THE REGULATION OF POLYCLONAL MITOGEN-STIMULATED HUMAN GAMMA-INTERFERON PRODUCTION.

by

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DECLARATION

The results presented in this thesis were obtained entirely by myself with the exception of those presented in Table 2.1 in chapter 2 which were produced by Miles Wilkinson.

DEDICATION

To my mother and father for their unstinting belief in the importance of a good education.

Flagging spirits were revived by:
Judy Garland (Carnegie Hall, April 23rd, 1961)
and
Janis Joplin, who frequently reminded me to
"Get it while you can" and "Try just a little bit harder".

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ABBREVIATIONS

ADR : activator of DNA replication

AMD : actinomycin D

cDNA : copy DNA

CHX : cycloheximide

Con A : concanavalin A

DG : diacylglycerol

EGTA : ethyleneglycol-bis-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid

Fc : Fc receptor for immunoglobulin

FCS : foetal calf serum

FITC : fluoroscein isothiocyanate

H2 : murine major genetic histocompatability region

HLA : human leukocyte antigen

^{3}H-Thy : tritiated-thymidine

IFN : interferon

Ig : immunoglobulin

IL-1 : interleukin 1

IL-2 : interleukin 2

IP_3 : inositol triphosphate

kd : kilodalton

Kd : constant of dissociation

LGL : large granular lymphocytes

LPS : lipopolysaccharide from E. Coli (endotoxin)

Ly : lymphocyte

Ly.H : high density lymphocyte

Ly.L : low density lymphocyte

MAF : macrophage activation factor

mez : mezerein

MHC : major histocompatability complex

MLR : mixed lymphocyte reaction

MLTC : mixed lymphocyte tumour cell culture

Mo : monocytes/macrophages

moab : monoclonal antibody

mRNA : messenger RNA

NBS : newborn serum

ND : not done

NK : natural killer (cells)

NW : nylon wool

PBML : peripheral blood mononuclear leukocytes

PBS : phosphate buffered saline

PGE : prostaglandin of the E series

PHA : phytohaemagglutinin

p.i. : post induction

PKC : protein kinase C

PtdInsP: phosphatidyl inositol phosphate

RITC : rhodamine isothiocyanate

RSV : respiratory syncytial virus

SEA : staphylococcus enterotoxin A

SFV : Semliki Forest virus

SRBC : sheep red blood cell

T3-Ti : T-cell antigen receptor plus associated T3 cell surface marker

TCA : trichloroacetic acid

TCAF : T-cell activating factor ("IL-4A")

Tel : teleocidin

TPA : 12-O-tetradecanoyl phorbol acetate

LIST OF PUBLICATIONS

1) Croll, A.D., Wilkinson, M.F., & Morris, A.G. (1985) Interleukin 2 receptor blockade by anti-Tac antibody inhibits IFN-gamma induction. Cellular Immunology 92, 184.

2) Croll, A.D., Wilkinson, M.F., & Morris, A.G. (1986) Gamma-interferon production by human low-density lymphocytes induced by T-cell mitogens. Immunology 58, 641.

3) Croll, A.D. & Morris, A.G. (1986) The regulation of human interferon-gamma production by interleukins 1 and 2. Lymphokine Research (in press)

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SUMMARY

The regulation of human interferon-gamma production by peripheral blood mononuclear leukocytes, stimulated by polyclonal T-cell activators (mitogens), was investigated because of its possible importance as a regulator of the immune response and because it usually accompanies lymphocyte activation.

Low density lymphocytes, enriched for large granular lymphocytes, were shown to be capable of IFN-gamma production in the absence of macrophages, unlike T-cells, but with interaction of two subsets of this low density population being required for optimal production. It is suggested that a non-T cell low density population can act as accessory cells for T-cells in the absence of macrophages.

The action of both positive and negative modulators of IFN-gamma production were investigated. The importance of IL-1 production was demonstrated by the depressive effects of anti-IL-1 antibody and the ability of purified IL-1 to reverse the depressive effects of macrophage-depletion on T-cell activation.

Blockade of the IL-2 receptor by monoclonal antibodies inhibits IFN-gamma production, as does treatment with prostaglandin E_2, known to inhibit IL-2 production. The receptor blockade is reversible by pure IL-2 as is the PGE_2 inhibition. IL-1 and IL-2 alone rarely induced any IFN-gamma. These data imply that for maximal IFN-gamma production the interaction of at least two other protein factors (IL-1, IL-2) with mitogen-stimulated T-cells is necessary, and that other factors may act as down-regulators.

A variety of cell-surface molecules involved in MHC restriction and also the T11 antigen were also shown to have regulatory effects. Those of the T11 pathway may involve effects on calcium and IL-2 levels.

T-cell activation could also be triggered by calcium ionophore plus tumour promoter. Activation of the IL-2 and IFN-gamma genes by this method was shown to be coordinate and not to require protein synthesis. Thus many regulatory effects on IFN-gamma production probably act at a post-transcriptional level.

CHAPTER 1.

INTRODUCTION.

The aim of this thesis is to investigate at least some of the major factors involved in regulating the production of immune interferon (IFN-gamma) by human peripheral blood mononuclear leukocytes (PBML). In this introduction the activity and possible functions of IFN-gamma, both in vitro and in vivo. will be briefly discussed in order to demonstrate its possible importance as a regulator of the immune response, and hence the necessity for investigating the control of its production. Since IFN-gamma is chiefly produced by T-cells in the course of normal T-cell responses, i.e. in an antigen-specific MHC restricted fashion, T-cell activation will also be reviewed.

1) WHAT ARE INTERFERONS?

IFN-gamma is a member of a group of molecules, the "interferons", which were initially identified and defined by their ability to induce in cells an antiviral state which inhibits viral replication (Isaacs & Lindenmann, 1957). However, interferons (IFNs) also have a variety of non-antiviral effects including possible immunoregulatory roles (Kirchner, 1984). IFNs are a heterogeneous family of proteins, dividable into two groups (I and II) which differ in terms of structure, function, producer cell type, and mode of induction. The viral IFNs alpha and beta are related proteins, usually induced in vivo by viral infection of cells (Baron & Buckler,1963), whilst IFN-gamma (type II) is induced by immune stimulation of

peripheral blood mononuclear leukocytes (PBML; Kirchner,1984). Alpha and beta were originally known as type I IFNs, but throughout this thesis will be referred to as IFN-alpha/beta where the exact type has not been specified or where a mixture of the two types exists. IFN-alpha and IFN-beta were originally distinguished as being produced by fibroblasts and lymphoid cells respectively, but these cells are not the only producers of these IFN types. For example, in mouse fibroblast cultures IFN-alpha and IFN-beta appear to be produced concomitantly, their production seeming to be linked (Yamamoto & Salvin,1980). IFN-gamma, also known as immune IFN, is the predominant form produced by stimulated PBML (Youngner & Salvin,1973).

IFN-gamma was first described in 1965 as being produced in lymphocyte cultures stimulated by the T-cell mitogen PHA (Wheelock,1965). IFN-gamma is distinguishable from IFNs alpha and beta in several ways, being sensitive to pH 2, heat labile at 56°C, and antigenically different, antibodies to IFNs alpha or beta failing to neutralise antigen-induced IFN-gamma (Youngner & Salvin,1973). However, while as a general rule IFN-alpha is acid stable, a novel human IFN has been described, though not yet adequately characterised, with the antigenic reactivity of IFN-alpha but which is acid labile (Balkwill et al..1983). IFNs alpha/beta and IFN-gamma also bind to different receptors, human IFN-gamma not competing for binding with IFN-alpha-A, whereas all IFNs alpha and beta do compete (Branca & Baglioni,1981). The structure and position of

the IFN-gamma gene also differs from IFNs alpha and beta. More than ten distinct non-allelic genes coding for IFN-alpha proteins (80-95% homologous) are found on the short arm of chromosome 9, plus the gene for IFN-beta$_1$ which is 40-50% homologous to the IFN-alpha genes and was thought for a long time to be the only IFN-beta gene (Shows et al.,1982; Trent et al.,1982). The single IFN-gamma gene, however, is found on the long arm of chromosome 12 (Trent et al.,1982). Furthermore, IFN-gamma has no sequence homology to either IFNs alpha or beta and also has three introns whereas alpha and beta$_1$ have none (Gray & Goeddel,1982). This suggests that while there may be a close evolutionary link between IFNs alpha and beta, IFN-gamma is not related and possesses certain properties distinct from IFNs alpha and beta.

Cloned IFNs alpha and beta$_1$ consist of 166 amino acids whilst IFN-gamma is composed of 146 amino acids (Gray & Goeddel,1982). Very recently a second IFN-beta type has been described, HuIFN-beta$_2$, whose gene structure is radically different from IFN-beta$_1$ in that it contains three introns and is located on chromosome 7 (Sehgal et al.,1986).

T-cell derived IFN-gamma, purified to homogeneity, consists of two active species (20kD and 25kD), but these were shown to have identical amino acid sequences and differed only in the extent of glycosylation of the 17kD protein (Rinderknecht et al.,1984). The asparagine residue at position 28 (numbering based on the recombinant cDNA sequence) is glycosylated in both species, but at position

will therefore tell us more about T-cell activation as well.

i) Mitogens.

A considerable amount of research has been devoted to determining what signals are involved in initial T-cell activation and subsequent processes, mostly using mitogens (polyclonal activators of T-cells). Most studies assume that mitogen- and antigen-induced events are very similar. This appears to be the case since there is no evidence of any qualitative difference in the biochemical and physicochemical changes induced by specific and non-specific activators. The differences in time and degree of response appear to be quantitative in nature and are due to the percentage of cells initially triggered by the activator.

The anti-human T-lymphocyte monoclonal antibody OKT3 has potent mitogenic properties at nanogram concentrations, but is inhibitory at higher levels (Van Wauwe et al.,1980). It acts by binding to the trimolecular T3 structure (Tsoukas et al.,1984) always closely associated with the T-cell antigen receptor (Ti) whose alpha and beta chains define antigen and MHC specificity (Saito et al.,1984; Yaque et al.,1985). Recently a putative second type of T-cell antigen receptor has been identified, composed of the gamma and "delta" chains, whose function is unknown (Brenner et al.,1986).

Other mitogens include the plant lectins (proteins which bind carbohydrates) PHA and Con A. Lectins which bind to

D-mannose and D-glucose residues on T-cells are generally mitogenic and induce IFN-gamma production, and sometimes low levels of IFN-beta too (Ito et al.,1984). Phytohaemagglutinin (PHA) is derived from the kidney bean (Nowell,1960) and appears to bind to the carbohydrate moieties of T3 (Valentine et al.,1985) or Ti (Kanellopoulos et al.,1985). It may also bind to another T-cell marker T11, also known as the E (sheep erythrocyte) receptor (O'Flynn et al.,1985a). Concanavalin A (Con A) also causes non-specific T-cell activation by binding to the T3 structure (Kanellopoulos et al.,1985). PHA and Con A bind to a large and partially overlapping range of glycoproteins (Henkart & Fisher,1975; Diller-Centerlind et al.,1980) only some of which are responsible for T-cell activation.

Human B-cells respond poorly if at all to T-cell mitogens (Janossy & Greaves,1971; Schuurman et al.,1980). Human B-cells will proliferate in response to certain B-cell mitogens (e.g. Cowan I strain Staphylococcus aureus protein A) but require T-cell derived factors induced by T-cell mitogens to differentiate into immunoglobulin secreting cells (Dosch et al.,1980; Saiki & Ralph,1981; Falkoff et al..1982).

The mitogen Staphylococcal enterotoxin A (SEA), different from protein A, is derived from bacterial cell walls and has similar properties to the plant lectins. At picogram levels it is a more powerful inducer than PHA or Con A (Carlsson & Sjogren,1985). The ability of microgram concentrations of OKT3 to inhibit proliferation triggered

by all these mitogens is consistent with the view that the T3 complex functions as a signal transducer in the early stages of T-cell activation (Van Wauwe et al.,1984).

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ii) Macrophages and IL-1.

Many workers have shown that T-cell activation requires two signals: firstly, triggering of the T-cell antigen receptor (Ti) and secondly, the presence of macrophages as accessory cells. Binding of mitogen to T3-Ti causes early changes in cytoplasmic free calcium ion concentration and membrane potential (Tsien et al.,1982; Weiss et al..1984). Macrophages act in two ways, "passively" presenting antigen or mitogen and "actively" producing IL-1 (Bendtzen & Petersen,1984). In the relative but not complete absence of macrophages mitogens can induce the first stage of activation, associated with cellular enlargement or blastogenesis, with RNA and protein synthesis occurring as

response to antigen or mitogen stimulation (Archer et al.,1979; Sonnenfeld et al.,1979; Klein et al.,1982; Valle et al.,1975). T-cell lines proliferating in vitro are also capable of IFN-gamma production when stimulated by mitogen (Matsuyama et al.,1982) or the appropriate antigen (Morris et al.,1982). Thus when a murine cytotoxic T-cell line is exposed to its specific target (influenza-infected P815 cells) it produces IFN-gamma in an H-2 restricted fashion, but does not respond to free virus (Morris et al..1982). Furthermore T-cell lines do not require macrophages as accessory cells, unlike T-cells freshly derived from human peripheral blood which need macrophages for optimal T-cell activation (Unanue et al.,1984) and subsequent IFN-gamma production (Epstein et al.,1971).

Whilst human PBML mainly produces T-cell derived IFN-gamma, it must be remembered that some IFN-alpha may also be produced by macrophages (Roberts et al.,1979).

Alloantigens also stimulate IFN-gamma production, as demonstrated in both murine (Kirchner et al.,1979) and human mixed lymphocyte cultures (Perussia et al.,1980). Highest yields are obtained if there are differences in class II rather than class I surface antigens in both mice (Kirchner et al.,1979) and humans (Andreotti & Cresswell,1981). As in mitogen and conventional antigen stimulation, the main IFN type produced is gamma and the response is both more rapid and potent if the responder lymphocytes have already been pre-sensitised to the stimulator lymphocytes, i.e. a secondary MLR (Perussia et al.,1980). T-cells are again the producers with

macrophages as accessory cells (Perussia et al.,1980; Ito et al.,1980).

Tumour cells can also induce IFN production when mixed with fresh leukocytes from non-sensitised donors in vitro (mixed lymphocyte-tumour cell culture, MLTC). However, in the human system the main product is IFN-alpha rather than IFN-gamma and the producer cells are non-T, non-B null cells which do not require macrophage assistance (Trinchieri et al.,1977 & 1978; Djeu et al.,1980), characterised as large granular lymphocytes (Timonen et al.,1980).

In general it appears that IFN-gamma is produced in almost all cases in which T-cell activation has occurred, as measured by proliferation, and relies upon the prior induction of the lymphokines (mediators of cellular immunity produced by lymphocytes) IL-1 and IL-2 (Lotz et al.,1986a&b). Proliferation is not an absolute requirement for IFN-gamma production (Kirchner et al.,1979) and visa versa (Wilkinson et al.,1985).

In view of the apparent importance of T-cell activation and these other lymphokines in IFN-gamma production, these topics are discussed next, separately and in detail.

3) T-CELL ACTIVATION AND THE LYMPHOKINE "CASCADE".

In recent years it has become clear that a group of proteins called lymphokines, which transmit growth and differentiation signals between haematopoietic cells, play a central role in the immune system (Marx,1983). Lymphokines were originally described as non-antibody

mediators of cellular immunity produced by activated lymphocytes (Dumonde et al.,1969). Similar substances produced by monocytes/macrophages should strictly speaking be termed monokines.

Activation of T-lymphocytes occurs when antigen is presented by macrophages to the appropriate T-cell antigen receptor. In addition to antigen-presentation, the macrophage also supplies an augmentary activation signal in the form of the monokine interleukin 1 (IL-1). This stimulates production of T-cell growth factor, known as interleukin 2 (IL-2), by activated T-cells which in turn stimulates IL-2 receptor bearing T-cells to proliferate. Since only antigen-stimulated T-cells normally express IL-2 receptors, IL-2 stimulated T-cell proliferation is clonally restricted to activated cells. IL-2 is also thought to stimulate production of IFN-gamma, which is itself a lymphokine with further stimulatory effects, some of which may amplify the response to the antigen.

There thus appears to be a cascading loop of lymphokine action, each of which amplifies the response to the triggering antigen. The initial response to the antigen is very specific and limited to the very few T-cells whose antigen-receptor binds the antigen. Once these cells are activated, however, they can respond to the non-specific effects of lymphokines and a large expansion of the antigen-specific cell population can occur. IFN-gamma is thus a reliable and relatively early marker of T-cell activation which is independent of proliferation. A greater understanding of the regulation of its production

will therefore tell us more about T-cell activation as well.

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resting T-cells move into the G_1 phase of the cell cycle (Williams et al.,1984). However, lymphocytes do not enter the S phase, in which DNA synthesis occurs, unless a second signal is presented.

Experiments with macrophage-depleted lymphocytes show that the events of primary T-cell activation can be simulated in the absence of macrophages by using sepharose-bound OKT3 plus purified IL-1 (Williams et al.,1985; Palacios et al.,1985a; Schwab et al.,1985). It is thought that the macrophage Fc receptors by binding soluble OKT3 facilitate crosslinking of the T-cell antigen receptor, thereby causing the T-cells to internalise T3-Ti and be activated (Kan et al.,1984&1986). Facilitating crosslinking of the T-cell receptor appears to be one role of macrophages in T-cell activation. This Fc binding can be simulated by linking soluble OKT3 to sepharose beads, since the required crosslinking does not occur if soluble OKT3 is used without macrophages or if monovalent OKT3 Fab fragments are used (Ledbetter et al.,1986). Mitogen responsiveness appears to involve multivalent ligands, experiments with PHA and Con A suggesting that mitogenic ligands must be at least divalent (Lindahl-Kiessling,1972; Wands et al.,1976). For both PHA and Con A the binding of the ligand is virtually complete within 30 minutes (Ling & Kay,1975), the extent to which proliferation is optimal depending on mitogen concentration and length of exposure.

IL-1 seems to act as a general stimulator, initiating cell-cycle progression of triggered T-cells, into G_1 and then S (Davis & Lipsky,1986), which has to precede IL-2

driven proliferation (Sheurich et al.,1985). IL-1 production is thought by some to be required for optimal IL-2 receptor expression (Kaye et al.,1984). Others suggest that T3-Ti crosslinking is a sufficient signal for IL-2 responsiveness and that IL-1 acts chiefly by triggering IL-2 production, since IL-2 receptor expression can occur in the absence of IL-1 (Tsoukas et al..1985: Meuer & Buschenfelde,1986). It is still not clear which of these two signals, crosslinking or IL-1, is the the main trigger for IL-2 receptor expression. Possibly both are involved. Recently it has been shown that recombinant IL-1 can help induce both IL-2 secretion and IL-2 receptor expression by the IL-1 sensitive EL4 thymoma cell line (Lowenthal et al.,1986). IL-1 can synergise with either increased calcium ion levels, using ionomycin, or protein kinase C (PKC) activation, caused by a tumour promoter, to secrete high levels of IL-2, but only with tumour promoter will IL-1 induce IL-2 receptor expression. Thus Lowenthal suggests that IL-1 activates the EL4 cell line by a third intracellular pathway involving neither calcium nor PKC activation (Lowenthal et al., 1986).

The trigger for IL-1 release by macrophages in some cases is direct stimulation by antigen (and probably also mitogen), but other soluble antigens which also trigger T-cell proliferation do not cause IL-1 release (Wallis et al.,1986). These antigens may induce some additional T-cell signal or alternatively may induce production of membrane bound rather than secreted IL-1 (Kurt-Jones et al.,1985a). This may explain the observation of Haq et al.

(1984) that macrophages, but not the less differentiated monocytes, can support T-cell proliferation without secreting IL-1. The magnitude of the T-cell response to antigen has been shown to be proportional to the level of membrane IL-1 and class II major histocompatability complex (MHC) antigen (Ia) expression (Kurt-Jones et al.,1985b).

Class II MHC expression by macrophages is required for antigen/mitogen triggered T-cell proliferation, anti-MHC class II antibodies inhibiting IL-2 production (Larsson & Coutinho,1984). These cell surface molecules may act not only as restricting elements for antigen recognition by the T-cell receptor, a requirement overcome by mitogens, but may also be directly involved in mediating activation signals to the macrophage for IL-1 production (Larsson & Coutinho,1984; Durum & Gershon,1982). Class II MHC expression seems to be the major requirement for any cell type to act as an accessory cell, such as macrophages and B-cells (Grey & Chesnut,1985). Since class II expression is usually transient, regulating the level of expression on the cell surface may be crucial in controlling the level of immune response (Unanue et al..1984).

At least two forms of IL-1, alpha and beta, have been discovered (Allison,1985; Billiau et al.,1985; Wood et al.,1985) which are both synthesised as large (33kD) precursors before cleavage into active forms of about 17.5 kD (Giri et al.,1985). Many different cell types can produce and respond to IL-1, so that it appears to be an important communication molecule with multiple activities

(Durum et al.,1985). Both IL-1 alpha and beta bind to the same high affinity plasma membrane receptor (80kD) found on both lymphoid and fibroblast/epithelial cell types (Dower et al.,1985; Kilian et al.,1986; Matsushima et al.,1986).

Activated macrophages also produce prostaglandins, another group of substances with multiple activities, which may act as inhibitors of both IL-1 production and T-cell proliferation (Kunkel et al.,1986a; Makoul et al.,1985). IL-1 and prostaglandin E_2 (PGE_2), both produced by macrophages, act antagonistically (Hayari et al.,1985) with PGE_2 seeming to depress the stimulatory effects of IL-1 and also inhibiting class II MHC expression (Snyder et al.,1982).

iii) Calcium ion flux and protein kinase C activation.

The early stages of T-cell activation, discussed above, can be bypassed without using conventional mitogens. Phorbol esters and calcium ionophores act synergistically by mimicking the membrane processes and signals thought to occur during normal T-cell activation (Truneh et al.,1985a; Isakov & Altman,1985). An influx of calcium ions across the cell membrane, causing a rise in cytoplasmic free calcium ion concentration, provides one essential signal. Activation and association with the cell membrane of the enzyme protein kinase C is the other signal. Both these events are usually triggered by perturbation of the T-cell receptor, either by antigen or mitogen (Imboden et al.,1985). As discussed above,

Lowenthal has suggested that IL-1 acts by causing a third intracellular signal which can synergise with either calcium ion flux or PKC activation (Lowenthal et al.,1986).

In resting T-cells protein kinase C (PKC) is inactive. T-cell activation leads to a transient production of diacylglycerol in the cell membrane which helps activates PKC by increasing its affinity for calcium ions, i.e. the other signal. This activation pathway is described in more detail in chapter 5. Calcium ionophores mimic the T3-Ti induced calcium flux whilst phorbol esters mimic diacylglycerol by binding to and activating PKC (Kikkawa et al.,1983). The calcium ion chelator EGTA inhibits mitogen activation, but this effect is bypassed if the phorbol ester TPA is added, presumably because it replaces PKC's need for calcium ions to become active (Gelfand et al.,1985).

IL-2 induced proliferation, however, is independent of any calcium ion influx (Mills et al.,1985b; O'Flynn et al.,1985b) and ionophore/phorbol ester triggering cannot replace the proliferative signal of IL-2 binding to its receptor (Albert et al..1985).

Voltage-gated potassium ion channels, the predominant ion channel in T-cells, also seem to be involved in T-cell activation since potassium ion channel blockers inhibit PHA induced mitogenesis (Chandy et al.,1984; De Coursey et al.,1984). The relationship between potassium and calcium ion fluxes across the cell membrane is not understood.

iv) The T11 antigen-independent activation pathway.

The T3-Ti complex may not provide the only T-cell activation pathway. The 50 kD T11 cell surface molecule, also known as the sheep erythrocyte (E) receptor, has also been shown to cause T-cell activation in the absence of antigen and macrophages when the appropriate T11 epitopes are bound by monoclonal antibodies (Meuer et al.,1984; Fox et al.,1985). T11 and T3 antigens can mobilise calcium independently (O'Flynn et al.,1986) with the T11 glycoprotein being functionally linked to a calcium channel (Alcover et al.,1986). The two activation pathways appear to be linked, however, since non-mitogenic anti-T3 antibodies can block activation via T11 (Fox et al.,1986). Antibodies against other T11 epitopes than those that cause activation can similarly inhibit mitogen induced T-cell activation via T3-Ti (Reed et al.,1985b). Also the anti-T11 moab specific for the 9-1 epitope plus anti-T3 will costimulate macrophage-depleted lymphocytes which are unresponsive to anti-T3 alone (Yang et al.,1986). This costimulation of T-cells is substantially enhanced by exogenous IL-1. Thus the T11 pathway does seem to have some involvement in T-cell activation via the T3-Ti antigen receptor.

Recently a lymphokine has been isolated from T-helper cells after antigen-specific triggering that appears to bind to T11 (Milanese et al.,1986a). This 10-12kD protein, tentatively named T-cell activating factor (TCAF), stimulates resting T-cells in the absence of macrophages, causing proliferation and IL-2 receptor expression. TCAF

is distinct from IL-2 in terms of size and from IL-1 which is not produced by T-cells. Its true role in T-cell activation and that of the T11 activation/suppression pathway have yet to be determined.

v) IL-2 and IL-2 receptors.

Two distinct factors are required for induction of T-cell growth, namely IL-1 and IL-2 (Larsson et al.,1980). Both helper and cytotoxic T-cells can produce IL-2 but helper T-cells may be the major source (Robb,1984). However, the ability of most cytotoxic T-cell clones both to respond to and also to produce IL-2 weakens the idea of a strict functional division of T-cell types (Andrus et al.,1984).

Human IL-2 consists of 133 amino acids, plus a 20 residue cleaved signal sequence, and a single disulphide bond. Any heterogeneity in molecular weight seems to be due to variation in the degree of glycosylation of threonine 3 (Robb et al.,1984a). IL-2 is derived from a single gene with three introns and a 5' flanking region with homology to the promoter region of the IFN-gamma gene (Hardy et al.,1985). Similarly only a single IL-2 receptor gene has been identified, coding for a 272 amino acid precursor of two domains separated by a putative 19 residue transmembrane region (Cosman et al.,1984; Leonard et al.,1984; Nikaido et al..1984).

The immune specificity of T-cell clonal expansion is guaranteed by the absence of IL-2 receptors from resting T-cells and the restriction of IL-2 receptor expression to

antigen activated T-cells (Cantrell & Smith,1983). Mitogens, however, by activating non-specifically cause a much higher percentage of the total T-cell population to express IL-2 receptors, which accumulate asynchronously. A critical threshold of IL-2 receptor density appears to be required before the commitment to cell cycle progression and DNA synthesis takes place. But once maximal IL-2 receptor expression occurs, continued proliferation is proportional to IL-2 concentration and time of exposure to IL-2 (Cantrell & Smith,1984).

IL-2 receptor expression is usually transient, peaking at 48-72 hours post-induction before declining. Maintenance of IL-2 receptor expression depends upon reintroduction of antigen (Andrew et al.,1985; Kaplan et al.,1984; Lowenthal et al.,1985b) or mitogen (Depper et al.,1984). Addition of exogenous IL-2 will not prevent the fall in IL-2 receptor levels. Reason dictates that IL-2 receptor expression should be transient in order to ensure that an antigen-specific clone does not expand too much. Thus induction combined with down-regulation of IL-2 receptor levels is likely to play a crucial role in controlling the cellular immune response.

In addition to its effects on T-cell growth, IL-2 also promotes proliferation of activated B-cells via surface receptors similar to those of activated T-cells (Waldmann et al.,1984; Mingari et al.,1984; Tsudo et al.,1984), though at 2000 per B-cell there are only half as many as on T-cells (Lowenthal et al.,1985a). However, IL-2 is thought to act at a later stage of B-cell proliferation

(48h) than B-cell growth factor (within 24h) and to play an auxiliary role (Boyd et al.,1985; Suzuki & Cooper,1985). As in the case of T-cells, macrophages are required as accessory cells for induction of IL-2 receptors on B-cells, purified IL-1 being capable of partially replacing this function (Shirakawa et al..1986).

The monoclonal antibody anti-Tac has been shown to bind to the IL-2 receptor (Leonard et al.,1982) and thereby inhibit various aspects of T-cell activation attributed to IL-2 binding (Depper et al.,1983). But quantitative binding assays with radiolabelled IL-2 and anti-Tac indicate that activated T-cells have 10-20 times more binding sites for anti-Tac than for IL-2 (Robb et al.,1984b). By using very high concentrations of labelled IL-2, it has been shown that a class of low affinity IL-2 receptors exists whose association constant is 5000 times lower than the high affinity receptors. Anti-Tac binds equally well to both, however, hence the initial discrepancy between IL-2 and anti-Tac binding assays. Despite there being many more low than high affinity sites, they are thought unlikely to act at in vivo IL-2 concentrations since <1% would be occupied whilst the high affinity sites would be saturated (Robb et al.,1984b). About 50% of IL-2 bound to high affinity sites is internalised within 10 minutes at 37°C and thereafter degraded with a half-life of 60-80 minutes (Fujii et al.,1986).

Studies with anti-Tac indicate that IL-2 up-regulates the expression of its own receptor once it has been

induced (Welte et al.,1984). In the absence of mitogen stimulation exogenous IL-2 is mitogenic only for any Tac positive cells already present and does not appear to induce IL-2 receptors on resting cells lacking this molecule (Taylor et al.,1986a).

Purified macrophage-depleted T-cells can be induced to express IL-2 receptors if PHA and recombinant IL-2 are added together, but this does not trigger endogenous IL-2 production (Katzen et al.,1985; Wakasugi et al.,1985). Thus IL-2 can induce its own receptor on T-cells in combination with PHA, but macrophages are also required to provide an additional signal (probably IL-1) for IL-2 production. The mitogens Con A and OKT3, however, are entirely dependent on macrophages for both IL-2 receptor expression and IL-2 production, even if exogenous IL-2 is added as well (Wakasagi et al.,1985).

The addition of exogenous IL-2 to T-cells and thymocytes which are already activated has been shown to increase the level of Tac expression by several groups (Reem & Yeh,1984; Welte et al.,1984; Depper et al.,1985; Reem & Yeh,1985). This upregulation of Tac levels requires de novo RNA and protein synthesis (Depper et al.,1985), IL-2 augmenting IL-2 receptor mRNA expression as well as other messages associated with T-cell activation. Transcription assays on isolated nuclei show that the rise in IL-2 receptor mRNA levels is due at least partly to activating transcription of the gene itself. However, as in the case of unstimulated PBML, IL-2 alone cannot induce IL-2 receptor expression in unresponsive T lymphoblasts

(previously activated T-cells), but acts by ensuring optimal expression when an initial activating signal such as PHA is also present (Depper et al.,1985).

However, Smith and Cantrell (1985) have demonstrated that whilst the addition of exogenous IL-2 to activated cells leads to a possible 8-10 fold enhancement of Tac expression (high and low affinity receptors) there is a simultaneous 20-30% diminishment of detectable high affinity binding sites. Thus whilst IL-2 apparently helps upregulate its own receptor levels, in fact it seems to cause a rise in the ratio of low to high affinity receptors (Smith & Cantrell,1985). Only in the case of Con A activated thymocytes has it been demonstrated to the contrary that IL-2 also augments the level of high affinity receptors (Reem et al..1985).

Both high and low affinity receptors share at least three different antigenic determinants, suggesting that they are structurally related and that any differences may be due to a change in configuration (Lowenthal et al.,1985c). This is supported by the finding that addition of a monoclonal antibody (PC61) for the IL-2 receptor causes an accelerated dissociation of IL-2 from receptors of both types, possibly by altering their conformation and hence their affinities (Lowenthal et al.,1985). Labelled IL-2 normally dissociates from the low affinity receptors with a half-life of 60 seconds (80 to 90% of total bound IL-2 is released by these receptors) and from high affinity with a half-life of 60-90 minutes. Binding of PC61 reduces these rates to 16 and 120 seconds

respectively. Other peptide hormones (e.g. epidermal growth factor and insulin) are reported to have similar high and low affinity receptor systems and it is thought that the two types can convert from one to the other. When L-cells displaying only murine low affinity IL-2 receptors are fused with membranes from human T-cells some of the murine IL-2 receptors (p55 chains) are converted to dramatically higher affinity forms (Robb,1986). Thus the same molecule (p55) can form low or high affinity receptors depending on the environment, cofactors present in the IL-2 receptor positive human T-cell line possibly crossing species lines to combine with the murine chain and convert it to form high affinity binding sites.

It has been suggested that IFN-gamma also increases the level of IL-2 receptor expression (Johnson & Farrar,1983) and may even induce IL-2 receptors to appear on macrophages (Herrman et al.,1985; Holter et al.,1986a). However, others have found no such evidence for IFN-gamma action in IL-2 receptor expression (Welte et al.,1984) and IFN-gamma does not appear to play a role in mitogen stimulated proliferation since anti-IFN-gamma antibodies have no effect on T-cell proliferation (Welte et al.,1984: Wilkinson et al.,1985).

vi) IFN-gamma production.

Binding of IL-2 to its receptor not only stimulates T-cell proliferation but also induces expression of at least 4 out of 7 cell cycle dependent genes, including c-myc and the IL-2 receptor (Kaczmarek et al.,1985). A

heat labile protein has been isolated from the cytoplasm of PHA stimulated, but not resting, PBML which can induce DNA synthesis in isolated quiescent nuclei (Gutowski et al.,1984). This activator of DNA replication (ADR) may be a cytoplasmic transducer of IL-2, there being a close relationship between ADR levels and IL-2 induced proliferation. Thus antigen/mitogen activate resting T-cells causing them to shift from the G_0 phase of the cell cycle to G_{1a} and express IL-2 receptors. IL-2 binding then triggers a shift from G_{1a} to G_{1b} and ADR formation. ADR upon reaching the nucleus would stimulate DNA replication (S phase).

There is also increasing evidence that IL-2 mediates IFN-gamma production. Blockade of IL-2/IL-2 receptor interactions inhibits various T-cell responses (Depper et al.,1983) and this may include IFN-gamma production (Vilcek et al.,1985; Croll et al.,1985) since T-cells are thought to be the major producers of IFN-gamma (Pasternack et al.,1984; Morris et al.,1982). Exogenous IL-2 acts synergistically with sub-optimal doses of mitogen to boost IFN-gamma yields (Kasahara et al.,1983a; Vilcek et al.,1985) but exogenous IL-2 alone induces at most only low levels of IFN-gamma from resting PBML and must be added at very high concentrations (Vilcek et al.,1985; Le et al.,1986; Blanchard et al..1986).

It is the aim of this thesis to investigate the role of IL-2 and other immune mediators in the regulation of IFN-gamma production in the context of T-cell activation.

4) PROPERTIES OF INTERFERON-GAMMA.

Although IFN-gamma appears to share some of the biological effects of IFN-alpha and IFN-beta, it also has certain unique properties and effects upon cells of the immune system and other cell types (Vilcek,1982; Vilcek et al.,1983; Trinchiera & Perussia,1985). The ability of IFN-gamma to mediate a variety of functions makes it a possibly important regulating factor of the immune, hematopoietic, and other systems. Its anti-viral activity may not therefore be its most important function. The availability of homogeneous recombinant IFN-gamma, free from the contamination of other lymphokines in the crude preparations used in earlier studies, has made possible most of the recent advances in the study of its properties. These are briefly reviewed here as an indication of IFN-gamma's possible importance in the immune response.

When protein extracts of cells induced with IFN-alpha or IFN-gamma are analysed by 2D-gel electrophoresis, it is clear that both IFNs induce the synthesis of several common polypeptides. These probably include those induced enzymes which lead to the development of the anti-viral state (Baglioni,1979; Lengyel,1982). This suggests that they share some common mechanisms of action, but IFN-gamma also induces synthesis of a further 12 distinct proteins (Weil et al.,1983). The synthesis of those proteins that are induced by both types of IFN (alpha and beta) has also been found to be regulated differently. IFN-alpha uses translational inhibition to regulate the steady state

levels of two induced proteins (mol.wts. of 67 and 56 kD), whilst IFN-gamma does not (Sen & Rubin,1984). Thus even in the shared anti-viral function of the two IFN types, the molecular mechanisms involved are not identical.

All three types of IFN enhance the expression of class I MHC antigens and beta-microglobulin, with IFN-gamma having the most potent effects in vitro (Ball et al.,1984), and in vivo (Momburg et al.,1986a). Treatment of cytototoxic T lymphocyte targets with either IFN-gamma or IFN-alpha/beta markedly augments cytotoxicity, presumably due to the enhancement of MHC class I antigens demonstrated by immunofluorescence (Blackman & Morris,1985). However, only IFN-gamma has significant effects on expression of class II antigens, IFN-alpha and IFN-beta enhancing weakly or not at all (Dolei et al.,1983; Rosa & Fellous,1984). Recombinant IFN-gamma increases the synthesis and expression of the HLA-DR (Ia-like) antigens, as well as beta-microglobulin, on human melanoma cells when 400 times higher doses of IFN-alpha had no effect (Basham & Merigan,1983). Recombinant IFN-alpha and IFN-beta induce HLA-A,B on monocytes and myeloid leukemic cell lines but not HLA-DR, unlike recombinant IFN-gamma (Kelley et al.,1984). IFN-gamma has been shown to enhance expression of class II antigens or induce them de novo in a large number of cell types, including myelo-monocytic cells (Koeffler et al.,1984; Virelizier et al.,1984), lymphoid cells (Wong et al.,1984), mast cells (Koch et al..1984). endothelial cells (Geppert & Lipsky,1985), fibroblasts (Collins et al.,1984), astrocytes (Lampson & Fisher,1984;

Fierz et al.,1985), and a large series of tumor-derived cell lines (Wong et al.,1983). IFN-gamma causes similar effects in vivo. injection in mice leading to widespread and selective induction of class I and class II antigens in a variety of tissues (Skoskiewicz et al.,1985; Momburg et al.,1986b).

The ability of IFN-gamma to induce the expression of class II antigens on a variety of cells suggests an immunoregulatory role when one considers the importance of class II molecules in antigen presentation (Unanue et al.,1984; Janeway et al.,1984). IFN-gamma treatment renders macrophage lines capable of antigen presentation (Zlotnik et al.,1983), an effect antagonised by the macrophage product PGE_2, though probably by its inhibitory effects on T-cells rather than on macrophages (Zlotnik et al.,1985). IFN-gamma treatment also renders endothelial cells susceptible to the cytotoxic effect of class II specific T lymphocytes (Pober et al.,1983). IFN-gamma treatment of macrophages presenting antigen accelerates immune proliferation of responding T-cells in response to soluble antigen in a manner which appears dependent on the level of macrophage DR (class II antigen) expression (Becker et al.,1985). However, induction of Ia expression on murine macrophages by IFN-gamma is antagonised by other immune response products, such as IgG and IgE (Virgin IV et al.,1985) and IFNs alpha and beta (Ling et al.,1985; Inaba et al.,1986). Thus any regulatory effects of IFN-gamma in vivo probably interact with other immune regulators.

All three types of IFN have also been shown to modify activities of several cellular components of the immune response in vitro. In the case of macrophages IFN-gamma boosts the level of IL-1 production (Arenzana-Seisdedos et al.,1985) and HLA-DR and DC expression (Virelizier et al.,1984), suggesting that it could amplify T-cell activation by a feedback loop to the beginning of the lymphokine cascade. It also boosts macrophage IgG Fc receptor expression (Perussia et al.,1983; Fertsch & Vogel,1984) which could increase the level of Fc-FcR interactions between T-cells and macrophages important in the phagocytosis of antibody coated bacterial and viral particles (Chang et al.,1985). IFN-gamma also appears to be an important macrophage activating factor (MAF), inducing cytotoxicity of human monocytes against tumour targets at levels as low as 0.3-1U/ml, IFNs-alpha/beta showing no MAF activity even up to levels of 200U/ml (Le et al.,1983). Monoclonal antibodies against IFN-gamma neutralise all MAF activity in conditioned medium from mitogen stimulated lymphocytes in both the human (Le & Vilcek,1984) and murine systems (Spitalny & Havell,1984), suggesting that it is the major MAF in such preparations. Other T-cell derived factors, however, also exhibit MAF activity (Lee et al.,1986).

All three IFN types markedly augment T-cell cytotoxicity (Blackman & Morris,1985; Lindahl et al.,1972; Vose et al.,1983) and IFN-gamma also appears to be involved in enhancement of NK cell activity (Braakman et al..1986: Peterman et al.,1984), although the nature of this

involvement with respect to IL-2's effects on NK activity is unclear (Trinchieri et al.,1984; Shaw et al..1985: Lanier et al.,1985). It is generally agreed that IL-2 augmented NK activity is mediated at most only partly by IFN-gamma since it is only partially inhibited by antibodies against the latter (Mukaida et al..1986: Braakman et al.,1986). However, IFN-gamma does enhance IL-2 mediated generation of activated killer cells (Itoh et al.,1985b) and helps maintain NK-like cytotoxicity (Peterman et al.,1984).

In addition to its effects on cellular responses, IFN-gamma effects humoral responses too. Soluble T-cell factors induce primary splenic B-cells to proliferate and differentiate into antibody secreting cells (Jelachich et al.,1984). Such action is partly due to IFN-gamma which can substitute for a late helper factor (T-cell replacing factor) which acts synergistically with other helper factors (IL-1 and IL-2) in the stimulation of a B-cell antibody response in vitro (Leibson et al.,1984). IFN-alpha also helps to stimulate immunoglobulin production in vitro (Nebauer et al.,1985).

IFN-gamma also directly induces maturation of resting B-cells in vitro. producing surface phenotype changes and immunoglobulin secretion (Sidman et al.,1984). But it is far from the only B-cell maturation factor, antibodies against IFN-gamma having no effect on other such factors produced by T-cells.

5) THE *IN VIVO* ROLE OF IFN-GAMMA ?

IFN-gamma clearly exhibits a variety of immunomodulatory effects *in vitro* in addition to its antiviral properties. Since IFN-gamma is produced by antigen stimulated T-cells on an immune specific basis it seems not unreasonable to assume that it plays some role in the immune response other than purely anti-viral. IFNs alpha and beta, however, can be made by many cell types and appear to be chiefly induced by viral infection. Although they have definite *in vitro* regulatory effects, the *in vivo* relevance of these effects to immune regulation is not clear. The induction of IFN production of all three types by some bacteria and bacterial products suggests that they may also help fight bacterial infection in some cases. Thus the use of Staphylococcal enterotoxin A (SEA) in this study will also provide some information about bacterial induction of IFN-gamma.

Even in the case of IFN-gamma there is little direct evidence for an *in vivo* immunomodulatory role. What evidence there is has recently been reviewed (De Maeyer *et al.*,1985) but is largely inferential. For example, murine recombinant IFN-gamma protects mice *in vivo* against local and systemic infection by the bacterium *Listeria monocytogenes* (Kiderlen *et al.*,1984), so it can clearly help combat both viral and non-viral infection. The effects of *in vivo* defects in IFN-gamma production have been taken as evidence for an *in vivo* role. Mice strains which are low producers of IFN-gamma (e.g. BALB/c) tend to be more susceptible to many infections compared to high

producing strains such as C57BL/6 (Virelizier,1982; Cheers & McKenzie,1978; Kirchner et al.,1978). The susceptibility of human neonates to viral and bacterial infections may be due to impaired T-cell immunity caused by defective IFN-gamma production (Wakasugi & Virelizier,1985). The low NK activity and HLA-DR levels of neonates appear to be consequences rather than causes of reduced IFN-gamma secretion since exogenous IFN-gamma can enhance both (Kaplan et al.,1982; Kelley et al..1984). More direct evidence about endogenous IFN-gamma's role requires experiments using antibodies to IFN-gamma in infected animals.

Its major non-antiviral role in vivo may prove to be its effects on macrophages. Activation of macrophage cytotoxic activity may be the major mechanism for dealing with many bacterial infections and enhancement of MHC class II antigen expression may be essential in regulating T-cell activation by macrophages. Basal levels of IFN-gamma may exist prior to activation of the lymphokine cascade which could help initiate an immune response leading to further lymphokine production. It has been argued that IFN-gamma is produced continuously in low "physiological" amounts which increase occasionally when infection induces an acute response (Bocci,1985).

6) OBJECTIVES AND COMMENTS ON THE EXPERIMENTAL SYSTEM EMPLOYED IN THIS STUDY.

i) Aims.

IFN-gamma's anti-viral effects alone make it worthy of study, but if in addition it has in vivo immunomodulatory effects then further study of its induction will contribute to our understanding of the immune reponse. IFN-gamma is thought to be produced at the end of a cascade of other substances. If this is indeed the case, how do the lymphokines and other regulatory molecules and pathways that make up this "cascade" interact? The primary aim of this study is to examine the interactions involved in T-cell activation using IFN-gamma production as a measure of their effects. This is a very active field of research with respect to other aspects of T-cell activation, e.g. T-cell proliferation, but rather less work has been devoted to the regulation of IFN-gamma production. However, some of the work on T-cell activation by other researchers over the past three years has had direct relevance to the work described here and is therefore discussed in that light.

Areas worthy of investigation include:

1. Can non T-cells produce IFN-gamma?
2. How are IL-1 and IL-2 involved in IFN-gamma production, i.e. does IFN-gamma production really rely upon the lymphokine "cascade"?
3. What cell surface molecules can regulate IFN-gamma production? The recent description of a putative lymphokine (TCAF) that acts via a T-cell

activation/suppression pathway (T11) distinct from that activated by the T-cell antigen receptor makes the role of this pathway of increasing interest. How does it affect IFN-gamma production and how are these effects regulated?
4. How is transcription of the IFN-gamma gene regulated compared to production of the protein itself?

ii) The experimental system.

Most of the major fundamental work on the immune response has made use of the advantages of the murine system over research using human material. For example, the use of mice from the same inbred strain, genetically identical in the MHC (H2) region of the genome, was essential for unravelling the role of MHC antigens in the immune response. In comparison work on human material, as here, has clear disadvantages. Most work is obviously in vitro and may therefore not necessarily be relevant to the in vivo situation. There is also the problem of variation between immune responses of different individuals to the same stimulus. In this study buffy coats (i.e. peripheral blood mononuclear leukocytes) from many different donors were used with no possibility of repeating experiments with cells from the same MHC genotype as there would be with mice strains. There was considerable variation between the response of different donors to the same mitogenic stimulus under the same conditions (Table 1). Whilst differences between donors was a major factor in this variation, the practical limitation on experiments which made it difficult to measure only the peak level of

Table 1.1

THE RESPONSE OF DIFFERENT DONORS PBML TO THE SAME STIMULUS.

Donor	IFN-gamma Titre (U/ml)	Donor	IFN-gamma Titre (U/ml)	Donor	IFN-gamma Titre (U/ml)
1.	160	8.	1600	15.	40
2.	630	9.	630	16.	1200
3.	130	10.	630	17.	250
4.	160	11.	160	18.	160
5.	40	12.	320	19.	160
6.	63	13.	55	20.	450
7.	80	14.	600	21.	2000

Range = 40-2000 U/ml.

Mean titre = 453 U/ml.

Standard deviation of titres = 524

a) PBML at $5x10^5$ cells/ml were induced with 20ng/ml of SEA. The cells were incubated at 37°C and the IFN-gamma titre of the supernatant measured 3 days post-induction.

IFN-gamma production probably also contributed to the variation in titres. IFN-gamma levels were always measured in the cell supernatant three days after induction, since time courses showed that this was usually the peak period of production. However, there was almost certainly some difference in the speed of response between donors with some donors producing peak levels earlier than others (probably between 2 and 4 days, 3 days post induction being the mean). However, whilst optimisation of IFN-gamma levels, by determining the peak level from a time course, might have partly reduced apparent donor variation it also would have greatly increased the number of samples and assays required for each experiment. Furthermore donor variation was not such a problem if one concentrated on relative rather than absolute levels of IFN-gamma production in different regulatory situations, i.e. absolute differences in level between donors are not as important as whether a certain set of conditions depress or elevate IFN-gamma production in all donors. When this proves to be the case then inferences can be made about regulatory effects for all donors since the response will not be specific to a single donor, T-cell line, or mouse strain.

However, variation in donor responses does necessitate frequent repetition of experiments if small shifts/effects are being examined where the results are not clear-cut. In this study effects were usually shown to be reproducible for many different donors, not just two or three, before firm conclusions were drawn.

The major and decisive advantage of the human system lies in the increasing availability of purified and recombinant human interleukins, plus antibodies against them, and the large range of antibodies available for cell surface markers of different cell types and subsets. Clinical research and investigation has been the spur for developing these powerful tools for further research. Far fewer such developments have occurred in the murine system so that in consequence the human T-cell response is in some ways the more thoroughly defined.

CHAPTER 2.

INTERFERON-GAMMA PRODUCTION BY LOW DENSITY LYMPHOCYTES.

Introduction.

A variety of T-cell types have been found capable of producing interferon-gamma (Archer et al., 1979), including both Lyt 2+ and Lyt 2- cells in the mouse (Guerne et al.,1984) and the T4 and T8 subsets in man (Biondi et al.,1984). However, probably all require the presence of accessory macrophages for significant interferon-gamma production (Bendtzen & Petersen,1984; Bruszewski et al.,1984; Chang et al.,1982; Unanue et al.,1984). There is also now evidence to suggest that non-T cells can also produce IFN-gamma. In particular, it has been shown that human low-density lymphocytes produce IFN-gamma and a variety of other lymphokines, including IL-1, IL-2, colony-stimulating factor, and B-cell growth factor, in response to T-cell mitogens (Kasahara et al.,1983b; Djeu,1983; Scala et al.,1984; Pistoia et al.,1985; Procopio et al.,1985). This IFN-gamma production is often ascribed to large granular lymphocytes (LGL), which make up a large proportion of the low density fraction of lymphocytes and possess natural killer cell characteristics (Burns et al.,1985). LGL, which make up less than 5% of the total peripheral blood mononuclear leukocytes, have a distinct morphology of high cytoplasmic to nuclear ratio with a granular cytoplasm. Nearly all natural killer (NK) cell activity can be attributed to the LGL population (Timonen et al.,1981), and they bear the

surface markers Leu 7, Leu 11, and OKM1 (Scala et al.,1985; Abo et al.,1984), Leu 7 being expressed later in the path of differentiation of NK cells (Phillips & Lanier,1985). NK cells have spontaneous cytolytic activity against a variety of cells, unlike cytotoxic T-cells whose action is specific, MHC restricted, and takes several days to develop (Herberman & Ortaldo,1981). NK cells may therefore play an important role in immune surveillance in a primary broad range defence system which can respond almost immediately to foreign materials, helping to control infection until the more potent and specific immune system begins to respond.

In this chapter IFN-gamma production by LGL enriched low-density lymphocytes, in response to the mitogen SEA, was examined. Unlike high density T lymphocytes, low-density cells were found not to require macrophages for IFN-gamma production. However, this IFN production appeared to be partially dependent upon the interaction of two subsets separable by sheep red blood cell rosetting.

RESULTS.

Low density lymphocytes are high-level producers of IFN-gamma independent of macrophages.

Macrophage-depleted lymphocytes were prepared by replating PBML on plastic petri dishes, discarding adherent cells or retaining them as purified macrophages, and overnight incubation with silica to eliminate phagocytic cells (see methods). This method produced >99% pure lymphocyte preparations as judged by non-specific esterase staining.

Separation of lymphocytes into differing density sub-populations was achieved by centrifugation at 550G for 30 minutes over a six-step discontinuous buffered Percoll/RPMI gradient, the steps of which varied by 2.5% Percoll concentrations from 40 to 52.5% Percoll (see methods).

As can be seen in table 1 (data provided by M.F.Wilkinson), when macrophage-depleted lymphocytes were separated on a stepped gradient the low density lymphocytes from fractions 1 and 2, comprising 6% of the total lymphocytes, were the best IFN-gamma producers on a per cell basis, as induced by SEA. The interferon produced was shown to be IFN-gamma for both total lymphocytes and low-density cells by its 99% neutralisation with anti-IFN-gamma antiserum (80U/ml and 320U/ml reduced to <2U/ml respectively). Addition of macrophages made little difference to IFN-gamma production by low-density cells, but boosted production five- to 10-fold for the denser and total lymphocytes. This has also been shown to be the case

TABLE 2.1

PRODUCTION OF IFN-GAMMA BY LYMPHOCYTES OF DIFFERENT DENSITIES INDUCED BY SEA.

	IFN-gamma titre (U/ml)	
Cells	Alone	With macrophages
Lymphocytes	50	2000
Macrophages	<8	-
Fraction 1 (3%)	800	130
Fraction 2 (3%)	400	500
Fraction 3 (27%)	80	200
Fraction 4 (53%)	40	200
Fraction 5 (14%)	20	200

a) Macrophages were added back to the macrophage-depleted cells in the column headed "with macrophages" at a macrophage:lymphocyte ratio of 1:10.

b) The percentage of the total lymphocyte population present in each fraction is indicated in brackets.

c) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

d) Data obtained from M. Wilkinson.

for induction by Con A and PHA (Croll et al.,1986a).

In subsequent experiments low-density cells were usually isolated from the top two steps of the gradient, chiefly from the interface between the 42.5% and 45% steps. A single 46% Percoll cushion was also used to isolate low-density cells (Ly.L). Cells isolated from the bottom three steps or below the 46% interface were considered high density, chiefly T-cells (Ly.H).

Comparison of the level of IFN-gamma production by these various fractions, for several different donors, confirmed that the low-density fraction is enriched for high level producers in the absence of macrophages (Tables 2 & 3). Addition of purified macrophages, from the same donor, to the fractions shows that Ly.L IFN-gamma production is relatively macrophage independent compared to the unfractionated lymphocytes and especially when compared with the high density fraction (Ly.H). The latter are particularly macrophage-dependent for significant IFN-gamma production. When the total lymphocyte populations were reconstituted from the low- and high-density cells, the requirement for macrophages was restored to that found in the unfractionated lymphocyte population. Thus, only low-density lymphocytes are macrophage-independent

The removal of B lymphocytes by nylon-wool filtration, which removes both macrophages and B-cells, does not alter the observation that it is the low-density cells that can produce significant IFN-gamma levels without macrophages (Table 3). The low-density fraction contained up to 20%

TABLE 2.2

PRODUCTION OF IFN-GAMMA BY LOW AND HIGH DENSITY LYMPHOCYTES INDUCED BY SEA.

	IFN-gamma titre (U/ml)					
	Donors					
	1		2		3	
Cells	Alone	With Mo	Alone	With Mo	Alone	With Mo
PBML	400	800	630	1000	630	1000
Ly.	200	1000	100	250	100	800
Ly.Low	320	250	400	1000	2000	800
Ly.High	10	100	16	100	32	200
Ly.Rec.	130	1000	80	200	130	400

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) Macrophages were added back to cell populations in the columns headed "With Mo" at a macrophage:lymphocyte ratio of 1:10.

c) Ly.= Unfractionated Lymphocytes.

Ly.Low = low density lymphocytes.

Ly.High = high density lymphocytes.

Ly.Rec. = recombined high and low density lymphocytes.

TABLE 2.3

PRODUCTION OF IFN-GAMMA BY LOW AND HIGH DENSITY LYMPHOCYTES DEPLETED OF MACROPHAGES AND B CELLS.

	IFN-Gamma titre (U/ml)			
	Donors			
	1		2	
Cells	Alone	With Mo	Alone	With Mo
PBML	100	200	160	400
Ly.	40	160	50	160
Ly.Low	320	130	160	160
Ly.High	100	400	80	160
Ly.Rec.	1300	1300	80	400
NW-	130	200	8	100
NW-.Low	80	100	60	120
NW-.High	16	100	4	8

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) Macrophages were added back to cell populations in the columns headed "With Mo" at a macrophage:lymphocyte ratio of 1:10.

c) Ly.= Unfractionated Lymphocytes.

Low = low density lymphocytes.

High = high density lymphocytes.

Ly.Rec. = recombined high and low density lymphocytes.

NW- = nylon wool depleted lymphocytes.

SIg+ cells, reduced to <2% by nylon-wool treatment. Therefore, B lymphocytes are not involved in macrophage-independent IFN-gamma production by low-density cells.

Differing lymphocyte-macrophage ratios or mitogen concentration did not effect the relative lack of macrophage-dependence of Ly.L compared to Ly.H (Tables 4 & 5). However, Ly.L fractions often did benefit from the addition of macrophages to a limited extent, IFN-gamma production being boosted 2-3 fold compared to 5-10 fold for Ly.H. This effect may have been due to high-density T-cells contaminating the low-density cell preparation.

When the levels of IFN-gamma production of low density cells and PBML were compared over a time course (Table 6) the low density cells were found to respond to SEA stimulation much more rapidly. Within 24 hours of induction low density cells were producing IFN-gamma levels (40U/ml) not attained by PBML, from which they were derived, until 3 days after induction. Thus Ly.L produce readily detectable levels of IFN-gamma much more rapidly than PBML, although both cell populations reach peak production at a similar time, 3-4 days post induction.

Co-operation between subsets of low-density lymphocytes is necessary for maximal IFN-gamma production.

In order to purify further the low-density fraction and remove any T-cells, which compose the majority of the high density population, the Ly.L were further fractionated by high-affinity sheep red blood cell (SRBC) rosetting into

TABLE 2.4

THE EFFECT OF INCREASING MITOGEN CONCENTRATION ON IFN-GAMMA PRODUCTION BY LOW AND HIGH DENSITY LYMPHOCYTES.

	IFN-Gamma titre (U/ml) SEA concentration (ng/ml)					
	10		30		100	
	Alone	With Mo	Alone	With Mo	Alone	With Mo
Donor 1 Ly.Low	200	150	50	80	150	250
Ly.High	4	32	<2	50	20	250
Donor 2 Ly.Low	130	500	100	200	63	400
Ly.High	20	250	32	250	32	320

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) Lymphocyte to macrophage ratio was 10:1 when macrophages were added in those columns headed "With Mo".

c) The low density fraction contained only 5% of the total lymphocyte population for both donors.

TABLE 2.5

THE EFFECT OF VARYING MACROPHAGE/LYMPHOCYTE RATIOS ON IFN-GAMMA PRODUCTION BY LOW AND HIGH DENSITY LYMPHOCYTES.

	IFN-gamma titre (U/ml) Percentage of macrophages added.			
	Without Mo	3% Mo	10% Mo	30% Mo
Donor 1				
Ly.Low	130	320	400	320
Ly.High	5	250	630	160
Donor 2				
Ly.Low	63	160	80	100
Ly.High	13	200	200	150

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) The low density fraction contained only 5% of the total lymphocyte population for both donors.

TABLE 2.6

TIME COURSE OF SEA-INDUCED IFN-GAMMA PRODUCTION BY PBML AND LOW DENSITY LYPHOCYTES.

IFN-gamma titre (U/ml)

Time after induction.

	0h.	18h.	24h.	2 days	3 days	4 days	5 days
LGLs	6	25	40	100	400	800	550
PBML	<2	3	3	8	50	80	63

a) Macrophage-depleted LGLs were isolated from PBML and contained only 8% of the total lymphocyte population.

b) All cells were induced with 20ng/ml of SEA at $5x10^5$ cells/ml.

E+ and E- cells. The E+ cells bore the sheep erythrocyte receptor, the high affinity form being found on T-cells, whilst the E- fraction was further enriched for non-T cells (see characterisation of cell fractions section). The IFN-gamma production of these two populations on a per cell basis was lower than that of unfractionated low-density cells (Tables 7 & 8). The non-rosetting fraction generally produced more IFN-gamma than the rosetting fraction. In order to check whether this reduction of IFN-gamma production by the subfractions was due to cell damage during the fractionation procedures, reconstitution experiments were performed. It was found that reconstitution of the low-density population by mixing E+ and E- cells largely restored IFN-gamma production to its unfractionated level. This was found to be the case in four out of five donors.

In additional experiments, macrophages were added back to the E+ and E- fractions in order to examine the role of these fractions in macrophage independency of low density cells (Table 8). Addition of macrophages to E+ and E- cells boosted IFN-gamma levels considerably, 10-fold or greater for E+ cells and 5-fold or greater for E- cells. This was in contrast to the unfractionated low density population whose level of production was far less macrophage dependent, with at most a 4-fold increase. In the one experiment in which E+ and E- were mixed and macrophages added, IFN-gamma production was partially restored to its unfractionated level and macrophage independence regained. Thus cooperation between the E+ and

TABLE 2.7

IFN-GAMMA PRODUCTION BY SUB-FRACTIONS OF LOW DENSITY LYMPHOCYTES.

	IFN-gamma titre (U/ml) Donors				
	1	2	3	4	5
PBML	400	800	320	200	320
Ly	120	260	75	8	80
Low	320	500	205	5000	400
Low E-	100	250	105	40	120
Low E+	55	160	125	18	5
Rec E-/E+	225	320	325	40	220

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) PBML = Peripheral Blood Mononuclear Leukocytes.

Ly = Macrophage-depleted lymphocytes.

Low = Low density lymphocytes.

Low E- = Non E-rosetting low density lymphocytes.

Low E+ = E-rosetting low density lymphocytes.

Rec E-/E+ = Reconstituted low density lymphocytes (E- and E+ cells are recombined in the proportions in which they were obtained after sheep erythrocyte rosetting).

TABLE 2.8

MACROPHAGE ASSISTANCE OF LOW DENSITY LYMPHOCYTE SUB-FRACTIONS IN IFN-GAMMA PRODUCTION.

	IFN-gamma titre (U/ml)					
	Donors					
	1		2		3	
	Alone	With Mo	Alone	With Mo	Alone	With Mo
PBML	320	-	32	-	55	-
Ly	80	800	20	160	6	2000
Low	400	350	100	400	1100	3200
Low E-	120	600	32	170	60	1600
Low E+	5	400	3	100	50	450
Rec E-/E+	220	110	ND		ND	

a) All cells ($5x10^5$ cells/ml) were induced with SEA at 20ng/ml.

b) PBML = Peripheral Blood Mononuclear Leukocytes.

Ly = Macrophage-depleted lymphocytes.

Low = Low density lymphocytes.

Low E- = Non E-rosetting low density lymphocytes.

Low E+ = E-rosetting low density lymphocytes.

Rec E-/E+ = Reconstituted low density lymphocytes (E- and E+ cells are recombined in the proportions in which they were obtained after sheep erythrocyte rosetting). ND = Not Done.

c) Macrophages isolated from the same donor were added in those columns headed "With Mo" at a macrophage:lymphocyte ratio of 1:10

d) Donor 1 is the same as donor 5 on table 2.7.

E- fractions appears to be a requirement for optimal IFN-gamma production in the absence of macrophages acting as accessory cells.

Characterisation of cell fractions.

In order to characterise the various cell fractions, the expression of three leukocyte surface markers (Leu 11, OKT3, and OKM1) by the cells was examined by immunofluorescence. Leu 11 is a marker found on LGL, OKT3 is found on T-cells, and OKM1 is found on macrophages and some null (i.e. non-T) cells. Unfractionated lymphocytes were 11% Leu 11+ and 84% OKT3+ (Table 9). A typical low-density preparation was 23% Leu 11+ and 38% OKT3+, showing enrichment for the LGL marker. If only the top two steps of the gradient were used for the low density fraction, preparations containing up to 70% Leu 11+ cells could be obtained, but the third step was usually included to provide sufficient cells for all the fractionation procedures. After rosetting, the E- fraction was further enriched for Leu 11+ cells and the E+ fraction strongly enriched for T-cells. Using different coloured conjugates specific for Leu 11 and OKM1 (fluorescein-labelled anti-murine IgM against the Leu 11 moab and rhodamine-labelled anti-murine IgG against the OKM1 moab), the expression of both markers on the same cell was examined. OKM1 was found to be generally coexpressed with Leu 11. A typical low-density fraction contained 42% Leu 11+/OKM1+ cells, but after rosetting the E+ fraction contained 10% Leu 11+/OKM1+ cells and the E- fraction

TABLE 2.9

CELL SURFACE MARKERS AND CYTOTOXICITY OF THE LYMPHOCYTE FRACTIONS

	% cells bearing markers	
	Leu 11+	OKT3+
Total Ly.	11%	84%
Ly. Low	23%	38%
Low E- fraction	50%	36%
Low E+ fraction	7%	79%

a) All above fractions isolated from a single donor in which the low density cells made up 45% of the total lymphocyte population.

	Leu 11+/M1+	M1+ only
Ly.Low	42%	0%
Low E- fraction	60%	5%
Low E+ fraction	10%	1%

b) All above fractions were isolated from a single donor with the low density fraction containing 16% of the total lymphocytes.

CYTOTOXICITY

Ly.Low	70% Leu 11+ , 31% T3+	82% cytotoxicity
Ly.High	2% Leu 11+ , 42% T3+	16% cytotoxicity

c) Ly.Low contained 13.5% of the total lymphocyte population. An effector to target ratio of 30:1 was used for cytotoxicity measurements, using ^{51}Cr labelled K562 target cells.

contained 60% Leu 11+/OKM1+ with 5% OKM1+ only cells. Thus, the low-density fracion is enriched for the LGL marker Leu 11 and somewhat depleted of T-cells. Subsequent rosetting leads to a Leu 11-enriched E- fraction and an OKT3-enriched E+ fraction.

Non-specific esterase staining to detect cells of the monocyte/macrophage lineage and the addition of indian ink to detect phagocytic cells both confirmed macrophage depletion. Lymphocytes and low-density fractions were both shown to contain less than 0.2% macrophages.

Enrichment for natural killing in the low-density cells, LGL possessing NK activity, was confirmed by means of an 18-hour ^{51}Cr-release assay, using K562 target cells at an effector:target ratio of 30:1. Low-density cells were found to elicit 82% cytotoxicity and high density cells only 16% cytotoxicity, showing clear enrichment for NK-activity in the low-density cells.

The low-density fraction was also enriched for B cells, containing up to 20% SIg+ cells as detected by FITC-labelled anti-human IgG. Nylon wool filtration reduced this to <2%.

DISCUSSION : Chapter 2

There have been numerous demonstrations that induction of IFN-gamma by antigen or mitogen in unfractionated lymphocytes is macrophage-dependent. However, the results described in this chapter show that lymphocyte preparations enriched for low density cells do not require macrophages for IFN-gamma production, unlike high density cells which make up the majority of lymphocytes and are strongly macrophage-dependent. Djeu has also shown that low density lymphocytes can produce IFN in response to a variety of inducers, including SEA, PHA, and Con A, in a macrophage-independent fashion (Djeu,1983). It is possible, therefore, that low density preparations are enriched for cells able to produce IFN-gamma independent of the accessory cell function supplied by macrophages. These are unlikely to be T-cells, which usually require the presence of accessory cells for activation, but may be natural killer or null cells (also known as large granular lymphocytes = LGL) which are reported to produce a variety of lymphokines (Kasahara et al.,1983b; Scala et al..1984: Pistoia et al.,1985) including IFN (Djeu,1983).

The low density preparations examined in this chapter were clearly shown to be depleted of macrophages (<0.2% esterase positive) and enriched for NK activity (82% cytotoxicity against an NK target cell, K562) and the NK cell marker, Leu 11 (up to 70% Leu 11+). The high density population, however, were depleted for NK activity and contained only 2% Leu 11+ cells. Measurements of IFN-gamma production by cells from increasing density percoll steps

revealed that only low density lymphocytes produced high levels of IFN-gamma in the absence of macrophages (Table 2.1). The high density lymphocytes were in comparison highly macrophage dependent (Table 2.2) and contained mainly T-cells.

B cells are of a similar density to LGL, but B cell depletion did not alter the observation that the low density cells are the significant IFN-gamma producers without macrophages compared to the high density cells (Table 2.3). Therefore the macrophage-independent IFN-gamma production of low density lymphocytes is more likely due to LGL and not to B cells. The relative macrophage-independence of low density cells was unaffected by changes in mitogen concentration (Table 2.4) or increasing macrophage/lymphocyte ratios (Table 2.5).

However, it is also possible that the low density preparations are enriched for non-macrophage accessory cells which interact with any remaining T-cells also present in the low density preparation. The possibility was investigated by sub-fractionating the low density cells by high affinity sheep red blood cell rosetting, thus separating T-cells, which express a high affinity E receptor, from null cells, most of which express a low affinity form (Kay et al.,1977) of the E receptor. The E+ subfraction produced in this way consisted largely of OKT3+ cells (79%) and produced little IFN-gamma unless macrophages were added (Table 2.8). The E- subfraction, which contains most of the Leu 11+,OKM1+ cells but also significant numbers of OKT3+ cells, produced less

IFN-gamma than the unfractionated low density population, but rather more than the E+ cells (Tables 2.7 & 2.8). When the low density population was reconstituted by mixing E+ and E- cells, IFN-gamma production was almost restored to the level produced by unfractionated low density cells. The reduction of IFN-gamma production by low density cells was thus not due to damage caused by the fractionation procedure itself. Therefore it would appear that some form of cooperation in IFN-gamma production takes place in the absence of macrophages between two low density lymphocyte populations separable by rosetting. However, the identities of the cell types involved is not clear.

There is ample evidence from studies with T-cell clones to show that T-cells can produce IFN-gamma (Matsuyama et al.,1982; Pasternack et al.,1984; Morris et al.,1982), but there is also evidence to indicate that Leu 11+,OKM1+ cells may also be capable of IFN-gamma production (Kasahara et al.,1983b). In this chapter both E+ and E- cells required macrophages for optimal IFN-gamma production, although E- cells were still capable of significant production in their absence (Table 2.8). A possible explanation for these observations is that Leu 11+ cells, which make up the majority of the E- fraction, may themselves produce IFN-gamma without the necessity for macrophages, but in addition can act as accessory cells for the production of IFN-gamma by low density T-cells, which make up the majority of the E+ fraction. This explains the required cooperation for maximal IFN-gamma production between E+ and E- cells.

Other reports of lymphokine production by low density cells are of considerable interest. Kasahara et al. have shown that macrophage and T-cell depleted LGL produce greater levels of IL-2 and IFN-gamma in response to mitogens than PBML, and that the IL-2 production was not enhanced by addition of macrophages or purified IL-1, unlike T-cell populations (Kasahara et al.,1983). However, the same workers found that depletion of M1+ and T3+ cells did reduce IFN-gamma production by LGL significantly, suggesting that there was still some T-cell production in their low density fraction despite macrophage depletion by adherence and nylon columns prior to percoll gradient fractionation. The results described in this chapter suggest that E- cells could act as accesssory cells for low density T-cells in the absence of macrophages, thus explaining the apparent production of IFN-gamma by low density T3+ cells found by Kasahara in macrophage depleted populations.

LGL are a very heterogeneous population and the interactions between various subsets therefore complex. Kasahara attributes IL-2 production by LGL to an atypical T-cell subset (OKT3-,Leu1-,OKT11+) rather than the myeloid subset (OKM1+), since depletion of OKT11+ cells using moab plus complement greatly reduces IL-2 production. The myeloid subset or an OKT8+ subset appear to regulate this production (Kasahara et al.,1983). Scala et al. have also found that an OKT8+ subset helps regulate IL-1 production by LGL, OKT8+ depletion boosting IL-1 production, which is attributed to a B73.1+ (= Leu 11),OKM1+,DR+ subset of LGL

(Scala et al.,1984). This IL-1 producing subset would be equivalent to the E- subset examined in this chapter, possibly IL-1 production by E- cells being required by E+ cells (chiefly T3+) for optimal IFN-gamma production by the unfractionated low density population. IL-1 production is shown in chapter 3 to be an important accessory function of macrophages for IFN-gamma production.

Subsets of LGL have also been shown to exhibit accessory cell functions, T11+,DR+,OKM1+ LGL acting as accessory cells for T-cell proliferation in response to soluble polyclonal stimulants (Staphylococcus protein A and Streptolysin O) and to surface antigens in mixed leukocyte reactions, whilst OKM1-,DR- LGL do not (Scala et al.,1985). This is in contrast to the findings of Brooks and Moore (1986) who found that purified populations of LGL (sorted with a fluorescence activated cell sorter) were unable to present Streptolysin O but could stimulate mixed lymphocyte reactions. Perhaps LGL can only act as accessory cells when antigen uptake and processing is not required, but class II MHC expression and IL-1 production are, as in the case of mitogen stimulation rather than antigen stimulation. The T11+,DR+,M1+ LGL also exhibited most of the NK activity, and are therefore equivalent to Leu 11 cells characterised in this chapter, since the most potent NK activity of NK cells is associated with Leu 11 expression (Abo et al.,1984; Itoh et al.,1985; Seki et al.,1985). Interactions between subsets of LGL have also been shown to be important for optimal induction of autologous mixed lymphocyte reactions (Scala et al.,1985).

Thus there is accumulating evidence for a cell type which, although it shares at least one marker (OKM1) with macrophages, is distinct from them (i.e. non-adherent, non-phagocytic, esterase negative, Leu 11+) but able to carry out accessory functions, such as IL-1 production. In the case of IFN-gamma production, the results described in this chapter, and those of other workers, suggest that the Leu 11+,OKM1+ cells of the E- fraction (which can also contain T11+ cells since the E receptor/T11 of NK cells is of low affinity compared to that of T-cells) may therefore act as accessory cells for the T3+ cells of the E+ fraction. The IFN-gamma production of the E- fraction, however, is most likely to be due to Leu 11+ cells since these make up the majority of the fraction. Some IFN-gamma production by T11-,T3+ cells cannot be excluded, however. Whether Leu 11+ cells can replace macrophages in other immune responses is as yet unclear, but such an accessory function might be important in the early stages of an immune response.

LGL enriched cell preparations have been shown to react to a wide range of immune stimuli (mitogens, viruses, bacteria, mycoplasma-contaminated tumour cells) and are thus uniquely equipped to respond to almost all immune stimulating agents by rapid production of IFN (Djeu,1983). The type of IFN produced, however, depends on the stimulus, mitogen stimulation leading mainly to IFN-gamma production (SEA stimulated LGL produced IFN which was >99% neutralised by anti-IFN-gamma antisera, e.g. 320U/ml to <2U/ml) but viral and bacterial stimulation cause

IFN-alpha production, whilst T-cells produce only IFN-gamma (Djeu,1983).

It is shown in this chapter that LGL produce readily detectable levels of IFN-gamma within 24 hours (Table 2.6), unlike PBML (40 vs. 3U/ml respectively). Such early production by LGL could have regulatory effects since levels of IFN-gamma as low as 10U/ml induce IL-1 secretion by human monocytes (Arenzana-Seisdedos et al.,1985) and augment antigen presentation by human astrocytes (Fierz et al.,1985). However, since LGL form such a small percentage of the total lymphocytes, a few days post-induction their contribution to total lymphokine production during an immune response would be negligible. It has therefore been suggested that their importance as lymphokine producers may lie in acting as a rapid macrophage-independent first line of defence in response to a wide variety of immune stimuli. Their early release of low but detectable levels of IFN-gamma could help to activate T-cells, macrophages, and other NK cells, thus helping to start a cascade of immunological responses to infection (Djeu,1983). The possibility that they can act as accessory cells for T-cells might also be important in this respect as a faster initial alternative to macrophage activity. The granularity of LGL suggests a secretory function which may be involved in rapid lymphokine release compared to T-cells. The spontaneous NK cytotoxicity of LGL, being much more rapid than development of cytotoxic T-cell activity, would also be compatible with a role in the first line of defence against infection (Herberman &

Ortaldo,1981). In rats LGL are localised in the spleen, rather than with T-cells in the lymph nodes, which may act as a preferential site of LGL function, clearing blood-borne elements susceptible to LGL killing (Rolstad et al.,1986).

However, the contribution of LGL to IFN-gamma production by unfractionated PBML is negligible by 3 days post-induction. Since nearly all the experiments in subsequent chapters use unfractionated PBML, or the total lymphocyte population, the contribution of low density cells to IFN-gamma yields can be ignored.

In view of the possible interactions between LGL and T-cells the former's origin and lineage become important considerations. LGL seem to have characteristics of both T-cells and cells of the myeloid lineage. Expression of OKM1 and the ability to produce IL-1 (Scala et al.,1984) suggest an association with macrophages. However, OKM1 expression and IL-1 production (Durum et al.,1985) are not restricted to the myeloid lineage.

Despite the clear differences to mature T-cells, there is a body of evidence to suggest a relationship between NK and T-cells (Grossman & Herberman,1986). Some NK cells express T8 (Kasahara et al.,1983b) and low affinity SRBC receptors (Kay et al.,1977), for example. NK cells as well as T-cells respond to IL-2, with resulting stimulation of cytotoxic activity and proliferation (Lanier et al..1985: Ortaldo et al.,1983; Shaw et al.,1985; Trinchieri et al..1984; Miyasaka et al.,1984). The mechanism for promoting proliferation in LGL and T-cells seems identical

since anti-Tac (an anti-IL-2 receptor moab) inhibits proliferation of both (Abo et al.,1983; Ortaldo et al.,1984) but anti-Tac cannot inhibit IL-2 augmentation of NK activity (Ortaldo et al.,1984; Mukaida et al.,1986). Possibly IL-2 enhances NK activity by binding to a low affinity receptor that is not recognised by anti-Tac, though whether enhanced NK activity is the result of subsequent IFN-gamma production (Ortaldo et al.,1984: Itoh et al.,1985) or is independent of it (Mukaida et al.,1986) is not yet certain. It is also interesting to note that some T-cells share the LGL morphology and bind specifically to NK target cells, though without killing them (Velardi et al.,1985), whilst other T-cells exhibit potent NK-like cytotoxicity but not the LGL morphology (Schneider et al.,1984).

Observations such as these have led several authors to suggest that NK cells are a type of pre-thymic, pre-T cell (Mackay et al.,1985; Kaplan,1986). Grossman and Herberman (1986) suggest that the point of departure between T and NK lineages occurs when the T-cell receptor gene is rearranged and expressed, the phenotypic plasticity of the two lineages, exhibited in culture, decreasing as maturation proceeds.

If LGL are related to the T-cell lineage, as opposed to being a monocytic or independent lineage, then their apparent ability to produce IFN-gamma and other T-cell derived lymphokines is less surprising.

In conclusion, three points emerge from the results in this chapter. Firstly, the rigorous removal of macrophages

demonstrates that low density lymphocytes, unlike high density lymphocytes, can make IFN-gamma in the absence of macrophages. The level of this production is relatively independent of macrophages compared to that by high density cells which make up the majority of the total lymphocyte population. Secondly, in the absence of macrophages, more than one cell type is necessary for optimal IFN-gamma production by low density cells. Finally, whilst many authors ascribe IFN-gamma production by low density cells to Leu 11 enriched LGL, the results in this study suggest that other mechanisms may exist, low density T-cells possibly producing IFN-gamma if OKM1+,Leu 11+ cells act as accessory cells.

CHAPTER 3.

REGULATION BY INTERLEUKINS (IL-1 AND IL-2) AND PROSTAGLANDIN E_2 OF IFN-GAMMA PRODUCTION.

Introduction.

A variety of molecules produced by cells of the immune system, collectively described as lymphokines or monokines depending on their source, are believed to be involved in regulating the activities of immune cells, including that of production of IFN-gamma (Marx,1983). This may itself have important regulatory functions in the immune response. The major accessory role of macrophages in T-cell activation are believed to be IL-1 production and crosslinking of the T3-Ti complex (Williams et al..1984: Williams et al.,1985; Palacios,1985; Kaye et al.,1984), leading to a calcium flux which results in T-cell activation. The importance of IL-1 as a regulatory molecule is suggested by the fact that IL-1-like molecules can be produced by and responded to by many different cell types (Durum et al.,1985). Prostaglandins, particularly E_1 and E_2, are another major group of cell-regulatory molecules produced by many cell types, including macrophages (Roth et al.,1985; Khansari et al.,1985). In the latter case, however, PGE_2 release appears to act as a suppressive mechanism inhibiting IL-2 production (Chouaib et al.,1984) and class II MHC antigen induction (Zlotnik et al.,1985).

IL-2 production by activated T-cells stimulates division of cells bearing IL-2 receptors. By this interaction IL-2

is believed to regulate T-cell proliferation, IFN-gamma production (Croll et al.,1985; Vilcek et al.,1985; Kasahara et al.,1983; Reem and Yeh,1984), expression of its own receptor (Katzen et al.,1985), and the specificity of the immune response (antigen specific T-cell clonal expansion being guaranteed by the normal limitation of IL-2 receptor expression to antigen-activated T-cells). In the mouse, for example, IL-2 can replace Lyt 1+ helper T-cells in mitogen-induced IFN production by Lyt 2+ T-cells (Torres et al.,1982) and is also needed by immature mouse T-cells if IFN-gamma production is to occur in response to mitogen (Klein and Bevan,1983).

However, early work on lymphokine interaction made use of relatively impure lymphokine preparations which probably contained other lymphokines too, making results from such experiments equivocal. More recently the availability of purified and recombinant interleukins, plus antibodies against them and their receptors, has introduced much greater certainty in the interpretation of experiments involving lymphokine interactions. In this chapter the roles of both IL-1 and IL-2 (i.e. natural ligands as opposed to artificial ligands, such as antibodies against cell surface molecules which are discussed in chapter four) in the stimulation of IFN-gamma production were investigated in a number of situations where IFN-gamma production by mitogen induction was modified.

RESULTS

a) The Effects of IL-1 on IFN-gamma Production by Macrophage-Depleted PBML.

Depletion of macrophages from human PBML has been found to reduce considerably IFN-gamma production by mitogen stimulated lymphocytes (see Chapter 2). In order to determine the possible role of IL-1 in macrophage assistance of IFN-gamma production, PBML were depleted of macrophages by replating and silica treatment and the effects of adding back purified IL-1 determined.

Replating PBML three times at hourly intervals on plastic petri dishes removed the majority of adherent cells, i.e. macrophages. Silica was then added at 200μg/ml and the cells incubated overnight to eliminate the few remaining phagocytic cells. The lymphocytes were then isolated over Ficoll at 350G to remove the silica and cell debris (as described in the methods section). It was important to remove the majority of macrophages by adherence first as silica treatment may cause the release of macrophage-derived factors into the surrounding medium when the cells are destroyed. This method produced >99% pure lymphocyte preparations as judged by non-specific esterase staining for macrophages.

As can be seen in Table 1, macrophage depletion usually reduced IFN-gamma yields by 10-fold or more, confirming the importance of macrophage-lymphocyte cooperation for IFN-gamma production. The addition of exogenous IL-1 (6U/ml) at the time of induction was found greatly to increase IFN-gamma production by macrophage-depleted

Table 3.1

THE EFFECTS OF IL-1 ON IFN-GAMMA PRODUCTION BY PBML AND MACROPHAGE-DEPLETED LYMPHOCYTES.

	IFN-Gamma Yield (U/ml)			
	Donors			
Cells	1	2	3	4
PBML	400	4000	1600	4000
PBML + IL-1	220	4000	ND	3200
Ly.	35	320	40	120
Ly. + IL-1	200	1100	200	2000

a) All cells were induced with SEA (20ng/ml).

b) Purified IL-1 was used at 6U/ml.

lymphocytes (Ly.), by 5-fold or more, in response to SEA. The addition of exogenous IL-1 to PBML (i.e. not macrophage-depleted), however, had little if any effect on the cells response to SEA with no increase in IFN-gamma yields. Thus exogenous IL-1 does not boost IFN-gamma yields generally except when the lymphocytes have been depleted of macrophages. This suggests that IL-1 production is one of the accessory functions of macrophages for IFN-gamma production. In the case of PBML adding exogenous IL-1 has no effect presumably because optimal levels of IL-1 are produced by the macrophages present.

Using six different donors and a range of IL-1 concentrations (Table 2) it was found that the optimal level of IL-1 for maximum stimulation of IFN-gamma production varied from donor to donor. As little as 0.6U/ml could boost IFN-gamma yields, whilst the highest levels of IL-1 used (20U/ml) did not always lead to the greatest IFN-gamma yields. In the case of PHA (donor 6, table 2) as opposed to SEA induction 2U/ml of IL-1 were sufficient for the optimal effect. Although the addition of IL-1 did boost yields it was not always to the level produced by undepleted PBML.

PBML uninduced by mitogen produced little IFN-gamma and the addition of IL-1 without mitogen generally (4 out of 5 cases) caused only low level production (Table 2). This suggests that IL-1 alone is not a sufficient signal for significant IFN-gamma production compared to that provided by mitogen. However, in the case of a few donors (e.g.

Table 3.2

THE EFFECTS OF IL-1 ON IFN-GAMMA PRODUCTION BY MACROPHAGE-DEPLETED LYMPHOCYTES.

	IFN-Gamma Yield (U/ml) Donors					
Cells	1	2	3	4	5	6
PBML	4800	5700	1200	60	200	280
Ly.	32	120	160	3	35	32
Ly.+ IL-1 (0.6U/ml)	120	250	ND	ND	ND	ND
Ly.+ IL-1 (2U/ml)	320	250	6000	80	55	250
Ly.+ IL-1 (6U/ml)	320	160	4000	25	135	250
Ly.+ IL-1 (20U/ml)	2000	500	500	25	100	250
Uninduced PBML + IL-1 (6U/ml)	1000	ND	60	250	<3	5
Uninduced PBML	250	3	<3	ND	<3	6

a) Donors 1 to 5 were induced with SEA (20ng/ml) and donor 6 with PHA (10μg/ml).

b) ND = Not Done.

c) Ly.= Lymphocytes, i.e. macrophage-depleted PBML.

donors 1 and 4 in table 2) IL-1 alone did induce some IFN-gamma production, perhaps because these donors PBML were already partially activated by some endogenous infection. This is an example of one of the problems with work on the human system, i.e. variation in the response of different donors PBML to the same stimulus due to differences in the state of their immune systems.

This boosting effect of IL-1 on lymphocyte responses was found to be the case for a variety of different mitogens (Tables 2 & 3). The effects of macrophage depletion and IL-1 on tritiated-thymidine incorporation, as a measure of mitogenesis, were also examined. Removal of macrophages inhibited incorporation in the case of SEA and OKT3 (reproducible for three different donors for each), but had the reverse effect when Con A or PHA were used as T-cell activators (though only a single donor was examined). Reduction of IFN-gamma titre was also less severe for these mitogens, suggesting that mitogens may differ in their mechanism of T-cell activation. Addition of exogenous IL-1 boosted tritiated-thymidine incorporation only in the case of SEA but raised IFN-gamma production for all four mitogens. The IFN induced by each mitogen was shown to be IFN-gamma by its 99% inhibition by anti-IFN-gamma antisera.

Thus, using a range of mitogens and donors, exogenous purified IL-1 was capable of at least partially replacing the activity of macrophages in activating T-cells in response to mitogens, and the effect was neither donor nor mitogen specific.

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Thus, using a range of mitogens and donors, exogenous purified IL-1 was capable of at least partially replacing the activity of macrophages in activating T-cells in response to mitogens, and the effect was neither donor nor mitogen specific.

Table 3.3

THE EFFECTS OF IL-1 ON IFN-GAMMA PRODUCTION AND ^{3}H-THYMIDINE INCORPORATION BY MACROPHAGE-DEPLETED LYMPHOCYTES STIMULATED BY DIFFERENT MITOGENS.

	Mitogen			
	SEA (20ng/ml)		PHA (10μg/ml)	
Cells	IFN yield	^{3}H-Thy.inc	IFN yield	^{3}H-Thy.inc
PBML	2000	46,397	160	142,025
Ly.	80	25,806	55	193,965
Ly. + IL-1	3200	37,417	250	187,165
	OKT3 (10ng/ml)		Con A (10μg/ml)	
	IFN yield	^{3}H-Thy.inc	IFN yield	^{3}H-Thy.inc
PBML	55	41,826	125	91,769
Ly.	6	3,110	55	139,749
Ly. + IL-1	120	4,118	100	169,737

a) The same donor was used for PHA and Con A, and different donors for SEA and OKT3.

b) IFN-gamma titre (U/ml) was measured on day 3, and ^{3}H-Thy. (tritiated-thymidine) incorporation over day 3-4 as the mean of four wells (Standard Deviation less than 15%).

c) IL-1 was used at 6U/ml.

d) Similar results for IFN-gamma titre and ^{3}H-Thy.inc. were obtained for three different donors for both SEA and OKT3 induction (representative data shown). Only a single donor was examined for PHA and Con A (data shown).

b) The Effects of anti-IL-1 Antibody on IFN-gamma Production.

A commercially available polyvalent antibody for IL-1 was used to determine the effect of reduction of free endogenous IL-1 during T-cell activation. Using a range of concentrations it was found that when added at the time of induction anti-IL-1 could greatly reduce the subsequent level of IFN-gamma production (Table 4), an effect reproducible for eleven different donors. This effect was clearly dose dependent for SEA with a reduction of IFN-gamma titre of over 90% at the highest antibody concentration. Induction by PHA could be similarly reduced, but the effect was found to be far more variable between donors than in the case of SEA.

The effects of adding anti-IL-1 at various times post-induction (Table 5) for one donor were also examined. The greatest reduction of subsequent IFN-gamma titre was observed when the antibody was added three hours post-induction rather than at induction itself. Addition at subsequent times had a reduced effect, suggesting that endogenous IL-1 was most important for subsequent IFN-gamma production in the first 24 hours of induction. When added 48 hours post-induction anti-IL-1 had a much reduced, for SEA, or no effect, for PHA, on the IFN-gamma titre.

Anti-IL-1's effects on SEA induction were examined for several donors (Table 6) and reductions of over 80% of IFN-gamma production were obtained in all cases at the

Table 3.4

THE EFFECTS OF ANTI-IL-1 ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-Gamma Yield (U/ml)	
	SEA (20ng/ml)	PHA (10μg/ml)
Mitogen alone	800	120
With anti-IL-1 (10%)	32	28
With anti-IL-1 (3%)	120	16
With anti-IL-1 (1%)	1000	25
With anti-IL-1 (0.5%)	800	20

a) Single Donor examined (due to shortage of anti-IL-1).

b) Anti-IL-1 added at induction.

Table 3.5

THE EFFECT OF ANTI-IL-1 ADDED POST INDUCTION ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-Gamma Yield (U/ml) 3 days post-induction.	
	SEA (20ng/ml)	PHA (10μg/ml)
Mitogen alone	1200	400
With anti-IL-1 at induction	100	200
With anti-IL-1 3h. p.i.	55	100
With anti-IL-1 16h. p.i.	160	250
With anti-IL-1 24h. p.i.	200	200
With anti-IL-1 48h. p.i.	550	400

a) Anti-IL-1 used at 5%.

b) p.i. = time of addition post induction.

c) Different donor used to that in Table 3.4.

Table 3.6

THE EFFECT OF IL-1 ON ANTI-IL-1 MEDIATED INHIBITION OF IFN-GAMMA INDUCTION BY SEA.

	IFN-Gamma Yield (U/ml)			
	Donors			^{3}H-Thy
	1	2	3	3
Mitogen alone	3200	2300	120	65,862
With anti-IL-1 (5%)	270	400	18	32,908
With anti-IL-1 (1%)	2000	1600	100	ND
With anti-IL-1 (5%) +IL-1	400	800	32	34,157
With anti-IL-1 (1%) +IL-1	2200	3200	160	ND
With IL-1	4000	2500	160	51,472

a) SEA used at 20ng/ml.

b) IL-1 used at 5U/ml.

c) ^{3}H-Thy.(Tritiated Thymidine) incorporation measured over day 3-4 as the mean of four wells (Standard Deviation less than 20%).

higher antibody concentration. Addition of purified IL-1 at least partially reduced the effect of the antibody suggesting that the antibody's inhibitory effects are indeed IL-1 mediated. Addition of purified IL-1 without the antibody had little effect on SEA induction, as found earlier (Table 1), presumably because endogenous IL-1 levels were sufficient for normal levels of induction. In the case of the one donor examined anti-IL-1 was also found to reduce the level of tritiated-thymidine incorporation by 50%. This is an effect similar to that found after macrophage depletion (Table 3). Addition of exogenous IL-1 as well raised the IFN-gamma titre only slightly for this donor and also had only a minimal effect on the depression of tritiated-thymidine incorporation.

Con A induction of IFN-gamma is also significantly depressed by anti-IL-1 (Table 7) in a dose dependent fashion, although only two donors were examined. Purified IL-1 again partially reversed the effect of the antibody. When PHA was used as the inducer, however, the effects of the antibody varied between donors (Tables 4, 5 & 8) and were not usually as pronounced as in the case of SEA. Once again though the addition of purified IL-1 at least partially reversed the antibody's effects whilst having little effect on the IFN-gamma titre otherwise (Table 8).

These experiments with anti-IL-1 suggest that endogenous IL-1 levels during induction have an important effect on subsequent IFN-gamma production. This was found to be reproducible for several mitogens and was not donor specific.

Table 3.7

THE EFFECT OF IL-1 ON ANTI-IL-1 MEDIATED INHIBITION OF IFN-GAMMA INDUCTION BY CON A.

	IFN-Gamma Yield (U/ml)	
	Donors	
	1	2
Mitogen alone.	1000	320
With anti-IL-1 (5%)	32	80
With anti-IL-1 (1%)	220	250
With anti-IL-1 (5%) + IL-1	55	250
With anti-IL-1 (1%) + IL-1	18	200
With IL-1	32	180

a) Con A used at 20μg/ml.

b) IL-1 used at 5U/ml.

Table 3.8

THE EFFECT OF IL-1 ON ANTI-IL-1 MEDIATED INHIBITION OF IFN-GAMMA INDUCTION BY PHA.

	IFN-Gamma Titre (U/ml) Donors.		
	1	2	3
Mitogen alone	1000	16	400
With anti-IL-1 (5%)	360	12	200
With anti-IL-1 (1%)	450	18	180
With anti-IL-1 (5%) + IL-1	450	32	320
With anti-IL-1 (1%) + IL-1	1000	80	320
With IL-1	1000	180	320

a) PHA used at 10μg/ml.

b) IL-1 used at 5U/ml.

c) The Effects of Anti-Tac (an IL-2 Receptor specific antibody) on IFN-Gamma Production.

In an analogous set of experiments to those described above the role of IL-2 in the stimulation of IFN-gamma production was investigated by the use of a monoclonal antibody (anti-Tac). This antibody binds to the human IL-2 receptor (Leonard et al.,1982) and inhibits various aspects of human T-cell activation. including T-cell proliferation and the generation of cytotoxic T-cells in mixed lymphocyte cultures (Depper et al.,1983). PBML from different donors were treated with PHA, Con A, and OKT3 at different concentrations, alone or in the presence of anti-Tac (1/1000 dilution of the ascitic fluid).

When PHA was used as the mitogen anti-Tac had a marked depressive effect on the IFN-gamma yield (Table 9), measured on both day 2 and day 3 post-induction in order to catch the peak IFN-gamma yields. The depressive effect of anti-Tac decreased with increasing mitogen concentration. However, anti-Tac had little effect on tritiated-thymidine incorporation, a measure of mitogenesis, at all PHA concentrations.

IFN-gamma levels induced by Con A were similarly reduced in the presence of anti-Tac (Table 10). Unlike PHA, however, there was also marked inhibition of tritiated-thymidine incorporation in all the donors examined. Once again the depressive effects decreased with increasing mitogen concentration.

OKT3-induced tritiated-thymidine incorporation and

Table 3.9

THE EFFECTS OF ANTI-TAC ON IFN-GAMMA INDUCTION BY PHA.

	^{3}H-Thy. inc. (cpm)		Day 2 IFN Yield (U/ml)		Day 3 IFN Yield (U/ml)	
Mitogen Conc.	Mitogen only	With anti-Tac	Mitogen only	With anti-Tac	Mitogen only	With anti-Tac
Donor 1						
0.3µg/ml	20,976	18,665	1,000	250	4,000	400
1.0µg/ml	20,034	23,644	2,000	250	400	320
3.0µg/ml	34,121	30,691	500	250	50	63
Donor 2						
0.3µg/ml	46,337	41,491	200	40	200	13
1.0µg/ml	43,227	39,764	160	16	100	6
3.0µg/ml	57,446	56,539	63	40	40	13
Donor 3						
1.0µg/ml	ND		ND		200	40
3.0µg/ml	ND		ND		27	4
Donor 4						
0.3µg/ml	ND		ND		250	10
1.0µg/ml	ND		ND		800	<3
3.0µg/ml	ND		ND		8	4

a) Anti-Tac was added at 1/1000 dilution of ascitic fluid.

b) ^{3}H-Thy (Tritiated-Thymidine) incorporation was measured over day 3-4 as the mean of 4 wells (Standard Deviation less than 20%)

c) ND = Not Done.

Table 3.10

THE EFFECTS OF ANTI-TAC ON IFN-GAMMA INDUCTION BY CON A.

	^{3}H-Thy. inc. (cpm)		Day 2 IFN Yield (U/ml)		Day 3 IFN Yield (U/ml)	
Mitogen conc.	Mitogen only	With anti-Tac	Mitogen only	With anti-Tac	Mitogen only	With anti-Tac
Donor 1						
1µg/ml	2,900	462	<3	<3	<3	<3
3µg/ml	7,459	550	<3	<3	4	<3
10µg/ml	12,516	9,693	80	10	32	4
Donor 2						
1µg/ml	6,216	2,015	<3	<3	<3	<3
3µg/ml	7,964	4,050	10	<3	4	<3
10µg/ml	27,369	15,640	32	20	20	6
Donor 3						
1µg/ml	8,660	3,243	<3	<3	<3	<3
3µg/ml	22,070	13,312	25	<3	20	<3
10µg/ml	74,662	32,200	32	16	25	20

a) Anti-Tac was added at 1/1,000 dilution of ascitic fluid for donors 1 and 2, and 1/10,000 for donor 3.

b) ^{3}H-Thy (Tritiated-Thymidine) incorporation was measured over day 3-4 as the mean of 4 wells (Standard Deviation less than 20%)

IFN-gamma production were also reduced by anti-Tac (Table 11).

Similar results were obtained with Con A and OKT3 (Table 12) at a lower concentration of anti-Tac (1/10,000). Due to limited supplies of anti-Tac it was not possible to determine its effects at higher concentrations on mitogenesis and IFN-gamma production. This would have been of particular interest in the case of PHA, anti-Tac having little effect on mitogenesis at 1/1,000 dilution but still inhibiting IFN-gamma production, even though both processes are believed to be IL-2 mediated. Thus the effects of anti-Tac were found to be reproducible for each mitogen over a range of donors, at least four in each case.

The depressive effects of anti-Tac on IFN-gamma induction by three different mitogens were shown to be IL-2 mediated by the addition of purified human IL-2 together with anti-Tac at the time of induction (Table 13). The inhibition of IFN-gamma production by anti-Tac was shown to be reversible if exogenous IL-2 was also added. The extent of this reversibility varied with the mitogen and its concentration. In the case of PHA inhibition was nearly completely reversed. Indeed at the lowest PHA concentration IL-2 boosted IFN-gamma production above that of mitogen induction alone. However, in the case of Con A and OKT3, IL-2 only partially restored IFN-gamma levels.

A second monoclonal antibody against the IL-2 receptor (from Becton-Dickinson) was also tested. This too

Table 3.11

THE EFFECTS OF ANTI-TAC ON IFN-GAMMA INDUCTION BY OKT3.

	^{3}H-Thy. inc. (cpm)		Day 2 IFN yield (U/ml)		Day 3 IFN yield (U/ml)	
OKT3 conc. (ng/ml)	OKT3 only	With anti-Tac	OKT3 only	With anti-Tac	OKT3 only	With anti-Tac
Donor 1						
10	10,969	3,568	10,000	320	13	<2
30	11,819	3,272	1,000	10	32	<2
100	9,602	2,950	13	50	50	25
Donor 2						
10	ND	ND	1,200	4	ND	ND

a) Anti-Tac was added at 1/1,000 dilution of ascitic fluid.

b) ^{3}H-Thy (Tritiated-Thymidine) incorporation was measured over day 3-4 as the mean of 4 wells (Standard Deviation less than 20%)

c) ND = Not Done.

Table 3.12

IFN-GAMMA PRODUCTION WITH DIFFERENT ANTI-TAC CONCENTRATIONS.

	Day 2 IFN-gamma yield (U/ml)		
Mitogen conc.	Without anti-Tac	With anti-Tac (1/10.000)	With anti-Tac (1/1,000)
Con A (µg/ml)			
3	10	<3	<3
10	32	6	20
OKT3 (ng/ml)			
10	10,000	500	320
30	1000	15	10
100	13	4	10

a) Donor 2 from Table 3.10 was used for Con A and the same donor as used in Table 3.11 for OKT3.

Table 3.13

THE EFFECTS OF IL-2 ON ANTI-TAC MEDIATED INHIBITION OF IFN-GAMMA PRODUCTION.

		IFN-Gamma Yield (U/ml)		
Mitogen conc.	Donor	Without anti-Tac	With anti-Tac	With anti-Tac and IL-2
PHA (μg/ml)				
0.3	1	16	<3	160
1.0	1	160	<3	160
3.0	1	500	320	500
Con A (μg/ml)				
1	3	25	<3	12
10	4	250	25	100
OKT3 (ng/ml)				
10	3	800	50	100
10	4	10	<3	40

a) Anti-Tac was added at 1/1,000 dilution of ascitic fluid.

b) Purified IL-2 was used at 100 (Genzyme) U/ml.

inhibited IFN-gamma induction, by PHA and SEA, with greater effect at lower mitogen concentrations (Table 14). Thus the inhibitory effect is not antibody specific (both may bind to the same epitope), but probably due to interference with IL-2/IL-2 receptor interactions.

The reversal effect of addition of IL-2 is specific to that interleukin since IL-1, used at a concentration that boosts IFN-gamma production by macrophage-depleted lymphocytes, has little effect on the inhibitory effect of either IL-2 receptor antibody (Table 15).

As a control for possible nonspecific effects of ascites preparation on IFN-gamma production, mouse IgG and a monoclonal antibody W634 (Barnstaple et al.,1978) against a species antigen (coded for on chromosome 11 and found on all human cells) were tested for their effects on IFN-gamma induction (Table 16). Concentrations similar to that used for anti-Tac experiments (1/1,000 anti-Tac contained approximately 6-8ug/ml) had no significant effect on IFN-gamma production. The effects of monoclonal antibodies for cell surface molecules associated with the immune system are discussed in chapter four.

d) The Effects of Prostaglandin E_2 (a Macrophage Product) on IFN-gamma Production.

In view of the reported depressive effects of PGE_2 on IL-2 production (Chouaib et al.,1984) its effects on IFN-gamma production were examined. When added at induction at a range of concentrations (physiological levels are estimated to peak at 30ng/ml in the

Table 3.14

THE EFFECTS OF ANTI-IL-2 RECEPTOR ANTIBODY ON IFN-GAMMA PRODUCTION.

Mitogen concentration	Day 3 IFN-gamma yield (U/ml) anti-IL-2 receptor concentration None	1/1,000	1/10,000
PHA (μg/ml)			
1	160	<3	<3
3	40	<3	<3
10	200	200	160
SEA (ng/ml)			
20	100	40	25

a) PBML from a single donor were used.

Table 3.15

THE EFFECTS OF IL-1 ON ANTI-TAC-MEDIATED INHIBITION OF IFN-GAMMA PRODUCTION.

	IFN-gamma yield (U/ml)		
Mitogen	Without anti-Tac	With anti-Tac	With anti-Tac and IL-1
PHA (1µg/ml)	18	<2	<2
PHA (3µg/ml)	14	<2	<2
SEA (20ng/ml)	35	12	8
		With anti-IL-2-R	With anti-IL-2-R and IL-1
PHA (3µg/ml)	14	8	<3
SEA (20ng/ml)	35	14	25

a) PBML from a single donor were used.

b) Anti-Tac was used at 1/1,000 dilution.

c) Anti-IL-2-R was used at 1/10,000 dilution.

d) IL-1 was used at 6U/ml.

Table 3.16

THE EFFECTS OF IgG AND W634 BINDING ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-gamma titre (U/ml)	
Antibody	Donor 1	Donor2
Mitogen only	800	1600
W634 (3µg/ml)	1300	2000
(1µg/ml)	3200	1000
Mu IgG (5µg/ml)	ND	1000
(1µg/ml)	ND	800

a) SEA (20ng/ml) used as mitogen.

b) W634 was used in ascites form and is specific for the species antigen S.A.1 found on all human cells.

inflammatory state; Snyder et al.,1982) and with a range of different mitogens, PGE_2 was found to cause significant inhibition of IFN-gamma production (Table 17) for three of the mitogens. SEA induction, however, was not significantly inhibited. Across a range of different donors PGE_2 consistently inhibited IFN-gamma levels induced by PHA, but inhibited SEA induction in only three out of six donors (Table 18). The reduced effect of PGE_2 on the more potent mitogen SEA, compared to the other inducers used, suggests that sub-maximal mitogen stimulation is required if PGE_2 is to cause significant inhibition. It was also found that μg levels of PGE_2 were required for any effect on SEA induction and for consistent effects on induction by other mitogens, since PGE_2 effects varied from donor to donor.

Since PGE_2 is known to inhibit IL-2 production, and that this lymphokine has an important role in optimal IFN-gamma production, the effects of exogenous IL-2 on PGE_2 mediated inhibition were examined. Exogenous IL-2 was found to reverse the inhibitory effects of PGE_2 on a range of donors using PHA as the inducer (Table 19). In only two out of six cases, however, were IFN-gamma levels fully restored.

The effects of both IL-1 and IL-2 on PGE_2 inhibition were tested using a range of mitogens (Table 20). Purified IL-1 was used at a concentration that boosted IFN-gamma production by macrophage-depleted lymphocytes. When added at induction IL-1 had no effect on the inhibition but IL-2 at least partially reversed it. In the case of PHA

Table 3.17

THE EFFECTS OF PGE_2 ON IFN-GAMMA PRODUCTION IN RESPONSE TO DIFFERENT MITOGENS.

IFN-gamma yield (U/ml)

	PGE_2 concentration (ng/ml)				
Mitogen	None	3	30	300	3000
PHA (3μg/ml)	930	80	67	54	13
Con A (20μg/ml)	27	8	2	<2	<2
OKT3 (10ng/ml)	14	<2	<2	<2	<2
SEA (20ng/ml)	400	960	750	1000	ND

a) The same donor was used for Con A and OKT3 with different donors for PHA and SEA.

b) Conditions were set up in triplicate and the mean titre calculated.

c) ND = Not Done.

Table 3.18

THE EFFECTS OF PGE_2 INHIBITION ON PHA AND SEA INDUCED IFN-GAMMA PRODUCTION BY PBML.

	IFN-gamma titre (U/ml)					
	Donors					
Mitogen	1	2	3	4	5	6
PHA (10µg/ml)	250	12	40	16	25	1600
PHA + PGE_2	25	<2	<2	12	<2	<2
SEA (20ng/ml)	250	160	160	450	320	2000
SEA + PGE_2	250	16	100	80	80	4000

a) PGE_2 was used at 1µg/ml.

Table 3.19

THE EFFECT OF IL-2 ON PGE_2 MEDIATED INHIBITION OF IFN-GAMMA PRODUCTION.

	IFN-gamma titre (U/ml)					
	Donors					
	1	2	3	4	5	6
PHA (3µg/ml)	250	320	80	8	14	25
PHA + PGE_2	16	160	<2	<2	8	<2
PHA + PGE_2 + IL-2	160	800	16	5	32	32

a) PGE_2 was used at 3µg/ml.

b) Recombinant IL-2 was used at 2U/ml.

Table 3.20

THE EFFECTS OF IL-1 AND IL-2 ON PGE_2 MEDIATED INHIBITION OF IFN-GAMMA PRODUCTION BY PBML.

IFN-gamma yield (U/ml)

and tritiated-thymidine incorporation (cpm)

	Culture conditions			
Mitogen	Mitogen only	With PGE_2	PGE_2 + IL-1	PGE_2 + IL-2
PHA	250	25	20	80
(3µg/ml)	116,712	150,779	ND	337,614
Con A	10	<2	6	250
(5µg/ml)	88,364	86.593	ND	188.726
OKT3	25	3	<2	160
(1ng/ml)	72,570	47,789	ND	259,944
SEA	200	200	400	500
(1ng/ml)	143,446	182,325	ND	256,811

a) PGE_2 was used at 3µg/ml.

b) IL-1 was used at 10U/ml.

c) Recombinant IL-2 was used at 2U/ml.

d) IFN titre was measured on day 3 and tritiated-thymidine incorporation from day 3 to 4.

e) All results came from a single donor.

stimulation the IFN-gamma levels were partially restored by the addition of exogenous IL-2, but for Con A and OKT3 stimulation they were elevated far above the yield produced by mitogen stimulation alone. This is consistent with the stimulatory properties of exogenous IL-2 when stimulation is sub-maximal (see next section).

Despite its inhibitory effects on IFN-gamma levels, PGE_2 did not inhibit mitogenesis, as measured by tritiated-thymidine incorporation (Table 20). The presence of exogenous IL-2 boosted tritiated-thymidine incorporation 2-3 fold for all mitogens, except SEA.

e) <u>The Effects of IL-1 and/or IL-2 on IFN-gamma induction by mitogens and on IFN-gamma production without mitogen stimulation.</u>

When high titres of IFN-gamma were induced by mitogens, in the absence of inhibitory factors, the addition of exogenous IL-2 had little or no effect on the titre produced (Table 21). This was seen in the case of both PHA (donor 1) and OKT3 (donor 2). However, when IFN-gamma production by mitogen stimulated PBML was "low" (<100U/ml), as with donor 3 using Con A and PHA, exogenous IL-2 considerably boosted IFN-gamma production.

Without mitogen IL-2 induced IFN-gamma production only when added at high (20U/ml) concentrations (Tables 22 & 23). Yet when added at the same high concentration in the presence of mitogen IL-2 had least amplificatory effect (Table 22). However, it should be noted that only a single donor was examined in this case.

Table 3.21

THE EFFECTS OF IL-2 ON MITOGEN STIMULATED IFN-GAMMA PRODUCTION BY PBML.

		IFN-gamma yield (U/ml)		
Mitogen	Donor	Mitogen only	IL-2 only	Mitogen + IL-2
PHA (1µg/ml)	1	1000	32	1600
OKT3 (1ng/ml)	2	250	32	400
Con A (5µg/ml)	3	6	ND	160
PHA (3ug/ml)	3	16	ND	1000

a) Recombinant IL-2 was used at 2U/ml.

Table 3.22

THE EFFECTS OF IL-2 ON IFN-GAMMA PRODUCTION BY PBML WITH AND WITHOUT MITOGEN.

	IFN-gamma yield (U/ml) Exogenous IL-2 concentration			
	None	0.2 U/ml	2.0 U/ml	20 U/ml
No mitogen	25	100	32	4000
PHA (1μg/ml)	1000	3200	1600	1000

a) PBML from a single donor were used.

Table 3.23

THE EFFECTS OF IL-2 ON IFN-GAMMA PRODUCTION BY PBML WITH AND WITHOUT IL-1 IN THE ABSENCE OF MITOGEN.

	IFN-gamma yield (U/ml) Exogenous IL-2 concentration.			
	None	0.2 U/ml	2.0 U/ml	20 U/ml
No IL-1	<2	3	5	20
With IL-1 (6U/ml)	<2	18	<2	18

a) PBML from a single donor were used.

IL-1 in the absence of mitogen rarely induced any IFN-gamma production by PBML, as described earlier in the chapter. It also had little effect on IL-2 induced IFN-gamma production, in the absence of mitogen (Table 23). Thus the two interleukins do not appear to act synergistically, in the absence mitogen, and both require mitogen as an additional signal for significant IFN-gamma induction, compared to the levels induced by mitogen alone. The two situations in which exogenous interleukins have a stimulatory effect are for macrophage-depleted lymphocytes, in the case of IL-1, and low level mitogen stimulation, in the case of IL-2.

DISCUSSION : Chapter 3.

Because of the breadth of data covered in this chapter the discussion has been broken down into the following sections: 1) the role of macrophages and IL-1; 2) the role of IL-2; 3) the action of PGE_2.

1) THE ROLE OF MACROPHAGES AND IL-1 IN IFN-GAMMA INDUCTION.

Many workers have investigated the role of macrophages in T-cell activation and the importance of IL-1 in macrophage/T-cell interactions. Such studies, however, have concentrated on such markers of T-cell activation as IL-2 production and IL-2 receptor expression with little attention being paid to IFN-gamma production.

The primary signal for T-cell activation is thought to be the binding of antigen or mitogen to the T-cell receptor, or the closely associated T3 molecular complex. Activation by antigen (monoclonal) does not appear to differ from activation by mitogen (polyclonal) in any qualitative fashion, only quantitatively. If macrophages are not also present, however, both early (IL-2 production, IL-2 receptor expression, and RNA synthesis) and late (DNA synthesis) T-cell responses are prevented (Williams et al.,1984). Several groups have shown that purified IL-1, usually obtained from mitogen-stimulated macrophage supernatants, can partially restore T-cell responses to mitogens (Williams et al.,1984) or to anti-T3 monoclonal antibodies (Schwab et al.,1985; Williams et al.,1985; Palacios,1985a) in the absence of macrophages,

but full activation seems to require the accessory cell membrane, pesumably to present antigen in the context of class II MHC (Unanue et al.,1984). Both IL-1 and IL-2 have been shown to be involved in IL-2 receptor expression (Schwab et al.,1985; Williams et al.,1985). but some extra macrophage signal is still needed for IL-2 production with IL-1 seeming to amplify rather than induce IL-2 production (Palacios,1985a). In a recent study using an IL-1 sensitive cell line (EL4 thymoma cells) it was shown that IL-1 is involved in the induction of both IL-2 secretion and IL-2 receptor expression, apparently by a third intracellular pathway that can synergise with either increased intracellular calcium ions levels or protein kinase C activation for IL-2 production, and with activation of protein kinase C only for expression of IL-2 receptors (Lowenthal et al..1986).

The exact role of IL-1 in IFN-gamma production is also not clear, but the results obtained in this study strongly suggest that it is an important regulatory factor. Macrophage-depletion from PBML clearly reduces IFN-gamma induction at least 10-fold (Table 1), just as other workers have found for IL-2 induction. Bearing in mind the importance of IL-2 production and binding to its receptor for maximal IFN-gamma production (also shown in this chapter) it is not surprising that if induction of IL-2 and its receptor are reduced by macrophage-depletion then IFN-gamma titres would also be greatly reduced as a consequence. Similar to the situation found by others for IL-2 production in macrophage-depleted populations, in

this study it was found that the addition of purified exogenous IL-1 could at least partially restore IFN-gamma production (Table 1). The lymphocyte preparations contained less than 0.5% macrophages, as shown by non-specific esterase staining, and yet the addition of exogenous IL-1 clearly boosted IFN-gamma production for a variety of mitogens (Table 3) and donors (Table 1 and 2). This boosting effect, however, was not related to the IL-1 concentration in any clear cut manner, the dosage effects varying from donor to donor (Table 2). The maximum limits of impurity claimed by Genzyme for the IL-1 used were 1.0% IL-2 and 1U/ml of IFN, with an IL-1 activity of >100U/ml (one unit is defined as that amount of IL-1 required to double the proliferative response of mouse thymocytes stimulated with 1µg/ml of PHA). It is reasonable therefore to assume that any effects were IL-1 mediated rather than due to impurities, especially since IL-1 had no effect when IL-2 did. Therefore when IL-1 did show effects it would not be due to IL-2.

When exogenous IL-1 was added with mitogen to PBML it had no effect on IFN-gamma levels. This lack of stimulation is presumably because the macrophages already present in PBML produce sufficient endogenous IL-1 for optimal subsequent IFN-gamma production, any exogenous IL-1 added therefore having no additional effect. Thus exogenous IL-1 is not a general stimulator of IFN-gamma production but acts only when the cell population is macrophage-depleted.

Addition of IL-1 to PBML in the absence of mitogen

induced significant IFN-gamma levels in only a few donors (Table 2) and at most only very low levels in the remainder (Table 2 and 23). Why some donors responded to IL-1 alone is not clear. Possibly some lymphocytes in these preparations were already preactivated by some natural infection of the donor, making the cells more responsive to IL-1. In general, however, IL-1 was not a sufficient signal alone for lymphocyte activation, as others have shown (e.g. Palacios,1985a).

The lymphocyte populations in these experiments (i.e. macrophage-depleted PBML) still produced low but significant levels of IFN-gamma (usually 100U/ml at most) even though the titres were much reduced compared to the undepleted population. This suggests that either not all lymphocytes require accessory cells, as appears to be the case for the low density lymphocytes discussed in chapter 2, or that very low levels of residual macrophages remain after depletion (<0.5%) and that these were sufficient to support low level IFN-gamma production, or that non-macrophage accessory cells are present.

If IFN-gamma production by macrophage-depleted cells is chiefly attributable to low density lymphocytes, then the addition of exogenous IL-1 at least partly replaces the macrophage requirement of the higher density T-cells for activation. In this case IFN-gamma levels are boosted to an intermediate level and not fully restored to levels produced if macrophages are present. This agrees with the findings of Williams that adding IL-1 restores indicators of T-cell activation, notably IL-2 and IL-2 receptor

expression, to intermediate levels in accessory cell depleted T-cell populations, which otherwise show no signs of activation in response to PHA (Williams et al.,1984). A similar study by Williams (Williams et al.,1985) with a monoclonal antibody for the T-cell receptor (therefore arguably more akin to antigen stimulation than with PHA) came to similar conclusions, providing the antibody was sepharose-bound and not just in soluble form. This sepharose binding is thought to facilitate crosslinking of the T3-Ti complexes on the T-cell surface, thus mimicking the action of the Fc receptor on macrophages, leading to T3-Ti modulation as one signal for IL-2 production (Schwab et al.,1985).

Even if the depleted preparations used in this study still contained sufficient residual macrophages for low-level T-cell activation, it is still clear that IL-1 can greatly boost IFN-gamma production in this situation. This suggests that endogenous IL-1 production is an important role of macrophages in response to T-cell mitogens for maximal IFN-gamma production. It could be argued of course that the exogenous IL-1 simply substitutes for some other macrophage function, but the depressive effects of the anti-IL-1 antibody on IFN-gamma production described in this chapter suggests that this is not the case and that endogenous IL-1 production is indeed important.

It is possible that endogenous IL-1 does not have to be actively secreted for macrophages to support T-cell proliferation (Haq et al.,1984), but is active in a

membrane bound form known to be present on murine macrophages (Kurt-Jones et al.,1985a&b) and on B-cells (Kurt-Jones et al.,1985c). These can act as antigen-presenting cells under different circumstances from macrophages.

IL-1's effects on macrophage-depletion were found to vary in extent between the different mitogens used (Table 3). Depletion clearly reduced the IFN-gamma titre and tritiated-thymidine incorporation using SEA or OKT3 as stimulators. The effect on mitogenesis was especially noticeable for OKT3 stimulation, reflecting the importance perhaps of macrophage Fc receptors for T3-Ti crosslinking as a signal for IL-2 production (Schwab et al..1985). Exogenous IL-1 had little effect on OKT3 stimulated lymphocyte mitogenesis, but partially restored the level of mitogenesis when SEA was the mitogen. This may reflect a difference in activation between SEA and OKT3, the latter being very dependent on macrophage Fc receptors for crosslinking. PHA and Con A in contrast were independent of macrophages for T-cell activation in terms of proliferation, although their induction of IFN-gamma levels was reduced by macrophage depletion. Exogenous IL-1 served only to restore IFN-gamma levels with little effect on proliferation. These results are in agreement with the findings of Lederman that macrophage-depletion reduced SpA-induced (Staphylococcus aureus protein A) tritiated-thymidine incorporation by 80%, and depressed the calcium ion flux, compared to PBML, but had no effect on proliferation or calcium flux if PHA was the mitogen

(Lederman et al.,1984), even though SEA is a stronger inducer of IFN-gamma and IL-2 than PHA or Con A (Carlsson and Sjogren,1985). PHA has also been found to cause blastogenesis of T-cells in the absence of macrophages (Maizel et al.,1981), although further progression through the G_1 phase of the cell cycle requires macrophages or a source of IL-1. It would appear then that PHA and Con A have effects independent of macrophages, including the ability to induce a shift from the resting G_0 stage of the cell proliferation cycle to an activated G_1 stage, whilst SEA and OKT3 require the presence of macrophages for proliferation.

The differences in responses between mitogens noted in this and other studies may reflect their differing binding specificities. OKT3 triggers cell activation by binding to the T3 complex of the T-cell receptor with 40-50,000 homogeneous receptor sites per cell (Van Wauwe et al.,1980), while the mitogenic lectins bind to carbohydrate moieties D-mannose and D-glucose (Ito et al.,1984) and therefore bind to a large and partially overlapping range of lymphocyte glycoproteins (Diller-Centerlind et al.,1980) with up to 10 million "receptor" sites per cell. However, only 10% or less of these sites are likely to be responsible for initiating proliferation, with other lectin induced changes mediated by other sites. Thus PHA causes disproportionately greater uridine uptake and inositol incorporation than OKT3 (Walls et al.,1984) and may activate the T11 pathway (O'Flynn et al.,1985a). Con A is known to bind to T3 but PHA may not

(Kanellopoulos et al.,1985), or may bind to a 20 kDa member of the T3 complex (Valentine et al.,1985). But despite these differences in activation pathways the results in this chapter suggest that all four mitogens examined require IL-1 for optimal IFN-gamma production, which presumably occurs further on in the cell proliferation cycle than G_1, beyond a stage requiring macrophages.

The importance of endogenous IL-1 production is supported by the effects of anti-IL-1 polyclonal antibody on IFN-gamma induction by SEA (Table 6), Con A (Table 7), and PHA (Table 8) and that they could be reversed by the addition of exogenous IL-1, confirming that the antibody's effects were mediated by blocking IL-1 action. In the case of SEA, anti-IL-1 depressed proliferation and its effects were found to be dose dependent, suggesting that IFN-gamma production is proportional to the level of IL-1. Similarly, the proliferative response of mouse thymocytes in the presence of excess IL-2, but without mitogen, has also been found to be proportional to the level of IL-1 present (Mannel et al.,1985). Anti-IL-1 had its greatest depressive effects if added within 24 hours of induction (Table 5) suggesting that high endogenous IL-1 levels are necessary only in the early stages of IFN-gamma production. Indeed, the proliferative responses of T-cells to mitogens are known to depend on macrophages only in the early stages of activation, whilst B-cell activation is continuously dependent on accessory cells (Thiele and Lipsky,1982). Other inhibitory effects of antibodies

against IL-1 include blocking of purified T-cell responses to autologous monocytes pulsed with tetanus toxoid (Chu et al.,1984) or inhibition of mouse thymocyte responses to IL-1 (Mannel et al.,1985). Thus the results described in this section concur with findings of other workers and also strongly suggest that endogenous IL-1 production is one of the major roles of macrophages in the production of IFN-gamma.

2) THE ROLE OF IL-2 IN IFN-GAMMA INDUCTION.

The recent availability of both purified and recombinant IL-2 and a monoclonal antibody for the IL-2 receptor known as anti-Tac (Leonard et al.,1982), which binds to the receptor and inhibits various aspects of human T-cell activation (Depper et al.,1983), make it possible to determine the various roles of IL-2 with greater certainty. Other workers have used purified IL-2 in a variety of experiments that suggest that exogenous IL-2 can regulate IFN-gamma production (Kasahara et al..1983a: Reem and Yeh,1984), but anti-Tac had not previously been used to examine the role of endogenous IL-2.

In this study anti-Tac was shown to have a marked depressive effect on IFN-gamma production by PBML in response to several mitogens (Tables 9 to 11). Its effects on proliferation, however, varied between mitogens. There was marked depression of tritiated-thymidine incorporation for Con A and OKT3 stimulation but little effect for PHA at the same concentration of antibody. This lack of effect on proliferation, despite reduced IFN-gamma production, is

not unexpected in view of the finding that there is no strict correlation between IL-2 stimulation of IFN-gamma production and cell proliferation (Kasahara et al..1983a). Higher concentrations of anti-Tac might have suppressed PHA induced proliferation, however.

The reversal of anti-Tac's inhibitory effects by the addition of exogenous IL-2 (Table 13) strongly suggest that the antibody's inhibitory effects are indeed mediated by blocking of IL-2/IL-2 receptor interactions, as opposed to some other mechanism, such as down regulation of IL-2 receptor expression. Depper et al. (1983) have shown that anti-Tac does not inhibit IL-2 production but does block both mitogen and antigen induced T-cell proliferation, inhibit cytotoxic T-cell formation, and reduce T-cell dependent PWM activated B-cell Ig production. The fact that the antibody does not inhibit IL-2 production but that its inhibitory effects can be reversed by adding highly purified IL-2 are consistent with the view that the antibody reacts with the IL-2 receptor. A second anti-IL-2-receptor antibody was also found to inibit IFN-gamma production (Table 14). Depper also found that anti-Tac was least able to inhibit responses associated with the greatest proliferation. In this study PHA-induced proliferation was generally 2-5 times greater than that induced by Con A and OKT3, but was inhibited proportionately to a much lesser extent, in agreement with Depper. This effect and the failure of anti-Tac to inhibit IFN-gamma production completely could be due to several factors.

Firstly, IL-2 has a much greater affinity for its receptor than does anti-Tac (Kd=10^{-12}mol/l for IL-2 and 10^{-9}mol/l for anti-Tac)(Leonard et al.,1982) and the 1,000 fold difference means that IL-2 binding is strongly favoured over anti-Tac. Thus anti-Tac probably does not block binding of all endogenously produced IL-2. This is particularly the case for PHA which, judging from its stronger induction of proliferation, induces greater levels of IL-2 than Con A or OKT3.

Secondly, factors other than IL-2 may also be responsible for stimulation of IFN-gamma production, although IL-2 binding to its receptor is a major factor judging from the results obtained.

Thirdly, non T-cells (e.g. NK cells) may also produce IFN-gamma in response to mitogens, possibly in an IL-2 independent manner.

The effects of anti-Tac were further shown to be IL-2 specific by the failure of IL-1 to reverse inhibition (Table 15), and non-specific effects of the antibody seem unlikely in view of the failure of mouse IgG or W634 to depress IFN-gamma levels when used at similar concentrations to anti-Tac (Table 16).

When IL-2 was added to mitogen-induced PBML in the absence of any inhibitory factors, such as anti-Tac or PGE_2, the IFN-gamma titre was boosted significantly only if there had been only "low" level induction (i.e. <100U/ml of IFN) by the mitogen (Table 21). Thus exogenous IL-2 acts synergistically with "low" level mitogen stimulation, as with Con A (also see Kasahara et

al.,1983a), but not if the mitogen had delivered a strong signal for IFN-gamma production (Tables 21 and 22). Welte has shown that OKT3 at concentrations too low to be mitogenic (25pg/ml) together with IL-2 (20U/ml) lead to Tac expression and proliferation when separately they have no effect (Welte et al.,1984). It is suggested by Welte that at such low concentrations OKT3 cannot cause Tac expression but can still somehow pre-activate T-cells to become IL-2 responsive, so that exogenous IL-2 can lead to Tac expression and proliferation.

In the absence of mitogen IL-2 stimulated only low levels of IFN-gamma (Tables 21 and 23) unless used at high concentrations (20U/ml) or the PBML already produced low levels of IFN-gamma in the absence of stimulation (Table 22). The addition of IL-1 made no appreciable difference to IL-2 induced IFN-gamma production, suggesting that an additional signal is required to the two interleukins for cell activation. IL-2 may, however, be mitogenic for a population of T-cells in the presence of macrophages, or IL-1, which are not Tac +ve (Roosnek et al.,1986).

Vilcek finds that exogenous IL-2 can stimulate a good IFN-gamma yield, but it has to be added at concentrations (1000U/ml) far higher than normal physiological levels of 5-30U/ml (Vilcek et al.,1985). He also found, as in this study (Croll et al.,1985), that anti-Tac treatment inhibited IFN-gamma production induced by mitogen, but not by IL-2 stimulation (unsurprising in view of IL-2's greater affinity for its own receptor). He also found, as shown in Table 21, that doses of Con A which induce

little IFN-gamma on their own increased IFN-gamma induction by exogenous IL-2, even when the latter was added at the physiological levels used in this study. The high levels of IL-2 needed to activate PBML in the absence of mitogen may be necessary because of the very low number of pre-activated (i.e. Tac +ve) cells present in otherwise unstimulated PBML. Alternatively, large IL-2 concentrations may activate Tac -ve cells or cells expressing low-levels of Tac. Taylor has found that IL-2 alone is mitogenic only for Tac +ve lymphocytes (Taylor et al.,1986a), since IL-2 alone appears to induce proliferation by expanding only that small fraction of cells in a resting population (1 to 5%) already expressing the IL-2 receptor (Tac +ve). Thus after 24 hours of IL-2 stimulation less than 8% of PBML are Tac +ve compared to 25-50% with PHA stimulation. IL-2 does not appear to induce IL-2 receptor expression on cells previously lacking it, unlike mitogen, and thus acts as a secondary rather than primary signal for IFN-gamma production and general T-cell activation. Virtually all T-cells that produce IFN-gamma after PHA stimulation are reported to express IL-2 receptors (Palacios,1984), whilst both Tac +ve and -ve cells can produce IL-2. Removal of IL-2 from cultures of continuously proliferating T-cells causes them to stop cycling and enter the dormant Go phase, mitogen and macrophages being needed to reactivate them (Bettens et al.,1984). Having stressed the importance of IL-2 binding to its receptor as a requirement for IFN-gamma production it is not, however, by itself sufficient to

cause it. Growing T lymphoblasts (i.e. previously activated T-cells) in IL-2 containing medium already express IL-2 receptors, but they do not constitutively produce IFN-gamma or its mRNA unless mitogen is also added (Wilkinson and Morris,1984; Siggens et al..1984). suggesting that mitogen provides signals additional to induction of IL-2 and its receptor.

3) THE ACTION OF PGE_2 .

Having investigated the role of macrophages and IL-1 on IFN-gamma production, the effects of PGE_2 were examined. PGE_2 is also produced by macrophages (Roth et al..1985). although apparently by a different subset to the major IL-1 producers (Khansari et al.,1985). In vivo prostaglandins act as local rather than systemic hormones, with those of the E series potentiating inflammation and those of the F series counteracting it. Anti-inflammatory drugs such as aspirin are thought to act by interfering with prostaglandin synthesis (Ninneman,1984).

When added at induction PGE_2 significantly inhibited IFN-gamma induction by PHA, OKT3, and Con A, but had little effect when SEA was the inducer (Table 17). This inhibitory effect was evident over a range of concentrations (3 to 300ng/ml) down to levels found physiologically. Basal level of PGE are estimated to be no greater than 0.1ng/ml in serum, but traumatic injuries (e.g. burns) result in large prostaglandin release with 1-3ng/ml in serum (Ninneman,1984) and even higher local concentrations of 30ng/ml at inflammatory sites (Snyder et

al.,1982). Thus responses of lymphocytes to prostaglandin concentrations of 3 to 300ng/ml (10nM to 1μM) may reflect in vivo effects, despite being apparently above physiological levels, since high local concentrations may exist at sites of inflammation and during close macrophage-lymphocyte contact.

The effects of PGE_2 were found to vary to some extent between donors (Table 18) but μg/ml concentrations gave consistent inhibition of ten-fold or greater (Tables 18 to 20) for all mitogens except SEA. Even when SEA and PHA induced similar IFN-gamma titres in the same donors (Table 18, donors 1 and 6; Table 17 and 20) PGE_2 significantly reduced IFN-gamma titres in the case of PHA but had little or no effect on SEA induction. Disparate effects between mitogens were not due to donor variation but may have been the result of different activation pathways or SEA inducing higher IL-2 levels than the other mitogens.

Other workers have also found PGE_2 effects to be suppressive, probably by inhibiting IL-2 production (Makoulet et al.,1985; Wolf et al.,1985). Whether PGE_2 acts by inducing suppressor cells (Chouaib et al.,1984; Leclerc et al.,1984), perhaps by enhancing OKT8 +ve cell proliferation (Gualde and Goodwin, 1982), or by directly inhibiting IL-2 production and IL-2 dependent proliferation (Tilden and Balch,1982) is not clear. However, it has been claimed that T-cells incubated with PGE_2 produce two peptides (PITS alpha and beta) which are potent inhibitors of T and B cell responses (Roger et al.,1984). Also a subset of T-cells exist (10-30% of all

T-cells) which bind PGE_2 and exert strong suppressive activity on T and B cell proliferation (Fisher et al.,1985).

In view of the reported suppressive effects on IL-2 production and the latter's role in IFN-gamma production, exogenous IL-2 was added to PGE_2 suppressed cells. It clearly reversed the inhibitory effects of PGE_2 for a range of donors, although IFN-gamma levels were not fully restored in all cases (Table 19). The concentration of IL-2 used (2U/ml) did not usually boost IFN-gamma production, unless the mitogenic stimulation was "low" level (Table 21), suggesting that its effects on PGE_2 inhibition were not simply due to non-specific boosting but to replacement of depressed endogenous IL-2 production.

However, it must be noted that PGE_2 did not exhibit any depressive effects on cell proliferation in this study, even though IFN-gamma levels were depressed (Table 20). This reflects the lack of a strict correlation between IL-2 stimulated IFN-gamma production and proliferation (Kasahara et al.,1983a), also found with PHA and anti-Tac. It is possible that higher levels of IL-2 are needed for IFN-gamma stimulation than for proliferation. Exogenous IL-2 not only reversed PGE_2 inhibition of IFN-gamma levels but also boosted proliferation above that induced by mitogen alone.

Other inhibitory actions of prostaglandins may play a role in depressing IFN-gamma production. PGE_2 affects macrophages too, inhibiting full induction of class II MHC

antigens (Zlotnik et al.,1985) with the implication of reduced antigen presentation (Beller,1984). However, Zlotnik found that despite reduced Ia levels antigen presentation was not impaired, probably because most T-cells only need fairly low Ia levels on the surface of presenting cells. Thus IL-2 producing T-cells are probably more sensitive to PGE_2 than antigen presentation.

PGE_2 also depresses NK cell activity (Zielinski et al.,1984), although IFN-gamma secretion appears to antagonise this effect (Tracey et al.,1982; Dore-Duffy et al.,1983). There is also a body of evidence that prostaglandins are antagonists of IL-1 production and effects on B-cells (Goldings,1986) and murine thymocytes (Hayari et al.,1985). PGE_2 treatment has been found to reduce the natural cytotoxicity of large granular lymphocytes by reducing their IL-1 production (Herman and Rabson,1984) and the IL-1 production of LPS-stimulated peritoneal macrophages (Kunkel et al.,1986). In the latter case inhibitors of the cyclooxygenase pathway that leads to PGE_2 production, such as indomethacin, reduced PGE_2 production up to 40 fold while boosting IL-1 levels up to 6 fold. IL-1 was also found to boost PGE_2 levels, suggesting that IL-1 can regulate its own production via a partially self-induced inhibitor.

However, in this study exogenous IL-1, when added at stimulatory levels for lymphocytes, had no effect on PGE_2 mediated inhibition of IFN-gamma production (Table 20). Thus the possible effects of PGE_2 on IL-1 discussed above do not appear to be a major inhibitory mechanism in the

case of IFN-gamma levels. PGE_2 suppression of T-cell IL-2 production appears to be the main inhibitory pathway in this case. Any effects on human peripheral macrophages (as opposed to murine peritoneal cells examined by Kunkel) appear negligible in comparison, since only exogenous IL-2 was necessary to reverse the inhibition.

If PGE_2 has a true endogenous regulatory role in the immune system, it may be the down-regulation of IL-2 levels, and hence IFN-gamma, after the transient IL-2 response to an immune stimulation. At the peak of an immune response PGE_2 effects may be negligible due to high IL-2 levels and possible IFN-gamma mediated inhibition of prostaglandin synthesis by macrophages (Dore-Duffy *et al.*,1983). Exogenous PGE_2 effects on IFN-gamma levels do, however, suggest that this possible regulatory pathway requires further investigation to determine if it does occur *in vivo* as oppose to *in vitro*.

CHAPTER 4.

REGULATION OF IFN-GAMMA PRODUCTION BY CELL SURFACE MOLECULES BINDING SPECIFIC LIGANDS.

Introduction

An ever increasing group of cell surface molecules have been implicated in regulation of the immune response. Some are receptors for molecules involved in cell-cell communication, such as the IL-2 receptor discussed in the previous chapter, or receptors for IL-1 (Dower et al.,1985) and IFN-gamma (Orchansky et al.,1986). T-cells possess other cell-surface molecules which are important in cell-cell recognition and signal transduction. These are involved, for example, in the interaction between antigen presenting cells and T-cells, leading to antigen-specific T-cell activation via the T-cell antigen receptor. T-cells appear to recognise antigen only on the surface of cells bearing the appropriate self major histocompatability complex (MHC) gene product, which they were "restricted" to recognise in the thymus. Regulation of the level of MHC expression on antigen presenting cells may be involved in regulating the immune response (Unanue et al.,1984).

In recent years a series of molecules have been defined on the T-cell surface, using monoclonal antibodies (moabs), and described in terms of clusters of differentiation, or CDs (Bluestone & Hodes,1983; Townsend,1985). The most interesting of these are T3, T4, T8, and T11. These molecules work by binding to other cell-surface structures or ligands which may or may not

yet be identified. In this chapter the various monoclonal antibodies which bind to these cell-surface structures are used as model ligands. Although obviously they are not the natural ligands, unlike purified interleukins which deliver physiological signals, they are presumed to mimic a physiological ligand and do have their advantage of specificity and simplicity. The use of moabs as agonists to induce activation, growth, or differentiation of lymphocytes has recently been reviewed by Clark and Ledbetter (1986).

The human T4 (CD4) and T8 (CD8) molecules and their murine equivalents may play a role in T-cell recognition by enhancing the overall avidity of cell-cell interactions through specific binding to non-polymorphic cell surface ligands, possibly the non-polymorphic regions of class II and class I MHC molecules respectively (Reinherz et al.,1981; Reinherz et al.,1983). Moabs against MHC molecules and the T-cell subset markers specific for them have been used to investigate their functions in a variety of studies.

Another T-cell marker T11 (CD2) known as the sheep red blood cell (or E) receptor is found on all T-cells, like the T-cell antigen receptor and associated T3 complex (T3-Ti). Like T3-Ti, the E receptor also appears to be involved in T-cell activation and has been described as an alternative antigen-independent activation/suppression pathway (Meuer et al.,1984). It has been shown that some moabs binding to T11 can suppress IFN-gamma production (Wilkinson & Morris,1984a), IL-2 production (Tadmori et

al..1985), and IL-2 receptor expression (Reed et al..1985a&b), whilst moabs binding to other epitopes can lead to T-cell activation (Meuer et al.,1984; Fox et al..1985; Wilkinson & Morris,1984b). Thus some moabs may deliver signals different from those delivered by the physiological ligand to the same receptor. The natural ligand of T11 and the in vivo role of the T11 pathway are not yet certain, but recently a new lymphokine ("IL-4A" = T-cell activation factor) has been described, derived from antigen specific activation of T-helper cells, which seems to bind to T11 and cause T-cell activation (Milanese et al..1986a). Furthermore, the presence of the T11 molecule as the first T-cell marker to be expressed by thymocytes has led Reinherz to suggest the T11 pathway is important for imparting self-tolerance and MHC restriction in the thymus (Reinherz,1985).

Not only may the various T-cell and macrophage cell surface molecules be involved in cell-cell interactions, but it has also been suggested that they can act as transmembrane signal receptors, or transducers. On T-cells T11 may act as a "negative signal receptor" (Palacios & Martinez-Maza,1982), although the description of "IL-4A" suggests otherwise, and T4 may have a similar role, in addition to its involvement in accessory cell interaction (Bank & Chess,1985). Similarly, human HLA-DR antigens and their murine equivalent (H2 I-A) on macrophages and B cells may act as signal transducers, as well as restriction elements (Durum & Gershon,1982; Palacios,1985b).

In this chapter the ability of these cell surface molecules to regulate IFN-gamma production was investigated, using a variety of monoclonal antibodies as model ligands since to use the natural ligands would not usually be possible. This is in contrast to the previous chapter where purified or recombinant versions of the soluble mediators, which are the natural ligands, are used.

RESULTS

Effects of IL-1 and IL-2 on inhibition of IFN-gamma production by Moab 9.6.

The monoclonal antibody 9.6, which binds to the T-cell marker T11, was found to inhibit IFN-gamma production if added at the time of induction at a sufficient concentration (1µg/ml). IL-1, despite its effects on low IFN-gamma yields by lymphocytes, did not reverse this inhibition, over a range of concentrations, for both PHA and SEA induction (Table 1). This was found to be the case for a total of seven donors (Table 1 is representative) and at the highest concentrations (20U/ml) IL-1 even seemed to increase the level of inhibition caused by 9.6. Thus the inhibitory effects of 9.6 are not mediated by preventing IL-1 production.

Recombinant IL-2, however, was able to reverse 9.6 mediated inhibition (Table 2). Both IFN-gamma yields and proliferation, as measured by tritiated-thymidine incorporation, were depressed significantly by 9.6. Addition of IL-1, at a concentration that reversed the effects of macrophage depletion, had little effect on this depression, whilst recombinant IL-2 clearly reversed both depressive effects for three different mitogens. The addition of recombinant IL-2 at induction at 2 U/ml of biological activity often restored tritiated-thymidine incorporation to a level above that stimulated by mitogen alone. This is most clearly seen when OKT3 is the T-cell activator, 9.6 causing a 25-fold drop in mitogenesis but IL-2 more than reversing this. The depressive effects on

TABLE 4.1

THE EFFECTS OF IL-1 ON THE INHIBITION OF IFN-GAMMA PRODUCTION BY AN E-RECEPTOR SPECIFIC MONOCLONAL ANTIBODY (9.6).

	IFN-gamma Yield (U/ml)	
	PHA (10μg/ml)	SEA (20ng/ml)
Mitogen only	1000	250
Mitogen + 9.6	20	40
Mitogen + 9.6 + IL-1 (0.6U/ml)	20	32
Mitogen + 9.6 + IL-1 (2 U/ml)	40	25
Mitogen + 9.6 + IL-1 (6 U/ml)	8	10
Mitogen + 9.6 + IL-1 (20 U/ml)	6	8

a) 9.6 was used at 1μg/ml.

b) PBML from a single donor were used.

Table 4.2

THE EFFECTS OF IL-1 AND IL-2 ON INHIBITION OF IFN-GAMMA PRODUCTION AND PROLIFERATION BY 9.6.

	Mitogen					
	PHA (10μg/ml)		OKT3 (10ng/ml)		SEA (20ng/ml)	
Donor 1	IFN	^{3}H-Thy	IFN	^{3}H-Thy	IFN	^{3}H-Thy
Mitogen only	35	83,413	25	48,722	100	129,011
Mitogen +9.6	<2	51,068	<2	1,820	32	51,764
Mitogen +9.6+IL-1	<2	35,373	<2	1,654	50	62,691
Mitogen +9.6+IL-2	12	128,126	16	85,843	200	119,369

Donor 2	IFN	IFN	IFN
Mitogen only	320	800	6000
Mitogen +9.6	60	3	1200
Mitogen +9.6+IL-1	160	<3	2500
Mitogen +9.6+IL-2	320	100	4000

a) IFN-gamma titre was measured in U/ml 3 days post-induction. ^{3}H-Thymidine incorporation was measured for donor 1 over a period of 24 hours, 3-4 days post-induction, as the mean of four wells. Standard deviation was 10% or less.

b) 9.6 was used at 1μg/ml.

c) Purified IL-1 was used at 5U/ml and recombinant IL-2 at 2U/ml.

mitogenesis are less severe when SEA or PHA are used as mitogens. This effect of IL-2 on 9.6 inhibition appeared to be neither mitogen nor donor specific and suggests that depressive effects of 9.6 on IFN-gamma production are mediated by inhibition of IL-2, but not IL-1, reducing its production or somehow interfering with its effects.

Effects of Moabs binding to T4 and T8 surface markers on IFN-gamma production.

OKT4 and OKT8 monoclonal antibodies were added to PBML at a range of concentrations 30 minutes prior to induction and the effects on subsequent IFN-gamma titres measured (Table 3). At concentrations of up to 100ng/ml neither moab had any significant effect on IFN-gamma induction by SEA, but at 500ng/ml IFN-gamma production was reduced, 3-fold in the case of OKT8 and 10-fold for OKT4, or the two moabs combined. Immunofluorescence using a fluorescence activated cell sorter (FACS) analyser showed that IFN-gamma induction was inhibited at concentrations (500ng/ml or greater) of moab equal to, in the case of OKT8, or less than, in the case of OKT4, those which caused significant binding to the cell surface (Figs 4.1 & 4.2). The moabs were titrated out and the level of fluorescence at each concentration (from 1ng/ml to 5µg/ml) compared to that caused by non-specific binding of the FITC-conjugate alone. In the case of OKT4 there is no significant binding of the moab at less than 500ng/ml and definite binding only at 1-5µg/ml (Fig 4.1). In the case of OKT8 significant binding of the moab occurred only at

TABLE 4.3

THE EFFECTS OF VARYING CONCENTRATIONS OF OKT4 AND OKT8 ON IFN-GAMMA PRODUCTION BY PBML.

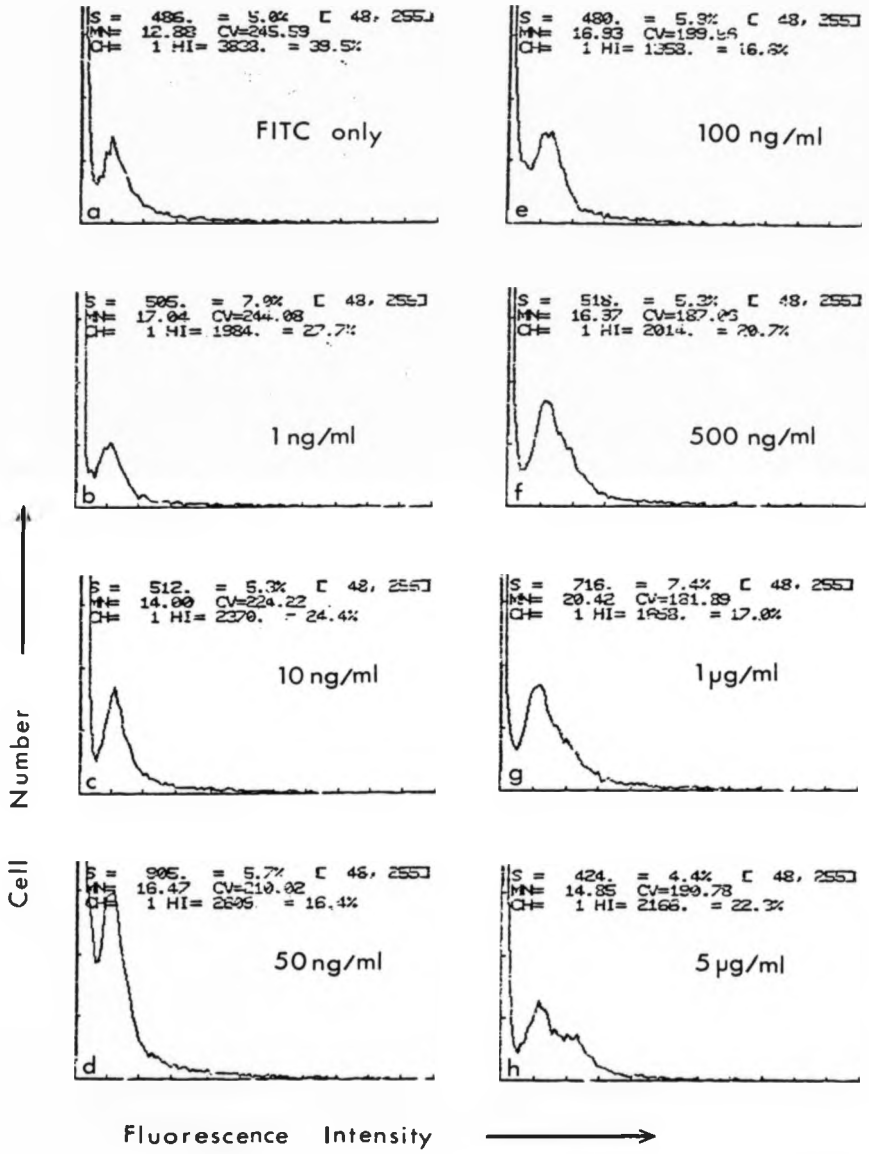
	IFN-gamma Titre (U/ml) Moab concentration (ng/ml)			
	10	50	100	500
OKT4	100	50	80	10
OKT8	130	80	130	40
OKT4 + OKT8	160	80	80	16
Mitogen only	80	-	-	-

a) Moab added 30 minutes prior to induction.

b) SEA (20ng/ml) was used to induce the PBML from a single donor.

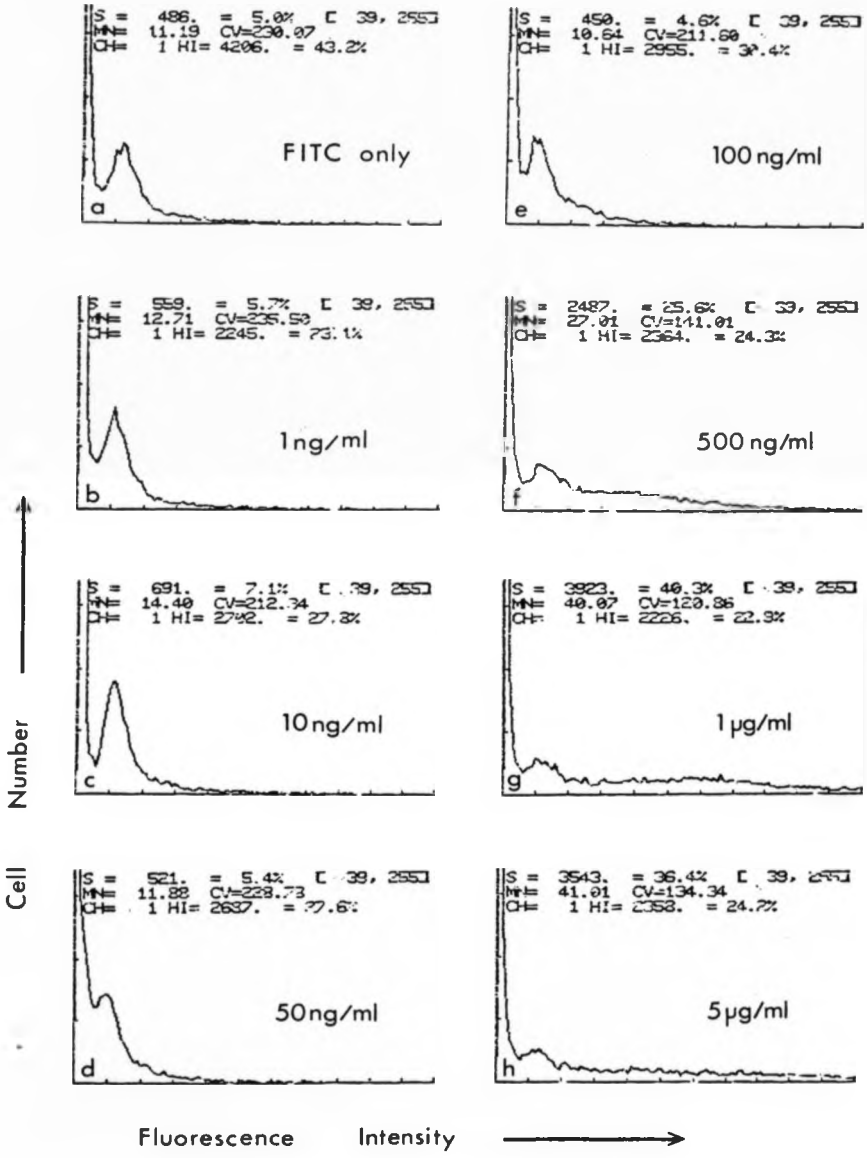
c) Using immunofluorescence both moabs were found to bind at concentrations of 30ng/ml or greater. No binding was detectable at <30ng/ml.

Figure 4.1 OKT4 BINDING TO PBML.



Binding of FITC to PBML pre-incubated with OKT4 at increasing concentration.

Figure 4.2 OKT8 BINDING TO PBML.



Binding of FITC to PBML pre-incubated with OKT8 at increasing concentration.

concentrations of 500ng/ml or greater (Fig 4.2), i.e. the same range of concentrations as those which cause inhibition of IFN-gamma induction. Thus inhibition occurs at concentrations of both moabs equal to or less than those required for significant binding as revealed by fluorescence, and is not due to some non-specific effect caused by a great excess of antibody.

These inhibitory effects of OKT4 and OKT8 were demonstrated across a range of donors (Table 4). OKT4 was found to be a more potent inhibitor than OKT8 at the same concentration, particularly when effects on proliferation are compared. OKT3 was also found to be inhibitory at these high concentrations, despite its stimulatory effects at lower levels.

However, this inhibition of T-cell activation seems to be dependent upon the time of addition of the moab relative to induction, based on results from two donors. When OKT4 and OKT8 are added one hour post-induction, as opposed to one hour prior to induction, there is a difference in the levels of inhibition (Table 5). In the case of OKT8 mediated inhibition IFN-gamma levels were unaffected or at most reduced by 50% if the SEA had been added first, compared to >10-fold inhibition when PBML were pretreated with OKT8. Inhibition by OKT4 was still significant (7-fold or greater) even if added one hour post-induction, however. OKT4 thus seems to be a rather more potent inhibitor than OKT8. Different inhibitory mechanisms may be involved or the difference in effects may reflect different involvement of the two T-cell

TABLE 4.4

THE EFFECTS OF OKT4 AND OKT8 ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-gamma Titre (U/ml) Donors						
	1	2	3	4	5	6	6 (prolif.)
Mitogen alone	130	160	1000	500	400	320	103,115
With OKT4	6	80	40	50	80	4	7,360
With OKT8	40	80	160	160	120	8	52,216
With OKT4 + OKT8	25	250	25	5	ND	ND	ND
With OKT3	ND	ND	ND	ND	32	3	1,616

a) Moab (500ng/ml) was added 30 minutes prior to induction with SEA (20ng/ml).

TABLE 4.5

THE EFFECTS OF OKT4 AND OKT8 BINDING TO PBML BEFORE AND AFTER INDUCTION.

	IFN Titre (U/ml) Donors	
	1	2
SEA only	2100	8000
SEA then OKT4	320	320
SEA then OKT8	4000	4000
OKT4 then SEA	80	1000
OKT8 then SEA	250	250

a) The SEA was added one hour before or one hour after the moab.

b) Moab was used at 500ng/ml and SEA at 20ng/ml.

subsets in IFN-gamma production. However, these conclusions are tentative since only two donors were examined in this situation.

Effects of moab binding to MHC class I and II molecules on IFN-gamma production.

The addition of moabs against class I MHC cell surface molecules at the time of induction caused a severe reduction of the IFN-gamma titre for two donors (Table 6). Both BBMI (specific for the beta-2-microglobulin chain of MHC class I cell surface complexes) and W6/32 (specific for a determinant common to most heavy chains of HLA-A, B, or C class I antigens; Barnstaple et al.,1978) caused significant inhibition of IFN-gamma production across a range of concentrations. W6/34 antibody is thought to bind to a widely distributed tissue common or species antigen (SA1), coded for on chromosome 11, with no connection to MHC antigens, coded for on chromosome 6. Neither W6/32 or murine IgG had any depressive effect on IFN-gamma production when used at similar concentrations to W6/32 or BBMI. Thus the depressive effects of W6/32 and BBMI are unlikely to be the non-specific result of binding to just any cell surface molecule, since W6/34 has no effect, or due to the presence of IgG somehow inhibiting T-cell activation. Their inhibitory effects are thus probably due to their interaction with class I HLA complexes, consisting of the 43kd heavy chain plus the 12kd beta-2-microglobulin chain.

Antibodies against class II HLA-DR antigens, found on

TABLE 4.6

THE EFFECTS OF ANTIBODIES AGAINST CLASS I HLA MOLECULES ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-gamma Titre (U/ml)	
Antibody	Donor 1	Donor 2
Mitogen alone	800	1600
BBMI (1µg/ml)	<2	80
(0.5µg/ml)	<2	50
(0.3µg/ml)	32	1000
W6/32 (1µg/ml)	40	160
(0.5µg/ml)	16	100
(0.3µg/ml)	4	320
W6/34 (3µg/ml)	1300	2000
(1µg/ml)	3200	1000
Mu IgG (5µg/ml)	ND	1000
(1µg/ml)	ND	800

a) SEA (20ng/ml) was used as the inducer for PBML throughout.

b) BBMI was used in the form of an ascites supernatant and binds to beta-microglobulin.

c) W6/32 (ascites supernatant-IgG_2) binds to HLA class I heavy chain.

d) W6/34 (ascites supernatant-IgG_2) binds to a species antigen (SA1) found on all human cells.

macrophages, B lymphocytes, and some other cells, also inhibited IFN-gamma production by PBML from six different donors (Table 7). The effect was dose specific, although the actual protein concentration of the anti-DR preparations was not measured. The level of inhibition was reduced if the anti-DR moab was added 24 hours post induction rather than at the same time as the mitogen. Thus interactions involving HLA-DR antigens required for optimal IFN-gamma production would appear to occur in the first 24 hours of induction. These inhibitory effects were shown to be neither donor nor mitogen specific. However, the level of inhibition was found to be greater for SEA than PHA induction (90% versus 50% inhibition).

TABLE 4.7

THE EFFECTS OF HLA CLASS II (DR) SPECIFIC ANTIBODIES ON IFN-GAMMA PRODUCTION BY PBML.

	IFN-gamma Titre (U/ml) Donors			
	1	2	3	4
SEA alone	2000	1200	600	320
SEA + anti-DR (1/1000)	120	400	ND	ND
SEA + anti-DR (1/100)	55	50	800	100
SEA + anti-DR (1/10)	55	32	100	40
SEA + anti-DR (1/100) 24h p.i.	ND	ND	800	120
SEA + anti-DR (1/10) 24h p.i.	ND	ND	600	160

a) Anti-DR supplied by the Dr.W.Bodmer (ICRF).

	Donors	
	1	2
SEA (20ng/ml)	320	400
SEA + anti-DR (1/100)	32	32
SEA + anti-DR (1/10) 24h p.i.	120	250
PHA (10μg/ml)	40	45
PHA + anti-DR (1/100)	20	20
PHA + anti-DR (1/100) 24h p.i.	32	40

a) Different anti-DR (DAKO) used from above.

DISCUSSION : Chapter 4.

The results in this chapter clearly demonstrate that a variety of cell surface molecules, other than receptors for interleukins 1 and 2, can regulate IFN-gamma production. To a great extent, however, involvement of these cell surface molecules in regulating IFN-gamma levels may reflect their overall importance in T-cell activation (Bluestone & Hodes,1983; Townsend,1985).

The growing evidence for the T11 pathway as an alternative antigen-independent activation/suppression pathway to the T3-Ti pathway made the possible role of interleukins in regulation of its effects on IFN-gamma production an obvious candidate for investigation. A variety of monoclonal antibodies to the T11 molecule (the E receptor) have been shown to suppress IL-2 production (Tadmori et al.,1985; Moretta et al.,1985) and IL-2 mRNA accumulation (Tadmori et al.,1986). OKT11A also inhibits expression of IL-2 receptors (Palacios & Martinez-Maza,1982; Reed et al.,1985a&b) and T-cell mitogenesis (Van Wauwe et al.,1981). There are an estimated $2x10^4$ binding sites/cell for OKT11A (Van Wauwe et al.,1981), which is similar to the number of binding sites reported for OKT3. The T11 moab 9.6 has also been shown to inhibit both RNA and DNA synthesis plus IFN-gamma production, as induced by several different mitogens, although tumour promoters can reverse these effects (Wilkinson & Morris,1984a).

However, binding of sheep erythrocytes to the E receptor can augment IFN-gamma production (Wilkinson &

Morris,1984b), whilst antibodies against epitopes other than those bound by SRBC, 9.6, or OKT11A can induce T-cell proliferation in the absence of antigen and/or presenting cells (Meuer et al.,1984; Yang et al,1986). At least three distinct epitopes exist, recognised by different moabs, and have been designated $T11_1$, $T11_2$, and $T11_3$. One of these epitopes, $T11_3$, is found only after activation or binding of moab to the $T11_2$ epitope, both being unrelated to the SRBC binding site, $T11_1$ (Fox et al.,1985). Activation via these epitopes can trigger cytotoxic cells, independently of antigen exposure (Siliciano et al.,1985), and IL-2 receptor expression on both T3+ and T3- thymocytes, although without inducing IL-2 production (Fox et al.,1985). The effects of the T11 pathway appear, therefore, to depend on the epitope(s) bound to. More recently a new lymphokine ("IL-4A") has been described which is produced by antigen specific activation of T-helper cells and leads to non-specific activation of resting T-cells by binding to the T11 molecule (Milanese et al.,1986a). "IL-4A" is apparently distinct from IL-1 or IL-2 in size and action. It stimulates IL-2 receptor expression by binding to T11 and does not require the presence of macrophages for its action, similar to the action of anti-$T11_{2+3}$. "IL-4A" also stimulates the cytotoxic activity of cytotoxic T-cell clones, inducing them to kill targets lacking the apropriate MHC molecules, and T3-,T11+ NK cell clones (Milanese et al.,1986b).

In this study the T11 moab 9.6 was used. It depressed IFN-gamma induction by several mitogens as has been shown

before. Addition of IL-1 at levels stimulatory for lymphocytes had no effect on this depression, but addition of IL-2 reversed both the depression of IFN-gamma levels and of tritiated-thymidine incorporation. This concurs with the findings of Reed et al. (1985a&b) and Tadmori et al. (1985 & 1986) that several different moabs blocked mitogen-induced IL-2 production and IL-2 mRNA accumulation, IL-2 receptor expression, and tritiated-thymidine incorporation, but the addition of IL-2 reversed these suppressive effects. Thus 9.6 depressed IFN-gamma levels are probably restored by exogenous IL-2 boosting the depressed levels of IL-2 and IL-2 receptor expression.

Tadmori et al.(1985) found that 9.6 could inhibit proliferation even when added 48 hours after stimulation and therefore is unlikely to act by blocking mitogen/antigen binding to the cells. However, Wilkinson et al.(1984a) found that 9.6 needed to be added within 15 hours of stimulation for significant inhibition of IFN-gamma production. Since IL-1 production occurs rapidly, within 3 hours of activation (Durum et al..1985). it seems unlikely that T11 depression, which can occur much later, acts via IL-1 levels. Hence, addition of exogenous IL-1 would not be expected to reverse any T11 mediated inhibitory effects. Since IL-2 binding to its receptor is required for optimal IFN-gamma production, as shown in chapter 3, and the T11 pathway is known to reduce levels of both IL-2 production and expression of its receptor, exogenous IL-2 is most likely to restore

IFN-gamma production by boosting depressed IL-2 levels and by increasing IL-2 receptor expression (Reem & Yeh,1984).

The way in which different T11 epitopes can transmit a positive or negative signal is not understood, although it is interesting to note that there are even two different mechanisms of T11 mediated activation, distinguishable by causing a calcium flux or not (Holter et al.,1986b). Modulation of the T3-Ti complex by a non-mitogenic anti-T3 moab can block activation via the T11 pathway, but T11 modulation by a non-mitogenic anti-T11 moab does not inhibit T-cell activation by OKT3 (Fox et al.,1986). Anti-T3 moab can costimulate macrophage-depleted T-cells if an anti-T11 moab against the appropriate epitope is also present, suggesting that the two pathways can be jointly involved in T-cell activation (Yang et al.,1986). The pathways are clearly interactive and Reinherz (1985) has suggested that regulation of T11 induced thymocyte expansion by the T3-Ti antigen/MHC receptor pathway, T11 appearing earlier than T3-Ti in thymocyte development, can explain MHC restriction in the thymus. Thymocytes whose T3-Ti has a high affinity for self MHC molecules (i.e. autoreactive) found on dendritic cells in the thymus would be inactivated via the T11 pathway so that they cannot respond to IL-2. T3-Ti activation needs IL-1, which is not present in the thymus, but T11 activation is independent of IL-1. Thymocytes lacking autoreactive T-cell receptors would not have their T11 pathway inhibited and could therefore repond to IL-2 and expand in number.

As yet, however, the natural ligand(s) for T11 has not

yet been positively identified. The hormone early pregnancy factor (EPF) can inhibit certain immune reactions, possibly via T11 since it blocks SRBC rosetting (Whyte & Heap,1983). Possibly EPF, by acting via T11, helps regulate the maternal immune system during pregnancy to aid survival of the fetal "allograft". If EPF actually does regulate immune function in vivo, and this is far from certain, it may well act by regulating IL-2 and IFN-gamma levels to some extent. A 42,000 m.wt. glycoprotein has also been isolated which may be the target structure (T11TS) for T11, since it blocks rosette formation and competes with anti-T11 moabs in binding the E receptor (Hunig,1985). Possibly T11 and T11TS are complementary cell interaction molecules involved in regulating T-cell proliferation. The most likely in vivo T11 ligand, however, is the newly described lymphokine "IL-4A" which acts as a non-specific T-cell activator (Milanese et al.,1986a). Its actual role in vivo has yet to be shown. however, since it is not known how the apparent non-specific amplificatory effects of IL-4A would be regulated and how antigen specificity would be maintained.

Considering the importance of "restriction" in the immune response (the ability of T-cells to recognise antigen only in the context of the MHC molecules to which they are exposed in the thymus), the role of the cell surface molecules involved in mitogen activated IFN-gamma production was examined. Numerous studies show that T-cell subsets can be distinguished on the basis of their

expressed cell-surface phenotype, and that there is a strong association between cell-surface phenotype and the class of MHC product recognised (Bluestone & Hodes,1983). Thus T-cell populations in man which are class II reactive appear to express the T4 (62,000 m.wt.) glycoprotein and are usually T helper cells (Dalgleish,1986). Class I reactive T-cells, however, predominantly express the 33,000 m.wt. T8 cell surface marker and are usually cytotoxic or suppressor T-cells. T4 and T8 may act as stabilising elements, enhancing the overall avidity of cell-cell interactions through specific binding to non-polymorphic regions of class II and class I molecules respectively (Reinherz et al.,1984; Maddon et al..1985) when the T-cell's receptor has low affinity for its ligand (polymorphic MHC region plus antigen).

Monoclonal antibodies against T4 and T8 have been used to examine their function, e.g. anti-T8 inhibits cytolytic function of T-cells (Reinherz et al.,1981) and moabs against the murine equivalent of T4 (L3T4) or T8 (Lyt 2) inhibit antigen induced lymphokine production by class I or class II MHC reactive clones (Lancki et al.,1984). In this study moabs against OKT4 and OKT8 both inhibited mitogen induced IFN-gamma production, when added at 500ng/ml. OKT4 was found to be a more potent inhibitor than OKT8. This may be because T8 positive cells are a smaller subset (35%) of the total T-cell population. Alternatively, the difference in level of inhibition could be due to differing roles in lymphokine production, or differing levels of production by the two subsets. The

moabs differed also in their effects relative to the time of induction. OKT8 caused inhibition only when added prior to or very shortly after induction. If added even one hour after induction the inhibitory effects were greatly reduced (>10-fold). OKT4 mediated inhibition, however, was only slightly reduced by addition one hour post-induction. Why there should be this difference in effect according to the time of addition is unclear. Possibly OKT8 acts by blocking cell-cell interactions necessary for induction, whilst OKT4 mediated inhibition is due instead to T4 acting as a negative signal transducer (Bank & Chess,1985). Alternatively, the difference may reflect different roles of the subsets in IFN-gamma production as has been suggested for the murine system (Torres et al.,1982). OKT8 has been shown by other workers to inhibit IL-2 production and IL-2 receptor expression by OKT8+ cells, but not OKT4+, if added less than 2 hours after stimulation. Thereafter it failed to suppress IL-2 receptor expression, although IL-2 production is still reduced up to 20 hours post induction (Welte et al.,1983). In the same study it was shown that OKT8 did not interfere with binding of the OKT3 used as inducer and therefore acts either as a negative signal or blocks cell interactions needed for activation.

Perturbation of T4 also seems to deliver a negative signal, inhibiting helper cell functions, proliferation, and lymphokine release. Bank and Chess (1985) have found that the moab OKT4c can inhibit proliferation irrespective of whether Ia +ve macrophages or Ia -ve U937 cells are

used as the accessory cells. This makes it less likely that T4 is purely a receptor for non-polymorphic regions of class II (Ia) molecules, but instead can act as a transducer of negative signals. OKT4 inhibition would therefore be due to T4 perturbation rather than blocking T4-MHC interactions. Perhaps T4-Ia interaction in the absence of antigen leads to a negative signal, but the presence of antigen changes this to a positive signal.

Recently it has been shown that anti-T4 and T8 moabs specifically inhibit all types of cytotoxicity of T4+ and T8+ CTL respectively, regardless of whether the target cells expressed class I or class II antigens (Fleischer et al.,1986). Thus T4 and T8 binding may have a regulatory, inhibitory effect so that high affinity binding of CTL to a relevant target cell is required to stimulate a strong positive signal from the T-cell antigen receptor. This would overcome the weak negative signal transmitted by perturbation of T4 or T8, which would otherwise prevent lysis of low affinity irrelevant targets. Inhibition of IFN-gamma production by OKT4 and OKT8 could therefore be due to such negative signals caused by perturbation of T4 and T8 molecules. The observation that OKT4 still causes significant inhibition one hour post induction (up to 24 hours post-induction according to Banks & Chess,1985) make it less likely that it acts by interfering with mitogen binding or macrophage/T-cell interactions needed for activation. This may not be the case for OKT8, however.

Further interest in the T4 molecule has been stimulated by the discovery that it acts as the receptor for LAV (or

HTLV III) virus, since preincubation of T4+ cells with OKT4 blocks cell infection by this virus (Klatzman et al.,1984). Recent sequencing of the genes encoding T4 (Maddon et al.,1985) and T8 (Littman et al.,1985; Sukhatme et al.,1985) proteins reveal significant homology with members of the immunoglobulin superfamily, which includes MHC antigens and the T-cell receptor (Williams,1984). Both have V-like domains homologous to equivalent regions of both immunoglobulin and the T-cell antigen receptor chains, but T4 also has a J-like region making it, at 435 amino acids, twice the size of T8 (Maddon et al.,1985). Possibly T4 reflects a more primitive gene that evolved before the emergence of rearrangement mechanisms, found in immunoglobulins and the T-cell antigen receptor, similar to receptors operative in more primitive cellular immune responses. Thus a growing body of work, plus the findings described in this chapter, suggest that T4 in particular may have regulatory functions beyond that of restriction alone.

Antibodies against MHC molecules, both class I and II, were also shown in this chapter to inhibit IFN-gamma production, possibly by interfering with restriction requirements. Class I MHC molecules are expressed on all cells, which is hardly surprising since any cell can be parasitised and thus should be a potential lytic target for the class I restricted cytotoxic T-cells (T8). Class II MHC molecules, however, are found only on antigen-responsive and accessory cells (e.g. B cells and macrophages), since these are the only targets for helper

T-cells (T4). If there was only a single class of restricting element then cells not related to the immune system would compete for helper T-cell recognition of antigen presenting cells, decreasing the level of activation considerably.

In this study moabs against both the class I heavy chain (W6/32 against HLA-A, -B, and -C gene products) and the associated beta-2-microglobulin chain inhibited IFN-gamma induction 10-fold or more when added at induction. These effects were shown to be specific since similar concentrations of murine IgG or a moab against a species antigen, found on all cells, had no effect on induction. Taylor et al have also shown that HLA class I molecules have a functional role in human T-cell activation, moabs against HLA-A, -B, and -C gene products (including W6/32) inhibiting both OKT3- and ionomycin- (a calcium ionophore) induced proliferation of both T4+ and T8+ enriched populations by reducing IL-2 secretion and IL-2 receptor expression (Taylor et al.,1986b). Since it was also shown that anti-class I moabs still caused inhibition when added some 40 hours post-induction, and since they also inhibited ionophore induced proliferation, it seems unlikely that these moabs interfere with antigen recognition but that HLA class I molecules are somehow directly involved in early events of lymphocyte activation and proliferation (e.g. protein kinase C activation; see chapter 5).

Similarly, moabs against HLA class II DR molecules, found on macrophages and B cells, could inhibit the level

of IFN-gamma production by 10-fold or more. The effect was dose dependent and also greatly reduced if the anti-DR moab was added 24 hours post-induction rather than at induction itself. This reduced effect post-induction is probably due to the importance of restriction during T-cell activation, rather than in subsequent stages of activity. Thus anti-DR moab's inhibitory effects on IFN-gamma levels are most likely to be due to their interference with initial macrophage/T-cell interaction. However, restriction may not be as important when polyclonal activating mitogens are used, as opposed to specific antigen recognition by T-cells. In mice anti-I-A (equivalent to human DR) moabs have been shown to inhibit lectin induced IL-2 production, the addition of IL-1 reversing this inhibition, but not if the induction was antigen specific (Larsson & Coutinho,1984). This suggests that class II antigens play two roles in lymphokine induction, being involved both as restricting elements in antigen recognition, a requirement overcome by lectins, and in directly mediating activation signals to macrophages at the level of IL-1 production. Other workers have found that I-A molecules may have a role in signal transduction, since anti-DR moabs can stimulate human macrophages to secrete IL-1 (Palacios,1985b) and IL-1 can replace the requirement for I-A positive cells in the proliferation of antigen primed mouse T-cells (Durum & Gershon,1982). Perhaps I-A recognition is required by T-cells not to trigger the T-cell but for the T-cell to trigger the macrophage to secrete IL-1.

In vivo treatment of mice with anti-I-A antibodies down-modulates Ia expression by their cells and has been shown to obliterate the antigen presenting ability of their spleen cells (Aberer et al.,1986). The depressive effects of anti-HLA moabs on human PBML function has also been demonstrated by Akiyama et al. (1985) who found that the depressive effects on proliferation varied between the types of mitogen used. Anti-class II moabs blocked proliferation in response to OKT3 and PWM, but not PHA and Con A, whilst anti-class I blocked responses to all four mitogens. Findings such as these, and the effects on IFN-gamma production described in this chapter, suggest that both class I and II antigens have an important role in the sequence of events following interaction of mitogens with cells. Antibodies against them may disturb macrophage/T-cell interactions, or perhaps mitogen binding, the effects varying between different mitogens possibly because different binding sites are involved or because mitogens may differ in their requirements for the presence of cells expressing MHC for lymphocyte activation. Differences such as these may explain the difference in level of inhibition of IFN-gamma production by anti-DR between SEA and PHA induction, the relative level of inhibition being less when PHA was the inducer.

Whatever the exact role of class II restriction there is evidence for a relationship between Ia or DR expression and IFN-gamma levels. Because macrophage Ia expression is facultative, unlike class I expression, the regulation of the level of Ia in antigen presenting cells may be crucial

for controlling the level of the immune response (Unanue et al.,1984). Prostaglandin E_1 can suppress murine, but not human, macrophage Ia expression (Kunkelet et al.,1986), whilst IFN-gamma can induce I-A expression, both on cells which normally express it and also cells that do not, and thus augment antigen presentation and hence the immune response (Ameglio et al.,1983; Kelley et al.,1984; Fierz et al.,1985; Becker,1985; Geppert & Lipsky,1985; Virelizier et al.,1984; Janeway et al.,1984). Recombinant IFN-gamma has been shown to act at the level of transcription, inducing HLA-DR gene expression and boosting the level of class I HLA mRNA (Koefler et al.,1984; Collins et al.,1984). Similarly IFN-gamma can induce class I HLA and beta-2-microglobulin expression on cells which are usually class I negative, such as human amnion cells (Hunt & Wood,1986) and neuroblastoma cell lines (Lampson & Fisher,1984). IFN-gamma has also been shown to have in vivo effects, since its injection into mice leads to increased levels of antibodies in response to antigen (Nakamura et al.,1983) and increased (at least 4-fold) levels of class I and II expression in a variety of tissues (Skoskiewicz et al.,1985; Momburg et al.,1986a&b). IFN thus appears to be both a modulator, able to increase the level of an MHC gene product already expressed, and a real inducer, able to induce de novo activation of both classes of MHC genes (Rosa & Fellous,1984). The importance of Ia antigen expression in T-cell activation and consequent IFN-gamma production, combined with the latter's effects on boosting Ia antigen

expression, and hence increased T-cell activation and IL-1 production by macrophages, would indicate the existence of an amplification loop in the immune response.

In conclusion, the results described in this chapter show that the binding of ligands to a variety of cell surface molecules can regulate mitogen activated IFN-gamma production. The T11 pathway was shown to be capable of depressing IFN-gamma production, probably via depressive effects on IL-2 production. What significance the T11 pathway has for immune regulation *in vivo* has yet to be demonstrated, however. Cell surface molecules involved in MHC restriction on both macrophages and T-cells were also shown to have effects on IFN-gamma induction. Inhibitory effects of moabs against class I and II molecules, as well as T4 and T8, were probably due to the involvement of these molecules in the macrophage/T-cell interaction during the initial stages of T-cell activation. However, these effects varied, depending on the cell surface molecule involved and also the mitogen used. Furthermore T4 may have a regulatory role beyond its involvement in restriction since OKT4 has inhibitory effects long after induction. Possibly OKT4 can act as a negative signal transducer in a similar way to the T11 molecule. Its membership of the immunoglobulin "superfamily", with structural similarities to the T-cell antigen receptor, adds circumstantial evidence for this possibility. It must be reiterated, however, that all of the regulatory effects described in this chapter are probably the consequence of overall inhibitory effects on T-cell activation being

carried through to the level of IFN-gamma production. It should also be borne in mind that some of the conclusions drawn come from results obtained from an artificial model system, i.e. monoclonal antibodies binding to cell surface molecules. The depressive effects of moabs against these cell surface molecules suggest, if nothing else, that separation techniques involving them may cause some degree of inactivation.

CHAPTER 5.

THE INDUCTION OF IFN-GAMMA AND IL-2 PRODUCTION AND THEIR mRNAs BY PHORBOL ESTERS AND A CALCIUM IONOPHORE.

Introduction.

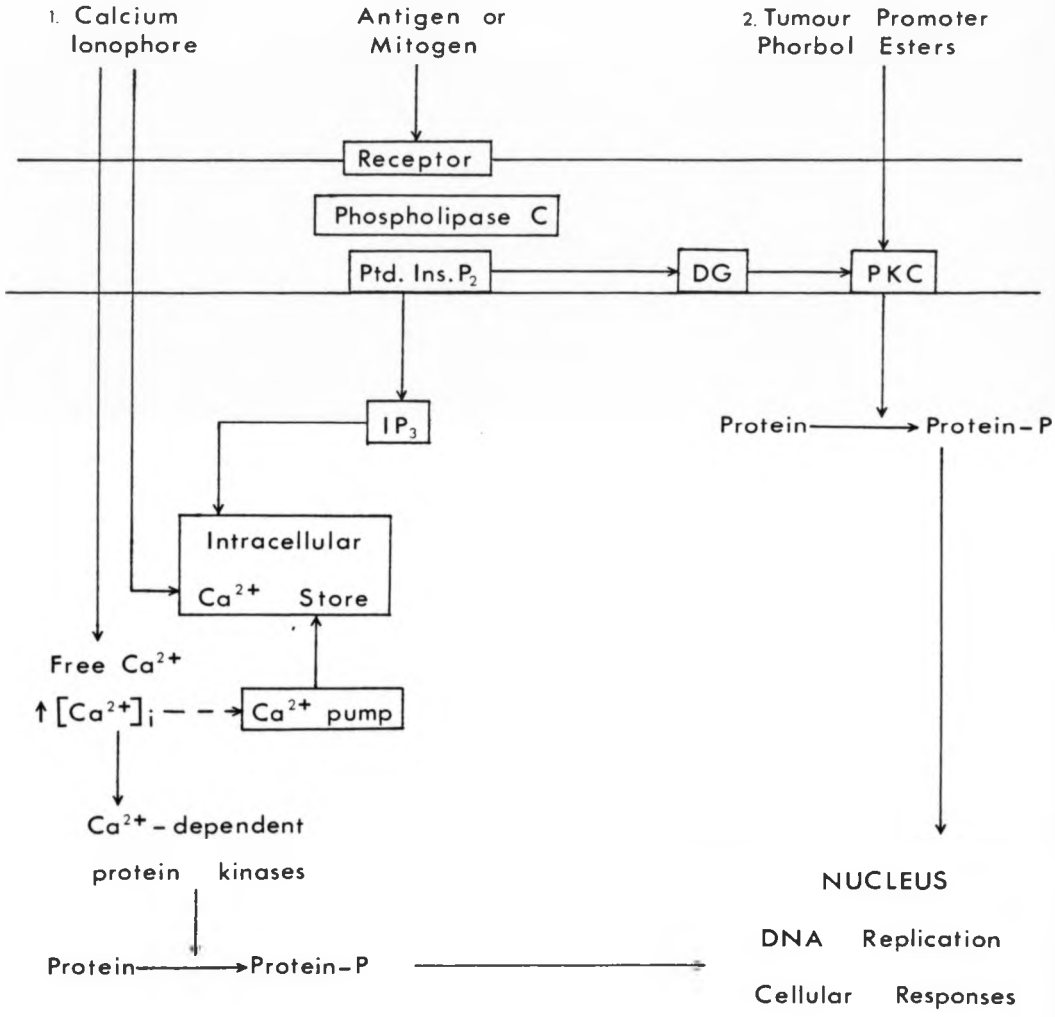
Studies by several groups have shown that an increase in the concentration of cytoplasmic free calcium ions is associated with the transmembrane signal delivered by the T-cell antigen receptor in response to the binding of mitogenic lectins (Tsien et al.,1982), antibodies against the T3 complex (Oettgen et al.,1985), or the appropriate antigen (Nisbet-Brown et al.,1985; Shapiro et al.,1985). A direct role for an increase in calcium concentration in cellular activation is supported by the activation of T-cells by calcium ionophores such as A23187 and the inhibition of T-cell activation by blocking calcium flux with EGTA (Weiss et al.,1984).

A second "signal" implicated in transmembrane signalling is the activation of the enzyme protein kinase C (Wolf et al.,1985) usually by the binding of calcium (May et al.,1985), but also by the binding of phorbol esters to the enzyme (Castagna et al.,1982; Kikkawa et al.,1983; Niedel et al.,1983). This may explain the synergism of phorbol esters with mitogens for T-cell activation (Ashman et al.,1984) and their ability to partially replace macrophage functions in lymphocyte activation (Koretzky et al.,1982).

When cells are stimulated by external ligands (mitogens or antigens) binding to the T-cell receptor, both "signals", i.e. increase in calcium ion concentration and

activation of protein kinase C. are normally triggered (see discussion and recent review by Isakov et al.,1986). However, experimentally these two synergistically acting signals can be mimicked by using non-physiological chemicals which trigger one signal or the other (Figure 5.1). Thus in combination calcium ionophores and phorbol esters can mimic antigenic/mitogenic stimulation. bypassing the requirement for an antigenic/lectin signal at the onset of lymphocyte activation (Truneh et al.,1985a&b; Isakov & Altman,1985). In this chapter, their ability to induce IFN-gamma production in the absence of mitogen was investigated. The involvement of calcium ion flux and protein kinase C as signals for IFN-gamma and IL-2 gene activation was also examined. Using cDNA probes made it possible to look at gene activation independently of downstream effects which could modulate supernatant levels, such as those examined in previous chapters, and in the absence of protein synthesis. Furthermore cDNA probes are both very specific and very sensitive, i.e. the mRNA can be detected long before the protein it codes for. The results suggest that the two genes are coordinately induced by calcium ion flux and tumour promoters, that macrophages may play a role in regulating messenger RNA levels, and that protein synthesis is unnecessary.

Figure 5.1 A SIMPLIFIED MODEL OF T-CELL ACTIVATION.



Interaction between the T-cell receptor and a ligand activates phospholipase C, which hydrolyses Ptd Ins P_2 into IP_3 and DG. These act synergistically to elicit physiological responses. The intracellular signals mediated by IP_3 and DG can be independently mimicked by Ca^{2+} ionophores and phorbol ester tumour promoters, respectively.

RESULTS

Synergy between A23187 and phorbol ester.

Separately the calcium ionophore A23187 and phorbol esters mezerein, teleocidin, and TPA induced low levels of IFN-gamma production at the concentrations used. Higher levels of A23187 (10μM) usually led to cell death. Together, however, the ionophore and phorbol ester acted synergistically to induce high levels of IFN-gamma, and also IL-2 (Table 1). This IFN activity was shown to be due to IFN-gamma by its 99% inactivation by anti-IFN-gamma anti-serum, which reduced the A23187/Mezerein induced IFN activity from 1260U/ml to <2U/ml. Higher concentrations of TPA and teleocidin (30ng/ml or greater) were needed for the same level of induction caused by 10ng/ml of mezerein, with A23187. In view of the reported ability of phorbol esters to replace the accessory function of macrophages, the ability of IL-1 to synergise with A23187 was examined. Unlike phorbol esters, however, IL-1 failed to act as a co-inducer with ionophore, even when used at levels which reverse the effects of macrophage depletion (Table 2).

The levels of IFN-gamma induced by A23187 and mezerein 3 days post-induction were frequently significantly much greater than induced by the powerful mitogen SEA (Tables 1 and 3). Depletion of macrophages greatly reduced SEA induction but generally had little effect on A23187/mezerein induction of IFN-gamma production (Table 3). In only one out of five donors was the level of IFN-gamma reduced by a comparable amount (>10 fold) for both methods of induction. In the remaining donors it can

Table 5.1

SYNERGY OF CALCIUM IONOPHORE AND PHORBOL ESTERS FOR BOTH IFN-GAMMA AND IL-2 INDUCTION.

	IFN-gamma titre (U/ml)				
	Donors				
	1	2	3	4	5
SEA (20ng/ml)	1000	400	320	320	500
A23187	<2	40	10	<2	<2
Mezerein	<2	32	25	ND	ND
Teleocidin	<2	3	<2	ND	ND
TPA	<2	3	<2	ND	ND
A23187 + Mez.	180	6000	5000	1600	4000
A23187 + Tel.	<2	50	120	500	25
A23187 + TPA	320	120	8	200	1200

	IL-2 titre (U/ml)	
A23187	<2	<2
Mezerein	<2	<2
A23187 + Mez.	16	20

a) PBML used at $5x10^5$ cells/ml.

b) IFN-gamma titres were measured on day 3 and the IL-2 titres on day 1 post-induction.

c) A23187 was used at 1μM. All phorbol esters were used at 10ng/ml.

Table 5.2

THE EFFECTS OF A23187 AND IL-1 ON IFN-GAMMA PRODUCTION.

	IFN-gamma titre (U/ml)	
	Donor 1	Donor 2
SEA (20ng/ml)	320	500
A23187 (1μM)	<2	<2
" + IL-1 (1 U/ml)	<2	<2
" + IL-1 (3 U/ml)	<2	<2
" + IL-1 (10 U/ml)	<2	<2
" + Mez. (10ng/ml)	1600	4000

a) PBML used at $5x10^5$ cells/ml.

b) IFN-gamma titre measured on day 3.

Table 5.3

THE EFFECTS OF MACROPHAGE DEPLETION ON IFN-GAMMA PRODUCTION INDUCED BY MEZEREIN AND A23187.

	IFN-gamma titre (U/ml) Donors				
PBML	1	2	3	4	5
SEA (20ng/ml)	160	2000	250	800	160
A23187 + Mezerein	2000	12000	6000	1000	250
Lymphocytes					
SEA	40	8	20	160	40
A23187	<2	12	<2	<2	<2
Mezerein	<2	<2	<2	<2	<2
A23187 + Mez.	2500	3200	400	1200	3200

a) PBML and Ly used at $5x10^5$ cells/ml.

b) A23187 was used at 1μM and mezerein at 10ng/ml.

Table 5.3

THE EFFECTS OF MACROPHAGE DEPLETION ON IFN-GAMMA PRODUCTION INDUCED BY MEZEREIN AND A23187.

	IFN-gamma titre (U/ml) Donors				
PBML	1	2	3	4	5
SEA (20ng/ml)	160	2000	250	800	160
A23187 + Mezerein	2000	12000	6000	1000	250
Lymphocytes					
SEA	40	8	20	160	40
A23187	<2	12	<2	<2	<2
Mezerein	<2	<2	<2	<2	<2
A23187 + Mez.	2500	3200	400	1200	3200

a) PBML and Ly used at $5x10^5$ cells/ml.

b) A23187 was used at 1μM and mezerein at 10ng/ml.

be seen that any reduction in IFN-gamma levels is far less when A23187 and mezerein are the inducers as opposed to the mitogen SEA. Thus mitogen induction is clearly macrophage-dependent but A23187/mezerein induction is probably not, implying that different activation pathways may be in operation for the two types of induction.

A23187 could not only act as a powerful co-activator but also reversed the depressive effects of the T11 pathway on induction of IFN-gamma by SEA (Table 4). The monoclonal antibody 9.6, which binds to the T11 cell-surface molecule, depressed IFN-gamma production by at least five-fold. A23187 induced little IFN-gamma on its own and did not act in synergy with SEA, as it does with mezerein, but was capable of at least partially reversing the inhibitory effects of 9.6. This was found to be the case in 70% of donors tested (10 out of 14) and is further evidence for a rise in cytoplasmic calcium ion concentration acting as a positive signal in T-cell activation. The effects of mezerein on depression of IFN-gamma production by 9.6 were not examined as the phorbol ester has known synergistic effects with SEA.

Medium containing the two inducers at the concentrations used for induction had no effect on the WISH cell IFN assay. Thus the high titres observed were not an artefact due to non-specific effects of the ionophore or tumour promoter on Wish cells.

Table 5.4

THE EFFECTS OF A23187 ON THE INHIBITION OF IFN-GAMMA PRODUCTION BY MONOCLONAL ANTIBODY 9.6

	IFN-gamma titre (U/ml) Donors				
	1	2	3	4	5
SEA (20ng/ml)	250	1000	160	2000	320
A23187 (1μM)	<2	<2	<2	55	10
SEA + 9.6 (1μg/ml)	18	200	16	50	10
SEA + 9.6 + A23187	55	1000	100	300	8
SEA + A23187	320	320	ND	ND	ND

a) PBML used at $5x10^5$ cells/ml.

b) In 10 out of 14 cases A23187 at least partly reversed the inhibitory effects of 9.6 (representative data shown).

The induction of IL-2 and IFN-gamma mRNA production by A23187 and mezerein.

Using cDNA probes for IL-2 and IFN-gamma mRNA the induction of both messages in cells treated with A23187 and mezerein under different conditions was studied. It was found that A23187 with mezerein induced high levels of both messages compared with little effect seperately (Table 5). Fresh PBML from pooled donors (up to four donors per experiment in order to get enough material) at $3x10^6$ cells/ml were cultured for one hour in either untreated medium or medium containing cycloheximide (200µg/ml). Cells were then induced with A23187 and/or mezerein and harvested 4 hours post-induction, a period which allowed steady-state levels of mRNA to increase to values which could be quantitated accurately while avoiding long term incubation in the presence of cycloheximide (CHX), a metabolic inhibitor. Their RNA was isolated and levels of IFN-gamma or IL-2 specific mRNA determined by dot-blotting (Thomas,1980) using labelled cDNA probes homologous to IFN-gamma (Siggens et al..1984) or IL-2 (Taniguchi et al.,1983). Since A23187 with mezerein plus CHX generally gave the greatest level of induction this level was assigned the arbitrary value of 1.0 and other levels given relative to this. Inclusion of standard samples containing 0.1, 0.01, and 0.001 ng of IFN-gamma DNA proved that the level of hybridisation was indeed proportional to the level of specific message expressed.

In two experiments A23187 and mezerein seperately

induced only low steady state levels of both IFN-gamma and IL-2 mRNA (Table 5, donors 1 and 2). In combination, however, A23187 and mezerein acted synergistically inducing levels of mRNA 5 to 10 times greater than either did alone. The synergy of the ionophore and phorbol ester for IFN-gamma gene expression may thus at least partly explain their powereful induction of IFN-gamma production.

The role of protein synthesis in activation of either gene was examined by addition of an inhibitor of protein synthesis, cycloheximide, one hour before induction. Protein synthesis was reduced by 99% as determined by ^{35}S-methionine incorporation (147,000 cpm in untreated cells was reduced to 1,950 in CHX treated cells). The levels in cells treated with inhibitor were usually either unaffected or even somewhat augmented (see in particular IL-2 mRNA levels induced by A23187 plus mezerein). Therefore both genes can be expressed in the absence of synthesis of proteins, such as interleukins 1 and 2. In the absence of T-cell induction, the presence of CHX was shown not to induce any IFN-gamma gene expression.

When mRNA production by PBML was compared to that by macrophage-depleted lymphocytes (Table 6) the level of expression of both genes was found to be higher in lymphocytes than PBML. However, the relative level of expression varied between experiments, with PBML expressing 9% of the level found in lymphocytes in one experiment but 58% in another, at 4 hours post-induction. In a third experiment results very similar to experiment 1 were obtained judging from the opaqueness of dots on

Table 5.5

INDUCTION OF IL-2 AND IFN-GAMMA mRNA.

	Experiment 1		2		3	
	IFN	IL-2	IFN	IL-2	IFN	IL-2
A23187 (1μM)	0.13	0.02	0	0.03	ND	ND
A23187 + CHX	0.22	0.13	0.06	0.07	ND	ND
Mezerein (10ng/ml)	0.15	0.10	0	0.07	ND	ND
Mez. + CHX	0.27	ND	0.04	0.11	ND	ND
A23187 + Mez.	0.90	0.51	0.39	0.68	0.95	0.95
A23187 + Mez.+ CHX	1.00	1.00	1.00	1.00	1.00	1.00
cpm equal to 1.00	(1,057)	(217)	(547)	(241)	(324)	(11)

a) RNA was harvested 4 hours post induction, with 1 hour pre-treatment with cycloheximide (CHX) at 200μg/ml where indicated.

b) Hybridised cpm for RNA samples (20μg) from PBML treated with A23187, mezerein, and CHX were arbitrarily assigned a value of 1.0 and levels of mRNA in other samples were then calculated relative to this sample. Each value is the mean of duplicate 20μg samples. The cpm equivalent to 1.00 for each experiment is given at the bottom of the table.

c) A standard of 0.1ng IFN-gamma mRNA = 1.06, 2.05, and 1.59 for experiments 1 to 3 respectively.

Table 5.6

INDUCTION OF IL-2 AND IFN-GAMMA mRNA BY A23187 PLUS MEZEREIN IN PBML VERSUS LYMPHOCYTES.

	Experiments			
	1		2	
	IFN	IL-2	IFN	IL-2
PBML at 4 h.	0.09	0	0.58	0.60
PBML at 24 h.	0	0.02	0	0
Ly. at 4 h.	1.00	1.00	1.00	1.00
Ly. at 24 h.	0.90	0.46	0.44	0.22
cpm equal to 1.00	(533)	(475)	(560)	(124)

a) All preparations were induced with A23187 (1μM) plus mezerein (10ng/ml).

b) Hybridised cpm for RNA samples (20μg dots) from 4 h. lymphocyte (Ly.) samples were assigned a value of 1.00 and levels of RNA in other samples calculated relative to this. The cpm equivalent to 1.00 for each experiment is given at the bottom of the table.

c) A standard of 0.1ng IFN-gamma mRNA = 1.04 and 1.6 for experiments 1 and 2.

autoradiographs after hybridisation of the probes, but due to the small amounts of RNA isolated it was not possible to count the level of hybridisation on the filter by liquid scintillation counting. When levels of mRNA were examined 24 hours post-induction the contrast between levels in PBML and lymphocytes was even clearer. At 24 hours PBML did not express detectable levels of either mRNA, whilst macrophage-depleted lymphocyte mRNA levels, although lower than at 4 hours, were still detectable at 24 hours. Thus macrophage-depleted lymphocyte populations express higher levels of both messages than PBML, and although the level of expression is reduced at 24 hours compared to 4 hours it is not wholly abolished as in PBML. Using this induction scheme macrophages are thus not required for expression of either gene.

Comparison of the level of message at different times post-induction also shows that induction of gene expression in PBML is both very rapid and transient using this method of activation, peaking in only a few hours and being shut down within 24 hours. This is in contrast to mitogen induction which gives maximal steady state mRNA levels at 1-2 days at $3x10^6$ cells/ml as used in this chapter (Siggens *et al.*,1984).

DISCUSSION : Chapter 5.

The ability of A23187 plus mezerein synergistically to induce the high levels of IFN-gamma found in this study is consistent with the findings of many other workers that such a combination can mimic antigenic/mitogenic stimulation (Truneh et al.,1985a). A23187 and phorbol esters are able to induce IL-2 receptor expression and IL-2 production (Isakov & Altman,1985). In the case of immature murine thymocytes this can be induced even before the cells have developed the ability to respond to mitogens (Lugo et al.,1986) and in a mutant T-cell line (S5) which lacks the T3 complex and also does not respond to mitogen stimulation (Weiss et al.,1984b). Thus ionophore plus phorbol esters can activate T-cells in a fashion that bypasses the T-cell receptor. They also act synergistically to trigger B-cell proliferation (Clevers et al.,1985). Another calcium ionophore called ionomycin has similar properties (Delia et al.,1984).

This synergy is thought to be due to the involvement of two signalling pathways in T-cell activation (Imboden et al.,1985; Isakov et al,1986). Perturbation of the T-cell antigen receptor stimulates a phosphodiesterase (phospholipase C) to hydrolyse phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) found in the cell membrane. The products of this hydrolysis are diacylglycerol (DG) and inositol tri-phosphate (IP_3) which act as second messengers triggering two signalling pathways (Berridge,1984). IP_3 formation leads to a rise of cytoplasmic calcium ion concentration, chiefly by release

of calcium from internal stores (Imboden & Stobo,1985). Activation of T-cells via the T3 molecular complex by mitogens (Fleischer,1984), OKT3 (Oettgen et al.,1985), or antigens (Nisbet-Brown et al.,1985) typically leads to a calcium ion flux across the cell membrane, providing one signal for activation. This rise in intracellular calcium ion concentration may activate calcium-dependent protein kinases, resulting in protein phosphorylation. Diacylglycerol is thought to act as the other "signal" by activating protein kinase C (PKC), increasing its affinity for calcium ions (Imboden & Stobo,1985; Nishizuka,1984). This activation model is shown in figure 5.1.

Activation via T3/Ti thus generates both calcium flux and activates protein kinase C, but this can be mimicked by an artificially induced calcium flux, using a suitable ionophore, combined with the addition of a phorbol ester which activates protein kinase C (Castagna et al..1982: Niedel et al.,1983; Kikkawa et al.,1983; Weiss et al.,1984b). PHA and OKT3 stimulate a transient redistribution, and consequent activation, of PKC from the cytosol to the cell membrane, reaching a maximum within 20 minutes of stimulation (Farrar & Ruscetti,1986). Activators of PKC lead to internalisation of the T3 complex (Cantrell et al.,1985) similar to that observed during normal T-cell activation. Transient PKC redistribution is also found in monocytes during activation (Meyers et al.,1985) and after IL-2 binds to its receptor (Farrar & Anderson,1985). It should be noted though that IL-2 induced proliferation is independent of a

calcium ion flux (Mills et al.,1985b: Gelfand et al.,1986), unlike mitogenesis, and ionophores plus phorbol esters cannot replace the IL-2 dependent triggering of the IL-2 receptor (Albert et al.,1985). A rise in cytoplasmic free calcium ion concentration acts as a signal for IL-2 production but not for expression of the IL-2 receptor (Mills et al.,1985a) which can occur even when calcium flux is blocked (Gelfand et al.,1986).

There is thus a considerable body of evidence for the existence of the two signal model of T-cell activation. In this study it was clearly shown that ionophore plus tumour promoter could also stimulate high level IFN-gamma production in the absence of any other signal, e.g. mitogen (Table 1). They also acted synergistically to induce IL-2 production when neither could alone (Table 1). While stimulation of IL-2 production by this method has been demonstrated by other workers, the effects on IFN-gamma production had not previously been investigated. They were found to be consistent with other effects on cell activation. Mezerein was the most potent co-activator with A23187 in this study, the combination often inducing significantly greater levels of IFN-gamma than the mitogen SEA. Higher concentrations of teleocidin or TPA were required for similar effects. This may reflect differential capacities between the tumour promoters for protein kinase C binding and activation. On its own A23187 induced only low levels of IFN-gamma at most, with optimal effects at 1μM. At higher concentrations it caused cell death, probably due to gross disturbance of mitochondria

morphology, which act as intracellular stores of calcium ions. Although A23187 alone can lead to blastogenesis of lymphocytes (Luckasen et al.,1974), with enlarged cytoplasm and increased tritiated-thymidine uptake similar to the effects of PHA (Maino et al.,1974), it was not a sufficient signal on its own for significant induction of IFN-gamma.

Similarly, on their own tumour promoters induced at most only low levels of IFN-gamma. Teleocidin and TPA, however, are reported to induce IL-2 receptor expression (Isakov et al.,1985; Ando et al.,1985a&b) and IL-2 independent proliferation (Isakov et al.,1985), presumably via PKC activation.

In view of the reported ability of TPA at least partially to replace macrophage involvement in T-cell activation (Ashman,1984; Koretzky et al.,1982: Davis & Lipsky,1985) it is possible that phorbol esters and IL-1 share to some unknown extent a similar mechanism of T-cell activation. However, IL-1 showed no ability to synergise with A23187 in the induction of IFN-gamma (Table 2), making it unlikely that IL-1 acts via protein kinase C activation in a similar fashion to phorbol esters. The exact role(s) of IL-1 in T-cell activation are still far from clear. It may induce some signal that interacts synergistically with diacylglycerol-activated PKC, as when resting macrophage-depleted T-cells are stimulated with sepharose bound OKT3 plus recombinant IL-1 (Manger et al.,1985). IL-1 may induce some as yet unknown "third" signal in addition to both calcium flux and PKC

activation. This possible third intracellular signal or pathway has been shown to be distinct from yet act synergistically with both calcium flux, inducing IL-2 secretion, and PKC activation, inducing IL-2 secretion and also IL-2 receptor expression (Lowenthal et al.,1986).

Depletion of macrophages prior to induction had different effects on activation by SEA as opposed to A23187/mezerein (Table 3), the latter being able to induce significant IFN-gamma levels in the absence of macrophages. However, there was still a reduction in two donors despite the lack of effect in the other three. This may have been due to differing stringency of depletion between donors. Far fewer macrophages are reportedly required for optimal proliferation in response to TPA than to SEA (Ashman,1984; Davis & Lipsky,1985). Reduction of A23187/mezerein induction occurred only in those donors where the response to SEA was most severely reduced (>10 fold) and not when the depletion may have been less severe (SEA response reduced only 4-5 fold). In the latter case there may have ben a sufficient residual level of macrophages for A23187/mezerein induction to be unaffected compared to SEA, whilst there was some effect when depletion was most stringent (donors 2 and 3). Thus although macrophages are not required for A23187/mezerein to induce significant levels of IFN-gamma, presumably because requirements for macrophage/T-cell interaction for mitogens are bypassed by acting at the level of the two basic transmembrane "signals", very low levels of macrophages may still be required for optimal response

(perhaps for IL-1 to provide a possible "third" intracellular signal). Macrophages may therefore provide signals additional to their involvement in antigen presentation to T-cells which A23187/mezerein cannot wholly replace.

The involvement of the T11 pathway in T-cell activation, discussed in chapter 4, has been shown by many workers, along with its ability to act as a positive or negative signal transducer according to which epitopes are bound by monoclonal antibodies. Differential effects on calcium flux may well be involved since it has been reported that T-cell stimulation via T11 usually involves a calcium flux (Weiss et al.,1984a; Holter et al.,1986) whilst suppression via T11 may block calcium entry (Palacios & Martinez-Maza,1982). In view of the depressive effects of the T11 monoclonal antibody 9.6, the role of calcium was investigated using A23187. In a total of 10 out of 14 experiments, A23187 at least partially reversed the inhibitory effects of 9.6 on SEA induction, but did not boost the level of IFN-gamma production induced by SEA alone. The ionophore would appear then to specifically reverse the inhibitory effects of 9.6 and does not simply act as a general booster of IFN-gamma production by mitogen induction. Presumably the calcium flux induced by SEA, in the absence of 9.6, is optimal for T-cell activation so that A23187 then has no additional effect. Tumour promoters may boost SEA induction, however, because PKC activation by SEA alone is not optimal. It has been suggested that T11 can act as a "negative signal receptor"

by turning off the process by which calcium enters the cell upon stimulation, e.g. OKT11A inhibits proliferation of T-cells stimulated by A23187 alone (Palacios & Martinez-Maza,1982).

The partial reversal of T11 inhibitory effects by A23187 tend to confirm that they are indeed at least partially mediated by inhibition of calcium flux. Also the importance of calcium influx at the commencement of T-cell activation would explain why 9.6 must be added shortly after induction for significant inhibition of IFN-gamma production (Wilkinson & Morris,1984a). However, the failure of A23187 to completely reverse the inhibition and its total lack of effect in some donors suggests that some other inhibitory effects are also involved. Antibodies against T11 are reported to block calcium flux if PHA is used, but not Con A or OKT3 (O'Flynn et al.,1985a), despite their inhibition of proliferation in all three cases (Palacios & Martinez-Maza,1982), suggesting that other, calcium-independent, signals are regulated by the T11 50kD surface protein. Further investigation of the T11 activation/inhibition pathway and its relationship to the T3-Ti pathway are clearly necessary. It has yet to be established, for example, what the in vivo role of T11 is, if any, and what its natural ligand is ("IL-4A" perhaps).

IFN-gamma and IL-2 gene induction.

In order to examine gene activation independently of the downstream effects of immune modulators cDNA probes were used. They also made it possible to examine gene

activation in the absence of protein synthesis. Using cDNA probes for both IL-2 and IFN-gamma mRNA, it was found that ionophore and mezerein separately induced only low level transcription of both genes compared to their synergistic effects. In combination A23187 and mezerein were potent inducers of both genes and thus high levels of IFN-gamma production are probably due to effects at the level of transcription. Also levels of mRNA production for both genes were not depressed by the presence of cycloheximide (CHX) at a concentration sufficient to reduce protein synthesis by >99%. This shows that synthesis of proteins, such as IL-1 or IL-2, is not a requirement for expression of either gene. Siggens et al. (1985) similarly showed that activation of the IFN-gamma gene by SEA could still occur without protein synthesis, at up to 76% of normal levels, but CHX abolished the synergistic effects of mezerein with SEA. This is in contrast to the findings in this study, using A23187 with mezerein, in which CHX had no depressive effect on induction of either gene. It has also been shown that exogenous IL-2 has little or no effect on IL-2 or IFN-gamma mRNAs, but does significantly increase c-myc, IL-2-receptor, and heat shock protein mRNAs (Granelli-Piperno et al.,1986). Thus protein factors appear to have no role in initial activation of either gene by A23187 and mezerein acting in synergy, although they may be involved in synergistic effects of tumour promoters with mitogens (Siggens et al.,1985). In some cases CHX even boosted the level of induction. This may have been due to partial "superinduction", a variety of

metabolic inhibitors having been shown to boost the level of IL-2 mRNA (Efrat et al.,1984; Efrat & Kaempfer,1984) and IFN-gamma mRNA expression (Siggens & Morris,1985), possibly by blocking production of a labile repressor protein. A longer period of induction is usually required for "superinduction" to become obvious, however. Other workers have found similar synergistic effects for IL-2 mRNA production using TPA and PHA (Hirano et al..1984: Efrat et al.,1982) and TPA with A23187 (Yamamoto et al.,1985). These effects appear to be specific since the level of "housekeeping" non-lymphokine gene expression, such as actin, microglobulin, and c-myb, has been found to be unaffected by signals which induce IL-2 and IFN-gamma genes (Wiskocil et al.,1985; Granelli-Piperno et al..1986).

Examination of mRNA levels in PBML compared to macrophage-depleted lymphocytes, at 4 and 24 hours post-induction, revealed that macrophage-depletion increases the level of gene expression. The level of mRNA in PBML peaked early with little still detectable at 24 hours compared to 4 hours post-induction. In comparison the mRNA levels, although reduced by 24 hours, were still detectable in macrophage-depleted lymphocytes. These results suggest that macrophages are involved in regulating expression of both genes since their absence both boosts the level of induction and retards the rapid shut-down of mRNA expression found in PBML. Some macrophage product may act to depress gene induction, only slightly at 4 hours, but significantly at 24 hours

post-induction. It would be useful to examine the effects of CHX on PBML at 24 hours to determine if mRNA levels are restored to those found in lymphocytes, although this may be too long an exposure to a metabolic inhibitor. The hypothetical inhibitory macrophage product may be the same as the putative labile repressor protein suggested to be involved in superinduction.

The rapid accumulation and subsequent shut-down of IFN-gamma gene expression observed here is reminiscent of that seen in preactivated lymphoblasts as compared to fresh lymphocytes. IFN-gamma mRNA peaking at 8 hours in the former and up to 4 days post-induction in the latter (Siggens et al.,1984). Also, levels of mRNA induced in lymphoblasts have been found to be up to five times greater per cell than induced in previously unstimulated T-cells (Granelli-Piperno et al.,1986). Thus activation by A23187 and mezerein may be as rapid as in metabolically active growing T-lymphoblasts because preliminary stages involved in activating resting T-cells are bypassed in both cases.

The importance of two signals in T-cell activation are thus confirmed in this study. Furthermore, various observations suggest that activation of IL-2 and IFN-gamma genes occurs coordinately when these signals are provided.

1. These signals can induce both genes in the absence of translation, suggesting that they act on transcription and that the protein product of one gene is not required for induction of the other gene.

2. The levels of mRNA for both genes are affected in a

similar fashion by macrophage depletion and the type of inducer(s) used. Situations which induce high or low levels of RNA for one gene have the same effect on the transcription of the other gene.

3. The mRNA levels for both genes follow a similar time course.

Very similar findings were made in a study using TPA plus mitogen to activate the T-cell line Jurkat in which coordinate expression of IL-2 and IFN-gamma genes also seems to occur (Wiskocil et al..1985). Grabstein et al. (1986) have also shown that IL-2 mRNA and IFN-gamma mRNA both show early accumulation after PHA stimulation, peaking simultaneously at 60 minutes. However, IFN-gamma mRNA exhibits a secondary greater peak at 20 hours post-induction subsequent to its earlier peak (Grabstein et al.,1986). The first IFN-gamma mRNA peak is probably independent of any IL-2 requirement, since it occurs coordinately, but the later peak would appear to be IL-2 dependent. However, no sign of this later peak was found using A23187 plus mezerein as inducers of PBML as opposed to PHA. This may have been due to some difference between mitogen activation and the artificial system of ionophore plus tumour promoter, although Efrat et al. (1982) also used PHA as the inducer and found that both messages levelled off by 16 hours post-induction.

Thus despite the apparent sequential nature of lymphokine production, with IL-2 binding to its receptor being a requirement for optimal IFN-gamma production, at the level of transcription at least the two lymphokines

appear to be coordinately regulated using the induction method described in this chapter. Both genes can be transcribed in the absence of protein synthesis and there does not seem to be any delay between appearance of each message. Efrat et al.(1982) also observed very similar kinetics for appearance of both messages, using PHA activated lymphocytes at a similar cell density to that used in this chapter and by Grabstein et al. (1986), beginning at 4 hours and levelling off at 16 hours. It should be noted that the kinetics of appearance of IFN-gamma mRNA in PBML are partly dependent on cell density during incubation with the inducer, as well as the nature of the inducer and the state of the cells, higher cell densities giving more rapid induction. The peak of IFN-gamma mRNA accumulation in response to mitogen reportedly occurs earlier (1-2 days versus 3 days) at $3x10^6$ cells/ml than at $5x10^5$ cells/ml (Siggens et al.,1984).

Possibly the two signals induce production of an intermediate substance which influences both genes or RNA processing and stability. It is interesting to note that there is a homologous sequence (83%) in the 5' flanking region of both IL-2 and IFN-gamma genes 300 bp upstream of the genes' promoter in a region of potential regulatory importance (Hardy et al.,1985). This common sequence is highly conserved, e.g. 85% homology in the IL-2 genes of mouse and man while the coding region is only 72% homologous (Fuse et al.,1984), and may well be involved in coordinate expression. The methylation state of the

IFN-gamma gene may also play a role in its regulation, since demethylation of some unknown sequences (not the gene itself, which can be active and methylated) restore the ability of a murine T-cell line to produce IFN-gamma in response to IL-2 (Farrar et al.,1985). Some of the deleterious effects of human T-lymphotropic viruses types I and III (AIDS) may be due to disruption of the regulation of IL-2 and IFN-gamma gene expression. In HTLV III (AIDS) infected cells IL-2 gene transcription, induced by PHA/TPA, is unaffected but IFN-gamma transcription is inhibited. The reverse is the case in HTLV I infected cells where the IFN-gamma gene is actively transcribed but the IL-2 gene is not (Arya & Gallo,1985). Such effects are surprising in view of the coordinate expression described in this study.

The depressive effects of 9.6 binding to T11 may also occur at the level of transcription, since 9.6 added within 4 hours of induction is reported to inhibit IL-2 mRNA accumulation, the level of inhibition being inversely proportional to the strength of the mitogenic signal (Tadmori et al.,1986). As in the case of Wisckocil et al. (1985), these effects on IL-2 gene expression are relatively specific since beta-actin gene expression is not affected.

In conclusion, the results described in this chapter demonstrate that mitogenic stimulation of IFN-gamma production can be mimicked by the combination of two signals, calcium flux and protein kinase C activation, using A23187 and phorbol esters. Using these activators

the requirements for macrophages as accessory cells can be at least partially bypassed and the inhibitory effects of the T11 pathway reversed, using A23187. High levels of IFN-gamma production are reflected by rapid induction of IFN-gamma and IL-2 gene expression, the two genes seeming to be coordinately inducible. This induction does not require the production of other lymphokines or proteins. Macrophages are also found to be involved in regulating the level of gene expression and its rapid shut-down. These findings are consistent with those of other workers using a variety of activation methods. Induction with A23187 plus mezerein therefore provides a useful model for examining the mechanisms by which calcium ion mobilisation plus protein kinase C activation leads to T-cell activation at the level of lymphokine protein and mRNA production.

CHAPTER 6.

GENERAL DISCUSSION AND CONCLUDING REMARKS.

In this concluding chapter the major points of the thesis and how they are interrelated will be discussed, along with possible further avenues of investigation suggested. The original aim of the thesis was to investigate at least some of the factors involved in regulating human IFN-gamma production because of the possible importance of this lymphokine in the immune response. IFN-gamma production also serves as a more specific marker of T-cell activation than proliferation. These two processes often occur together but are not in fact linked. Since no significant qualitative differences have been found between antigen and mitogen activation, polyclonal mitogen activation is a reasonable model for the specific activation caused by antigens. Furthermore mitogen-like activation may well occur in vivo, caused by bacteria and bacterial products, and is therefore worthy of investigation in its own right. However, it would also be possible to investigate antigen activation of PBML by using antigens to which the majority of the population have been exposed, such as the tuberculin purified protein derivative. This, however, would only provide information on secondary responses. Primary reponses are too small to examine easily in mixed T-cell populations.

PBML form a very heterogeneous population of cells and yet IFN-gamma production was originally ascribed only to T lymphocytes, with macrophages acting as accessory cells. More recently attention has focused on the non-T cell

natural killer population known as LGL, which are themselves a very heterogeneous population. They have been described as producing a variety of lymphokines and in this study it was shown that they can also produce IFN-gamma. Furthermore, unlike T-cells, they do not require macrophages for this production, perhaps because of their ability to produce lymphokines, such as IL-1, which are needed for optimal IFN-gamma production and which T-cells cannot produce. Optimal IFN-gamma production was shown to require the interaction of at least two LGL subsets. It is argued that Leu 11+ NK cells (E-) can not only produce IFN-gamma but may also act as accessory cells for low density T-cells (E+) in the absence of macrophages. This particular interaction of LGL subsets has not previously been described, although there is growing evidence for other LGL subset interactions. Thus not only can non-T cells produce IFN-gamma but they may also be able to regulate its production by T-cells. Since LGL form only a small fraction of the total PBML, how important is their contribution to the overall response? Can LGL act as accessory cells for all T-cells?

Since LGL do not express T3 and the associated T-cell antigen receptor, how are they activated by mitogens, which are thought to cause activation by binding to these structures? Presumably mitogens must activate Leu 11+ LGL via some other cell surface receptor, implying that the T3-Ti pathway is not the only one that leads to IFN-gamma production. Different receptors may be involved for different IFN types, since mitogens cause IFN-gamma

production but tumour cells cause IFN-alpha production. Since LGL may be related to T-cells, is IFN-gamma production triggered by essentially the same processes as in T-cells? The reported inhibitory effects of anti-Tac on LGL suggest that they resemble T-cells at least in this respect. A considerable amount of work on the interactions of the various LGL subsets hinted at in this study awaits. LGL are reported also to produce IL-1 and IL-2, but are these producer cells the same as those that produce IFN-gamma?

However, the bulk of IFN-gamma production by PBML is ascribable to T-cells. Most of the work described in this thesis was aimed at investigating the role of various regulatory molecules in IFN-gamma production. The recent availability of highly purified and recombinant interleukins at last makes it possible to investigate the proposed role of these lymphokines in T-cell activation in the absence of any other contaminating regulatory substances. The work described here to a large extent confirms the existence of the lymphokine "cascade" with regards to IFN-gamma production. IL-1 production was shown to be a major component of the accessory role of macrophages during mitogen activation since purified IL-1 could largely replace macrophages. This may not be the case for antigens, other non-secretory properties of macrophages such as antigen presentation also being important. There also appeared to be differences in the importance of macrophages and IL-1 between the different mitogens which require further investigation. We also need

to know more about IL-1's exact role(s) in T-cell activation. What are the processes involved in regulating IL-1 production? It is still not clear whether IL-1 acts by triggering or amplifying IL-2 production or IL-2 expression or both? The availability of recombinant IL-1 and probes for mRNA expression of the IL-2, IL-2 receptor, and IFN-gamma genes now make it possible to examine if IL-1 acts at the level of gene activation and also which of these genes are involved. We also need to know a lot more about the recently identified IL-1 receptor(s). Is its expression regulated in any way, as the IL-2 receptor is?

It had not previously been clearly demonstrated that IL-2 is involved in triggering IFN-gamma production, as proposed by the lymphokine cascade. Using anti-Tac to block IL-2 binding to its receptor, this study confirmed that IL-2 binding is definitely required for optimal IFN-gamma production. The reversal of this effect with IL-2 but not IL-1 confirms that anti-Tac's effects are indeed IL-2 mediated. However, the low affinity of anti-Tac for the IL-2 receptor compared to IL-2 itself meant that not all IL-2 binding could be blocked. Thus the effects of anti-Tac decreased with increasing strength of mitogen stimulation. It was thus not possible to determine if IFN-gamma production was wholly dependent on IL-2 binding. Are there any situations in which IL-2 or its receptor are not produced but IFN-gamma production can still occur (perhaps by LGL)? Thus are all the regulatory situations for IFN-gamma described in this study due to

effects on IL-2 production or IL-2 receptor expression carrying through to IFN-gamma production? IL-2 induced proliferation and IFN-gamma production presumably follow a common pathway up to some point. But clear differences have been demonstrated between the two processes since there is often no correlation between them, as shown in this study, and since blockage of either process has been shown not to necessarily inhibit the other. Just where do these pathways diverge? The results using cDNA probes demonstrates that IFN-gamma gene activation is a primary event in T-cell activation which does not rely on production of other proteins.

IL-2 alone was not found to be sufficient for IFN-gamma production. The low levels of IFN produced by high concentrations of IL-2 are thought to be due to the presence of a small fraction of activated T-cells expressing the IL-2 receptor in an otherwise unactivated PBML population. The presence of some T-cell activator, such as a very low level of mitogen, was required for exogenous IL-2 to boost IFN-gamma levels.

The effects of other physiological ligands were also investigated. Prostaglandin E_2 which is produced *in vivo* by a variety of cell types, including macrophages, was found to depress IFN-gamma production. It has been reported to depress IL-2 production as well and its effects on IFN-gamma production appeared to be a consequence of this. Thus PGE_2 appears to have antagonistic effects to IL-1, also a macrophage product. Perhaps these depressive effects of PGE_2 have some *in vivo*

role, helping to make T-cell activation transitory by depressing the immune response.

A variety of cell surface molecules connected with the immune system were also found to have effects on IFN-gamma induction. Monoclonal antibodies against these cell surface molecules were used as model ligands. Molecules involved in MHC restriction, T4 and T8 on T-cells and class I and II MHC molecules, and hence T-cell activation were not unsurprisingly also found to have regulatory effects on IFN-gamma production. Blockage of T-cell/macrophage MHC interactions with antibodies at the initial stages of T-cell activation depressed IFN-gamma induction. However, it may not be simply a case of antibodies blocking cell interactions. Perhaps they cause T4 and DR cell surface molecules to act as negative signal transducers.

The T-cell surface molecule T11 (CD2) is drawing increasing attention. In this study it was confirmed that anti-T11 moabs can inhibit IFN-gamma production. The reversal of this inhibition by exogenous IL-2 suggests that the effect is due to depressed IL-2 levels. The T11 molecule appears to mediate an alternative activation/suppression pathway to T3-Ti. Just what the role of this pathway is <u>in vivo</u> is far from clear, but it does seem to be partly linked to T3-Ti mediated activation in some fashion. The recent description of a proposed natural ligand for T11, which helps activate T-cells and has been tentatively named "IL-4A", makes the involvement of the T11 pathway in T-cell ativation of even greater

interest. Just how do the T11 and T3-Ti pathways interact in vivo? Would antibodies against "IL-4A" inhibit T-cell activation?

Now that the mystery of the identity of the T-cell antigen receptor has been essentially solved, attention should now switch to the intracellular processes mediated by antigen or mitogen binding. It has been shown, both in this study and by many others, that calcium ionophores and tumour promoting phorbol esters can mimic normal T-cell activation by causing a rise in calcium ion levels and activating protein kinase C. Very high levels of IFN-gamma are induced by these two substances acting synergistically. Does this induction require IL-1 and IL-2 production, or is the lymphokine cascade bypassed and IFN-gamma production activated directly? Experiments with anti-IL-1 and anti-Tac should give some indication, although very high affinity antibodies against IL-2 and its receptor will be needed to overcome the very high titres of IL-2 that A23187/mezerein probably also induces. The ability of A23187/mezerein triggering to largely bypass the macrophage requirement for optimal IFN-gamma production suggests that they do directly activate IFN-gamma production without any source of IL-1 being needed.

Unlike tumour promoters, IL-1 did not appear to act synergistically with A23187, suggesting that it does not act by protein kinase C activation. Also, IL-2 induced proliferation is reported to be independent of calcium ion flux, another difference from IFN-gamma production. So

just what intracellular events are triggered by IL-1 and IL-2, and how do they differ from those caused by the T3-Ti pathway? The ability of A23187 to reverse the inhibitory effects of the T11 pathway suggests that the latter has effects on calcium ion concentration.

The most interesting finding using this induction method was that although optimal IFN-gamma production requires the presence of IL-2, their genes are expressed coordinately rather than sequentially. Similar findings have been made by other groups using mitogens as inducers. Furthermore production of their mRNAs does not require protein synthesis. Thus IL-1 and IL-2 may be needed for production of IFN-gamma but not for activation of its gene. This suggests that IL-1 and IL-2 act as regulators of IFN-gamma beyond the level of transcription, at the level of translation, or possibly message processing or stability, or even secretion. Thus initial T-cell activation may trigger coordinate activation of several lymphokine genes, but translation of their messages seems to be sequential and rely on the lymphokine cascade. It is also possible, however, that IL-1 and IL-2 binding to their receptors trigger fresh bursts of gene activation, such as the reported double peak of IFN-gamma mRNA.

The coordinate induction may involve the concensus sequence reported to be upsteam of both the IL-2 and IFN-gamma genes, in which case any regulatory factor(s) which bind to this sequence must be identified.

The kinetics of IFN-gamma mRNA expression were more rapid than has been reported when mitogens are the

inducers. This may be due to A23187 and mezerein bypassing the initial steps of T-cell activation triggered via the T-cell receptor, such as $PtdInsP_3$ hydrolysis. Activation of both genes in PBML was shut down within 24 hours, neither message being detectable at this time. However, this appeared to be regulated by macrophages since their depletion both boosted the general level of induction and lengthened the period over which both mRNAs were detectable. Are macrophages down-regulators of IL-2 and IFN-gamma gene activation or message stability? This would be in contrast to their importance as accessory cells, acting as positive regulators of IFN-gamma protein production. Further experiments involving the effects of macrophage-depletion on induction of IL-2 and IFN-gamma mRNAs are required to check this possibility, since regulation of the termination of IFN-gamma production is as crucial as its activation. Would the addition of IL-1 or PGE_2 to lymphocyte cultures affect the level of these mRNAs? What about the effects of anti-T11 moabs or "IL-4A" on lymphokine gene activation?

Whether all of the regulatory situations which occur *in vitro* are also relevant *in vivo* is far from clear. Hopefully work on animal models and diseases of the human immune system will provide some of the answers. More clear cut information on the *in vivo* importance, or otherwise, of IFN-gamma should be obtained by studies with mice presently being made at the University of Warwick using recently prepared antibodies against murine recombinant IFN-gamma.

In conclusion, it is clear that regulation of IFN-gamma production is a complex process dictated by the interaction of a variety of natural regulatory molecules and cell types. The great heterogeneity of PBML means that these interactions are both complex and far from completely understood. The ability of LGL also to produce IFN-gamma makes the situation even more complicated. The results described in this study have confirmed the proposed importance of several positive immune modulators in IFN-gamma production, such as IL-1 and IL-2, and additionally indicated that several down-modulators may also be involved, such as PGE_2. Several cell surface molecules can also exert regulatory effects, probably via their involvement in MHC restriction during T-cell activation. The alternative T11 pathway may also be involved. It is also now possible to begin investigating some of these regulatory effects at the molecular level and a start has been made in this study. This avenue of research should prove particularly rewarding in the near future.

CHAPTER 7.

MATERIALS AND METHODS

MATERIALS

a) Mitogens :

1. Purified Staphylococcal enterotoxin A (SEA) was supplied by the Food and Drug Administration of the U.S.A. (Washington, D.C.).

2. Crude Staphylococcus aureus supernatant was supplied by Dr. A. Meager (National Institute of Biological Standards and Control, Holly Hill, Hampstead, London).

3. Purified phytohaemagglutinin (PHA) from Wellcome Reagents Ltd.

4. Concanavalin A (Con A) from Miles-Yeda Ltd.

b) Tumour Promoters :

1. Mezerein from L.C. Services Corporation.

2. 12-O-tetradecanoyl phorbol acetate (TPA) from Sigma Chemical Co. Ltd.

3. Teleocidin was provided by Dr. H. Fujiki (National Cancer Research Institute, Tokyo).

c) Polyclonal Antibodies :

1. Rabbit anti-human IL-1 binds to the common smaller molecular weight form of IL-1 (natural and recombinant) pIs 5.0 and 7.0 (17.5kD) and also to the larger molecular weight precursor (31-40kD). Supplied by Genzyme Koch-Light Ltd.

2. Sheep anti-human IFN-gamma serum was supplied by Dr. A.

Meager at the National Institute for Biological Standards and Control (Holly Hill, Hampstead, London) and prepared against >95% pure recombinant IFN-gamma obtained from Genentech Inc.

3. FITC (fluorescein isothiocyanate) conjugated goat anti-mouse IgG from Sigma Chemical Co. Ltd.

4. FITC conjugated goat anti-mouse IgM from Sigma.

5. RITC (rhodamine isothiocyanate) conjugated goat anti-mouse IgM from Sigma.

6. FITC conjugated sheep anti-human IgG from Wellcome Reagents Ltd.

d) Monoclonal Antibodies (Murine anti-human) :

1. OKT3 (IgG_{2a}) from Ortho Diagnostic Systems Ltd.

2. OKT4 (IgG_{2b}) from Ortho.

3. OKT8 (IgG_{2a}) from Ortho.

4. OKM1 (IgG_{2b}) from Ortho.

5. Leu 11b (IgM) from Becton-Dickinson Monoclonal Center Inc.

6. Anti-human IL-2 receptor from Becton-Dickinson.

7. Anti-Tac (human IL-2 receptor) was kindly provided by Dr.T.A.Waldman of the National Cancer Institute (Bethesda, MD, USA) in ascites form containing approximately 6-8mg/ml of IgG_{2a} antibody.

8. 9.6 (anti-E receptor; IgG_{2a}) from New England Nuclear Ltd.

9. Anti-BBMI (against beta-chain of MHC class I MHC antigens) was kindly provided by Dr.E.Jones (Biological Sciences Dept., Warwick University, Coventry) in ascites

form (IgG_2).

10. W6/32 (anti-class I heavy chains) was kindly provided by Dr.E.Jones (see above) in ascites form (IgG_2).

11. W6/34 (anti-species antigen 1) was kindly provided by Dr.E.Jones (see above) in ascites form (IgG_2).

12. Anti-DR was kindly provided by Dr.W.Bodmer of the Imperial Cancer Research Fund.

13. Anti-DR also from DAKO.

e) Interleukins :

1. Human Ultrapure Interleukin 1 from Genzyme Koch-Light Ltd.

2. Human Ultrapure Interleukin 2 from Genzyme Koch-Light Ltd.

3. Recombinant Human Interleukin 2 from Biogen.

f) Media :

Medium RPMI 1640, GMEM (Glasgow's modification of Eagle's Minimal Essential Medium), foetal calf serum and newborn calf serum were all obtained from Gibco Ltd. RPMI was buffered with 20mM Hepes (pH 6.9) and GMEM with bicarbonate (pH 7.0). All media was supplemented with penicillin (60μg/ml) and streptomycin (100μg/ml), both obtained from Glaxo Laboratories Ltd., plus 2mM glutamine. GMEM was also supplemented with non-essential amino acids (NEAA).

g) Cells :

1. Buffy coats were obtained from the U.K. West Midlands

Blood Transfusion Centre (Edgbaston, Birmingham) on the same day of use. The buffy coats were prepared in the presence of citrate by two centrifugation steps to remove the majority of the plasma and the red blood cells.

2. WISH human amnion cells from Flow Laboratories Ltd.

3. Sheep erythrocytes from Tissue Culture Services.

4. K562 human erythroleukemic cells from Ian Kimber at the Patterson Laboratories, Christie Hospital, Manchester.

5. CTLL from Dr.R.Wilsoncroft, St. Thomas Hospital, London.

h) Radiochemicals :

1. 5-^{3}H-Uridine (29Ci/mmol) from Amersham Radiochemicals PLC.

2. Methyl-^{3}H-thymidine (5-10Ci/mmol) from Amersham.

3. ^{51}Cr from Amersham.

4. ^{32}phosphate labelled dCTP and dGTP (3000Ci/mmol) from Amersham.

i) Other Materials :

Actinomycin D (Sigma)

Bovine serum albumen (Sigma)

Butanol (Fisons)

Caesium chloride (BRL)

Chloroform (May & Baker Ltd)

Complement (Rabbit; Pel Freeze)

Cycloheximide (Sigma)

DEPC (Diethyl pyrocarbonate; Sigma)

EDTA (Diaminoethanetetra-acetic acid, sodium salt; Sigma)

Eppendorf tubes, 1.5ml (Sarstedt)

Ethanol

Ficoll-Hypaque (Pharmacia)

Flat bottomed microtitre trays (Flow)

Formamide (Fisons)

Guanidinium isothiocyanate (Fluorochem Ltd.)

Hydrochloric acid 1M (BDH Chemicals Ltd)

Human IFN-gamma standard

International human IFN-gamma standard (Research Resources Section, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA).

Ionophore A23187 (Sigma)

2-mercaptoethanol (BDH Chemicals Ltd.)

Micropipette tips, 200µl & 1ml (LIP Ltd)

Microtiter trays: round bottomed (Flow Labs Ltd.) and flat bottomed (Sterilin)

Nitrocellulose filter membrane (Anderman & Co. Ltd)

Non-specific esterase staining kit (Sigma)

Nylon wool (Leuko Pak)

Percoll (Pharmacia)

Plastic universals and petri dishes (Sterilin)

Prostaglandin E2 (Sigma)

PBS (pH 7.4)

Salmon Sperm DNA (Sigma)

Sephadex G75 (Pharmacia Fine Chemicals Ltd.)

Scintillation fluid: EP or NA (Beckman Ltd.)

Sephadex G75 (Pharmacia)

Silica (Sigma)

Sodium hydroxide 1M (BDH Chemicals Ltd)

Trichloroacetic acid (May & Baker Ltd.)

Tris = TRIZMA base (Trishydroxymethylaminomethane: Sigma)

Trypsin (Sigma)

Ultracentrifuge tubes (MSE)

X-ray film (Fuji)

j) Buffers and Solutions.

1. Lytic Buffer:

4M guanidinium isothiocyanate

1M 2-mercaptoethanol

20mM sodium acetate pH 5 = (600µl 3M NaAc pH 6 + 110µl 3M NaAc pH 5)

Solution must be warmed at 37°C to dissolve the isothiocyanate, and then filtered through Millex or Nalgene filters. Store in the dark at room temperature.

2. Caesium chloride solution:

5.7M CsCl

Refractive index should be adjusted to 1.405. Filter through Millex filter before use.

3. DEPC treated water:

0.1% DEPC (Diethyl pyrocarbonate)

Autoclaved to remove residual DEPC.

4. Tris:

5mM Tris (pH 7.5) in DEPC treated water.

5. $T_{10}N_{150}E_1$:

10mM Tris (pH 7.5)

150mM NaCl

1mM EDTA (pH 7.5)

6. 20X SSPE:

   3.0M NaCl

   200mM NaH_2PO_4 (pH 7.4)

   20mM EDTA (pH 7.4)

7. 20X SSC:

   3M NaCl

   0.3M Na citrate (pH 7)

8. Denhardt's solution:

   0.02% Ficoll

   0.02% polyvinylpyrrolodine

   0.02% bovine serum albumen

9. 10X RP buffer:

   500mM Tris HCl (pH 7.5)

   500mM NaCl

   100mM $MgCl_2$

   70mM beta-mercaptoethanol

   500µg/ml bovine serum albumen

10. Phosphate buffered saline (PBS) pH 7.4:

    0.8% NaCl

    0.02% KCl

    8mM disodium hydrogen phosphate

    1.5mM potassium dihydrogen phosphate

11. RNA loading dye :

    5mM EDTA

    40% Ficoll

    0.05% bromophenol blue

k) Names and adresses of suppliers:

Amersham International : White Lion Rd., Amersham, Bucks HP7 9LL.

Anderman & Co. Ltd : Central Avenue, East Molesey, Surrey.

Beckman Ltd. : High Wycombe, Bucks.

Becton-Dickinson Monoclonal Center Inc. : Mountain View, CA, USA.

BDH Chemicals Ltd.: Poole, Dorset.

Biogen : Geneva, Switzerland.

BRL-Life Technologies Inc. : PO Box 6009, Gaithersburg, MD, USA.

DAKO : DAKOPATTS Ltd., Copenhagen, Denmark.

Fenwal Laboratories : Illinois, USA.

Fisons plc : Bishops Meadow Rd., Loughborough, Leics LE11 0RG.

Flow Labs Ltd. : Irvine Industrial Estate, PO Box 17 Second Avenue, Ayrshire, Scotland, KA12 8NB.

Fluorochem Ltd. : Peakdale Rd., Glossop, Derbyshire SK13 9XE.

Fuji Photo Film Co. Ltd : Tokyo, Japan.

Genentech Inc. : San Francisco, CA, USA.

Genzyme Koch-Light Ltd. : Hollands Rd., Haverhill, Suffolk CB9 8PU.

Gibco Europe Ltd. : PO Box 35 Trident House, Renfrew Rd., Paisley, Scotland, PA3 4EF.

Glaxo Laboratories Ltd. : Greenford, England.

L.C. Services Corporation : Woburn, MA, USA.

Leuko Pak : Fenwal Laboratories, Illinois, USA.

LIP (equipment & Services) Ltd : 111 Dockfield Rd.,

Shipley, W. Yorkshire.

May & Baker Ltd. : Dagenham

Miles-Yeda Ltd. : Box 37, Stoke Poges, Slough, Berkshire.

MSE (Measuring & Scientific Equipment Ltd) : Manor Rd., Crawley, W. Sussex.

New England Nuclear Ltd. : 2 New Rd., Southampton SO2 0AA.

Ortho Diagnostic Systems Ltd. : Station Rd., Loudwater, High Wycombe, Bucks HP10 9UF.

Pel-Freez Biologicals : PO Box 68, Rogers, AR, USA.

Pharmacia Ltd. : Midsummer Blvd. Centre, Milton Keynes, Bucks MK9 3HP.

Sarstedt : Germany

Sigma Chemical Co. Ltd. : Fancy Rd., Poole, Dorset BH17 7NH.

Sterilin : Feltham, England.

Tissue Culture Services : 10 Henry Rd., Slough, Berkshire SL1 2QL.

Wellcome Reagents Ltd. : 303 Hither Green Lane, London SE13 6TL.

METHODS

a) Cell preparation and culture

i) PBML.

Peripheral blood mononuclear leukocytes (PBML) were obtained by centrifugation over Ficoll-Hypaque of human buffy coat residues from normal blood. The cells were cultured in RPMI-1640 medium buffered with HEPES and supplemented with 10% foetal calf serum (FCS), penicillin (60μg/ml) and streptomycin (100μg/ml). This medium was used for all procedures and incubations involving PBML and fractions of PBML, unless otherwise stated.

ii) Lymphocytes.

Macrophage-depleted lymphocytes were prepared by replating PBML three times at hourly intervals on plastic petri dishes to remove adherent cells. Silica was then added at 200μg/ml overnight to eliminate the few remaining phagocytic cells. The lymphocytes were then isolated over Ficoll at 350G to remove the silica and cell debris. This method produced lymphocyte preparations containing <1% macrophages judged by non-specific esterase staining.

iii) Macrophages.

Macrophages/monocytes were isolated by virtue of their density and adherent properties. Since they are of lower density than the majority of lymphocytes, macrophages were initially enriched for by centrifugation of PBML on a 4 step Percoll/RPMI gradient consisting of 40, 43, 46, and

50% Percoll fractions. The Percoll was buffered by mixing 8 parts of 10x concentrated phosphate buffered saline (PBS) with 92 parts Percoll, before adding RPMI 1640/10% FCS for the required concentrations. After centrifugation the lowest and highest density fractions were discarded since they contain mainly platelets and lymphocytes, respectively. The middle two fractions which are enriched for macrophages were incubated (37°C) on FCS treated plastic petri dishes (prepared by a 4 hour treatment of the plates with FCS at 4°C). Non-adherent cells (lymphocytes) were removed at intervals over the next 18 hours (at least 5 times). All macrophage/monocyte preparations obtained by this method contained >99% macrophages as judged by the india ink test (1 hour incubation in 0.5% ink, followed by visual examination for any non-phagocytic cells remaining). Macrophages were removed from the FCS treated plates by incubation at 4°C for at least 30 minutes in chelating buffer (0.2% EDTA and 5% FCS in PBS). This caused resuspension of the adherent cells, aided by vigorous pipetting and several washes with PBS.

iv) Low density lymphocytes.

Separation of lymphocytes into differing density sub-populations was achieved by centrifugation at 550G for 30 minutes over a six step discontinuous Percoll/RPMI gradient whose steps varied from 40% to 52.5% Percoll. Cells were harvested from each layer and counted to determine the percentage of the total lymphocyte

population present in each step. Cells from the top one to three steps were used as the low density fraction, varying from 5 to 30% of the total lymphocyte population, the percentage depending on efficiency of separation. The low density lymphocytes were chiefly found at the interface between the 42.5 and 45% steps. In later experiments a single 46% Percoll cushion was used to isolate low density cells. Cells isolated from the bottom three steps or below the 46% interface were considered high density lymphocytes (i.e. chiefly T-cells).

v) High affinity sheep red blood cell rosetting.

The low density population was further fractionated into E+ and E- cells by high affinity sheep red blood cell (SRBC) or erythrocyte (E) rosetting which separates T from non-T cells by virtue of the high affinity sheep erythrocyte or E receptor found on all T-cells (Timonen et al.,1981). Lymphocytes were washed twice in PBS and resuspended in RPMI at $2x10^6$ cells/ml. SRBC, after three washes in PBS, were resuspended in RPMI at $3x10^8$ cells/ml. Equal volumes of lymphocytes and erythrocytes were mixed and then an equal volume of FCS added. After gentle pelleting (200G) the cells were incubated at 29°C for one hour. After gentle resuspension with a wide bore pipette, in order not to disturb any "rosettes" formed between T-cells and SRBC, the rosetted and non-rosetted cells were separated by Ficoll centrifugation (30 minutes at 500G). Only the denser rosetted E+ cells fell through the Ficoll to form a pellet, whilst the E- cells formed a layer above

the Ficoll. After checking visually that rosettes had actually formed, SRBC were removed from the rosetted lymphocytes by incubation in lysis buffer (0.14M ammonium chloride, 20mM tris, pH 7.5) for 10 minutes at 37°C, followed by three washes in PBS.

vi) Nylon wool column filtration.

Both adherent macrophages and B cells could be removed from PBML by nylon wool chromatography (Julius et al.,1973). For 10^8 cells a 10ml syringe was packed with 0.6g of nylon wool (sterilised as an assembled unit). The column was equilibrated with RPMI 1640/2% FCS medium for 45 minutes at 37°C, rinsed with 10ml of medium, and then 5ml of $1x10^8$ cells was loaded on. After 45 minutes at 37°C, the nylon wool non-adherent cells were eluted with warmed medium at a rate of one drop per second until no further cells were collected.

vii) Induction of IFN-gamma production and metabolic activation.

Cells were cultured at 37°C in fresh medium (RPMI 1640/10% FCS) at a cell density of $5x10^5$ cells/ml. Round-bottomed 200µl well microtiter trays (Flow Labs Ltd.) were used. Inducers were added at the concentrations stated in the results sections, usually those found to give optimum stimulation, i.e. SEA at 20ng/ml, PHA at 10µg/ml, Con A at 20µg/ml, and OKT3 at 10ng/ml. Supernatants were harvested 3 days post-induction, unless otherwise stated, since IFN-gamma production usually

peaked at this time. The level of general cell activation, as measured by ^{3}H-thymidine incorporation, was determined 3-4 days post-induction.

b) Characterisation of cell fractions using immunofluorescence.

Indirect immunofluorescence was used to characterise fractions by virtue of the cell surface markers that they expressed. This involved an initial incubation with the required mouse monoclonal antibodies and a second incubation with fluorescein (FITC) labelled goat anti-mouse Ig. An IgG specific FITC conjugate was used with all the monoclonal antibodies except Leu 11. This required an IgM specific FITC or rhodamine (RITC) conjugate. A 100µl volume of cells at 10^{7} cells/ml were incubated for 30 minutes at 4°C with 5µl of the mouse monoclonal antibody. Every 10 minutes the mixture was gently shaken. The cells were then washed 3 times in cold PBS and the entire procedure repeated with 50µl of FITC-labelled anti-mouse Ig. This dilution of conjugate usually ensured that non-specific binding was kept to a minimum, i.e. <5% of the cells appeared fluorescent in the reagent control. The cells were then resuspended in 80ul of glycerol:PBS for storage at -20°C or examination on a UV fluorescence microscope. The percentage of positive cells was determined by judging which cells were more fluorescent than control cells (treated only with FITC labelled anti-mouse Ig) and examining at least 200 cells per sample. However, if the cells were to be analysed by

FACS (fluorescence activated cell sorter) then they were fixed in a 3% paraformaldehyde solution instead.

A direct procedure was used to determine the percentage of human B-cells. Cells were incubated with FITC labelled rabbit anti-human IgG by the same procedure as above. A B-cell line was shown to be 100% positive by this method.

c) Assay Methods.

i) IFN assay.

Interferon was assayed by the INAS-50 method (Atkins et al.,1974) using Semliki Forest virus to challenge human amnion (WISH) cells. Viral replication was measured by tritiated uridine incorporation, the extent of which depended on the degree to which an IFN sample had induced an antiviral state.

WISH cells were grown in GMEM medium plus 10% newborn calf serum (NBS) at 37°C in 5% CO_2 humidified incubator. For an assay they were plated out in flat bottomed microtitre trays at approximately $4x10^4$ cells/well 18 hours before use in order to allow the cells to adhere to the well bottom surface. Cells were initially plated out in GMEM/10% NBS, but this was replaced with 180ul/well of GMEM/2% NBS (minimum maintenance level to reduce cellular RNA synthesis) before making dilutions of the IFN samples. To dilute out the samples 20µl volumes of the neat IFN samples were placed in duplicate wells and serial ten-fold dilutions, also in duplicate, were made across the plate. The WISH cells were then incubated for a further 12-18 hours in order to allow the antiviral state to be induced

proportional to the level of IFN in the sample.

A laboratory IFN-gamma standard was included to calibrate the assays, and this was itself calibrated against an international standard of human reference IFN-gamma (National Institute of Health, Bethesda, MD). Laboratory units of IFN were found to be equivalent to reference units.

The IFN dilutions were then aspirated off and the cells challenged with Semliki Forest virus (SFV) stock (L10 strain of the order of 10^8 pfu/ml) in the presence of 3μg/ml of actinomycin D (AMD) in fresh medium (2% NBS), adding 100μl per well. The purpose of the AMD was to inhibit cellular DNA-dependent RNA synthesis, yet allow virus-specific RNA synthesis. After 3 hours 10μCi/ml of ^{3}H-uridine in the the presence of AMD in fresh medium (2% NBS) was added, 100μl/well to give a total volume of 200μl in each well. The cells were incubated for at least a further 3 hours. The medium was then aspirated and the cell monolayers washed once with PBS, then twice with cold 5% trichloroacetic acid to precipitate the incorporated ^{3}H-uridine, and once with absolute ethanol. The cell sheets were then dried and 48μl of molar NaOH added to each well. The plates were left overnight at 37°C for hydrolysis to occur. Just prior to harvesting the samples, 52μl of molar HCl was added to each well to make the pH of the wells slightly acidic. The individual wells were then harvested and transferred to plastic scintillation vials before adding 2ml of acidified scintillation fluid (EP, Beckman). Incorporation of ^{3}H-uridine was assessed by

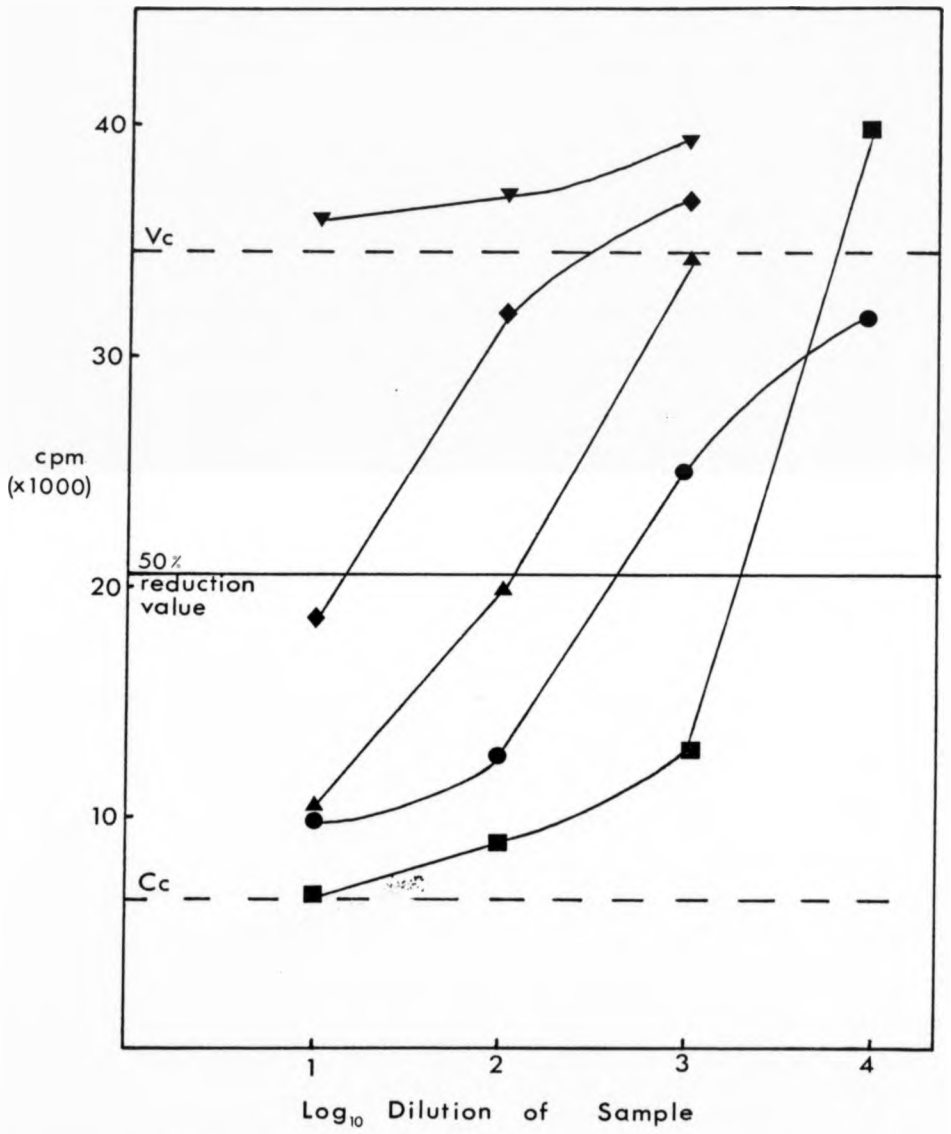
counting samples in an LKB "Rackbeta" scintillation counter.

As well as IFN samples, each plate also containd a laboratory IFN-gamma sample and controls. A "virus" control (4 wells) contained challenge virus but no IFN was added to the wells, thus giving a measure of maximum incorporation of radioactive label. A "cell" control (4 wells) consisted of wells where neither IFN nor virus was added, thus giving a measure of background incorporation of ^{3}H-uridine by the AMD-treated cells alone.

Raw results were obtained as readings of counts per minute (cpm) for each well, in duplicate. Using the mean cpm value for each set of duplicates, the level of incorporated label was plotted against $\log_{10}$ dilution value of the sample (Fig. 7.1). The number of U/ml of IFN in a sample is the reciprocal of the dilution causing a 50% reduction in viral nucleic acid synthesis. The 50% reduction value was the midpoint between the virus control (Vc = maximum incorporation by the virus) and the cell control (Cc = residual incorporation by cells alone). An appropriate dilution of virus was used to ensure that the virus control was at least 5 times the value of the cell control. The IFN titre was thus the reciprocal value of the dilution at which the titration curve dissected the horizontal 50% endpoint line. A typical IFN assay plot is shown in figure 7.1.

Neutralisation assays were carried out by adding 20µl of anti-IFN-gamma sera to the WISH cells (final dilution of 1/300), in addition to the 20µl IFN sample, before making

Figure 7.1 A TYPICAL INTERFERON ASSAY.



● = IFN standard = 2.7 log units = 500 Units/ml

▲ = SEA sample = 2.1 = 120 U/ml

▼ = A23187 = <0.3 = <2 U/ml

◆ = Mezerein = 1.2 = 16 U/ml

■ = A23187 plus Mezerein = 3.3 = 2000 U/ml

the dilutions. Any residual IFN activity was then assayed. The anti-IFN-gamma antiserum was prepared against recombinant IFN-gamma and had no effect on IFNs alpha and beta.

A greater than two-fold difference in titre was considered to be statistically significant, since in a series of replicate titrations of the same standard sample 95% of the titres obtained lay within a two-fold range. On the basis of this assumption, there was a 95% probability that the values obtained for a single sample titrated twice within the same assay would be within a two-fold difference. Thus any greater deviation than this constitutes a significant difference between IFN titres.

ii) IL-2 Assay.

IL-2 activity was assayed using the IL-2 dependent cell line CTLL. These were grown in RPMI 1640/10% FCS plus 10^{-5} 2-mercaptoethanol, buffered with bicarbonate. Periodic addition of IL-2 containing conditioned media (obtained from activated PBML) was required to sustain cell growth. CTLL were used for an assay three days after the last use of conditioned media. They were then resuspended in fresh media. IL-2 containing samples were diluted in duplicate, usually using half-log dilutions, across a series of 200µl microtitre round-bottomed wells containing an appropriate volume of medium. CTLL were added after the IL-2 samples, 2000 CTLL per well. The CTLL were then incubated at 37°C for 24 hours. The stimulatory effects of any IL-2 in the samples were determined from the level of ^{3}H-thymidine

incorporation, added at 10μCi/ml to all wells and incubated for a further 18 hours. The CTLL were then harvested using a MASH II cell harvester and cell-associated radioactivity measured using non-aqueous (NA) scintillation fluid. Several wells contained no IL-2 to measure minimum CTLL proliferation whilst commercial IL-2 was added to some wells to stimulate maximal proliferation. The number of U/ml of IL-2 in a sample was the reciprocal of the dilution causing half maximal incorporation.

iii) Assay of RNA and DNA synthesis.

The level of RNA production or cellular proliferation, as reflected by the level DNA synthesis, were measured by labelling the cells (quadruplicate wells) with ^{3}H-uridine or ^{3}H-thymidine at 2.5μCi/ml for a 24 hour period 3-4 days after induction. The cells were then harvested using the MASH II cell harvester and cell associated radioactivity measured using NA scintillant.

iv) NK cytotoxicity.

K562 cells were used as NK susceptible targets in order to measure cytotoxicity. They were incubated with 200μCi of ^{51}Cr at $2x10^{6}$ cells/ml for 2 hours. They were then washed 3 times in PBS, left for one hour, and washed once more in PBS. The K562 cells were then placed in 200μl microtitre wells at $5x10^{4}$ cells/ml with the cells whose cytotoxicity was to be measured at various effector:target ratios (usually 30,10, and 3:1), total volume of 200μl.

After incubation at 37°C for the appropriate time, 150ul of supernatant from each well was collected and counted on a gamma counter. The percentage of ^{51}Cr release was calculated by the formula :

$$\%\text{cytotoxicity} = \frac{\text{Experimental cpm - Spontaneous cpm}}{\text{Total cpm - Spontaneous cpm}} \times 100$$

Total cpm was determined as the amount released by incubating labelled K562 cells with 1M HCl. Spontaneous release was measured in wells containing no effector cells.

d) IFN-gamma and Interleukin 2 mRNA induction and estimation.

i) Induction of mRNA for IFN-gamma and IL-2.

For mRNA preparations much larger numbers of cells needed to be processed than required for measuring IFN-gamma production. Hence 50ml aliquots of cell suspensions (3 x 10^6 cells/ml), usually pooled from several donors, were cultured in large petri dishes (10cm diameter; Sterilin Ltd.) with addition of inducers and cycloheximide (CHX; Sigma) as appropriate. Crude SEA (used in these experiments because of the limited availability of pure SEA) was used at 0.5% by volume, A23187 at 1μM, and mezerein at 10ng/ml. CHX (added 1 hour before addition of inducers) was used at 200μg/ml, a concentration which inhibits protein synthesis by more than 99% in these cells, as assessed by ^{35}S-methionine incorporation.

ii) Isolation of RNA.

RNA was isolated by the guanidinium isothiocyanate-caesium chloride method (Morser et al.,1979). Cells were harvested 4 or 24 hours post-induction by scraping them off the plate with rubber policemen in the presence of PBS, twice. All PBS washes and the initial supernatant were spun at 3K for 5 minutes in a Chilspin. The cell pellet was then washed once in PBS before resuspending it in 3.5ml of lytic buffer (guanidinium isothiocyanate) and vortexing to ensure complete lysis. This was loaded onto a 1.5ml cushion of 5.7M caesium chloride (RI=1.405) in MSE (111) 5.5 ml polycarbonate ultracentrifuge tubes and centrifuged at 40,000 rpm for 22-24 hours in an MSE "Superspeed 65" ultracentrifuge, at 21°C, using an MSE 6x5.5 swing-out rotor. Lysis buffer and caesium chloride were then aspirated, and each RNA pellet was dissolved into 300µl of 5mM Tris buffer. The RNA was extracted three times with equal volumes of a 1:4 mixture of butanol/chloroform, the RNA contained in the upper aqueous phase and the caesium chloride being discarded with the non-aqueous phase. The RNA was then precipitated by adding 30µl of 3M NaCl to the 300µl of RNA/Tris solution, plus 2.5 volumes of absolute ethanol. then placed at -20°C for at least 20 hours.

All aqueous solutions (apart from the lysis buffer) used throughout the RNA extraction were made up in water which had been pretreated with diethyl pyrocarbonate (DEPC) in order to destroy any contaminating RNases.

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To measure yields of total RNA, precipitated RNA was pelleted by centrifugation at 10,000g for 10 minutes in an Eppendorf "Microfuge". The pellet was washed once in 80% ethanol, before repelleting then freeze-drying. The pellet was then dissolved in 100µl of DEPC-treated distilled water. A dilution of this was assayed for absorption at 260nm, in a Unicam SP500 spectrophotometer, in quartz cuvettes. An O.D. (optical density) of 1.0 was equivalent to 40µg/ml of RNA in the sample. Typically from 150 to 500µg of RNA was obtained from $1.5x10^8$ lymphocytes. The RNA solution could be stored long-term at -70°C.

iii) Quantitation of mRNA levels.

The level of IFN-gamma or IL-2 mRNA in the total RNA of each sample was then determined by dot-blot hybridisation analysis (Thomas,1980). Nitrocellulose filters were presoaked in 20xSSC (1xSSC is 0.15M NaCl, 15mM sodium citrate, pH 7.0) for 2 hours and then 20µg duplicates of each RNA sample were dotted onto them using a dot-blot manifold. Before dotting each RNA sample was made up to 100µl with water and denatured at 65°C for 10 minutes. The sample was transferred to an ice/water slurry to prevent renaturation and 50µl of cold 20xSSC added per 100µl of sample. After dotting the samples the filter was air-dried and then baked at 80°C for 2 hours in a vacuum oven to fix the RNA.

The filter was then soaked in 6xSSC for 5 minutes before incubating with a prehybridisation solution, sealed in a plastic bag, for 4 hours in a 42°C shaking water bath. The

prehybridisation solution consists of 50% deionised formamide (pH 6.8), 5xDenhardt's solution, 5xSSPE, 0.1% SDS, 1µg/ml poly A, and heat-denatured salmon sperm DNA at 100ng/ml. It was used at 0.3ml per cm^2 of nitrocellulose filter. This was then changed for a hybridisation solution containing heat-denatured, nick-translated cDNA probe homologous to IL-2 or IFN-gamma mRNA (Siggens et al.,1984). The IL-2 probe was kindly provided by Dr.T.Taniguchi (Taniguchi et al.,1983). In all other respects the hybridisation mixture was identical to that used for prehybridisation, and was used at 0.15ml per cm^2 of filter. Hybridisation was allowed to proceed for 15 hours at 42°C. The prehybridisation stage helped to block any non-specific binding of the labelled probe.

After hybridisation the filters were washed twice in 3xSSC at room temperature, then once in 0.5xSSC at 65°C for 45 minutes in a shaking water bath. Filters were then air-dried and the positions of the signals were located by autoradiography for 1-3 days, depending on the specific activity of the probe used, against Fuji RX X-ray film, using a Dupont Cronex "Lighting-plus" intensifying screen, at -70°C. The filters were then cut into sections and each sample quantitated by liquid scintillation counting.

iv) Preparation of radiolabelled probe by nick-translation.

For both IFN-gamma and IL-2 probes, 50ng of the appropriate cDNA in 3-7µl of water was denatured in boiling water for 2.5 minutes and immediately quenched in

ice slurry. The following components were then added on ice: 2µl of 10xRP buffer, 2µl of 10xdNTP mix (2mM of each unlabelled dNTP), 2µl of OCT primer (prepared as described in the Maniatis Cloning Manual p130), 2µl of water, 4µl (40µCi) of ^{32}phosphate dCTP or dGTP, and finally 2µl of Klenow enzyme (Amersham). After a brief vortex, the mixture was then incubated for at least 8 hours at 19±1°C. After this time 0.5ul of 10% SDS and 10µl of salmon sperm DNA (2mg/ml) were added, plus 10µl of loading dye. Nick-translated DNA was then separated from unincorporated label and deoxynucleotide triphosphates by chromatography on a Sephadex G-75 column (a slurry equilibrated in TNE) run in TNE buffer. The first eight fractions (7 drops each) were collected and the 3 or 4 "hottest" fractions mixed and used as probe. Typically, the specific activity of nick-translated probe prepared in this fashion was in the order of 1-5x10^{7} cpm. After denaturing by boiling the probe was ready for use in the hybridisation mix. Probe was used as soon as possible after preparation, usually within 2 days.

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