesis (Table 13). Furthermore supernatants from CD4+ enriched populations pulsed with fractions of Mr >200kDa failed to suppress NAL blastogenesis. These results confirm previous reports that such suppression is due to the activation of CD8+ lymphocytes (Wadee & Rabson, 1983; Bloom & Mehra, 1984; Ottenhoff *et al.*, 1989; Ebert *et al.*, 1991).

Since the lymphocyte suppressor supernatants were only produced by CD8+ cells, and because these supernatants inhibited lymphocyte proliferation (Table 13) it was of interest to investigate whether such supernatants inhibited the proliferative responses of lymphocyte subtypes. The results clearly demonstrate that lymphocyte suppressor supernatants inhibit the proliferative responses of CD4+ lymphocytes (Table 14). This

effect was not observed when lymphocyte control supernatants were used (Table 14). When lymphocyte control or suppressor supernatants were incubated with CD8+ cells, no effect on the proliferative responses of these cells was observed (results not shown).

To partially characterise the suppressor molecules produced by CD8+ lymphocytes by salt fractionation, a 60 and 75% solution of ammonium sulphate was added to the supernatants. The results reveal that the suppressor molecules were not precipitated by high salt and remained suspended in the supernatant, suggesting that the suppressor molecules were probably not of a proteinaceous nature (Table 15).

Further characterisation of the components present in CD8+ supernatants confirmed that the suppressor molecules were secreted as glycolipids of which the carbohydrate was the active component (Table 16). This was confirmed by destroying carbohydrates present on isolated glycolipids.

To confirm the carbohydrate nature and not the proteinaceous nature of the active components in the lymphocyte suppressor supernatants, experiments were undertaken whereby such supernatants were differentially modified to destroy proteins or carbohydrates or both. The results clearly indicate that the active component is a carbohydrate since destruction of proteins had no effect on the suppressor ability of these supernatants (Table 16). This is further confirmed by SDS-PAGE analysis

demonstrating the staining of large amounts of carbohydrate and little protein in the lane containing lymphocyte suppressor supernatants produced by CD8+ cells (Figure 6C).

It is beyond the scope of this thesis to establish the exact chemical nature of the carbohydrate identified and since the major thrust of the project is to evaluate immune suppression in tuberculosis, the characterisation attempts of the carbohydrate did not proceed any further from here.

Experiments with nitrocellulose membranes onto which these molecules were transferred indicate suppressor activity in the region of Mr ~122-148kDa, which corresponded to Schiff-staining areas in this region (Figure 6ii C; 7). Proteins identified by SDS-PAGE were of Mr ~67 and ~150kDa (Figure 6i A-D) and did not suppress MN cell blastogenesis (Table 17). The same was true of carbohydrate staining bands of Mr ~90, ~55 and ~50kDa detected in the supernatants of lymphocyte control cultures as well as lymphocyte suppressor supernatants (Figure 6ii A-D). The results of the effects of these molecules on lymphocyte blastogenesis is demonstrated in Table 17.

Since the possiblity exists that failure of suppression by these proteins and carbohydrates may have been due to lack of transfer, nitrocellulose strips were stained for proteins following Western blotting. Our results confirmed successful transfer of these components (results not shown). In addition, entire nitrocellulose strips from control supernatants separated

by SDS-PAGE were not immunosuppressive (results not shown) nor were strips from control lanes that were transferred as shown in Figure 7.

Such findings are confirmed by the results presented in Table 18 indicating that areas corresponding to the 122-148kDa region of the immunosuppressive glycolipid fail to have any effect on lymphocyte blastogenesis should they be derived from any other combination other than CD8+ cells pulsed with mycobacterial fractions of Mr >200kDa.

To confirm that suppressor molecules were carbohydrates, lymphocyte suppressor supernatants in saline were concentrated, delipidated and dialysed in distilled water. Carbohydrates were then isolated from the lipids by boiling in HCl according to the method of Lowenstein (1969). Isolated carbohydrates were shown to suppress MN cell blastogenesis optimally at concentrations of 100-250ng/ml in the presence of the mitogens and 500ng/ml when MN cells were incubated with PPD (Figure 8). Concentrations below 1ng/ml were without effect.

Of interest is the finding that MN cells required a minimum pulse with suppressor carbohydrates for 4 hours for active suppression (Table 19). These results demonstrate that inhibition of MN cell blastogenesis is initiated early during cell activation.

To assess the effects of the suppressor carbohydrate in systems other than those used up to this point, experiments utilised one-way mixed lymphocyte cultures using two donors. The results (Table 20) clearly indicate that the carbohydrate and the lymphocyte suppressor supernatants from which these were derived inhibited the blastogenesis of responder cells to irradiated MN cells from a non-identical HLA mismatched donor.

Other workers have described the release of soluble suppressor molecules in several systems (Greene *et al.*, 1981; Deal *et al.*, 1989; Yee *et al.*, 1990). These include their release by MN cells activated with Con A (Greene *et al.*, 1981), by UV-induced T suppressor cells (Yee *et al.*, 1990) and by splenic T cells injected with high doses of *M.bovis* BCG (Deal *et al.*, 1989). Characterisation of such suppressor molecules has identified them as soluble saccharides with Mr 30-40kDa (Greene *et al.*, 1981); Mr 45-60kDa (Yee *et al.*, 1990); 50-70kDa (Colizzi *et al.*, 1984) and 60-80kDa and 100-200kDa (Tokura *et al.*, 1987). Other reports suggest the presence of polysaccharides derived from *C.albicans* (Fischer *et al.*, 1978) and an 85kDa uromodulin isolated from the urine of pregnant women (Muchmore *et al.*, 1987) to be immunosuppressive.

Thus the results presented here demonstrating the existence of an immunosuppressive glycolipid of Mr~122-148kDa produced by CD8+ lymphocytes are by no means novel. They do however suggest a role for this to occur in mycobacterial infections.

Various components of mycobacteria themselves have been shown to be immunosuppressive. These include arabinomannan (Ellner & Daniel, 1979), lipoarabinomannan (Kaplan *et al.*, 1987), phenolic glycolipid (Prasad *et al.*, 1987) and D-arabino-D-galactan (Ellner & Wallis, 1989). Our findings demonstrate that lymphocyte suppressor supernatants retain their suppressor ability after passage through a Con A column (results not shown) thus indicating that the suppressor carbohydrate is not a mannoside. These results therefore indicate that once CD8+ cells are activated by mycobacterial lipids, they release suppressor glycolipids of which the carbohydrate fraction is the active component.

Since the results have indicated that both MN cell and NAL proliferation was inhibited by lymphocyte suppressor supernatants or carbohydrates it was of interest to examine the effect of such supernatants and carbohydrates on cytokine production.

Initial studies investigated the effect of supernatants on cytokine production by MN cells incubated in the presence of LPS together with various supernatants. Of interest is the finding of a differential action of lymphocyte suppressor supernantants on the various cytokines produced (Figure 9). Whilst IL-1, IL-2, TNF-alpha and IFN-gamma production was significantly suppressed, the production of the other cytokines (IL-4 and IL-6) increased significantly ($P < .005$). Of interest also was the observation that the production of IL-1ß, TNF-alpha and IL-2

was suppressed as early as 4 hours in culture. The technique used in these assays measured cytokine release by LPS-stimulated cells in the presence of lymphocyte control or suppressor supernatants. It could therefore be argued that these supernatants themselves could be contributing to the amounts of the various cytokines detected. This possiblity has been addressed by the fact that the results of the 4 hour experiments exclude the amounts of the various cytokines present in the lymphocyte control or suppressor supernatants. Generally these were of negligible amounts (results not shown). Cytokines detected after 24 and 48 hours however, could not have the cytokines, albeit in low amounts, present from the lymphocyte control or suppressor supernatants since the cultures were washed after 4 hours and reconstituted with cRPMI alone. This was done for all successive time periods.

Several studies have demonstrated reduced levels of IL-1 production by activated monocytes from patients with mycobacterial infections (Horwitz *et al.*, 1984; Watson *et al.*, 1984; Chensue *et al.*, 1986; Montreewasuwat *et al.*, 1987), even though others have demonstrated a direct stimulation of IL-1 release by mycobacterial protein antigens (Wallis *et al.*, 1986). These results indicate that supernatants derived from CD8+ lymphocytes (lymphocyte suppressor supernatants) incubated with high molecular weight mycobacterial components significantly inhibit the production of IL-1ß by monocytes activated with LPS (Figure 10).

With regard to IL-6, several investigators have found that this cytokine displays different relative activities with different target cells (Wong & Clark, 1988). In terms of its activities on the production of cytokines, IL-6 has been demonstrated to inhibit LPS induced TNF production by cultured human monocytes (Aderka *et al.*, 1989) and both IL-1 and TNF by human blood MN cells (Schindler *et al.*, 1990). The results are consistent with these findings in demonstrating that lymphocyte suppressor supernatants and carbohydrates increased the production of IL-6 by monocytes, NAL's and MN cells; whilst suppressing the production of IL-1ß and TNF-alpha (Figure 9; 10; 11; 12I, II, III).

These findings also support those reported by others (Horwitz *et al.*, 1984; Watson *et al.*, 1984; Chensue *et al.*, 1986; Montreewasuwat *et al.*, 1987) suggesting that the failure to activate mononuclear phagocytes in mycobacterial infections stems from an inability to produce cytokines necessary for cellular activation. It is also possible that the reduced production of cytokines IL-1ß and TNF-alpha may be a direct consequence of an increase in the levels of IL-6 as suggested by others (Schindler *et al.*, 1990). Recent evidence has indicated that the production of IL-6 is related to the presence of IL-4 (Smeland *et al.*, 1989) whereby IL-4 induces the selective production of IL-6. The results of the present study support such a theory by demonstrating that lymphocyte suppressor supernatants and carbohydrates increase the production of IL-4 together with IL-6 (Figure 9; 12II, VI).

Further evidence implicating the regulatory roles of the cytokines IL-4 and IL-6 arise from time course studies. Whilst increases in IL-4 and IL-6 were evident as early as 4 hours and remained elevated for 24 hours of culture, levels of IL-1ß, TNF-alpha, IFN-gamma and IL-2 decreased concomitantly (Figure 9; 10; 11; 12).

It is possible therefore that raised levels of IL-6 may act as a feedback loop to inhibit further production of IL-1ß and TNF-alpha. In the case of mycobacterial diseases this may be particularly relevant where mycobacterial products, following degradation by monocytes would activate a subpopulation of CD8+ suppressor lymphocytes to release suppressor glycolipids. These glycolipids could, in turn, act on other monocytes and lymphocytes increasing IL-6 secretion thus inhibiting the production of IL-1ß and TNF-alpha.

To confirm the role played by IL-6 on the production of IL-1ß and TNF-alpha by monocytes, experiments were undertaken whereby rIL-6 was added to MN cells and monocytes stimulated with LPS which resulted in a direct inhibition of monokine production (Figure 13C; 15D; 18D; 21).

The effect of IL-6 on the production of IL-1ß by LPS stimulated MN cells appears to be dose dependent with optimal inhibition of IL-1ß production obtained with 50U/ml; IL-6 did not significantly influence the production of IL-1ß by resting MN cells (Figure 13D).

Further evidence confirming the role of IL-6 on the production of IL-1ß by LPS stimulated MN cells is represented by the use of anti-IL-6 antibodies. Figure 14 demonstrates that the addition of anti-IL-6 antibodies to the lymphocyte suppressor supernatants restores IL-1ß production by MN cells with complete restoration observed at 100U/ml. To prove that suppression of IL-1ß production was indeed due to the increased production of IL-6 by MN cells in response to lymphocyte suppressor supernatants or carbohydrates, studies were undertaken utilising rIL-6 in place of lymphocyte suppressor supernatants. The results clearly indicate that the addition of exogenous IL-6 resulted in a direct inhibition of monokine production (Figure 13C; 15D; 18D; 21).

To extend the studies on the effects of IL-6 and the influence of anti-IL-6, studies included anti-IL-6 antibodies incubated with lymphocyte suppressor supernatants or carbohydrates (Figure 16; 17). Such experiments provide clear evidence to support the view that the production of IL-6 is stimulated by lymphocyte suppressor supernatants or carbohydrates and that its production by LPS stimulated MN cells can be inhibited by the addition of specific anti-IL-6 antibodies (Figure 16b). In similar experiments the presence of IL-6 resulted in the detection of decreased levels of TNF-alpha with dose kinetics similar to those seen with the effects on IL-1ß production (results not shown). Using optimal doses of rIL-6 and anti-IL-6 antibodies the results confirm that IL-6 inhibits TNF-alpha production and the presence of anti-IL-6 antibodies restores this production

(Figure 18D: 19b; 20B). Raised levels of IL-6 therefore correlate with reduced levels of IL-1ß and TNF-alpha as demonstrated in Figure 21. Similar findings have been reported by other workers who showed a direct correlation between IL-6 and the cytokines IL-1 and TNF (Schindler, *et al.*, 1990).

Of interest was the finding that IFN-gamma, a monocyte activator, could not restore monocyte production of IL-1ß or TNF-alpha induced by IL-6 when added at the initiation of culture (Figure 17D, 20D). In these experiments IFN-gamma had no significant effect on IL-1ß (Figure 17C) or TNF-alpha (Figure 20C) production by MN cells alone or those stimulated with LPS. The addition of IL-6 on its own once again significantly suppressed the production of these two monokines (Figure 17B; 20B). Adding IFN-gamma (at doses previously optimised in this laboratory for monocyte activation) to cultures containing IL-6 again had no effect on monokine production (Figure 17D; 20D). As a control in this system these experiments included cultures that contained IL-6, IFN-gamma and anti-IL-6 antibodies. The results confirm that the inhibition of IL-1ß and TNF-alpha is due to IL-6 since anti-IL-6 antibodies reversed the effect (Figure 17E, 20E).

The roles played by IL-2 and IFN-gamma in mycobacterial infections have been well documented (Nogueira *et al.*, 1983; Shiratsuchi *et al.*, 1987; Makonkawkeyoon & Kasinrerk, 1989; Ottenhoff *et al.*, 1990). Such studies have suggested that the cellular unresponsiveness in mycobacterial infections may be

due to reduced IL-2 production. These defects could be restored with recombinant IL-2, IL-4 and IFN-gamma (Nogueira *et al.*, 1983; Shiratsuchi *et al.*, 1987). The present studies indicate that the production of these cytokines is markedly depressed when activated lymphocytes are incubated with supernatants containing lymphocyte suppressor molecules or carbohydrates (Figure 9; 11; 12), suggesting that such molecules induced by mycobacteria interfere with lymphokine production, resulting in immune suppression.

It has been well documented that IL-4 directly suppresses the production or secretion of various cytokines by both monocytes and MN cells. These included TNF-alpha, IL-ß, IL-6, IL-2 and IFN-gamma (Essner *et al.*, 1989; Peleman *et al.*, 1989; te Velde *et al.*, 1990; Cheung *et al.*, 1990; Gaya *et al.*, 1991). The results of the present study demonstrate that in addition to lymphocyte suppressor supernatants and carbohydrates resulting in an elevation of IL-6 production by LPS stimulated MN cells, the production of IL-4 was also elevated (Figure 9VI; 11III; 12VI) by MN cells and NAL's. These results therefore concur with the previous reports mentioned above by suggesting that in addition to the effects of IL-6, raised levels of IL-4 may also be playing a role in the suppression of other cytokine production.

Since IL-6 levels in LPS activated MN cell supernatants returned to control levels 48 hours after treatment with lymphocyte suppressor supernatants, whilst IL-4 remained raised at this

time (Figure 9II, VI; 11III, IV) experiments were undertaken to assess the direct effects of IL-4 on IL-6 production. Such experiments clearly demonstrate that IL-4 significantly suppressed the production of IL-6 (Figure 22). Even though these results appear to be in contradiction of the findings presented herein, they in fact add to the validity of the roles played by these two cytokines. In the present study IL-4 levels remained elevated at 48 hours of culture whilst IL-6 levels had declined at this time (Figure 9; 11) suggesting that IL-4 may in part be responsible for the decline of IL-6 at 48 hours. Even though the rise in IL-4 levels could be detected at 4 hours, the amounts released could possibly not be sufficient to affect other cytokine levels at this early stage. Nevertheless, the present studies indicate that IL-4 suppresses the production of IL-6 (Figure 22) and that this suppression can be reversed with the use of anti-IL-4 antibodies. Furthermore, the addition of rIL-4 to LPS stimulated MN cells in the presence of lymphocyte suppressor supernatants or carbohydrates resulted in a reduction of IL-6 produced (Figure 22d, e). However, on closer examination, these reduced levels of IL-6 were not significantly below the control values obtained with MN cells stimulated with LPS (Figure 22B). When these experiments were repeated using lymphocyte suppressor supernatants or carbohydrates similar effects were seen (Figure 22II) in that the addition of IL-4 resulted in minimally reduced levels of IL-6 (Figure 22d, e) which could be restored to increased levels with the addition of anti-IL-4 (Figure 22f, g). The systems used in these studies therefore suggest that even though IL-4 reduces the amount of

IL-6 produced this reduction is not absolute, leaving enough IL-6 in the culture system to influence the production of other cytokines. Conflicting data has been presented by Smeland *et al.* (1989) suggesting that IL-4 stimulated the release of IL-6 from resting B cells. However both the target cells used by these authors and the system employed in their experiments differ significantly from those reported in the present study making comparisons invalid.

The importance of IL-4 in mycobacterial diseases has been described by Yamamura *et al.* (1991). These investigators demonstrated that T helper cell clones which secreted IL-4 could be isolated from patients with lepromatous leprosy and is thought to be responsible for immune suppression in these patients.

To confirm the role played by IL-4 on the production of IL-2 and IFN-gamma by LPS stimulated MN cells, experiments were undertaken which employed the use of rIL-4 in culture. The addition of rIL-4 resulted in inhibition of IL-2 (Figure 23C) and IFN-gamma (Figure 26C), an effect which could be reversed with anti-IL-4 antibodies (Figure 25c). Similar findings were reported by other workers who showed that IL-4 induced suppression of IL-2 and IFN-gamma (Peleman *et al.*, 1989; Gaya *et al.*, 1991).

The addition of both rIL-4 and IL-6 to LPS stimulated MN cells did not significantly suppress the production of IL-1ß,

TNF-alpha, IL-2 and IFN-gamma over and above suppression seen with optimal amounts of rIL-4 (Figure 15E: 18E; 23E; 26d, e). Furthermore the addition of rIL-4 to LPS stimulated MN cells incubated with lymphocyte suppressor supernatants or carbohydrates did not result in enhanced suppression of IL-1ß, TNF-alpha, IL-2 and IFN-gamma production (Figure 15d, e; 18d, e; 24H, J; 26d, e). These findings suggest that the addition of rIL-4 to already suppressed cultures has no additive or synergistic effect. However IL-6 in this 24 hour supernatants was restored to normal levels (Figure 22F, d, e), thus confirming previous findings that IL-4 inhibited the production of IL-6 under these circumstances (te Velde *et al.*, 1990; Cheung *et al.*, 1990).

Of interest is the finding that the addition of either anti-IL-4 or anti-IL-6 antibodies alone or together to cultures incubated in the presence of lymphocyte suppressor supernatants or carbohydrates totally restored the production of IL-1ß, TNF-alpha, IL-2 and IFN-gamma by MN cells stimulated with LPS (Figure 16; 19; 25; 27a, b, c). Furthermore, the addition of anti-IL-4 antibodies to LPS stimulated MN cells cultured in the presence of lymphocyte suppressor supernatants or carbohydrates resulted in IL-6 levels remaining elevated (Figure 22f, g). This result again suggests a link between IL-4 and IL-6 production. Furthermore anti-IL-4 or anti-IL-6 antibodies do not crossreact with IL-4 and IL-6 respectively according to information provided by the manufacturers. To investigate this further, anti-IL-6 antibodies were added to PMA and ionomycin

stimulated MN cell cultures incubated in the presence of lymphocyte suppressor supernatants or carbohydrates. This stimulus was chosen due to its known properties of stimulating increased production of IL-4 (Hashimoto *et al.*, 1991). The results demonstrate that elevated levels of IL-4 did not develop in the presence of anti-IL-6 antibodies (Figure 28a). Since these findings strongly suggested a role for IL-6 in the production of IL-4 by stimulated MN cells, experiments were undertaken whereby different doses of rIL-6 were added to PMA and ionomycin stimulated MN cells at the initiation of culture. The results (Figure 29C) clearly demonstrate that rIL-6 enhances the production of IL-4 detected in 24 hour supernatants in a dose dependent manner. Furthermore, this effect was reversed by the addition of anti-IL-6 antibodies (Figure 29D). Other workers (Feldman *et al.*, 1991), using murine myeloid progenitor cells have shown that rIL-6 increases the expression of IL-4 on these cells. Such results may suggest that IL-6 itself could enhance IL-4 production.

These findings therefore indicate that the suppressor carbohydrate produced by activated CD8+ cells increases the production of IL-6. As IL-6 levels rise to critical concentrations, IL-4 production ensues. After 48 hours in culture IL-4 feeds back to inhibit IL-6 production. Together IL-4 and IL-6 suppress TNF-alpha and IL-1ß production after 4 hours in culture. It is possible that decreased levels of the latter cytokine (IL-1ß) then results in the concomitant suppression of IL-2 and IFN-gamma production. The results

presented here may also suggest that these inhibitory activities could be acting in concert since MN cells would interact with each other *in vivo*.

Some investigators have demonstrated that rIL-6 suppresses the production of IL-1ß and TNF-alpha mRNA (Aderka *et al.*, 1989; Schindler *et al.*, 1990) while others have shown that IL-4 may inhibit the secretion and production of these cytokines (te Velde *et al.*, 1990). Since results presented here demonstrated enhanced secretion of IL-4 and IL-6 with a decreased IL-1ß, TNF-alpha, IL-2 and IFN-gamma secretion, experiments were undertaken whereby LPS stimulated monocytes were incubated alone, in the presence of lymphocyte control or suppressor supernatants for 3½ hours. Messenger RNA levels were assessed according to the method of Correa-Rotter *et al.* (1992) thus eliminating the need for ß actin quantification.

The current study attempted to detect mRNA of IL-6 and IL-1ß to represent one of each cytokine that was either increased (IL-6) or decreased (IL-1ß) in response to lymphocyte suppressor supernatants or carbohydrates. Furthermore, at the time of the experimental work probes for the other cytokines were not freely available in this department. The results obtained demonstrate that IL-1ß mRNA is not suppressed in the presence of lymphocyte suppressor supernatants (Figure 31D). When IL-6 mRNA was assessed at the same time, mRNA levels were not increased (Figure 32D) indicating no change in the levels of mRNA produced. Schindler *et al.* (1990) demonstrated the

production of mRNA for IL-1ß was suppressed in the presence of IL-6. These results differ from those presented here. Possible reasons for such differences could be due to incubation times, eg. the total assay in the current study was 3½ hours whereas that reported by Schindler *et al.* (1990) was an hour extra. More specifically, in the experiments of Schindler *et al.* (1990) MN cells were preincubated for 30 minutes with IL-6 followed by a further 4 hours incubation after LPS or PHA stimulation. In the present study, purified monocytes were stimulated with LPS and incubated in the presence of lymphocyte control or suppressor supernatants at the initiation of culture. It is possible that suppressor molecules may decrease secretion of IL-1ß only and not necessarily affect mRNA production. This has also been suggested by Schindler *et al.* (1990a) who showed that the actual secretion of IL-1ß was dependent on the stimulus used. Although IL-1ß mRNA levels appeared to be the same at 8 hours, IL-1ß secreted was significantly increased when *S.epidermidis* was used as a stimulus rather than LPS. The differential findings of Schindler *et al.* (1990a) lend credibility to the present findings, indicating that the lymphocyte suppressor supernatant may act at a post-translational level. This is most probably due to an enhanced secretion of IL-6 whilst inhibiting the secretion of IL-1ß.

The present studies have indicated that *M.tuberculosis* organisms have the ability to evade host defences by activating suppressor cell mechanisms. One of the effects of such

suppressor activity is the suppression of monokine production. In particular the present study has demonstrated suppression of IL-1ß and TNF-alpha production by monocytes. The current studies were therefore extended to investigate the effects of lymphocyte suppressor supernatants and suppressor carbohydrates on the expression of HLA Class II (DR) antigens expressed by cultured monocytes. The expression of DR antigens was used in these assays as an indicator of monocyte activation. The expression of DR antigens on cultured monocytes used a series of two experimental systems with the final expression of HLA Class II antigens detected using a cellular ELISA that had previously been developed in this laboratory. Monocytes were activated with LPS, Baker's yeast and IFN-gamma as described in Section 3.37. Initial studies stimulated monocytes with LPS or Baker's yeast in the presence of lymphocyte control or suppressor supernatants or carbohydrates. These supernatants or carbohydrates were also added to unstimulated monocyte cultures. The results obtained demonstrate a marked inhibition of Class II antigen expression by lymphocyte suppressor supernatants or carbohydrates. Furthermore, suppression of DR expression by activated monocytes was evident at 5 or 7 days in culture (Figure 33C, D). Class II expression of resting monocytes was unaffected. In a second series of experiments, where monocytes were incubated in the presence of IFN-gamma, Class II expression of unstimulated monocytes was unaffected whereas lymphocyte suppressor supernatants or carbohydrates suppressed DR expression of monocytes activated with LPS and IFN-gamma (Figure 34).

Activation of monocytes with IFN-gamma alone, using the experimental system described, demonstrated similar effects to those seen in Figure 34 (results not shown).

Suppression of macrophage functions has been documented previously. Ellner & Daniel (1979) and Kleinhenz *et al.* (1981) have demonstrated that mycobacterial components, eg. arabinomannan, was capable of suppressing macrophage dependent antigen induced activation of human lymphocytes. These authors have suggested that this was due to a mechanism whereby suppressor macrophages were activated to down-regulate the immune response. Since IL-10 has been shown to inhibit Class II expression (de Waal Malefyt *et al.*, 1991), it is not clear whether the lymphocyte suppressor supernatants or carbohydrates activate IL-10 production by monocytes which then down-regulates Class II expression. To establish whether this decreased expression was due to increased levels of IL-6 produced, similar studies were undertaken using the cellular ELISA. Results not presented here, however, demonstrated that both rIL-4 and rIL-6 do not suppress the expression of Class II antigens by activated monocytes. Whether the lymphocyte suppressor supernatants or carbohydrates act in a similar manner to that described by Ellner & Daniel (1979) and Kleinhenz *et al.* (1981) or whether it prevents the macrophage from processing and presenting antigen has not yet been determined. However, the results of the present study suggest that the lymphocyte suppressor supernatants or carbohydrates may be acting at the level of inhibiting the presentation of antigen by monocytes

rather than a direct activation of suppressor monocytes since Class II expression of activated cells is decreased (results not shown). However since the lymphocyte suppressor supernatants or carbohydrates do not affect pre-activated cells (results not shown), the present studies suggest an additional role for lymphocyte suppressor supernatants and carbohydrates in inhibiting the activation of resting monocytes. This is important for the pathogenesis of tuberculosis. If the initial number of infecting organisms is small, macrophages activated by cytokines, such as IFN-gamma, are able to present the degraded antigen together with Class II antigens to lymphocytes and so activate the CMI response. However, if the infective dose is high, the lymphocyte suppressor supernatants or carbohydrates released could prevent further activation of the other macrophages and so cause immune suppression and consequently tuberculosis. Whether the inhibition of Class II expression is mediated by an interference in the actual synthesis of the Class II antigen or by an abrogation of transport to the cell surface is at present unclear. This aspect of the effect of the lymphocyte suppressor supernatants and carbohydrates obviously requires further study.

The interaction of mycobacteria with macrophages in relation to the expression of DR antigens by the latter has been the subject of investigation by several groups recently (Ellner & Daniel, 1979; Kleinhenz *et al.*, 1981; Tweardy *et al.*, 1984; Ellner, 1986). An examination of peripheral blood from tuberculous patients demonstrated that the macrophage population

expressing DR antigens in this group was considerably decreased in comparison to normal healthy controls (Tweardy *et al.*, 1984; Ellner, 1986). This was particularly true for patients who had a depressed response to PPD. Furthermore, when monocytes from these patients were grown in culture for 24 hours, their expression of Class II antigens rose to normal levels. Even though these authors have suggested that the increase in DR expression may be due to a maturation of cells in culture (Tweardy *et al.*, 1984), it is possible that a high concentration of the lymphocyte suppressor supernatants or carbohydrates present within the granuloma could inhibit the macrophages' ability to express DR on their surfaces. This is particularly relevant to the present study which demonstrates a significant reduction of Class II antigen expression in the presence of the lymphocyte suppressor supernatants or carbohydrates. It is possible that the macrophages in the presence of mycobacteria and in particular the lymphocyte suppressor supernatants or carbohydrates could present less antigen by inhibiting the expression of Class II antigens. Current immunological dogma states that activation of CD3+, CD4+ helper/inducer lymphocytes occurs by antigen presentation in the presence of Class II antigens. However antigen presentation to suppressor cells does not occur via Class II but rather by the expression of antigen in the presence of Class I antigens. In keeping with these findings, the studies of Tweardy *et al.* (1984) and Ellner (1986) clearly show the activation of suppressor cells by monocytes not expressing Class II antigens.

This lends support to the possibility of reduced cellular immune responses due to lymphocyte suppressor supernatants or carbohydrates.

Similar reports have demonstrated that mycobacteria themselves also have the ability of inhibiting HLA Class II expression. Kaye *et al.* (1986) showed that the expression of Ia by mouse peritoneal monocytes was increased by IFN-gamma and that this expression was suppressed by *M.microtii.* Similarly Collins *et al.* (1985) and Poulter *et al.* (1984) described a reduction of HLA-DR expression by cells from lesions of patients infected with *M.leprae.* Collectively, these results point to an additional mechanism adopted by mycobacteria to evade the host's immune system. By reducing HLA-DR expression the mycobacteria suppresses the host's ability to activate a cell mediated immune response and to irradicate the invading organism. With regard to the lymphocyte suppressor supernatants or carbohydrates described herein, the results suggest a model whereby activated CD8+ cells release this factor which could in turn inhibit activation of any other macrophages in the surrounding area either directly or by activation of IL-10 thus ensuring the survival of remaining mycobacteria which may subsequently be phagocytosed.

CONCLUSION

The present study is in accordance with previous work from this laboratory (Wadee *et al.*, 1980; 1983a) and extends the findings of these authors to suggest a mechanism of immune suppression resulting from the initial interaction of *M.tuberculosis* with peripheral blood monocytes. Initially the findings of the study clearly demonstrate that lipid components derived from high MW fractions (Mr >200kDa) of *M.tuberculosis* could activate suppressor lymphocytes. These fractions were obtained by separating sonicated *M.tuberculosis* extracts by column chromatography. On incubation with NAL's, these lipid components selectively activated CD8+ lymphocytes to produce a suppressor glycolipid of Mr ~122-148kDa. The active component in this glycolipid was a carbohydrate moiety that appeared to be bound to the lipid. Further characterisation of the carbohydrate was not performed because of a variety of reasons, the main issue being that it was beyond the scope of this thesis.

Suppressor supernatants or carbohydrates derived from CD8+ lymphocytes were capable of suppressing lymphocyte blastogenesis to both mitogens and antigens, and to inhibit the secretion of IL-1ß, TNF-alpha, IL-2 and IFN-gamma by both monocytes and lymphocytes. In addition, the CD8+ derived components increased the production of IL-6 and IL-4 by monocytes and lymphocytes. This effect on cytokine production could be reversed by the addition of antisera to IL-4 and IL-6. The results presented

here therefore suggest that activation of CD8+ cells by mycobacteria or their lipids could result in the release of carbohydrates that would directly increase the production of IL-4 and IL-6. These cytokines in turn would act as modulators of cellular functions resulting in an overall locally depressed cellular immunity.

Further investigations assessed cytokine production at the mRNA level and revealed that an increase in IL-6 and a concomitant decrease in IL-1 is probably affected at a post-translational level.

Preliminary investigations to assess the effects of the lymphocyte suppressor supernatants or carbohydrates on the expression of Class II antigens by cultured monocytes indicate an inhibition of DR expression by activated cells without any effect on resting monocytes. Such findings lead to the temptation of speculating that in the granuloma, macrophages could present less antigen by virtue of a decreased expression of Class II molecules. Macrophages that had ingested mycobacteria could thus not present such antigens. In this regard however the question of mycobacterial survival within macrophages prior to processing and presentation needs to be addressed.

APPENDIX 1

Phosphate Buffered Saline (PBS) 0.15M (pH 7.2)

1 000ml

NaCl	(Univar, South Africa)	8g
KCl	(Univar, South Africa)	0.2g
$Na_2HPO_4-2H_2O$	(BDH, Poole, England)	1.15g
KH_2PO_4	(BDH, Poole, England)	0.2g

Dissolve in 900ml distilled water.

Adjust the pH to 7.2.

Make up to 1 000ml with distilled water.

Sterilise by autoclaving.

APPENDIX 2

Sodium Carbonate Buffer 0.05M (pH 9.6) 1 000ml

Na_2CO_2	(BDH, Poole, England)	1.590g
$NaHCO_3$	(BDH, Poole, England)	2.930g

Make up to 900ml with distilled water.

Adjust pH to 9.6

Make up to 1 000ml with distilled water.

0.5% Bovine Serum Albumin (BSA)

Dissolve 0.5g BSA (Seravac Pentex Products, Illinois, USA) in 100ml sodium carbonate buffer.

Store at -20°C until needed.

APPENDIX 3

Peroxidase Substrate for ELISA

A) Substrate Buffer

Citric acid	(BDH, Poole, England)	1.029g/100ml
Na_2HPO_4	(BDH, Poole, England)	1.448g/100ml

Dissolve in distilled water.

Adjust to pH 5.0.

Make up fresh daily.

B) Substrate

Immediately prior to use add 15ul of cold 30% H_2O_2 (BDH, Poole, England) and one O-phenylenediamine tablet (Zymed Laboratories Inc., California, USA) per 12ml substrate buffer.

Keep the substrate solution entirely covered until used.

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