THE EFFECT OF INTERLEUKIN 4 ON PROTEIN KINASE ACTIVITIES ASSOCIATED WITH B LYMPHOCYTE PLASMA MEMBRANES

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by

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Biochemical Journal with the following additions :-

APC	Antigen presenting cells
ATP	Adenosine triphosphate
BCGF	B cell growth factor
CD23/FcRε	The low affinity receptor for IgE
sCD23	soluble CD23
Con A	Concanavalin A
DAG	Diacylglycerol
EGF	Epidermal growth factor
FCS	Foetal calf serum
FcRII	Fc receptor
FITC	Fluorescein isothyocyanate
G protein	Guanine nucleotide binding protein
Ia	Major histocompatibility antigens
Ifn-γ	Interferon-γ
IL-1, 2, 3, 4, 5, 6	Interleukin 1, 2, 3, 4, 5 and 6
rmIL-4	Recombinant mouse IL-4
rhIL-4	Recombinant human IL-4
Ig A, D, E, G, M	Immunoglobulin subclass A, D, E, G, M
IgF(ab)'$_2$	F(ab)$_2$ fragment of immunoglobulin
IP$_3$	Inositol 1, 4, 5 triphosphate
KOH	Pottasium hydroxide
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex

NADPH	Nicotinamide adenine dinucleotide phosphate
PDGF	Platelet derived growth factor
PI	Phosphatidyl inositol
$PtdInsP_2$	Phosphatidyl inositol *bis*phosphate
PHA	Phytohaemagglutinin
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Th1	Helper T cell subset 1
Th2	Helper T cell subset 2
TLE	Thin layer electrophoresis
TNP	Trinitrophenol

CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

A variety of ligands bind to unique cell surface receptors and cause rapid changes in cell metabolism. In many systems this has been acheived by the functional modification of existing proteins, most notably through phosphorylation. Phosphorylation of proteins has been observed in B lymphocytes on activation with antigen, lipolysaccharide and several lymphokines. Although many of the biological functions of the lymphokine IL-4 are well known, the signals transduced when IL-4 binds to its receptor are poorly understood. In the murine system it has been reported that IL-4 fails to induce the hydrolysis of inositol lipids, mobilisation of intracellular calcium or the activation of protein kinase C. However, the addition of IL-4 to isolated B cell membranes in the presence of γ-[^{32}P]-ATP leads to the phosphorylation of a 42 kDa protein. In the human system recent experiments suggest that the addition of IL-4 to resting human B cells results in the activation of a complex second messenger cascade involving the hydrolysis of inositol lipids, elevation of intracellular calcium and an increase in the level of cAMP.

Studies in this project have indicated that exposure of plasma membranes isolated from high density resting murine B lymphocytes to either purified or recombinant IL-4 in the presence of γ-[^{32}P]-ATP promotes the phosphorylation of a 42 kDa protein. The use of recombinant material negates the possibility that residual PMA in the purified IL-4 is responsible for the observed phosphorylation. Linear laser densitometric analysis of the autoradiographs suggested that IL-4 was enhancing the phosphorylation of this protein some two-fold. The 42 kDa protein could be detected in plasma

membranes from low density B cells that had been exposed to lipopolysaccharide for 24 hours, but not in membranes prepared from B cells exposed to the mitogen for 48 hours, or in cell lines possesing high numbers of IL-4 receptors. This suggests that the 42 kDa phosphoprotein is present only in membranes from resting B cells or B cells at early stages of activation but not in membranes from cells which are commited to the proliferative stages of the cell cycle. Experiments using 8-azido-γ-[^{32}P]-ATP as a photoaffinity label indicated that the 42 kDa phosphprotein did not bind ATP and was therefore a protein kinase substrate rather than a protein kinase itself. There was no evidence of the 42 kDa phosphoprotein in identical experiments with human B cells using human IL-4 as a stimulatory ligand.

It has been shown that culturing B cells with IL-4 increases the expression of IL-4 receptors without driving the B cells into the cell cycle. Plasma membranes prepared from murine B cells cultured for 24 hours with IL-4 were exposed to γ-[^{32}P]-ATP in the presence of IL-4. Phosphorylation of the 42 kDa protein was evident, but the profiles also illustrated the presence of a major autoradiographic signal of molecular weight 75 kDa. This phosphoprotein was unique to cells exposed to IL-4 and could not be detected in membranes from resting B cells, B cells exposed to lipopolysaccharide for 24 or 48 hours or B cells cultured in medium alone for 24 hours. The 75 kDa autoradiographic signal was resistant to treatment with alkali which suggested that it may be phosphorylated on tyrosine residues. Preliminary experiments with 8-azido-γ-[^{32}P]ATP indicated that the 75 kDa phosphoprotein may be able to bind ATP. Culturing high density resting B cells with IL-4 in the presence of 11B11, an anti-IL-4 monoclonal antibody, prevented the appearance of

the 75 kDa phosphoprotein indicating that binding of IL-4 to its receptor was required for the induction of the 75 kDa phosphoprotein. Interferon-γ inhibits many of the biological effects of IL-4 but it does not prevent the binding of IL-4 to its receptor. Exposure of membranes, prepared from resting B cells cultured in the presence of Ifn-γ, to γ-[^{32}P]-ATP resulted in phosphoprotein profiles essentially identical to that obtained with membranes from cells cultured with IL-4. Phosphoprotein profiles of membranes prepared from B cells cultured with both IL-4 and Ifn-γ were very similar to membranes prepared from cells cultured in medium alone, ie. there was no evidence of the 75 kDa phosphoprotein.

Phosphoprotein profiles of membranes from human tonsillar B cells cultured with IL-4 for 24 hours showed the presence of an alkali resistant 72 kDa signal. In membrane phosphoprotein profiles from human tonsillar B cells there is also an indication of the disappearence of a 94 kDa protein when the cells were cultured in the presence of IL-4. Membrane phosphoprotein profiles from human B cells cultured with phorbol ester and calcium ionophore, also show the presence of a 72 kDa alkali-resistant phosphoprotein signal. Human B cells isolated from peripheral blood were cultured with IL-4 and the membranes from these cells exposed to γ-[^{32}P]-ATP. In this case there was some evidence of a 72 kDa protein but this was not definitive.

These data are consistent with the activation of a protein kinase activity when IL-4 binds to its receptor. Two phosphoproteins have been identified, 42 kDa, unique to the murine B cell model, and 75 kDa, apparently present in both murine and human B cells. The molecular identities and biochemical functions of these phosphoproteins remains undefined.

1. INTRODUCTION.

1.1. The Immune System

The immune system exists as a protection against foreign antigens. It is highly complex, discriminating between self and non self, and involves multiple cellular interactions in addition to cellular communication mediated by soluble growth and differentiation factors. The principal cells involved in the humoral immune response are B lymphocytes, which produce antibody, and accessory cells from the myeloid lineage and T lymphocytes, which do not produce antibody themselves, but assist in antibody production. All three types of cell are derived from haematopoetic stem cells.

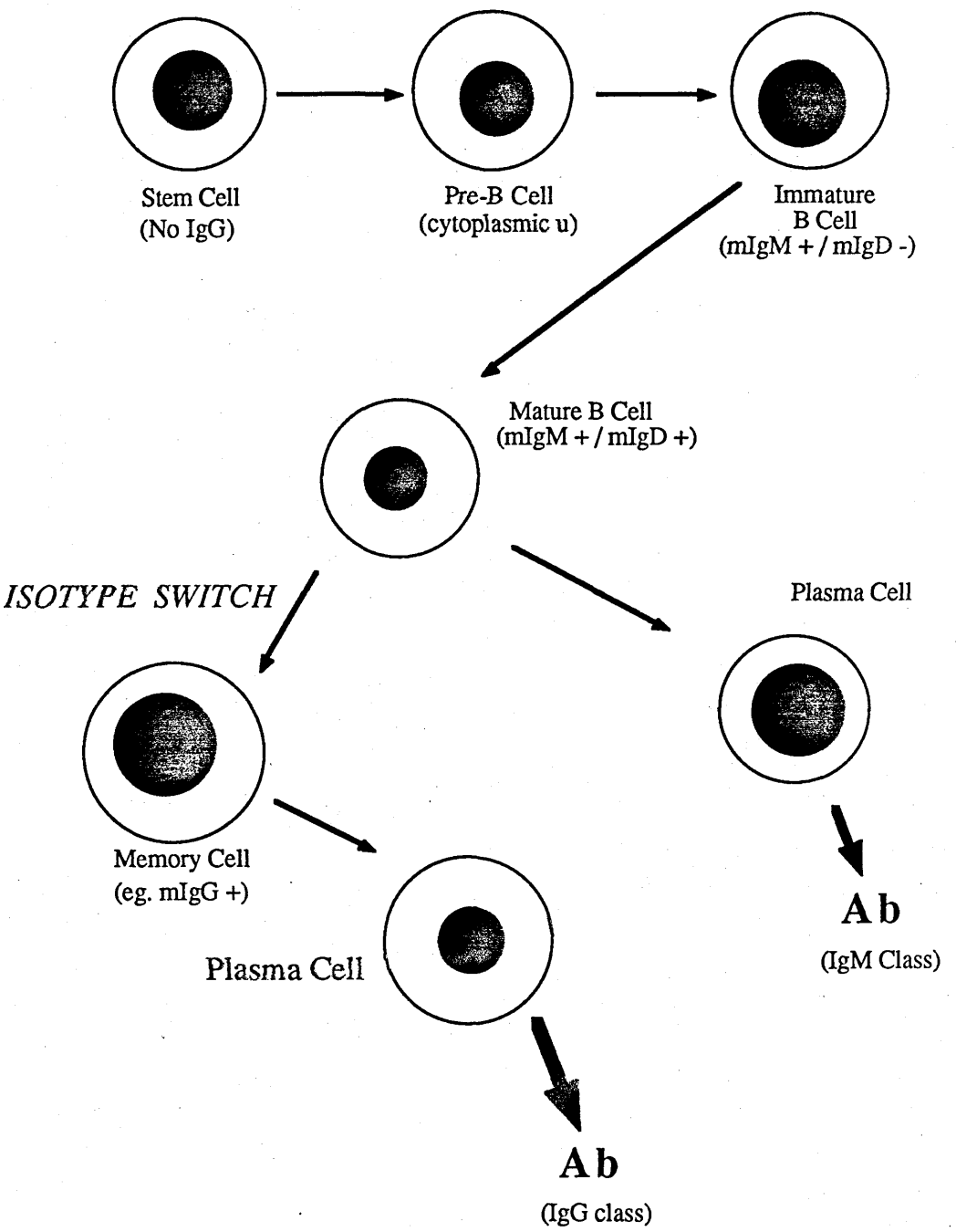
The immune system is capable of mounting a highly specific response against any foreign body. This specificity is determined by T and B lymphocytes. Antigen receptors on B lymphocytes have been well characterised and found to be identical to secreted immunoglobulin (Ig) except for an extra transmembrane portion (Rogers *et al.*, 1980). The recognition molecule on T cells is the membrane bound T cell antigen receptor which exists as a complex of several proteins (for a review see Davis & Bjorkman, 1988). The T cell antigen receptor recognises antigen peptides in combination with a molecule of the major histocompatibility complex (MHC). The antigen receptors on both T and B cells are clonally distributed.

1.1.1. *B Cell Development*

The development of B cells into mature antibody secreting cells can be divided into two stages (Figure 1). Pluripotential stem cells, which originate in the blood islands of all embryonic tissue, undergo a programmed proliferation and differentiation. This development occurs within primary lymphoid organs and is dependent on the microenvironment and independent of antigen.

Figure 1 B Cell Developement

Pluripotential stem cells undergo a programmed proliferation and differentiation to develop into mature B cells expressing membrane IgM and IgD. When antigen binds to a mature B cell the cell is stimulated to proliferate and differentiate into a high rate antibody secreting cell or a memory cell. The specificity of the immune response is based on clonal selection. Each B cell undergoes a unique set of V gene rearrangements to express only one structure of Immunoglobulin on its membrane (Burnet, 1959)


Stem Cell
(No IgG)
Pre-B Cell
(cytoplasmic u)
Immature
B Cell
(mIgM + / mIgD -)
Mature B Cell
(mIgM + / mIgD +)
ISOTYPE SWITCH
Plasma Cell
Memory Cell
(eg. mIgG +)
Plasma Cell
Ab
(IgM Class)
Ab
(IgG class)

Each immunoglobulin molecule consists of heavy and light chains. Both the heavy and light chains consist of a constant region and a variable region, which is the antibody combining site. It is the combining of the heavy and light chains that generates antibody diversity. Heavy chains are composed of V (variable), D (diversity), J (joining) and C (constant) exons. Three segments combine, V, D and J, to code for the V_H domain. One of several hundred V genes recombines with one of twelve D genes and one of four J genes to produce a functional VDJ gene. The VDJ gene then combines with a C gene, which determines isotype specificity, forming a complete heavy chain. Light chains possess V,J, and C exons, which combine in a similar manner to heavy chain genes producing a wide range of functional light chains. In the development of B cells each potential B cell undergoes a unique set of imunoglobulin V gene rearrangements. This means that each B cell produces only one particular structure of the V region of heavy and light chains (Burnet, 1959).

Pre B cells are characterised by the expression of μ heavy chains in the cytoplasm. On synthesis of light chains, either κ or λ, the immature B cell can express surface Ig. Mature B cells express both IgM and IgD and although quiescent are capable of responding to antigen.

1.1.2. *Clonal Selection*

The specificity of the immune response is based on clonal selection (Burnet, 1959). Each B cell is programmed to make one immunoglobulin molecule which is expressed on its surface as an antigen receptor. When antigen binds to the receptor the B cells are stimulated to proliferate and differentiate into antibody secreting cells and memory cells (Figure1).

Differentiation to memory cells involves a change of Immunoglobulin isotype. As the B cell matures class switching can occur eg. B cells expressing IgM can switch to IgG, IgA or IgE. Class switching occurs at the genomic level. The somatically recombined VDJ gene moves from a position 5' of Cμ and Cδ to a location 5' to another constant region gene (Shizimu & Honjo, 1984). Memory B cells are quiescent until they encounter antigen again when they have a choice to differentiate into antibody secreting cells or remain as memory cells.

1.1.3. *Proliferation and Differentiation*

Antibody production by B cells involves clonal expansion and differentiation into high rate antibody secreting cells. In the mouse 80-90% of B cells are in a resting or quiescent (Go) state (Monroe & Cambier, 1983a) and require the presence of growth factors in order to proliferate. The B cell response to antigen can be defined in three stages; activation, proliferation and differentiation. Each stage of the cell cycle of B cells is under the control of a variety of molecules, the properties of which have been elucidated in recent years. These factors are neither tissue, target or stage specific for their action on B lymphocytes (Gordon & Guy, 1987). Activation of quiescent (Go) B cells into the G1 phase of the cell cycle involves several changes in the B cell (Walker *et al*,1986) including enlargement of the cell, RNA synthesis and an increase in expression of proteins including several surface molecules eg. CD23 in humans. In mice entry into G1 is characterised by an increase in expression of major histocompatibility (MHC) class II antigens (Mond *et al*, 1981). Although an increase in MHC class II antigens is also observed in human B cells, the increase is not as pronounced (Godal *et al*, 1985). Entry to G1 phase is completed within 24 hours, and then progression factors move the cells into S phase and DNA synthesis (Gordon & Guy, 1987). The cell cycle has several sites where cells can potentially arrest or, depending on

external factors, proceed. Although these arrest points exist throughout the cell cycle the most important appear to be in the G1 phase. Figure 2 depicts a summary of the cell cycle.

Differentiation takes several days and results in the production of antibody (Melchers and Anderson, 1984). Several factors can induce differentiation directly from resting cells and in fact maturation appears antagonistic to replication (Melchers and Andersson, 1984). Pike and Nossal (1985a) have developed a system which selects single antigen specific B cells. These cells can be stimulated to proliferate and differentiate when they are cultured with antigen and B cell growth and differentiation factors. This system has allowed the actions of several growth factors to be identified. IL-1 and IL-2 have been shown to be both growth and differentiation factors for B cells in the presence of antigen and in combination result in an additive effect (Pike & Nossal, 1985b). This suggests that the action of individual factors is not confined to one stage.

1.2. Activation Through Membrane Immunoglobulin

The initial signal for B cell activation is the binding of antigen to membrane immunoglobulin (Ig) which is of two classes, IgM and IgD on virgin B cells. Most of the work done in this area makes use of anti Ig, which acts polyclonally, rather than using specific antigens. Crosslinking of membrane immunoglobulin by anti-immunoglobulin reagents results in signals being transduced across the membrane to the B cell (Parker, 1975). One of the consequences of anti Ig binding is a 5-10 fold increase in expression of surface MHC class II antigens (Mond *et al.*, 1981; Monroe & Cambier, 1983a). On antigen binding to membrane immunoglobulin the antigen is internalised, processed, and the processed antigen is then expressed

on the B cell surface in conjunction with MHC class II antigens (Chesnut & Grey, 1986). The increase in expression of MHC class II antigens (Ia) could be detected 6-8 hours after stimulation. Induction can be inhibited by actinomycin D (Monroe *et al.*, 1982) and cycloheximide (Mond *et al.*, 1981). Messenger RNA encoding the MHC antigens could be detected within 2 hours of stimulation (Cambier *et al.*, 1985). The increase in MHC class II antigen expression is significant as they are involved in the collaboration of T and B cells needed for final proliferation. Some data have suggested that an increase in MHC class II antigens allows a more efficient interaction with MHC restricted helper T cells (Bottomly *et al.*, 1983; Henry *et al.*, 1977) and increased ability to present antigens to them (Roehm *et al.*, 1984). Although anti Ig is required for activation of B cells this signal alone is not enough to drive the resting B cell to proliferate (Bretscher, 1975) or to make the B cell responsive to growth and differentiation factors (Snow *et al.*, 1983).

1.3. T-B Cell Interaction

When antigen binds to membrane immunoglobulin, signals are transduced to the B cell which, although not enough to drive the B cell into the cell cycle, leave the cell in an activated state poised to receive a major growth stimulus (Snow & Noelle, 1987) which will drive the B cell into the cell cycle. Early experiments by Mitchison (1971) demonstrated that T dependent antigens required an antigen mediated recognition between B and T cells. Since then the T-B interaction has been shown to be MHC class II restricted both *in vitro* (Andersson *et al.*, 1980; Jones & Janeway, 1981) and *in vivo* (Sprent, 1978). The receptors on T cells recognise the processed antigen and MHC class II antigens expressed on B cells. B cells therefore present antigen to T cells in a manner similar to antigen presenting cells (Lanzavecchia, 1985).

This cognate interaction of T and B cells results in the production of lymphokines from T cells and the B cells becoming responsive to the lymphokines and subsequently expanding and differentiating (Singer & Hodes, 1983).

The signals that are transduced when T and B cells interact are unknown and part of the signal may be through molecules that are in close proximity to the site of interaction. Baluyut & Subbaro (1986) using anti-Ia and anti-IgM antibodies have observed a synergistic effect on proliferation suggesting that some signals may be transduced via MHC class II antigens. This has been substantiated by reports that anti-Ia antibodies induced a rapid rise in cAMP levels in B lymphocytes within 60 seconds (Cambier & Ransom, 1987) and also induced the rapid translocation of protein kinase C from the cytosol to the nucleus (Chen *et al.*, 1986). The exact nature of the signals transduced when T and B cells interact are at present unclear but the main outcome of the interaction is the activation of B cells through the production of lymphokines.

1.4. Macrophage - T cell Interactions

When looking at T and B cell interactions many researchers have made use of the availability of T cell hybridomas, cloned T cell lines or *in vivo* primed T cells. This has meant that the question of whether resting B cells can initiate an immune response by providing the signals to activate resting T cells has not been answered. Cells of the myeloid lineage, such as macrophages, can internalise antigen, degrade it and re-express the processed antigen on the cell surface in conjunction with MHC class II antigens (Rosenthal & Shevach, 1973). These cells, known as antigen presenting cells (APC), interact in an MHC restricted manner with T cells. This interaction results in the activation of T cells and the production of lymphokines, IL-1

from APC and IL-2 from T cells (Melchers & Andersson, 1984).

Recently, Lassila *et al* (1988) performed an experiment in chickens studying the ability of resting B cells to activate resting T cells. In chickens it is possible to create a A-B chimerea consisting of T cells and APC's from host B type strain and B cells from an allogeneic donor A strain. Treating chickens neonatally with cyclophosphamide destroys B cell precursors but leaves T cells and APC 's intact. B cell precursors from another neonatal chicken (type A) were then introduced to the B cell depleted chicken, type B. The T cell independent response was normal in these chickens, but the T dependent response was deficient although the donor B cells were functional. The donor B cells differ in the class II region of MHC antigens from the T cells and APC's and, therefore, MHC T-B cell interactions will be difficult as there will be few helper T cells capable of recognising the antigen and MHC molecules expressed on the B cells. However it was found that the number of MHC specific interactions of T and B cells was sufficient for a normal response and it was the introduction of APC's expressing the same MHC as the donor B cells that restored the T cell dependent antibody response to normal. The explanation derived from this result was that antigen presenting cells other than B cells are required to activate resting T cells before the T cells can interact with the same antigen and MHC molecules expressed on B cells.

1.5. Excitation of B Cells by Other Modes

Although the most specific way to activate a B cell is by MHC restricted interaction with helper T cells there are other less specific ways to effect this stimulation. B cell populations are not homogeneous and therefore the signals required to activate a B cell may depend on the nature of the B cell population (Melchers & Andersson, 1984).

1.5.1. *Alloreactive T Cells*

Two classes of alloreactive T cells exist,

i) those that will excite a resting B cell expressing the appropriate class II MHC antigen only when specific antigen is also bound to that cell (Schreier *et al*, 1980).

ii) those that polyclonally excite a large proportion of B cells even in the absence of any antigen binding to surface immunoglobulin (Waterfield *et al.*,1979, Augustin & Coutinho, 1980)

1.5.2. *T Independent Antigens*

T independent antigens as their name suggests can activate B cells independently of T cells ie. they are non antigen specific and non MHC restricted. Originally two types of T independent antigen was identified, TI-1 and TI-2, based on the ability of the antigen to elicit an antibody response from B cells from three sources. B cells from normal adult mice, suckling mice and CBA/N mice with X-linked immunodeficiency syndrome (Mosier *et al.*, 1977).TI-1 antigens eg. haptenated lipopolysaccharide and *Brucella abortus*, have the ability to stimulate an antibody response in all three mice. TI-2 antigens eg. haptenated ficoll and dextran can only stimulate an antibody response in mature type B cells ie. from adult mice.

Pike and Nossal (1984) using single hapten specific B cells demonstrated that some T independent antigens may be partially dependent on T cells, although in a non MHC restricted manner. Individual antigens had different extents of requirement of T cell help and this was not related to TI-1 and TI-2 classifications. The antigens LPS and ficoll did not require any T cell help, whereas the polymerised flagellin of salmonella adelaide was

dependent on the presence of T cells and *Brucella abortus* was only partially dependent on T cell help. These results suggest that the original subclasses of T independent antigens may need further classification.

1.5.3. *Polyclonal Activators*

Polyclonal activators such as lipopolysaccharide (LPS), which excites mouse B cells, excite resting B cells irrespective of antigen specificity or MHC haplotype (Andersson *et al.*, 1972). LPS drives about 60% of B cells into S phase although this can be increased to 80% in the presence of dextran sulphate showing synergy in the action of the two mitogens (Wetzel & Kettman, 1981). Agents such as LPS may act by crosslinking putative receptors on the B cell surface as they are known to exist as large polymeric structures in solution (Galanos & Luderitz, 1975). No such receptors have been identified and the report that LPS can insert itself directly into the membrane suggests that it may not require surface receptors (Raetz *et al.*, 1983).

1.6. Control of B Cells by Lymphokines

One mechanism by which helper T cells help B cells is by the production of soluble molecules which deliver essential signals to the B cells by interaction with complementary cell surface receptors. These T cell products were first described in 1972 when it was shown that the supernatants from T cells could substitute for the cells themselves in T cell dependent B cell responses (Schimpl & Wecker, 1972). These lymphokines have now been shown to be pleiotropic (O'Garra *et al.*, 1988). Lymphokines act on a wide variety of cell types and have multiple effects on a single target cell. The availability of monoclonal antibodies, which have facilitated purification of the lymphokines and the availability of recombinant forms of the molecules, has to some extent helped to define the action of specific lymphokines. There has

been some problem with the nomenclature of lymphokines which has now been made clearer by the use of the term interleukin. The criteria for a molecule being termed interleukin is that the molecule has been cloned and the expressed molecule has been observed to have the same biological activities as the molecule purified from cell supernatants (Paul, 1988).

The molecules that were first identified by their effects on B cells were IL-4, IL-5 and IL-6. IL-4 will be discussed in detail later in this chapter. IL-5 has been shown to act as both a growth and differentiation factor on B cells. It induces the proliferation of activated normal murine B cells and BCL1 cells (Dutton *et al*, 1984). IL-5 promotes IgM and IgG secretion by preactivated murine B cells and BCL1 cells (O'Garra *et al.*, 1986) and enhances the production of IgA by LPS stimulated B cells (Yokota *et al.*, 1987; Coffman *et al.*, 1987). In the human system it has been demonstrated that IL-5 induces IgM (Azuma *et al.*, 1986) and IgA (Yokota *et al.*, 1987) secretion in staphylococcus aureus cowan 1 (Sac) activated B cells from peripheral blood. There does, however, seem to be some controversy over the proliferative effects of IL-5 in the human system, as it has recently been reported that recombinant human IL-5 does not have activity in many of the conventional B cell proliferation assays (Gordon & Guy, 1987). The effects of IL-5 are not confined to B cells as IL-5 has been shown to act as an eosinophil differentiation factor in both the murine and the human system (Yokota *et al.*, 1987). IL-5, in combination with IL-2, can induce cytotoxic T lymphocytes from thymocytes (Takatsu *et al.*, 1987).

IL-6 (BSF-2) was described as a B cell differentiation factor by its ability to induce antibody secretion in pre-activated normal and Epstein Barr virus transformed human B cells (Hirano *et al.*, 1987). It has also been

shown to support the growth of B cell hybridomas and plasmacytomas (Bazin & Lemieux, 1987; Van Damme *et al.*, 1987) and is identical to hybridoma growth factor (Van Damme *et al.*, 1987).

Other molecules that have been identified to have growth factor activity on B cells include a 12 kDa T cell product, described as low molecular weight BCGF and a high molecular weight factor (50-60 kDa) derived from human T cells and the Namalwa B cell line (Ambrus & Fauci, 1985).

There are several molecules that were originally described as having effects on cells other that B cells but have now been found to have effects on B cells. IL-1 can act on both pre B cells and activated mature B cells. Thus a murine pre B cell line expresses membrane immunoglobulin in response to IL-1 (Giri *et al.*, 1984). The addition of IL-1 to B cells treated with IL-4 and anti-Ig has been shown to induce proliferation (Howard *et al*, 1983). In studies with single antigen specific B cells IL-1 induced prolifereration and differentiation in these cells (Pike and Nossal, 1985b).

It has now been reported that IL-2 has a role in B cell proliferation and differentiation. Receptors for IL-2, recognised by the anti-Tac antibody, are expressed on activated human B cells (Mingari *et al.*, 1984; Jung *et al.*, 1984). IL-2 can induce proliferation of B cells in the presence of LPS and anti-Ig (Zubler *et al.*, 1984). Studies on antigen stimulated single B cells showed that IL-2 could induce proliferation and differentiation of B cells (Pike and Nossal, 1985b). IL-2 can also induce differentiation of B cells in the presence of anti-Ig and IL-4 (Nakanishi *et al.*, 1984). In the human system IL-2 induces proliferation (Jelinek *et al.*, 1986) and differentiation (Bich-Thuy and Fauci, 1985) of SAC activated B cells. The addition of IL-2 to

murine B cells, which express high affinity receptors for IL-2, results in the induction of NF-J_B. NF-J_B is a DNA binding protein which promotes the expression of J chains. IL-2, therefore, promotes high rate secretion of IgM from B cells.

Ifn-γ appears to exert both stimulatory and inhibitory effects on the response of B cells to antigen. The proliferative effects of IL-4 on B cells are inhibited by Ifn-γ (discussed later in this chapter, section 1.9.). Although some reports show that human B cells can proliferate in response to anti-Ig and Ifn-γ (Defrance *et al.*, 1986; Romagnani *et al.*, 1986; Defrance *et al.*, 1987b). In the murine system, Ifn-γ has been shown to enhance antibody production specifically of the IgG2a class in LPS activated B cells (Snapper & Paul, 1987).

As well as soluble molecules contributing to the growth and differentiation of B cells, antibodies to several surface antigens on B cells have been described as delivering stimulatory or inhibitory signals to the cells although the ligands for these surface molecules have not been identified. It is also now apparent that the B cell itself can regulate the immune response. An autostimulatory B cell growth factor (BCGF) has been detected which has a molecular weight of 25-35 kDa (Jurgensen *et al.*, 1986). Human CD23, and its mouse equivalent FcRε, is expressed on B cell membranes as a 45 kDa protein (Defrance *et al.*, 1987a). This protein can bind IgE and has been identified as the low affinity receptor for IgE (Walker *et al.*, 1986). Expression of CD23/FcRe is increased in the presence of IL-4 (discussed in detail later). The binding of CD23 antibody to the 45 kDa membrane protein results in its cleavage to a 35 kDa soluble protein (Gordon & Guy, 1987). This soluble protein acts as a growth factor for B cells stimulating G1 cells to enter S phase and G2 phase of the cell cycle (Gordon *et al.*, 1986). It has

been suggested that the CD23 molecule and the autocrine BCGF are the same molecule (Gordon and Guy, 1987). Binding of a T cell derived BCGF results in the release of soluble CD23 and it is possible that the BCGF has a protease activity which cleaves the CD23 molecule (Gordon and Guy, 1987).

It is clear that there are several soluble factors and surface molecules that effect the proliferation and differentiation of B cells. Figure 2 is an attempt to summarise the stages at which these different factors exert their effect. There are several differences in the action of some molecules in the mouse and human system. This could be accounted for to some extent by the different sources of B cells used. Experiments on human B cells have tended to concentrate on cells obtained from peripheral blood or tonsils whereas the spleen has been the main source of B cells in the mouse. The work defining the biological effects of lymphokines have so far been performed mostly *in vitro* and the relevance of most of these effects still have to be determined *in vivo*. *In vivo* the lymphokines have a short half life (minutes) and the actions of many of the lymphokines are dependent on the state of responsiveness of the B cell.

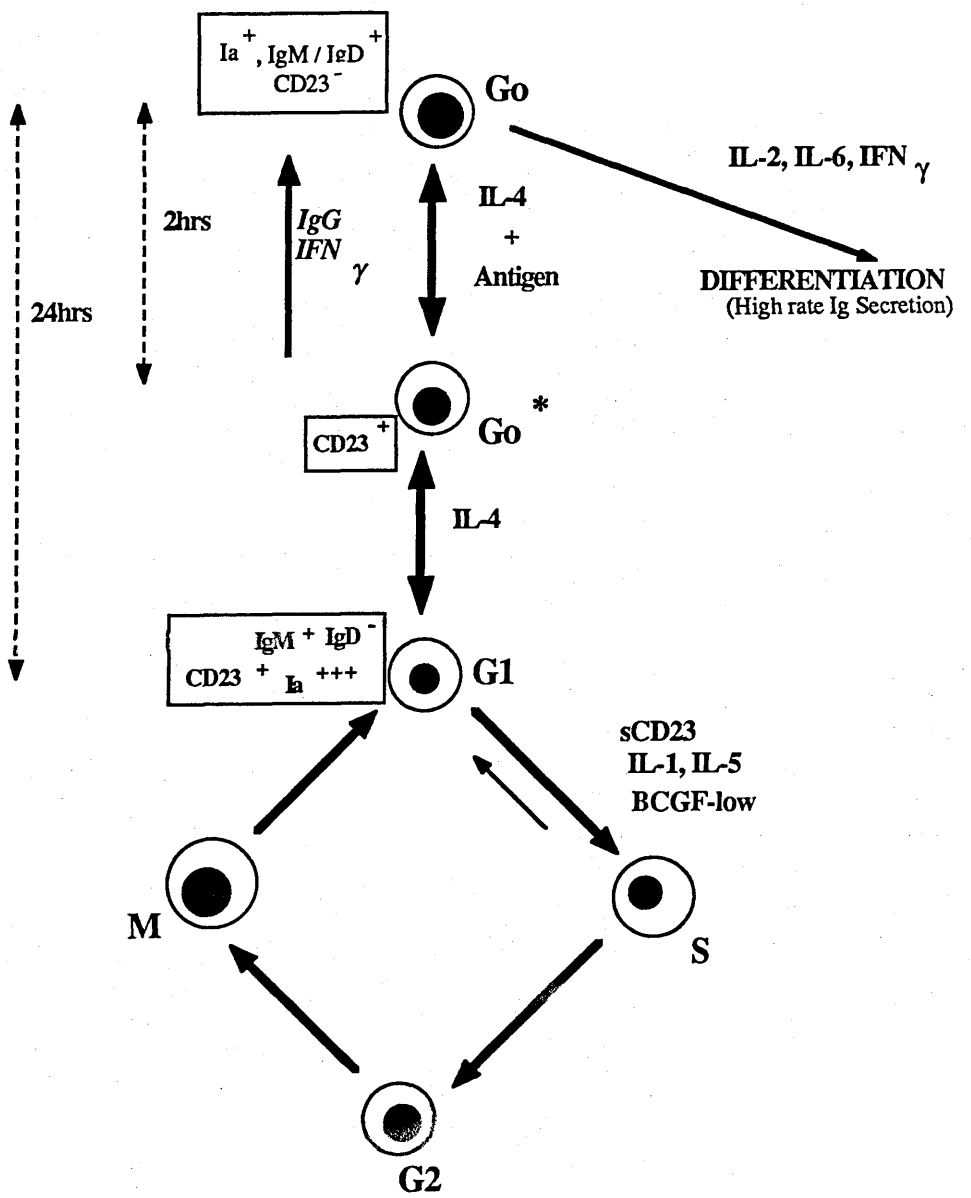
1.7. Interleukin 4

Interleukin 4 (IL-4) is a glycoprotein with a molecular weight of 20 kDa. It is synthesised by some activated T cells and exerts its effects on several different cells including B lymphocytes, T lymphocytes, mast cells (Mosmann *et al.*, 1986) and haematopoetic cells (Zlotnick *et al.*, 1986). Murine interleukin 4 was initially identified in the supernatants of EL-4, a thymoma cell line, that had been stimulated with phorbol myristate acetate (PMA) (Howard *et al.*, 1982) and has since been purified from this source (Farrar *et al.*, 1983; Ohara *et al.*, 1985). cDNA sequences for both murine (Noma *et al.*, 1986; Lee *et al.*, 1986a) and human (Yokota *et al.*, 1986) IL-

Figure 2 Proposed Sites of Action of B Cell Growth and Differentiation Factors

Antigen and IL-4 can act on resting B cells to allow entry into the G1 phase of the cell cycle (Howard *et al.*, 1982). This is characterised by cell enlargement and an increase in expression of MHC class II antigens and FcRe/CD23 (Walker *et al.*, 1986; Mond *et al.*, 1981). Activation to G1 is complete within 24 hours and can be inhibited by Ifn-γ (Rabin *et al.*, 1986; Mond *et al.*, 1986) and intact immunoglobulin (Sinclair, 1983). Progression factors, low molecular weight BCGF, IL-1, IL-5 and sCD23 have been reported to assist in driving the B cells into S phase and DNA synthesis (Gordon and Guy, 1987).

Differentiation of B cells to antibody secreting cells takes several days and several factors have been identified in playing a role in this, IL-2, IL-6 and Ifn-γ.


Ia+, IgM / IgD+
CD23-
Go
IL-2, IL-6, IFN γ
2hrs
IgG
IFN γ
IL-4
+
Antigen
DIFFERENTIATION
(High rate Ig Secretion)
24hrs
CD23+
Go*
IL-4
IgM+ IgD-
CD23+ Ia+++
G1
sCD23
IL-1, IL-5
BCGF-low
M
S
G2

4 have now been derived.

The nucleotide sequence derived for murine IL-4 encodes for a protein of 140 amino acids. The first 20 amino acids are highly hydrophobic and represent the signal peptide which allows the protein to be secreted. The secreted IL-4 consisted of 120 amino acids and has a molecular weight of 20 kDa which is consistent with that determined from EL-4 supernatants (Noma *et al.*, 1986). Treatment with endoglycosidase F reduces the molecular weight of the IL-4 fom 20 kDa to 15 kDa (Ohara *et al.*, 1987). From the sequence three potential sites for the attachment of N-linked sugars could be identified and the decrease in molecular weight observed with endoglycosidase F suggested that at least one of these sites must be glycosylated. Glycosylation of IL-4 does not appear to be necessary for the biological activity of the molecule (Ohara *et al.*, 1987). IL-4 also has six cysteine residues but the intact molecule cannot be labelled with ^{14}C-iodoacetamide which indicates that all the residues are involved in disulphide bonds. The disulphide bonds need to be intact for IL-4 to be biologically active (Ohara *et al.*, 1987).

Regions of the sequence determined for murine IL-4 contained significant homology to two other lymphokines, granulocyte-macrophage colony stimulating factor (GM-CSF) and γ-interferon. Noma *et al* (1986) suggested that these lymphokines may be distantly related which is particularly interesting as γ-interferon inhibits many of the biological effects of IL-4.

The nucleotide sequence derived for human IL-4 coded for a protein with 153 amino acids, with 24 amino acids being part of the signal peptide, leaving 129 amino acids as the secreted protein (Yokota *et al.*, 1986). There are seven cysteine residues in the human IL-4 compared to six in the mouse. The molecular weight of the protein was estimated to be 15 kDa although this does not take account two potential N glycosylation sites. From the amino

acid sequence human and murine IL-4 have approximately 50% homology but IL-4 exhibits complete species specificity (Mosmann *et al.*, 1986).

1.8. Biological Activities of IL-4 on B Lymphocytes

1.8.1. *IL-4 acts as a costimulant with anti-Ig*

Murine IL-4 was first described by its ability to act as a costimulator with anti-IgM to stimulate resting B cells to enter DNA synthesis (Howard *et al.*, 1982). As has been described previously (section 1.2.), anti-IgM binding to resting B cells primarily results in increased expression of MHC class II antigens. The action of anti-IgM alone moves the B cell from a resting Go state to G1 but is insufficient to drive the B cell into DNA synthesis (Bretscher, 1975). Howard *et al* (1982), using supernatant from PMA stimulated EL-4 cells, first discovered that IL-4 could act as the factor that drove the anti-IgM stimulated B cells into DNA synthesis. IL-4 has now also been shown to act as a costimulant with antigen in the proliferation of antigen specific B cells using TNP specific B cells (Stein *et al.*, 1986) . It has, however, been reported that human IL-4 displays little or no growth promoting activity with human tonsillar cells in the anti-IgM costimulatory assay (Defrance *et al.*, 1987b). Human IL-4 could induce proliferation of B lymphocytes that had been pre-activated for one day with insolubilised anti-IgM or for three days with staphylococcus aureus strain Cowan 1. The differences observed between murine and human IL-4 may be due to differences in species, sources of the cells or the procedures used for activation. This factor was originally called B cell growth factor (BCGF) and was initially thought of as a progression factor acting in the late G1 stage of the cell cycle. This was proved to be wrong as further work with IL-4 showed that it had several effects on small, resting B cells in the absence of anti-IgM (Rabin *et al.*, 1985; Rabin *et al.*, 1986a).

The first indication that IL-4 may not only act in late G1 came from experiments that demonstrated that a delay in the addition of IL-4 resulted in a

delayed entry into S phase and delays of 12 hours completely inhibited entry into DNA synthesis (Howard & Paul, 1983). Rabin *et al* (1985) further substantiated that IL-4 has effects on resting B cells by showing that culturing cells for 24 hours with IL-4 alone increases the cell volume. Treatment of B cells with IL-4 also appeared to induce a state of activation, as cells that had been precultured with IL-4 responded more promptly to subsequent culture with IL-4 and anti-IgM to enter S phase (Rabin *et al.*, 1985). B cells stimulated with IL-4 and anti-IgM normally do not begin to enter S phase until about 30 hours after stimulation, some not until 45 hours (Rabin *et al.*, 1986a; Defranco *et al.*, 1982) but 24 hours preculture with IL-4 accelerates this response by 12 hours. The presence of IL-4 would, therefore, recruit B cells quickly in response to an antigenic challenge. IL-4 is required to be present over a long period (24 hours) to induce the maximum response from B cells (Rabin *et al.*, 1986a). This suggests that when IL-4 is bound to its receptor it produces a cellular signal that is required over a long period of time. Resting B cells do possess receptors for IL-4 and part of the prompter response could be explained by the fact that culturing with IL-4 upregulates the expression of its own receptors (Park *et al.*, 1987a). From these experiments it appears that IL-4 plays a more complex role in B cell activation than first imagined. IL-4 has actions on both resting cells and also later in G1.

1.8.2. *IL-4 increases the expression of MHC classII antigens*

Murine IL-4 enhances the expression of MHC class II antigens (Ia) on normal B lymphocytes (Roehm *et al.*,1984; Noelle *et al.*, 1984). Human IL-4 had a similar effect on Burkitt lymphoma cell lines, but increased MHC class II expression was not observed on normal human B cells (Rousset *et al.*, 1988). It may be because human B cells endogenously express high levels of MHC class II antigens and, therefore, it would be more difficult to unequivocally demonstrate an increase (O'Garra *et al.*, 1988). On mouse B

cells there is a selective and dramatic 10-15 fold increase in MHC class II antigen expression as detected by radioiodination and immunoprecipitation (Roehm *et al.*, 1984; Noelle *et al.*, 1984). This increase can be detected 8-12 hours after stimulation with IL-4 (Noelle *et al.*, 1986; Oliver *et al.*, 1985). IL-4 induced MHC class II antigen expression is dependent on new transcription and a two fold increase in MHC class II antigen mRNA can be detected within 4-6 hours (Noelle *et al.*, 1986). The increase in transcription could be mediated by gene regulatory elements which are controlled directly or indirectly by IL-4 binding to its receptor. In two murine pre B cell lines IL-4 induced rapid transcription of MHC class II antigen mRNA within 1 hour independently of protein synthesis (Polla *et al.*, 1986). A nuclear protein which binds to two sites in the region of the MHC class II genes has now been identified (Boothby *et al.*, 1988). This protein is specific to B cells and the addition of IL-4 to normal mouse spleen cells increases the binding of this protein. The increased binding was concomitant with the increase in MHC class II gene transcription. Therefore, B cells contain a sequence specific DNA binding activity whose level is influenced by IL-4. Noelle *et al* (1986) reported that the observed increase in transcription of MHC class II antigen genes was not enough to account for the dramatic increase in the expression of MHC class II antigen on the membrane. IL-4 may not only affect transcription but other events may also be involved perhaps including post translational modifications.

The increase in MHC class II antigen expression by IL-4 could have significant effects on the B cell especially in the context of T-B interaction. Increased MHC class II antigen expression may enhance the capacity of B cells to act as antigen presenting cells and, therefore, increase the responsiveness of the interacting T and B cell.

1.8.3. *IL-4 reverses the inhibition of Fc receptors on B cell activation*

B cell activation is under the control of several molecules, one of which is the antibody secreted in response to antigen. The secreted IgG can bind to Fc receptors which are present on B cells (Dickler, 1976). This binding to Fc receptors produces signals that inhibit further proliferation of the B cell, thus acting in the manner of feedback inhibition (Sinclair, 1983). As has been discussed previously, anti-Ig F(ab)'2 fragments act as polyclonal activators for almost all B cells and at high concentrations can drive the B cell into DNA synthesis (Defranco *et al.*, 1985). Intact anti-IgG, which mimic the secreted antibody, are not mitogenic and can inhibit entry into DNA synthesis (Phillips & Parker, 1984). The intact anti-IgG crosslinks surface Ig and FcRII resulting in a strong negative signal which prevents activation of the B cell (Klaus *et al.*, 1984). It has been shown by O'Garra *et al* (1987) that this negative signalling can be overcome by IL-4 allowing some B cells to enter S phase, although not as many as in the presence of both IL-4 and anti-Ig F(ab)'2. The mechanism by which IL-4 overcomes the inhibition by IgG is unclear. Intact IgG, although preventing B cell activation, still induces the B cell to enter a transitional state which is characterised by an increase in expression of MHC class II antigens (Klaus *et al.*, 1984). A short lived breakdown of phosphatidylinositol polyphosphates ($PtdInsP_3$) has been reported which is sufficient to increase the concentration of calcium in the cell and may be enough to allow IL-4 to act as a costimulant (O'Garra *et al.*, 1987). Ligand binding to antigen activates phosphoinositide hydrolysis which results in the generation of two second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). Coupling of the receptor to $PtdInsP_2$ hydrolysis is via a guanine nucleotide binding (G) protein. It has recently emerged that the co-crosslinking of sIg and FcRII on B cells uncouples the antigen receptor from the G protein (Harnett & Klaus, 1988; Rigley *et al.*, 1989). Uncoupling of the receptor from the G protein means

that there is no enhancement of $PtdInsP_2$ hydrolysis and, therefore, no second messenger or signal transduction. This results in abortive activation. To overcome this inhibition, IL-4 must be substituting for this loss perhaps by acting through a different mechanism or by stimulating the same or a different G protein from that associated with the antigen receptor.

1.8.4. *IL-4 Induces Expression of the Low Affinity Receptor for IgE*

On activation of B cells several new molecules are expressed on the B cell surface. One of the earliest is a 45 kDa protein which has been identified as the low affinity receptor for IgE (FcRε or CD23) (Walker *et al.*, 1986). Hudak *et al* (1987) demonstrated that murine rIL-4 increased the expression of FcRε on splenic B cells 2-3 fold and 6 fold on a B cell hybridoma. Induction of FcRε (CD23) on human B cells by human IL-4 has also been shown (Defrance *et al.*, 1987a). In both cases IL-4 induced expression of FcRε/CD23 was inhibited by Ifn-γ (Bonnefoy *et al.*, 1988; Hudak *et al.*, 1987). IgE itself has been shown to upregulate FcRε by binding to the receptor and preventing turnover of the receptor and so allowing newly synthesised receptor to accumulate on the surface of the B cell (Lee *et al.*, 1986b). IL-4 appears to enhance receptor expression by increasing transcription (Cairns *et al.*, 1988).

Cloning of the human cDNA encoding CD23 has revealed that the N terminus of the receptor is found in the cytoplasm and the C terminus outside the cell (Yokota *et al.*, 1988). CD23 appears to have two different functions. CD23 acts as a differentiation antigen which is expressed on mature B cells but is lost after isotype switching. This suggested that CD23 plays a role in growth and differentiation of B cells and reports of sCD23 acting as an autocrine growth factor for B cells have confirmed this (Gordon *et al.*, 1988). Membrane FcRε/CD23 can be cleaved, possibly by proteolytic hydrolysis, to release a soluble molecule of 35 kDa which is subsequently

further cleaved to 25 kDa. Soluble FcRε (sFcRε) can bind IgE. Bonnefoy *et al* (1988), using monoclonal antibodies that could bind to soluble FcRε, developed a radioimmunoassay that demonstrated that as well as enhancing membrane FcRε expression IL-4 could also induce the release of sFcRε from normal B cells. This action was strongly inhibited by Ifn-γ which suggests that the action is specific to IL-4.

The second function of FcRε is its role in IgE production and importance in parasitic infections. Studies in both murine and human systems have indicated that B cells with low affinity receptors for IgE release factors that modulate IgE production, and sCD23 has been shown to act with sub-optimal concentrations of IL-4 to increase IgE synthesis (Pene *et al.*, 1988a). FcRε are found on eosinophils, monocytes and B cells which are all cells involved in the IgE response to parasitic infection. Secondly IL-4, which regulates IgE production, enhances the expression of FcRε on B cells and monocytes (DeFrance *et al.*, 1987a).

Yokota *et al* (1988) have recently addressed the question as to whether the two different functions of FcRε are exerted by the same molecule or different molecules. Comparison of antigenicity and the mRNA structure of FcRε in B cells, monocytes and eosinophils showed that two different species of FcRε existed which differed only in their N terminal amino acid sequence. One form of FcRε is expressed only in B cells and is expressed constitutively. The second molecule is found on B cells and monocytes but only in response to IL-4. The second molecule is thought to be responsible for IgE production. The observation of the existence of two different FcRε helps to some extent to explain how apparently the same molecule can have two different functions.

1.8.5. *IL-4 Induces Secretion of IgG1 and IgE from B Cells*

In the mouse the addition of several different lymphokines has been shown to induce changes in the Ig isotype secreted (Snapper *et al.*, 1988a).

Resting B cells express IgM and IgD on their surface and, upon culture with LPS, IgM is the predominant isotype secreted. LPS treated B cell blasts that express membrane IgG3 and membrane IgG2b, although in the minority, secrete significant amounts of IgG3 and IgG2b. IgG1 and IgG2a secretion is low and IgE is undetectable. The addition of IL-4 to LPS treated B cells promotes the secretion of IgG1 and IgE and partially supresses IgG3 and IgM (Vitetta *et al.*, 1985; Sideras *et al.*, 1985a/b; Noma *et al.*, 1986; Isakson, 1986). Other lymphokines that selectively stimulate secretion of specific isotypes are Ifn-γ which promotes IgG2a production (Snapper *et al.*, 1988c) and IL-5 which has been reported to stimulate IgA secretion (Mosman & Coffman, 1987).

The induction of IgG1 and IgE are differentially regulated (Snapper *et al.*, 1987). IL-4 stimulates IgG1 in a bimodal fashion. Addition of increasing amounts of IL-4 to LPS treated B cells dramatically increases IgG1 production peaking at 100 units/ml IL-4. Beyond this concentration, between 600-1000 units/ml IL-4 there was no induction of IgG1, then above concentrations of 1000 units/ml IL-4 IgG1 production was again stimulated. Enhancement of IgE production by IL-4 required higher concentrations, no enhancement of IgE was seen until 100 units/ml of IL-4 was present and induction was maximal at 10,000 units/ml. IgG1 and IgE secretion are, therefore, regulated differently. Studies with IL-4 on resting B cells demonstrated that IL-4 had preparatory effects on resting B cells which did not involve cellular proliferation. When resting B cells were cultured in the presence of IL-4 alone for 48 hours then washed and LPS added IgG1 secretion was enhanced but the presence of IL-4 after the 48 hours was inhibitory. IgE secretion although requiring the preparatory effects of IL-4 also required IL-4 after the 48 hours suggesting a two phase regulation (Snapper *et al.*, 1987). These results suggest that IL-4 and LPS are acting on the B cells independently of each other perhaps through different intracellular pathways.

Snapper *et al* (1988b) have reported an increase in the number of B cells expressing membrane IgG1 and IgE after 4 days of culture with LPS and IL-4. Cell sorting analysis indicated that B cells expressing IgG1 and IgE on the surface secreted these Ig isotypes. Co-expression of membrane IgG1 and IgE on B cells has also been observed but the B cells do not appear to secrete both isotypes simultaneously (Snapper *et al.*, 1988b).

IL-4 can act on $IgG1^-$ B cells to induce secretion of IgG1. Therefore, IL-4 promotes Ig class switching rather that promoting the proliferation and differentiation of B cells expressing membrane IgG1 (Isakson *et al.*, 1982). The concentration of IL-4 needed to promote isotype switching is greater than that observed for the other effects on B cells. It may be possible to obtain the high concentrations of IL-4 that are needed at the site of T-B cell interactions where the local concentration of IL-4 could be high and may be directionally secreted from the T cell to the B cell (Kupfer *et al.*, 1986). IL-4 induced isotype switching has been shown to be relevant *in vivo*. The IgE response was demonstrated *in vivo* by Finkelman *et al* (1986). Mice that were infected with the larvae of the helminth *Nippostrongylus brasiliensis* had a 100 fold increase in serum IgE which could be inhibited in the presence of anti-IL-4 antibody. This suggests that IL-4 plays a role in the biosynthesis of IgE *in vivo*.

It has been reported that IL-4 induces IgE secretion in normal human B cells fom peripheral blood and tonsils and a low induction of IgG is also observed (Pene *et al.*, 1988a). Although no polyclonal activator was present in these experiments the presence of monocytes and T cells was required which suggests that factors other than IL-4 were needed. IL-5 enhanced IL-4 induced IgE production particuarly at sub optimal levels of IL-4 (Pene *et al.*, 1988b). IL-5 alone was ineffective and IL-4 inhibited the induction of IgA by IL-5. These results are consistent with reports in murine systems of murine

IL-5 augmenting IL-4 stimulation of IgE in LPS activated B cells (Coffman *et al.*, 1987). The IL-4 induced IgE response was inhibited by Ifn-γ and by $F_{(ab)'2}$ fragments of anti-CD23 antibody (Pene *et al.*, 1988a). This observation is particuarly interesting as IL-4 has been reported to enhance the expression of membrane CD23 on B cells and induce its release as a soluble 25 kDa molecule. The sCD23 has been demonstrated to enhance IL-4 induced IgE synthesis at sub-optimal concentrations of IL-4 although sCD23 alone is ineffective. Safarti *et al* (1984) reported that sCD23 augments ongoing IgE synthesis by B cells from atopic individuals. There is a relationship between CD23 expression, release and the production of IgE but the mechanism at present is unclear.

1.9. Interferon-γ: A Potent Inhibitor of IL-4 Action on B Cells

Interferon-γ (Ifn-γ) inhibits the majority of the biological effects of IL-4 on B cells in both mouse and human systems (Rabin *et al.*, 1986b; Mond *et al.*, 1986). It does not appear to affect the action of IL-4 on other cell types but is specific to B cells. The proliferation of B cells in response to LPS is not affected by Ifn-γ and neither is the increase in cell volume and increase in expression of MHC classII antigens induced by anti-Ig. Ifn-γ is, therefore, exerting its effect directly on IL-4 responses. Ifn-γ is most effective when added simultaneously with IL-4 and has been shown to inhibit IL-4 induced increase in MHC class II expression, increase in cell volume, FcRε/CD23 expression, the costimulatory effect with anti-Ig and promotion of IgG1 in the mouse and IgE secretion in both mouse and human (Mond *et al.*, 1986; Rabin *et al.*, 1986b; Rousset *et al.*, 1988). Although Ifn-γ has a number of inhibitory effects on antibody production it also has some stimulatory responses. Ifn-γ can induce MHC antigen expression on macrophages and B cell tumours (Mond *et al.*, 1986). It has been reported that IL-2 can synergise with Ifn-γ to enhance B cell differentiation (Sidman *et al.*, 1984; Nakagawa *et al.*, 1985). Romagani *et al* (1986b) have observed

Ifn-γ acting as a costimulator with anti-IgM to drive human tonsillar cells to DNA synthesis although this could be explained if the cells were not resting. There has also been reports of Ifn-γ acting on resting B cells. Culturing resting B cells with Ifn-γ enhances the secretion of IgG2a on subsequent culture with LPS (Snapper *et al.*, 1988c). The enhancement of IgG2a appears to work in a similar manner to IL-4 inducing IgG1 secretion. IL-4 is inhibitory to the induction of IgG2a by Ifn-γ. It therefore appears that there is a reciprocal regulation between IL-4 and Ifn-γ. Lowenthal *et al* (1988) have demonstrated that there is no decrease in binding of IL-4 to its receptor in the presence of Ifn-γ. Ifn-γ does not act by binding to the IL-4 receptor or by preventing binding of IL-4 itself. Perhaps it is the intracellular signals produced by IL-4 on binding to its receptor that are disrupted by Ifn-γ, although the signal transduction of both pathways are unclear. The mutually exclusive effect of IL-4 and Ifn-γ may have a functional significance in that together they can regulate the B cell immune response.

Recently, proposals have been made that murine helper T cells can be subdivided into two classes, Th1 and Th2 (Mosman & Coffman, 1987). Looking at a wide range of T cell clones it was observed that they could be subdivided on the basis of the lymphokines that they produced. Th1 cells produce IL-2, Ifn-γ and lymphotoxin, whereas Th2 cells produce IL-4 and IL-5. Both types of cells can secrete IL-3 and GM-CSF. The two types of helper T cell proliferate in response to antigen and T cell growth factors in the same manner and no difference in surface markers has been identified. Therefore the interaction of a T cell subset (Th1 or Th2) would determine whether IL-4 or Ifn-γ was produced and hence determine the response elicited. A cellular basis for this hypothesis would be in the regulation of isotype switch (Stevens *et al.*, 1988). Stimulation of Th2 cells would release IL-4 which promotes IgG1 and IgE secretion which are both needed to respond against parasitic infections. Stimulation of Th1 cells on the other hand, would result in Ifn-γ production which enhances IgG2a secretion.

IgG2a is particularly effective in responses against viral infections and is highly effective at complement fixation, opsonisation and antibody mediated cellular cytotoxicity by macrophages (Snapper *et al.*, 1988c). This means that the sub division of helper T cells may be relevant in what type of infection the immune response is elicited against. The division of helper T cells into sub classes is not confirmed as some T cell clones do not fall into either class but secrete both IL-4 and Ifn-γ. *In vitro* experiments with human T cells has so far failed to identify different sub classes of helper T cells such as those demonstrated in the mouse (Maggi *et al.*, 1988) and these sub classes have also not been identified *in vivo*. It is therefore some what controversial as to whether the divisions of helper T cells exist or are relevant *in vivo*.

1.10. Receptors for Interleukin 4

Receptors for IL-4 have been identified by the binding of radiolabelled [^{125}I]-IL-4 to IL-4 responsive cells (Lowenthal *et al.*, 1988; Park *et al* .,1987a; Park *et al.*, 1987b; Ohara & Paul, 1987). IL-4 receptors have been identified on several haematopoetic cells including B and T lymphocytes, mast cells and macrophages (Ohara & Paul,1987) and also on cells from non haematopoetic lineage (Lowenthal *et al.*, 1988). The finding of IL-4 receptors on a wide range of cell types is consistent with the wide range of biological activities reported for IL-4. Resting B cells express low levels of receptor. Binding experiments on high density resting B cells from DBA/2 and C57BL/6J have reported numbers of 65 (Park *et al.*, 1987a) and 311 (Ohara & Paul, 1987) IL-4 receptors per cell, respectively. The number of receptors is increased 5-10 fold on activation of the cells with LPS (320 IL-4 receptors/cell in C57BL/6J mice and 1511 receptors/cell in DBA/2 mice), and also with anti-IgM (1560 receptors/cell in DBA/2 mice). The same was found on human cells, where resting B cells from peripheral blood expressed 140 receptors/cell which was increased to 710 receptors/cell on treatment with PHA (Park *et al.*, 1987b). IL-4 receptors are also increased on culturing with IL-4 itself. Upregulation of the receptors is detectable within six hours

and is maximal at eighteen hours (Ohara and Paul, 1987). This observation helps to explain the fact that IL-4 has effects on resting B cells. Continuously proliferating cell lines in both mouse and human express high numbers of IL-4 receptors.

Binding of [^{125}I]-IL-4 to its receptor was specific and only excess unlabelled IL-4 and 11B11, an antibody to IL-4, could prevent IL-4 binding to its receptor. Human IL-4 could not inhibit the binding of murine IL-4 to the murine IL-4 receptor and vice versa, which is consistent with the finding that no murine IL-4 receptors were found on human cells and no human receptors on mouse cells. IL-1, IL-2, IL-3, Ifn-γ, anti-LFA-1, anti-Lyb2 and several other growth factors did not compete with IL-4 for binding to the receptor. Ifn-γ, although it inhibits many of the biological effects of IL-4, does not bind to the IL-4 receptor, nor does it prevent the binding of IL-4 and, therefore, must exert its effect after receptor binding. It has been reported that anti-LFA-1 (Mishra *et al.*, 1986) and anti-Lyb2 (Subbarao and Mosier, 1983) could substitute for IL-4 in some of its biological functions and it was proposed that these molecules may be the receptor for IL-4. Arguments against this are that IL-4 has effects on both LFA-1$^-$ and Lyb2$^-$ cells and neither monoclonal antibodies can inhibit the binding of IL-4. It is, however, still possible that these molecules are indirectly involved in IL-4 binding to its receptor.

Kinetic studies indicated that the IL-4 receptor exists as a single class of high affinity receptor (Park *et al.*, 1987a; Park *et al.*, 1987b; Ohara and Paul, 1987). The existence of a lower affinity receptor cannot be excluded due to the concentration of ligand used in these experiments. The binding of IL-4 to its receptor was rapid and saturable at 4^{o}C and 37^{o}C. Maximum binding of [^{125}I]-IL-4 was achieved within 20 minutes with a $t_{1/2}$ of 1-2 minutes in the mouse (Park *et al.*, 1987a) and less than 30 minutes in the human (Park *et al.*, 1987b). The dissociation was slow with a $t_{1/2}$ of 4

hours (Lowenthal *et al.*, 1988) exhibiting a biphasic pattern corresponding to a fast and slow dissociating component. Lowenthal *et al* (1988) have demonstrated in HT-2 cells, a T helper cell line, that the receptor and ligand are rapidly internalised which is thought to be an initial step in signalling. It was found that the cells could internalise and accumulate more molecules of [^{125}I]-IL-4 than the number of receptors expressed on the surface of the cell at any one time. This suggested that the internalised IL-4 receptors were either re-cycled, new receptors were synthesised, or a pool of pre-synthesised receptors existed which were not at the cell surface. Which of these possibilities is correct still remains to be elucidated.

Affinity crosslinking experiments where [^{125}I]-IL-4 bound to receptors was crosslinked using bifunctional agents have identified the IL-4 binding components of the receptor. In the mouse, using DBA/2 mice, Ohara and Paul (1987) have identified a component with a molecular weight of 80 kDa which, when the molecular weight of IL-4 (20 kDa), was deducted gave a molecular weight of 60 kDa. Park *et al* (1987a), using five different cell lines which expressed IL-4 receptors, identified a crosslinked species of molecular weight 124 kDa. The IL-4 was hyperglycosylated and had a molecular weight of 49 kDa which left an IL-4 binding component of 75 kDa. Crosslinking studies have been performed on two human cell lines, Raji and gingival fibroblasts (Park *et al.*, 1987b). Both cell types showed a crosslinked component of 154 kDa, which represented a binding component of 139 kDa. Recently Galizzi *et al* (1988) performed affinity crosslinking experiments using [^{125}I]-IL-4 on a human burkitt lymphoma cell line, Jijoye, and identified two IL-4 binding components; 140 kDa equivalent to that found by Park *et al* (1987b) and a 70 kDa component. The murine IL-4 receptor from B cells has now been cloned and the nucleotide sequence determined and the estimated molecular weight for the receptor is 140 kDa (Drs D. Cosman and S. Gillis, Immunex Group, USA. personnal communication). As the IL-4 binding component in human cells has been estimated to be 139 kDa (Park

et al., 1987b) it may be therefore, that the receptors for IL-4 are of similar size in the murine and human system. At present the human IL-4 receptor has not been isolated and therefore, it is unclear whether the IL-4 binding components represent the whole IL-4 receptor or only a subunit which binds IL-4. The IL-4 binding component in the mouse was found to be 75 kDa, but this may have been a result of protease action as a 70 kDa component was also identified in human cells under certain conditions. It was not determined whether this 70 kDa protein was related to the 139 kDa IL-4 binding protein (Galizzi *et al.*, 1988). IL-4 shows species specificity and, since IL-4 itself appears to have significant homology between species, differences may exist in the murine and human receptors. It may be that the receptor is composed of more than one protein and the overall structure could be similar between species with differences existing in the IL-4 specific binding protein.

1.11. Signal Transduction

A variety of molecules can participate in the activation of resting B cells. Each molecule has different biological effects on the B cell and a unique receptor through which it acts. This suggests that each molecule may have a distinct mechanism of intracellular signalling and the overall activation of a B cell may require the initiation of several of these pathways in a particular combination. There have been numerous reports detailing mechanisms of signal transduction in non lymphoid tissue. It appears from these studies that a variety of ligands binding to unique receptors and eliciting a wide variety of biological responses have common pathways of signal transduction. Sutherland and Rall (1958) were the first to propose the concept of second messengers. This allows the rapid changes in cell metabolism, that are observed on ligand binding to receptor, by the functional modification of existing proteins. These functional modifications have been most notably acheived through phosphorylation of existing proteins which can either up or

down regulate the activity of these proteins. Several pathways of transmembrane signalling have now been identified and these are now being investigated in lymphocytes.

There are three points in T dependent B cell activation where signals can be transduced; at the initial binding of antigen to membrane immunoglobulin; at the MHC restricted T-B cell interaction and at the interaction of the many lymphokines with their receptors (Snow and Noelle, 1987).

1.12. Antigen Binding To Membrane Immunoglobulin Activates The PI Cycle

The first indication of the mechanism of transmembrane signalling involved when antigen binds to membrane immunoglobulin was the report by Maino *et al* (1975) that an increase in incorporation of [^{32}P] into phosphatidylinositol (PI) was observed in porcine B lymphocytes on activation with anti-Ig. Grupp *et al* (1987) have since reported an increase in PI metabolism using hapten-specific B cells. This suggested that the phosphatidyl inositol cycle may be involved in membrane Ig signalling. The PI response has been demonstrated in transmembrane signalling in a number of systems (Michell, 1982a; Michell, 1982b) including T lymphocytes (Fisher and Muller, 1968).

Further evidence for the involvement of the PI cycle in Ig signalling in B lymphocytes came from the observation that the tumour promoting phorbol diesters, eg. phorbol myristate acetate (PMA), could also increase the expression of MHC class II antigens on B cells (Monroe *et al.*, 1984; Lindsten *et al.*, 1984). Phorbol esters are known to activate protein kinase C

(PKC) directly, replacing diacylglycerol and, indeed the receptor for PMA has been found to be PKC itself (Parker *et al.*, 1984; Castagna *et al.*, 1982). Further evidence for the involvment of PKC came from two reports; Coggeshall & Cambier (1984) reported a rapid rise, within 30 seconds to 1 minute, in [^{32}P] incorporation into PtdOH, a phosphorylated product of DAG, on stimulation with anti-Ig, and Bjisterbosch *et al* (1985) observed the rapid formation of diacylglycerol (DAG). Based on this it was hypothesised that signalling by membrane Ig involved the hydrolysis of PtdIns, PtdInsP and $PtdInsP_2$ by phospholipase C (PLC) which results in the formation of diacylglycerol (DAG), the physiological activator of PKC, to stimulate PKC (Cambier & Ransom, 1987). Ransom *et al* (1986) and Bjisterbosch *et al* (1985) reported that release of $InsP_3$ is rapid while $InsP_2$ and InsP is slower indicating that of all the polyphosphatidylinositol phosphates, $PtdInsP_2$ is preferentially hydrolysed and this is consistent with observations made in non lymphoid systems.

In nearly all cases where ligand binding to receptor induced an increase in polyphosphoinositide hydrolysis, a rapid increase in intracellular free calcium has also been reported. Experiments loading cells with labelled calcium, [$^{45}Ca^{2+}$], showed that anti-Ig induced a rapid increase in intracellular free calcium within minutes of stimulation. This was substantiated by the use of calcium indicator dyes (Braun *et al.*, 1979, Pozzan *et al.*, 1982). The release of calcium was not induced by PMA which suggested that PKC was not involved (Ransom & Cambier, 1986) but it was induced by $PtdInsP_2$ hydrolysis. Again using permeabilised cells loaded with [$^{45}Ca^{2+}$] it was shown that $InsP_3$ induced significant mobilisation of calcium from intracellular stores (Ransom *et al.*, 1986).

Stimulation of B lymphocytes with anti-Ig was shown to induce a

rapid translocation of PKC, within 2 minutes, in both human (Nel *et al.*, 1986) and mouse (Chen *et al.*, 1986) cells. DAG exerts its second messenger function by directly binding to and activating PKC. The binding of DAG to PKC increases the affinity of PKC for calcium and this mediates the redistribution of PKC to the plasma membrane. The activation of protein kinase C results in the phosphorylation of several cytosolic and membrane associated proteins on serine and threonine residues (Nishizuka, 1984). Phosphorylation of these proteins can influence cellular metabolism by altering the functional activities of the proteins which can result in a stimulatory or an inhibitory effect. Enhanced or unique phosphorylation of several proteins associated with B cell plasma membranes has been demonstrated. Studies by several groups (Nel *et al.*, 1986; Hornbeck and Paul, 1986) showed that phosphorylation patterns of B cells were similar on treatment with either PMA or anti-IgM. This provides further evidence for the activation of PKC by anti-IgM. Burke *et al* (1989) have substantiated this by demonstrating that phosphorylation of the proteins was inhibited in the presence of PKC inhibitors. The molecular weights of these proteins range from 170 kDa to 15 kDa (Nel *et al.*, 1986; Burke *et al.*, 1989). The functions of the phosphorylated protein are unknown but they may play a role in B cell activation. Hornbeck & Paul (1986) identified four of the seven phosphorylated proteins that they observed to be associated with the cytoskeleton. These results suggested that phosphorylation may play a role in the initial cytoskeletal reorganistaion which induces crosslinking of membrane immunoglobulin. One of the earliest events on treatment with anti-Ig is the crosslinking and reorganisation of receptors into patches or caps (Braun & Unanue, 1980). The crosslinked receptor is then internalised and degraded (Antoine *et al.*, 1974). Aggregation of membrane Ig involves the attachment of the immunoglobulin to proteins in the cytoskeleton. Actin has been

identified as being a protein involved in aggregation (Bourguigon *et al.*, 1978, Flanagan & Koch, 1978) and also immunoprecipitation of Ig has revealed attachment to a 50 kDa protein (Unanaue *et al.*, 1971).

The consequence of anti-IgM stimulation of B lymphocytes is the induction of transcription of several genes. Transcription of the gene cfos is induced within 15 minutes, c-myc within 60 minutes and Ia within 120 minutes (Cambier & Ransom, 1987). The signals that are transduced between the activation of protein kinase C and the induction of these genes are unknown. Several proteins have been identified as substrates for protein kinase C but as yet the function of these proteins has not been determined. The only event which occurs is the membrane depolarisation which is observed at 3 minutes and is maximal at 60 minutes and can be induced by agents which activate PKC (Monroe and Cambier, 1983b). Valinomycin, a potassium ionophore, inhibits both the membrane depolarisation and also the increase in MHC class II antigens. This suggests that membrane depolarisation is involved in the signalling of anti-Ig inducing expression of MHC class II antigens but, as yet, the precise mechanism is unclear.

In several signal transduction pathways, G proteins have been found to be involved in regulation (for a review see Harnett & Klaus, 1988). The proteins exist in an $\alpha\beta\gamma$ heterotrimeric structure. When GTP binds to the α subunit the GTP-α dissociates from the β and γ and can directly modify the activity of its target. The activation is terminated by conversion of GTP to GDP by the GTPase activity of the α subunit which allows the reassociation of the α subunit with the β and γ. A role for G proteins has been implicated in the coupling of receptor to $PtdInsP_2$ hydrolysis in several systems. It has been shown in B cells that sIgM and sIgD receptors are coupled to the

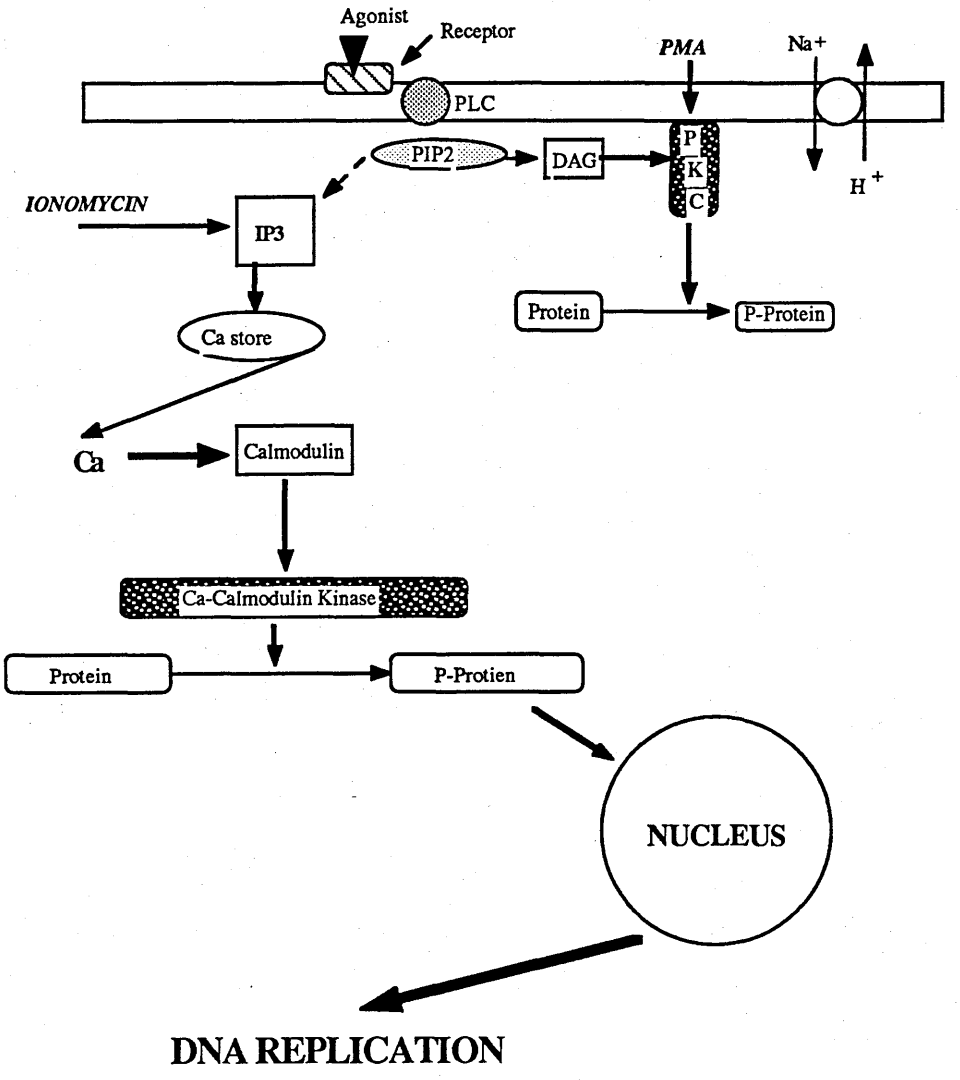
polyphosphoinositide-specific phosphodiesterase (PPI-PDE) by a pertussis toxin insensitive Gp (Gold *et al.*, 1987; Harnett & Klaus, 1988). Therefore binding of antigen to membrane Ig activates a PPI-PDE via a G protein. PDE then catalyses the hydrolysis of $PtdInsP_2$ to $InsP_3$ and DAG. $InsP_3$ induces the release of calcium from internal stores which activates calcium calmodulin dependent protein kinase and also acts with DAG to activate PKC. The activation of PKC results in the phosphorylation of several proteins which leads to membrane depolarisation and the induction of transcription of several genes (summarised in figure 3).

1.13. Lipopolysachariде

In a study by Bijsterbosch *et al* (1985) two polyclonal activators, LPS and PMA did not induce phosphoinositide hydrolysis or calcium mobilisation. Betel *et al* (1974) had earlier found that LPS did not stimulate incorporation of [^{32}P] orthophosphate into phosphatidylinositol. LPS does, however, induce the translocation of PKC (Chen *et al.*, 1986) and also membrane depolarisation (Kiefer *et al.*, 1980). It is possible that LPS bypasses phosphatidylinositol degradation and calcium mobilisation and directly activates PKC as has been suggested by Wightman & Raetz (1984). PMA has been shown to activate PKC directly, substituting for DAG and PKC is the cellular receptor for PMA (Parker *et al.*, 1984; Castagna *et al.*, 1982). It has now been shown that LPS activates a GTP binding protein (Jakeway & Defranco, 1986). The LPS response in WEHI-231, a B cell lymphoma, was inhibited by pertussis toxin, which inactivates a subset of G proteins. This result, combined with LPS inhibiting adenylate cyclase in $P388D_1$ cells, strongly suggests a role for a Gi receptor coupling protein. LPS, therefore by binding to a putative receptor activates a protein, Gi, which inhibits adenylate cyclase resulting in a decrease in cAMP levels. One of the consequences of decreased cAMP levels is an increase in the existing

Figure 3 The Signals Transduced Upon Antigen Binding

Ligand binding to the antigen receptor activates phospholipase C (PLC) via a G protein. Phospholipase C catalyses the hydrolysis of phosphatidylinositol 4, 5 bisphosphate ($PtdInsP_{2)}$ to yield two second messengers, diacylglycerol (DAG) and inositol 1, 4, 5 triphosphate (IP_3). IP_3 diffuses to the endoplasmic reticulum where it mediates the release of calcium (Ca^{++}) from intracellular stores. Calcium may act through calmodulin to activate calcium/calmodulin dependent protein kinases but it also acts in concert with DAG to mediate the translocation of protein kinase C (PKC) to the plasma membrane. Protein kinase C via phosphorylation can alter the functions of several proteins.


Agonist
Receptor
PMA
Na+
PLC
PIP2
DAG
P
K
C
H +
IONOMYCIN
IP3
Protein
P-Protein
Ca store
Ca
Calmodulin
Ca-Calmodulin Kinase
Protein
P-Protien
NUCLEUS
DNA REPLICATION

$PtdInsP_2$ hydrolysis as cAMP is an inhibitor of this pathway.

1.14. Signals Transduced upon Ifn-γ Binding to its Receptor

Interferon-γ (Ifn-γ) is a potent inhibitor of the majority of the biological effects of IL-4 on B lymphocytes. This inhibition is not a result of binding to the IL-4 receptor or preventing binding of IL-4. It is therefore interesting to consider the signal transduction pathways activated by IL-4 and Ifn-γ.

Experiments using bifunctional reagents to crosslink [^{125}I]-Ifn-γ to several human cell types have identified a binding component of 105-130 kDa (Pestka *et al.*, 1987; Rubinstein *et al.*, 1987), although, a larger component of 160-180 kDa was found on monocytes (Fischer *et al.*, 1989). On murine cells a slightly smaller Ifn-γ binding component of 90-110 kDa was determined (Wietzerbin *et al.*, 1986; Mariano *et al.*, 1987). Little is known of the initial events of cellular activation by Ifn-γ, although in human U937 cells, a monocyte cell line, rapid changes in intracellular calcium and membrane potential was reported (Klein *et al.*, 1987a; Klein *et al.*, 1987b). Ifn-γ also caused redistribution of PKC in these cells within 10 minutes, and the transcription of several Ifn-γ induced genes was blocked by inhibitors of PKC (Fan *et al.*, 1988). A role for intracellular calcium mobilisation and PKC has been inferred in the activation of murine macrophages with Ifn-γ (Celada and Schreiber, 1986). Binding of Ifn-γ to the human Ifn-γ receptor has been demonstrated by immunoprecipitation to result in the phosphorylation of the receptor (Mao *et al.*, 1987). Cambier & Ransom (1987) reported that Ifn-γ and anti Ia antibodies, which have similar biological effects to Ifn-γ, did not induce phosphatidylinositol hydrolysis or calcium mobilisation in B cells. It was noted, however, that the biological effects of

anti-Ia and Ifn-γ were similar to agents such as dibutyrl cAMP (dbcAMP), forskolin and cholera toxin. These agents are all known to elevate cAMP levels and it was hypothesised that anti-Ia and Ifn-γ may act in this manner ie. a β adrenergic like mechanism. Further evidence for this hypothesis came from the observation that anti-Ia antibodies induced a three fold increase in cAMP within 60 seconds in B cells. It was also reported that both anti-Ia and dbcAMP induce translocation of PKC to the nuclear envelope (Chen *et al.*, 1986). Recently Ostrowski *et al* (1988) observed that Ifn-γ induced the translocation of PKC from the cytosol to the membrane in a pre B cell line,7OZ/3. This activity was observed 20 minutes after stimulation and was not required for the enhanced Na+/H+ exchange demonstrated in these cells on treatment with Ifn-γ. The redistribution of PKC observed in other systems after stimulation of phosphatidylinositol hydrolysis is rapid (within two minutes).

The signals transduced when Ifn-γ binds to its complementary receptor appear to involve an increase in cAMP levels and a translocation of PKC but how these two events are connected is unclear. It may also be that different pathways of activation are used by different cell types.

1.15. Phosphotyrosine

Until recently, the only acid stable phosphoamino acids known were phosphoserine and phosphothreonine, but phosphorylation on tyrosine residues is now emerging as an important protein modification. Phosphotyrosine only accounts for 0.05% of the total acid stable phosphate in proteins while phosphothreonine and phosphoserine account for approximately 10% and 90% respectively (Sefton *et al.*, 1980). Despite this, protein tyrosine kinases are emerging as important regulatory enzymes.

Phosphotyrosine was first discovered as the product of a kinase activity that was associated with the retroviral transforming proteins (Eckhart *et al.*, 1979; Hunter & Sefton, 1980). This was substantiated when it was discovered that cells infected with certain retroviruses contained ten times the amount of phosphotyrosine of uninfected cells (Sefton *et al.*, 1980). Due to the discovery that tyrosine phosphorylation was associated with the transforming proteins of several RNA tumour viruses, it was hypothesised that it might be essential for transformation. Cells that were infected with virus mutants that were temperature sensitive for transformation showed that the increase in phosphotyrosine was correlated to transformation (Sefton *et al.*, 1980). Phosphotyrosine is not unique to retroviruses, but has also been detected in some cellular proteins and the receptors for several growth factors possess a tyrosine specific kinase activity. In many cases where cells are infected with retroviruses or treated with growth factors, tyrosine phosphorylation is the first detectable event. The fact that tyrosine phosphorylation plays a role both in viral transformation, which leads to unrestricted growth, and in the action of several growth factors suggests that it has a function in growth control.

1.15.1.*Association of Phosphotyrosine with RNA Tumour Viruses*

Genetic evidence indicates that the protein product of a single gene is sufficient to induce transformation of a virally infected cell to a malignant state (Bishop, 1983). This suggests that a single protein can induce a number of alterations to a cell. Rous sarcoma virus (RSV) is a sarcoma-causing virus of chickens. The virus has four genes, three which code for viral structural proteins and the other gene, *v-src*, which is responsible for transformation of the host cell (Copeland *et al.*, 1980). *V-src* codes for a phosphoprotein of 60 kDa, $pp60^{v\text{-}src}$. This protein was found to be tightly associated with a protein kinase activity *in vitro* which was specific for tyrosine residues

(Collett & Erikson, 1978; Levinson *et al.*, 1978). It has now been shown that pp60$^{v\text{-}src}$ itself possess the ability to transfer phosphate from ATP to tyrosine residues on proteins (Gilmer & Erikson, 1981; McGrath & Levinson, 1982). This was the first evidence that tyrosine phosphorylation was associated with the transforming gene product of RNA tumour viruses.

The major substrate of the pp60$^{v\text{-}src}$ protein tyrosine kinase was pp60$^{v\text{-}src}$ itself (Erikson *et al.*, 1979; Collett *et al.*, 1983). The autophosphorylation appeared to enhance the activity of the tyrosine kinase. The major site of phosphorylation *in vivo* was on tyrosine 416 in the carboxy terminal region (Smart *et al.*, 1981) although some phosphorylation has been observed on serine 17. This latter phosphorylation is believed to be due to cAMP dependent protein kinase (Collett *et al.*, 1979; Cross & Hanafusa, 1983). Early studies suggested that phosphorylation at tyrosine 416 may be responsible for transformation. This was disputed by studies using site directed mutagenesis to replace tyrosine 416 with either phenylalanine (Snyder *et al.*, 1983) or a short linker sequence (Cross & Hanafusa, 1983). This demonstrated that even without phosphorylation on tyrosine 416 the kinase was still active and the cells transformed. This does not mean that tyrosine phosphorylation plays no role in transformation as tyrosine phosphate, although reduced, was not totally absent due to phosphorylation of other tyrosine residues in pp60$^{v\text{-}src}$.

Pp60$^{v\text{-}src}$ was identified as an integral plasma membrane protein exposed at the inner membrane surface (Levinson *et al.*, 1981). Despite this, pp60$^{v\text{-}src}$ is synthesised on free ribosomes (Lee *et al.*, 1979; Levinson *et al.*, 1981) and released as a cytoplasmic protein. In the cytoplasm pp60$^{v\text{-}src}$ is associated with two proteins p89 and p50 (Hunter and Sefton, 1980).

It is then transported to the plasma membrane where it dissociates from the proteins and associates with the plasma membrane (Hunter and Sefton, 1980). The cytoplasmic form of $pp60^{v\text{-}src}$ has little tyrosine kinase activity (Courtneidge & Bishop, 1982).

The transforming proteins of several other retroviruses have since been shown to possess protein tyrosine kinase activity. The transforming protein of Abelson murine leukemia virus (*A-MuLV*) is a 120 kDa integral plasma membrane protein spanning the lipid bilayer (Witte *et al.*, 1979). In cells transformed with *A-MuLV* there is a ten fold increase in total cell phosphotyrosine and *in vivo* $p120^{gag\text{-}abl}$ was phosphorylated on serine, threonine and tyrosine residues. Immunoprecipitation of $p120^{gag\text{-}abl}$ has revealed a tyrosine specific protein kinase activity of which the major substrate was $p120^{gag\text{-}abl}$ (Witte *et al.*, 1980).

V-fps (an oncogene of chicken sarcoma viruses) and *v-fes* (an oncogene of feline sarcoma viruses) are proteins which are structurally, functionally and immunologically related (Beemon, 1981). Transformation by these proteins results in an increased phosphotyrosine content in cells (Cooper & Hunter, 1981a/b) and the proteins themselves possess a protein tyrosine kinase activity which results in autophosphorylation. These retroviral tyrosine kinases, like other retroviral transforming proteins, have been found to be associated with the plasma membrane.

V-yes from chicken sarcoma viruses has properties which are similar to $pp60^{v\text{-}src}$. It phosphorylates tyrosine and serine residues *in vivo* and tyrosine residues *in vitro* (Kawai *et al.*, 1980; Ghysdael *et al.*, 1981).

The only non retroviral transforming protein which was reported to be associated with tyrosine kinase activity *in vitro* is the middle T antigen of polyoma virus (Smith *et al.*, 1979; Eckhart *et al.*, 1979; Schaffhausen & Benjamin, 1979). Results now suggests that this tyrosine kinase activity is unlikely to be intrinsic to the middle T antigen as transformed cells contain no more phosphotyrosine than untransformed cells (Sefton *et al.*, 1980) and middle T antigen prepared by *in vitro* translation of polyoma specific mRNA has no protein kinase activity. The discovery that $pp60^{c\text{-}src}$ is stably associated with the middle T antigen in cell lysates suggests that this may have been the kinase responsible for the phosphorylation of middle T antigen observed *in vitro* (Courtneidge & Smith, 1984).

If protein tyrosine kinases play a role in viral transformation which leads to unrestricted growth they may play a role in the control of normal cell growth. This was substantiated by the fact that retroviral transforming genes, oncogenes, are derived from host sequences during the life cycle of the retrovirus. The cellular genes are termed proto oncogenes. A relationship between viral and cellular genes has been substantiated by the finding that the cellular homologue of $pp60^{v\text{-}src}$, $pp60^{c\text{-}src}$, is structurally similar to the viral protein (Takeya & Hanafusa, 1983). $Pp60^{c\text{-}src}$ has been conserved through evolution and was found to be expressed in every vertebrate species examined, suggesting that it may have an essential function in cellular metabolism (Bishop & Varmus, 1982). $pp60^{c\text{-}src}$ was identified as having a protein tyrosine kinase activity *in vivo* and is itself phosphorylated on serine and tyrosine residues (Collett *et al.*, 1979; Hunter & Sefton, 1980) which suggests that tyrosine phosphorylation may be involved in the control of normal cell growth. Transformation does not result from an overexpression of $pp60^{c\text{-}src}$ as cells that had ten times the amount of $pp60^{c\text{-}src}$ had a normal morphology (Parker *et al.*, 1984; Iba *et al.*, 1984). Therefore,

differences must exist between pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$, perhaps in enzyme activity or substrate specificity.

1.16. Phosphotyrosine in Normal Cell growth

Further evidence for the involvment of tyrosine kinases in cell growth has come from the finding that several receptors for mitogenic hormones possess intrinsic protein tyrosine kinase activity.

1.16.1.*Platelet Derived Growth Factor*

Platelet derived growth factor (PDGF) is the major growth factor in serum for cells of connective tissue origin and cultured glial cells (Deuel & Huang, 1983). Binding of PDGF to its receptor induces membrane ruffling, reduction in cell adhesion, changes in the actin cytoskeleton structure and an overall increase in mitotic activity (Deuel & Huang, 1983; Ek *et al.*, 1982). Cooper *et al* (1982) demonstrated that stimulation of cells with PDGF caused a two fold increase in total cell phosphotyrosine. Evidence from both *in vitro* and *in vivo* studies have shown that a protein tyrosine kinase activity is activated upon PDGF binding to its receptor (Frackelton *et al.*, 1983). Phosphorylation is rapid, occuring within five minutes, and the major protein phosphorylated has a molecular weight of 170-180 kDa which corresponds to the PDGF receptor itself (Ek & Heldin, 1982; Ek *et al.*, 1982; Nishimura *et al.*, 1982). Further evidence for the phosphorylation of the PDGF receptor has come from *in vivo* studies labelling PDGF stimulated fibroblasts with [^{32}P]-phosphate. Immunoprecipitation of proteins with anti phosphotyrosine antibodies revealed several tyrosine kinase substrates, but the major phosphorylated protein corresponded to the PDGF receptor. The protein tyrosine kinase appears to be its own substrate and this is consistent with other systems where protein tyrosine kinases are involved.

In the case of PDGF there is a strong correlation between PDGF binding, phosphorylation and the biological response induced by PDGF. The time for maximum phosphorylation to be reached and maximum binding are similar as are the concentrations of PDGF required for half maximum binding and tyrosine phosphorylation (Cooper *et al.*, 1982; Frackelton *et al.*, 1984). DNA synthesis has also been shown to correlate well to PDGF binding and phosphorylation (Huang *et al.*, 1982). The data are consistent with PDGF receptor kinase being the mediator of PDGF induced mitogenesis.

1.16.2.*Epidermal Growth Factor*

Epidermal growth factor (EGF) elicits a wide variety of cellular responses some occuring rapidly and others taking several hours, all of which culminate in the cell entering mitosis (Staros *et al.*, 1985). The most rapid response to EGF is the stimulation of a protein tyrosine kinase intrinsic to the EGF receptor itself (Cohen *et al.*, 1982). Two domains exist on a single polypeptide of the EGF receptor, one corresponding to the EGF binding site and the other to the EGF stimulated tyrosine kinase. *In vitro* studies following the incorporation of [^{32}P] from γ-[^{32}P]-ATP into A431, a cell line derived from a human epidermoid carcinoma, membrane proteins stimulated with EGF revealed the major substrates of the EGF stimulated tyrosine kinase to be proteins of molecular weight 150 kDa and 170 kDa (Carpenter *et al.*, 1978). The 170 kDa protein has been identified as the EGF receptor and the 150 kDa protein is a product of receptor degradation (Cohen *et al.*, 1982). Receptor autophosphorylation occurs on tyrosine residues 1068,1148 and 1173 at the carboxy terminus of the protein (Downward *et al.*, 1984a/b). Other substrates have been identified for the EGF stimulated tyrosine kinase which include several cytoplasmic proteins but their functions have not yet

been elucidated. The EGF tyrosine kinase is also capable of phosphorylating exogenous substrates.

A relationship between the EGF receptor kinase and mitogenesis is unclear and no direct correlation exists between receptor phosphorylation and DNA synthesis (Hunter & Cooper, 1981). One consequence of autophosphorylation of the receptor might be the enhancement of the EGF kinase activity (Staros *et al.*, 1985) which is consistent with the insulin receptor (Rosen *et al.*, 1983). Autophosphorylation may play a role in regulating the affinity of the receptor for hormone. It was observed that addition of tumour promoters to several cell types resulted in a loss of EGF binding and this was due to a decrease in the affinity of the receptor for EGF (King and Cuatrecausas, 1982; Friedman *et al.*, 1984). The loss of binding was accompanied by an increase in phosphothreonine and phosphoserine content of the receptor and a decrease in phosphotyrosine. It has been reported that tumour promoters replace diacylglycerol to activate the calcium-phospholipid dependent protein kinase C which phosphorylates proteins on serine and threonine residues (Nishizuka, 1984). Further evidence for the involvement of protein kinase C came from *in vitro* studies which demonstrated that PKC could phosphorylate the EGF receptor on the same threonine residue that was found to be phosphorylated in intact cells, threonine 654 (Hunter *et al.*, 1984). It was suggested from these studies that PKC might be a regulator of EGF receptor tyrosine kinase, acting in a negative feedback manner. These results are consistent with the observation that EGF stimulates phosphatidylinositol turnover which results in the activation of PKC (Moolenaar *et al.*, 1984). β adrenergic agonists that elevate cAMP levels, resulting in the activation of cAMP dependent protein kinase have also been shown to inhibit EGF binding (Pessin *et al.*, 1983). *In vitro* studies have demonstrated that cAMP dependent protein kinase can

phosphorylate the EGF receptor on serine in the presence of EGF but these results have not been confirmed *in vivo* (Ghosh-Dastidar and Fox, 1984).

1.16.3.*Insulin and Insulin Like Growth Factor*

Insulin initiates a series of biochemical events (Czech, 1977) involving phosphorylation and dephosphorylation of several enzymes (Cohen, 1982). The insulin receptor consists of two α, 135 kDa, subunits and two β, 95 kDa, subunits linked by disulphide bonds. The α subunit binds insulin and the β subunit has been shown to bind ATP acting as an insulin stimulated tyrosine specific protein kinase (Kasuga *et al.*, 1983). The kinase domain of the insulin receptor has similarities to the EGF receptor and the *src* family of protein kinases (Houslay, 1981). The major substrate of the kinase activity of the β subunit is the 95 kDa β subunit itself (Kasuga *et al.*, 1983). The autophosphorylation of the receptor has been reported to enhance the tyrosine kinase activity and this may overcome the need for the continuous presence of insulin (Yu & Czech, 1984). *In vivo* the β subunit is phosphorylated on serine in the basal state and treatment with insulin enhances phosphorylation of serine and induces tyrosine phosphorylation (Kasuga *et al.*, 1982).

Evidence for a role for tyrosine phosphorylation in insulin action comes from a mutant cell line which shows no receptor phosphorylation and fails to respond to insulin (Haring *et al.*, 1984). Involvement of phosphotyrosine in insulin action is substantiated by the observation that tumour promoters which inhibit the insulin stimulated tyrosine kinase also inhibit a number of insulin mediated effects (Takayama *et al.*, 1984). Consistent with the EGF receptor only a low level of phosphorylation is required to induce maximal biological effects (Haring *et al.*, 1982). *In vitro*

phosphorylation is exclusively on tyrosine (Czech, 1985). Phosphorylation is on tyrosine 1150 and 1316 although other tyrosine residues may be involved (Herrera & Rosen, 1986).

Control of the insulin tyrosine kinase activity appears to be modulated in the same way as the EGF tyrosine kinase. Tumour promoters and β adrenergic agents are antagonistic acting by inducing phosphorylation on serine residues of the β subunit resulting in decreased affinity of the insulin receptor for insulin and a reduced protein tyrosine kinase activity.

Insulin like growth factor (IGF1) can mimic some of the actions of insulin. IGF1 stimulates a tyrosine kinase activity which phosphorylates the β subunits of the IGF1 receptor and the structurally related insulin receptor (Rubin *et al.*, 1983).

1.17. Substrates for Protein Tyrosine Kinases

To identify the functions of tyrosine kinases it is important to identify the substrates and their functions. The major targets for many protein tyrosine kinases are themselves often resulting in an enhancement of the tyrosine kinase activity. Other phosphotyrosine containing proteins have been identified but their functions are unclear.

Several approaches have been made to identify targets for $pp60^{v\text{-}src}$. The two proteins that are associated with $pp60^{v\text{-}src}$ in the cytoplasm, pp89 and pp50 may be candidates. Pp89 is phosphorylated only on serine residues (Oppermann *et al.*, 1981). Pp50 although phosphorylated at serine in untransformed cells, is phosphorylated at both serine and tyrosine in RSV transformed cells (Hunter & Sefton, 1980). Pp50 would, therefore, be a

possible substrate but further studies have shown that its role in transformation is obscure.

Comparison of proteins in transformed and untransformed cells by 2D electrophoresis of [^{32}P] labelled cell extracts has identified several potential substrates. A protein, pp36 was identified in this manner (Radke & Martin, 1979). Pp36 exists in an unphosphorylated form in both chicken and mammalian cells, but transformation by RSV results in the phosphorylation of pp36 on both serine and tyrosine residues (Radke *et al.*, 1980; Erikson & Erikson, 1980; Cooper & Hunter, 1981b). Pp36 is an abundant peripheral membrane protein but its function is unknown (Cooper & Hunter, 1982). Some correlation has been described between pp36 phosphorylation and transformation (Cooper *et al.*, 1983a).

Enolase, phosphoglycerate mutase and lactate dehydrogenase, three glycolytic enzymes, have been identified as being phosphorylated on tyrosine residues in RSV transformed cells (Cooper *et al.*, 1983b). The relevance of tyrosine phosphorylation of these proteins is unclear as they are not thought to be important regulatory enzymes in glycolysis. A protein p42 was identified as a substrate of pp60$^{v\text{-}src}$ in chick cells, but not in mouse cells. A direct role for this protein in transformation is therefore questionable (Cooper *et al.*, 1984).

The only cytoskeletal protein found to contain significant amounts of phosphotyrosine is vinculin (Sefton *et al.*, 1981). Vinculin is concentrated in focal adhesion plaques and, therefore, tyrosine phosphorylation could be responsible for the disruption of the cytoskeleton which occurs rapidly on RSV transformation (Hanafusa, 1977).

Detailed analysis of phosphotyrosine containing proteins from several cell lines transformed by a variety of transforming agents have identified few

positive correlations between protein phosphorylation and any transformation parameter.

Phosphotyrosine containing proteins that are substrates for protein tyrosine kinases associated with normal cell growth have also been identified. Interestingly pp36 was shown to be phosphorylated on tyrosine in EGF stimulated A431 cells (Hunter & Cooper, 1981). Phosphorylation was on the same tryptic peptide as that defined in RSV transformed cells. Phosphorylation of pp36 has also been observed in some mouse 3T3 cells (Cooper *et al.*, 1982) but no correlation between pp36 phosphorylation and mitogenesis has been defined.

Other proteins identified as substrates for the EGF stimulated protein tyrosine kinase in a variety of 3T3 cell lines include at least five proteins in the molecular weight range 40-45 kDa (Cooper *et al.*, 1982). In chick embryo fibroblasts a variety of mitogens eg. TPA, PDGF, EGF and IGF-1 induced phosphorylation of a family of proteins in the range 40-42 kDa, now termed p42 (Nakamura *et al.*, 1983). P42 constitutes only 0.001% of total cell protein but it is highly conserved among vertebrates (Hunter & Cooper, 1985).

Phosphotyrosine containing proteins have been difficult to detect due to the low percentage of protein involved. The use of 2D electrophoresis can separate many phosphoproteins but minor proteins remain unresolved. Phosphotyrosine antibodies have identified several proteins as tyrosine kinase substrates but up to 40% of phosphotyrosine proteins are not identified. Protein tyrosine kinase substates have been difficult to detect due to the relative paucity of phosphotyrosine containing proteins and added to this is

the fact that tyrosine kinases have a tendency to be promiscuous in their activity.

1.18. Evidence for Phosphotyrosine in Cell Growth

The finding that the transforming proteins of several retroviruses, which lead to unrestricted growth, and the receptors for some growth factors possess tyrosine kinase activity has led to the idea that tyrosine phosphorylation may play a role in cell growth. Retroviral tyrosine kinases could be analagous to permanently activated receptors which neither respond to external signals or to down regulation. This hypothesis is substantiated by the observation that the transforming protein of RSV $pp60^{v\text{-}src}$ is similar to its cellular homologue (Takeya & Hanafusa, 1983). Since then several transforming gene products have been shown to be homologous to cellular proteins. Considerable homology has been observed between the EGF receptor and *v-erb-B*, a transforming gene product of erythroblastosis virus. It has been suggested that the gene for the EGF receptor is the cellular gene from which the oncogene was derived (Downward *et al.*, 1984b). The region of the EGF receptor between Leu 694 and Phe 937 has homology with several transforming proteins most of which have been found to express protein tyrosine kinase activity (Staros *et al.*, 1985). The oncogene of simian sarcoma virus, *sis* shows homology with the cellular gene encoding the PDGF receptor, although a direct relationship has not yet been identified (Waterfield *et al.*, 1983). The insulin receptor has some limited homology with the transforming protein, *v-ros*, from vr-2 sarcoma virus.

In vitro, purified protein tyrosine kinases are phosphorylated exclusively on tyrosine but it is emerging that, *in vivo,* phosphorylation takes place on serine and threonine residues as well as tyrosine. There have been

reports that EGF and insulin can enhance phosphatidylinositol turnover which leads to the activation of protein kinase C (Moolenaar *et al.*, 1984). There is also evidence *in vitro* that the tyrosine kinase encoded by *src*, $pp60^{v\text{-}src}$ (Sugimoto *et al.*, 1984) and *ros*, $pp68^{v\text{-}ros}$ (Macara *et al.*, 1984) can phosphorylate phosphatidyl inositol, phosphatidyl inositol 4 phosphate and 1,2 diacylglycerol. This increases PI turnover and results in activation of PKC which phosphorylates proteins on serine and threonine residues. These reports are supported by the observation that PI turnover is dramatically increased in cells transformed by RSV (Diringer & Friis, 1977) and UR2 sarcoma virus (Macara *et al.*, 1984). Phosphorylation of tyrosine kinases on serine and threonine appears to reduce the activity of the kinase; thus, PKC is acting as a negative control. PKC is known to have a role in cell growth and, therefore, the association of the two kinases may be important. As yet there has been no demonstration of the direct association of the two protein kinases and the report that the *abl* kinase from *E.coli* cannot stimulate $PtdInsP_2$ turnover either *in vitro* or *in vivo* would argue against a direct association (Foulkes & Rosner, 1985).

1.19. A Role for Tyrosine Phosphorylation in Lymphocytes

There is considerable support for the involvment of protein tyrosine kinases in the control of cell growth and recently several reports have supported a function for tyrosine phosphorylation in lymphocytes. Campbell & Parkhouse (1989) demonstrated by using anti-phosphotyrosine antibodies that mitogen activated murine B cells possessed tyrosine phosphorylated proteins that were not present in resting B cells.

Membranes from T and B lymphocytes have distinct patterns of tyrosine phosphorylation (Earp *et al.*, 1984; Earp *et al.*, 1985). In membranes from normal B cells two two tyrosine kinase substrates have been

identified, p61 and p55. These proteins have been found in membranes from human Raji cells, mouse spleen and rat spleen. Comparison of tryptic phosphopeptides of these proteins has revealed identical phosphotyrosine domains (Earp *et al.*, 1985). Two tyrosine kinase substrates have also been identified in T lymphocyte membranes, p64 and p58 (Earp *et al.*, 1984) these proteins have similar tryptic phosphopeptides but are distinct from the B cell proteins (Earp *et al.*, 1985). The T cell protein p58 has also been described by Casnellie *et al* (1983). Antibodies raised against the putative substrate site have been shown to inhibit tyrosine phosphorylation of exogenous substrates, suggesting that p58 may be be a tyrosine specific protein kinase. Casnellie *et al* (1982) have reported a protein tyrosine kinase activity in a lymphoma cell line, LSTRA, whose major substrate is a protein of molecular weight 58 kDa, later defined as $pp56^{LSTRA}$. Marth *et al* (1985) characterised a gene coding for a tyrosine kinase activity, IskT, expressed in lymphoid cells and overexpressed in LSTRA. The IskT protein is closely related to $pp60^{csrc}$ and was found to be analagous to the phosphoprotein, p58, described by Earp *et al* (1985). The gene for this protein was rearranged and expressed at high levels in LSTRA cells and it was thought, therefore, that it maybe this alteration that led to the transformation of the cells.

Reports of other phosphotyrosine containing proteins have included two proteins identified in human peripheral blood B lymphocytes (Nel *et al.*, 1984). These two proteins, p56 and p60 are phosphorylated after stimulation of the B cells with anti-Ig. A protein TPP-66 in human T lymphocytes showed enhanced phosphorylation on tyrosine in response to two mitogenic lectins, phytohemagglutinin (PHA) and concanavalin A (Con A) (Wedner & Bass, 1986). Phosphorylation was first observed two minutes after stimulation and increased over 120 minutes. Phorbol esters, which are

known activators of PKC, have recently been found to stimulate tyrosine phosphorylation in some cell types. Nel *et al* (1985) performed a study of tyrosine kinase activity in normal and chronic lymphocytic leukemia peripheral blood B lymphocytes stimulated with phorbol ester. They observed several tyrosine kinase substrates. In the triton soluble material two proteins with molecular weight 56 and 61 kDa, which is similar to the proteins identified before (Earp *et al.*, 1985, Nel *et al.*, 1984). Four phosphotyrosine proteins were also found in the soluble material, molecular weight, 75, 66, 43 and 28 kDa. The phosphoprotein profiles were similar in normal and leukemic cells. Phosphorylation was an early event and was followed by a mitogenic response within 24 hours.

Tyrosine phosphorylation has also been reported in association with the T cell antigen receptor (Klausner *et al.*, 1987, Samelson *et al.*, 1986). The antigen receptor complex on murine T cells consists of disulphide linked α and β chains which are non covalently associated with four polypeptides, gp26, gp21, p25 and p16. Stimulation of a murine T cell hybridoma with PMA induced phosphorylation of two proteins, p25 and gp21 on serine residues. Stimulation with antigen induced phosphorylation of gp21 on serine and a 21 kDa protein on tyrosine residues. The 21 kDa protein, previously unidentified was found to immunoprecipitate with the antigen receptor complex (Samelson *et al.*, 1986). These results show that two different protein kinases are being activated by antigen binding. T cells can also be activated by antibodies to the antigen receptor complex or Thy1, a molecule which is structurally independent of the antigen receptor. Antibodies to either these molecules activates both kinases (Klausner *et al.*, 1987). An induction of polyphosphoinositide hydrolysis, which leads to PKC activation, has already been implicated in the activation of T cells via the

T cell antigen receptor (Patel *et al.*, 1987). It is now evident that another kinase activity is also involved, a tyrosine specific protein kinase. The activation of two protein kinases has been observed before in the insulin and EGF receptors.

The ability of IL-2 to induce tyrosine phosphorylation has been determined in two IL-2 dependent T cell lines and in normal human T cells (Salzman *et al.*, 1988). The existence of phosphotyrosine containing proteins was assessed by using antibodies to phosphotyrosine in conjunction with immunoblotting. An increase in phosphorylation was observed in four proteins with molecular weight 100, 84, 57 and 38 kDa. The increases are detectable within 2.5 minutes and maximal between 5 and 15 minutes. A correlation between protein phosphorylation and incorporation of [^{3}H]-thymidine has been suggested. This suggests that IL-2 like many other polypeptide growth factors exerts its effect through activation of a protein tyrosine kinase. Whether the activity is intrinsic to the IL-2 receptor remains to be detemined.

1.20. Signal Transduction upon IL-4 Binding to its Receptor

Justement *et al* (1986) and Mizuguchi *et al* (1986) investigating the signals transduced when IL-4 binds to its receptor found that there was no evidence of enhanced phosphoinositide metabolism, calcium mobilisation, translocation of PKC and no membrane depolarisation in murine B cells. It has also been observed that IL-4 does not significantly increase protein phosphorylation in whole cells (Cambier & Ransom, 1987).

The only insight into the mechanism of action of IL-4 is its ability to induce phosphorylation of endogenous membrane proteins (Justement *et al.*, 1986). IL-4 specifically enhances phosphorylation of a 44 kDa (now 42 kDa)

protein in membranes from resting murine B cells. There may also have been some evidence of phosphorylation in the 60 kDa molecular weight range but this is thought to be an artefact. These experiments indicate that IL-4 binding to its receptor in murine B cells activates a protein kinase which is associated with the plasma membrane. Based on previous findings it is probably not PKC.

In contrast to this it has been demonstrated that binding of human IL-4 to its receptor on resting human tonsillar B cells initiates a complex second messenger cascade (Finney *et al.*, 1989). The signals involve a rapid transient generation of IP_3 which is mirrored by an increase in intracellular Ca^{2+} and followed by a large increase in cAMP. The increase in IP_3 induced by anti-μ is similar but more sustained than that induced by IL-4. The increase in calcium was transient returning to a basal level within 60 seconds after stimulation. Stimulation with IL-4 also showed an increase in cAMP levels but had no effect on cGMP. Phorbol diester and calcium ionophore which mimic the increase in hydrolysis of $PtdInsP_2$ could not increase cAMP levels or mimic the induction of CD23 by IL-4. This suggested that IL-4 although possibly acting by enhancing $PtdInsP_2$ hydrolysis may also involve another second messenger outwith this system. The involvement of two signal transduction pathways is not uncommon eg. EGF and insulin systems.

The data presented suggest that differences may exist in the signals transduced by IL-4 in the human and murine system. This is supported by the fact that human and murine IL-4 differ in their effects on target cells and exhibit species specificity (Park *et al.*, 1987a; Park *et al.*, 1987b; Coffey & Hadden, 1981).

1.21. Objectives

The sole clue to date on the signals transduced when IL-4 binds to its complementary receptor is the activation of a membrane associated protein kinase which phosphorylates a 42 kDa protein on resting murine B cells. PKC is thought not to be the kinase responsible which according to our current knowledge suggests two other possibilities, a cAMP or cGMP dependent protein kinase and a tyrosine specific protein kinase. As previously mentioned the products of several retroviruses and receptors of several growth factors have been associated with intrinsic tyrosine kinase activity. It is emerging that phosphotyrosine plays an important role in the control of cell growth.

The aim of this study was to assess the role of membrane associated protein kinases in the signals transduced when IL-4 interacts with its plasma membrane receptor on B lymphocytes. This work involved the analysis of any phosphoproteins induced by IL-4 on B cells at various stages of activation. The mitogen lipopolysaccharide was used to stimulate the B cells and B cell lines were also used. The ability of these phosphoproteins to bind ATP was also assessed to determine whether they possessed an intrinsic protein kinase activity or were protein kinase substrates. Attempts were made to identify the phosphoamino acids of these proteins and also the identity of these phosphoproteins.

Ifn-γ inhibits many of the biological effects of IL-4 and therefore the effect of Ifn-γ on the ability of IL-4 to induce protein kinase activity was determined.

These studies were performed on murine B lymphocytes from

BALB/c mouse spleen and human B lymphocytes from tonsils and peripheral blood. The ability of human and murine IL-4 to stimulate protein kinase activities was assessed and compared in murine and human B cells.

2. MATERIALS AND METHODS.

2.1.MATERIALS.

All routine chemicals were of the highest grade available and were supplied by Sigma Chemical Company or BDH Chemicals, both of Poole, Dorset, England except for the following.

2.1.1. Animals.

Mice	Male and female 8-12 week old BALB/c.
Rabbits	Male New Zealand Whites.

Mice and rabbits were supplied by the University of Glasgow Biochemistry department breeding colonies.

2.1.2. Human Tissue.

Peripheral blood	Male and female volunteers between 20 - 45 years old.
Tonsils	5-10 year old male and female tonsillectomy patients at Yorkhill Sick Childrens Hospital, Glasgow. (Courtesy of Mr Chandrachaud).

2.1.3. Cell Culture Materials.

RPMI-1640 medium	Gibco Ltd., Paisley, Scotland.
Glutamine	Gibco Ltd., Paisley, Scotland.
Penicillin/streptomycin	Gibco Ltd., Paisley, Scotland.
Foetal calf serum	Northumbria Biologicals.
Trypan blue	Sigma Chemical Co., St Louis, USA.
2-mercaptoethanol	BDH Chemicals Ltd, Poole.

2.1.4. B Cell Separation Materials.

Percoll	Sigma Chemical Co., St. Louis, USA.
Ficoll-Hypaque	Pharmacia Fine Chemicals, Uppsala, Sweden.
Sheep red blood cells	SAPU, Lanarkshire, Scotland.
2-aminoethylisothiouronium bromide (AET)	Sigma Chemical Co., St. Louis, USA.

T cell depletion kit — Seralab, Crawley Down, Sussex.
(comprises four rat monoclonal IgG_{2b} antibodies directed against T cell surface antigens, Thy1 on all mouse peripheral T cells, Lyt1 on helper T cells, L3T4 on helper T cells, Lyt2 on cytotoxic and suppressor T cells).

Guinea pig complement	Seralab, Crawley Down, Sussex.
Plasticware	Sterilin Ltd., Feltham, England.

2.1.5. Radiochemicals.

The following radiochemicals were obtained from Amersham International plc, Aylesbury, Bucks, UK.

2-[^{3}H]-Adenosine 5'-monophosphate	14 Ci/mmol
Uridine diphospho-D-[U-^{14}C]-Galactose	303 mCi/mmol
(Methyl-[^{3}H])-Thymidine	44 Ci/mmol
γ-[^{32}P]-Adenosine triphosphate	5000 Ci/mmol
[^{125}I] Na (Carrier Free)	100 mCi/ml
8-Azido-γ-[^{32}P]-ATP	8.6 Ci/mmol

(From ICN Radiochemicals, Irvine, CA).

2.1.6. Photographic Materials.

X-Omat-S X-ray film	Kodak (U.K.) Ltd., London.
Developer	Kodak (U.K.) Ltd., London.
Fixer	Kodak (U.K.) Ltd., London.

2.1.7. Enzymes and Substrates.

All fine chemicals listed below were obtained from Sigma Chemical Co., Poole, Dorset, England.

Lactate dehydrogenase EC1.1.1.27 (rabbit muscle type 11).

Pyruvate (sodium salt).

NADH (disodium salt).

Catalase EC1.11.1.6 (bovine liver).

Fumarase EC4.2.1.2 (pig heart).

Malic acid (monosodium salt).

Leucylaminopeptidase EC3.4.11.2 (porcine kidney microsomes).

Leucyl-2-napthylamide.

4-methylumbelliferone (sodium salt).

1-methyl-2-pyrollidone.

2-amino-2-methyl-propan-1-ol (free base).

β galactosidase E.C. 3.2.1.23 (jack beans).

β-N-acetyl glucosaminidase E.C. 3.2.1.30 (bovine kidney).

4-methylumbelliferyl-β-galactoside.

4-methylumbelliferyl-β-N-acetyl-glucosaminide.

5' nucleotidase E.C. 3.1.3.5 (snake venom).

Triethanolamine.

Adenosine 5' monophosphate.

Galactosytransferase E.C. 2.4.1.22 (bovine milk).

Uridine 5' diphosphogalactose (sodium salt).

Cytochrome C.

NADPH.

2.1.8. Ligands and Mitogens.

BSF-1	(IL-4 purified from EL4 supernatant) a generous gift from Dr R.J. Noelle, Dartmouth Medical School, Hanover, USA.
Recombinant mouse IL-4	Genzyme (Via A & J Beveridge, Edinburgh).
Recombinant human IL-4	Genzyme.
Recombinant mouse interferon γ	Holland Biotechnology bv, Lieden, The Netherlands.
11B11	(Rat monoclonal antibody to murine IL-4) A generous gift from Dr E.C. Snow, University of Kentucky, Lexington, USA).

The following were obtained from Sigma Chemical Company, Poole, Dorset.

Concanavalin A.

E.coli lipopolysaccharide O127:B8.

Goat anti-mouse IgM.

Phorbol 12-myristate 13-acetate.

Ionomycin (Colimycin, Calcium ionophore A23187).

2.1.9. Gel Electrophoresis Materials.

Acrylamide, N,N'-methylene bis acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate and sodium dodecyl sulphate (SDS) were obtained from BDH Chemicals, Poole, Dorset, U.K.

Low molecular weight marker proteins were purchased from Pharmacia Fine Chemicals, Hounslow, Middlesex, U.K. Coomassie brilliant blue R stain was from Sigma , and silver nitrate was purchased from Johnson Matthey Chemicals Ltd., London.

2.1.10. Antibodies.

Monoclonal anti rat IgG_{2b} FITC.	Seralab, Crawley Down, Sussex.
Simultest T and B cell test. (Anti-CD3 FITC and anti-CD19 PE).	Becton Dickinson.
Anti-CD23 (MHM6) mouse ascites.	A generous gift from Dr J. Gordon, University of Birmingham, UK.
Anti-mouse Fc-FITC.	ICN Immunobiologicals, Irivine, CA.
Monoclonal anti-mouse B cells (I-A).	Seralab, Crawley Down, Sussex.
Anti-mouse IgG-FITC.	ICN Immunobiologicals, Irvine, CA.
Anti-human IgM-FITC.	Sigma Chemical Co. Ltd.
Transferrin-FITC.	"
Insulin-FITC.	"
Anti-rat IgG-FITC.	"

2.1.11. Thin Layer Electrophoresis Materials.

All of the undernoted chemicals were from Sigma Chemical Company, Poole, with the exception of xylene cyanol which was purchased from Geo. T. Gurr Ltd.

O-phospho-L-tyrosine.
O-phospho-L-serine.
O-phospho-L-threonine.
Xylene cyanol.
ε-DNP lysine.
Ninhydrin.

Thin layer chromatography plates (type 100 cellulose).

2.1.12. Immunological Reagents.

Horse radish peroxidase conjugated anti rabbit immunoglobulin was supplied by ICN Biomedicals Inc. Normal rabbit and goat sera were obtained from the Scottish Antibody Production Unit, Carluke, Scotland, and the following reagents were from Sigma Chemical Company.

Bovine serum albumin.

Keyhole limpet haemocyanin (KLH).

Ovalbumin (OVA).

4-aminobenzylphosphonic acid.

o-phenylenediamine.

2.1.13. Substrate Peptides.

RR-SRC, Phosphate acceptor peptide and [Val^5]-angiotensin II were supplied by Peninsula Laboratories or Biomac Ltd., Glasgow.

2.1.14. Miscellaneous.

Ecoscint liquid scintillant was supplied by National Diagnostics, and Iodogen (1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril) was purchased from Pierce-Warriner Ltd., Chester, U.K.

2.2. BUFFERS

Phosphate Buffer Saline (PBS)

170 mM NaCl

3.4 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4

2.3. Preparation of B Lymphocytes

2.3.1. *Preparation of B Cells from BALB/c Mice.*

BALB/c mice were sacrificed by ether inhalation followed by cervical dislocation. The spleens were removed and teased into a single cell suspension in RPMI-1640 medium. Cells were then washed twice in RPMI-1640 medium at room temperature. Erythrocytes were removed by suspending cells in 5 ml of erythrocyte lysis buffer (17 mM Tris HCl, pH 7.2, 0.144 M ammonium chloride) at room temperature for 5 minutes. An equal volume of RPMI-1640 medium was added to the cells and they were carefully layered onto a 5 ml cushion of foetal calf serum and centrifuged for 5 minutes at 1000 rpm. Erythrocyte-depleted cells were then washed twice in RPMI-1640 medium. T cells were depleted by incubating cells for 40 minutes at 37°C with a cocktail of four rat IgG_{2b} monoclonal antibodies with specificities for T cell markers (anti-Thy-1, anti-Lyt-1, anti-Lyt-2, anti-L3T4), each diluted 1:500 in PBS, in the presence of guinea pig complement (diluted 1:40 with PBS). After T cell depletion the cells were washed and resuspended in 2 ml of a percoll solution of density 1.08 g/ml.

2.3.2. *Preparation of B Lymphoctes from Human Peripheral Blood and Tonsils*

AET Treatment of Sheep Red Blood Cells

Sheep erythrocytes were coated with 2-aminoethylisthiouronium bromide (AET). 5 ml of sheep erythrocytes were centrifuged and washed twice in RPMI-1640 medium. At the same time 102 mg of AET was dissolved in 10 ml distilled H_2O and adjusted to pH 9.0 with 5 M NaOH and then filter sterilised. 4 ml of the AET was added to the sheep erythrocytes and the suspension was incubated at 37°C for 20 minutes. After 20 minutes the cells were washed 5 times with RPMI-1640 medium and then a final volume of 9 ml of RPMI-1640 medium was added. The AET treated sheep red blood cells were stored at 4°C for up to a week. The cells were diluted

1: 5 before use.

Separation of B and T Lymphocytes

20 ml of human peripheral blood was removed by venipuncture and diluted 1:2 with RPMI-1640 medium. The diluted blood was layered onto an equal volume of Ficoll-hypaque and centrifuged for 15 minutes at 2500 rpm. Tonsillar cells were teased from the tonsils and then washed in incomplete medium, resuspended in 20 ml per tonsil and layered onto Ficoll-hypaque. Lymphocytes were removed from the interface and washed twice with RPMI-1640 medium. The cells were counted and resuspended in RPMI-1640 medium at 10^7 cells /ml and equal volumes of 2% AET treated sheep erythrocytes and FCS were added. This suspension was centrifuged at 800 rpm for 5 minutes and then left on ice for one and a half hours. After the incubation the pellet was gently resuspended by rocking. The sample was layered onto Ficoll-hypaque and centrifuged at 2500 rpm for 15 minutes. B lymphocytes were removed from the interface and washed twice in RPMI-1640 medium. The T lymphocytes which were rosetted by the sheep erythrocytes can be obtained by lysing the sheep erythrocytes with the addition of a few drops of sterile H_2O.

2.3.3. *Separation of B Cell Populations by Percoll Gradient Centrifugation*

Percoll gradients were prepared as described by Ratcliffe and Julius (1982). Percoll densities of 1.09, 1.08, 1.075, 1.065, and1.055 g/ml were prepared from an iso-osmotic stock (9 parts percoll : 1part 10 x PBS) using the formula:-

$$Vy = Vi \frac{(\rho i - \rho)}{(\rho - \rho y)}$$

where ρi = density of stock iso-osmotic percoll

ρ = density of diluted solution produced

ρy = density of diluting medium

Vy = volume of diluting medium

Vi = volume of stock iso-osmotic percoll

2 ml of each density of percoll solution were layered into a 15 ml nitrocellulose tube starting with 1.09 g/ml and decreasing in density to 1.055 g/ml with 1 ml of RPMI-1640 medium on top, the 1.08 g/ml contained the B cells to be separated. The gradients were centrifuged in a SW27 Beckman rotor at 1400g for 30 minutes. Separation on discontinuous percoll gradients depends on the size and density of the cells . The cells move until their density is equivalent to the percoll density. Resting B cells are small, high density cells and were collected at the 1.09/1.08 g/ml interface. On activation B cells increase in size and therefore their bouyant density falls (Rabin *et al* 1985). The larger, less dense B cells were collected at the 1.065/1.055 g/ml interface. After removing the B cells from the appropriate percoll interface the cells were washed three times in RPMI-1640 medium.

2.4. Cell Viability

Cell viability was determined by mixing 4 volumes of cell suspension diluted 1:100 to 1 volume of 0.05% (W/V) trypan blue. Viability was determined on the basis that dead cells take up trypan blue. Numbers of live and dead cells could be estimated by counting in a Neubauer haemocytometer. The average number of cells per 16 square grid was multiplied by 10^4 then the dilution factor taken into account to give the number of cells per ml of culture.

$$\% \text{ viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100$$

2.5. Cell Culture of Murine and Human B Lymphocytes from Spleen, Tonsils and Peripheral Blood

Cells were maintained in complete medium (RPMI-1640 (as described in table 1) supplemented with 10% (v/v) foetal calf serum , heat inactivated at

Table 1. Composition of RPMI-1640 Medium

Amino Acids	*mg/ml*	*Amino Acids*	*mg/ml*
L-Arginine	200.0	L-Lysine HCL	40.0
L-Asparagine	65.0	L-Methionine	15.0
L-Aspartic acid	20.0	L-Phenylalanine	15.0
L-Cystine (2HCL)	65.0	L-Proline	20.0
L-Glutamic acid	20.0	(hydroxy-L-proline free)	
Glycine	10.0	L-Serine	30.0
L-Histidine (free base)	15.0	L-Threonine	20.0
L-Hydroxyproline	20.0	(allo free)	
L-Isoleucine (allo free)	50.0	L-Tryptophan	5.0
L-Leucine	50.0	L-Tyrosine	15.0
(methionine free)		L-Valine	20.0
Vitamins		*Inorganic Salts*	
Biotin	0.2	$Ca(NO_3)_2.4H_2O$	100.0
D-calcium pantothenate	0.25	KCL	400.0
Choline chloride	3.0	$MgSO_4$	48.84
Folic acid	1.0	NaCl	6000.0
i-Inositol	35.0	Na_2HPO_4	800.0
Nicotinamide	1.0	$NaHCO_3$	2000.0
para-Aminobenzoic acid	1.0	*Other Components*	
Pyrodoxine hydrochloride	1.0	Glucose	2000.0
Riboflavin	0.2	Phenol red	5.0
Thiamine hydrochloride	1.0	Reduced Glutathione	1.0
Vitamin B12	0.005		

56°C for 30 minutes, 2 mM L-glutamine, penicillin (10^5 I.U./litre), streptomycin (100 mg/litre)) and 50 μM mercaptoethanol (Lemke and Opitz, 1976). Cells were cultured for the times indicated in the text and in figure legends and with the appropriate concentrations of polyclonal activators and interleukins. Cells were cultured in 25 cm^3 Costar flasks in a 95% O_2/5% CO_2 humid incubator. Preparation and maintenance of cells was carried out in a Laminar-flow hood.

2.6. Cell Lines

Daudi	human thyroid lymphoma (Klein, *et al.*, 1968).
Raji	human Burkitt lymphoma cell line (Pulvertaft, 1964).
$P388D_1$	mouse DBA/2 lymphoid macrophage-like cell line (Koren et al., 1975).

Cells were grown in Costar flasks in complete medium in a 95% O_2/5% CO_2 humid incubator at 37°C and sub-cultured every second day.

2.7. Measurement of Murine T Cell Depletion by Using Polyclonal Activators of T and B Cells

Concanavalin A (Con A) and lipopolysaccharide (LPS) are polyclonal activators of lymphocytes, they act by mimicing the *in vivo* stimulation of lymphocytes by specific antigens. Con A is a mitogen T cells and LPS is a mitogen for mouse B cells. This difference in lymphocyte specificity was used to determine if a population of lymphocytes contained T or B cells by their response to LPS or Con A. To determine if a purified population of B cells had been obtained aliquots of lymphocytes were taken before and after T cell depletion. Cells from these 2 populations were suspended at $5x10^6$ cells/ml in complete medium plus 50 μM mercaptoethanol and 100 μl added to each well of a 96 well costar plate. Each population was cultured with 100 μl containing 0, 5, 10 and 50 μg/ml of Con A or LPS. The cells were cultured for 18 hours then 1 μCi of [^{3}H]-methyl thymidine was added to each well and the cells were cultured for a further 6 hours. The cells were harvested onto nitrocellulose filters using a Titertek cell harvester and the

incorporation of [^{3}H]-methyl thymidine into the cells estimated by liquid scintillation counting in 3 ml of ecoscint.

2.8. Activation of Murine B Cells by Lipopolysaccharide

LPS is a polyclonal activator for B cells, it drives B cells into the cell cycle resulting in proliferation and differentiation. To determine the optimum concentration of LPS needed for B cell activation the following assay was carried out. Resting B cells were prepared (2.3.1. & 2.3.3.). Cells were suspended in complete medium plus 50 μM 2-mercaptoethanol at $5x10^6$ cells/ml. 100 μl of cell suspension was added to each well of a 96 well costar plate. 100 μl of complete medium containing LPS at 0, 2.5, 5, 10, 20 and 50 μg/ml was added to the appropriate well. Each concentration of LPS was tested in triplicate. The cells were incubated at 37°C for 18, 42, 66 and 90 hours, then 1 μCi of [^{3}H]-methyl thymidine was added to them. The cells were incubated for a further 6 hours and then harvested at 24, 48, 72 and 96 hours onto nitrocellulose filters by a Titertek cell harvester. The filters were counted in 3 ml of ecoscint on a Beckman LS 6800 scintillation counter.

2.9. Interleukin 4 Bioassay

The assay to asess the response of the murine B cells to recombinant murine IL-4 was performed according to the method of Ohara *et al* (1985). Resting B cells were prepared from BALB/c mice (2.3.1 & 2.3.2.). The cells were resuspended at $5x10^6$ cells/ml in complete medium plus 50 μM 2-mercaptoethanol and 100 μl was added to each well of a 96 well Costar culture plate. The B cells were incubated in either medium alone, 5 μg/ml goat anti mouse IgM, 100 u/ml rmIL-4, 5 μg/ml goat anti mouse IgM and 100 u/ml IL-4 or 10 μg/ml LPS for 48 hours then pulsed with 1 μCi/well [^{3}H] methyl thymidine for 6 hours. Cells were harvested by a Titertek cell harvester onto nitrocellulose filters and the radioactivity incorporated determined by liquid scintillation.

2.10. Preparation of Cells for Flow Cytomretry

To analyse cells by flow cytometry aliquots of approximately 1x 10^6 cells were removed from cultures, centrifuged and then washed twice in ice cold PBS. The cells were resuspended in PBS to give 1x10^6 cells/50 µl PBS. The appropriate antibody was then added to the cells as specified in the text and figure legends. Samples where antibodies conjugated directly to FITC were used were incubated for 20 minutes on ice. After incubation they were centrifuged, washed twice in ice cold PBS and finally resuspended in 0.5 ml of PBS for analysis by flow cytometry. In the cases where a two step reaction was required the first antibody was added to the cells and incubated for 20 minutes as before. The cells were then centrifuged, washed twice in ice cold PBS and resuspended in PBS containing the second antibody. The second incubation was again for 20 minutes on ice . After the incubation the cells were washed in PBS and then resuspended in 0.5 ml of PBS for analysis by flow cytometry.

In each sample 2.5 µl of propidium iodide (PI) (final concentration 1 µg/ml) was added to the cells before analysis. Dead cells were gated by their ability to take up PI and fluoresce in the red channel.

In each batch of cells analysed a control was included which had no antibodies added and this was used to correct for autofluorescence of the cells. Where a two step reaction was involved a control with second antibody was included to account for non specific binding.

2.11. Preparation of Plasma Membranes

Plasma membrane was prepared according to the method of Earp *et al* (1984). B cells were centrifuged at 1000 rpm for 7 minutes in a MSE Centaur centrifuge then washed twice with 10 ml of 0.15 M NaCl. The washed cells were gently resuspended in 2.5 ml homogenisation buffer (10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA) and homogenised at 4°C with 60 strokes of a glass hand pestle. To ensure that the cells had been broken a small sample was inspected under the light microscope. 2.5 ml of

homogenisation buffer containing 0.5 M sucrose was added to the broken cells and the homogenate centrifuged at 1000 rpm for 5 minutes to remove nuclei and unbroken cells. The supernatant was removed and centrifuged for 1 hour at 105,000 g in a SW65 Beckman rotor. The pellet was resuspended in 20 mM PIPES pH 7.0 and the protein concentration was estimated.

2.12. *In Vitro* Phosphorylation of Plasma Membrane Proteins

A modification of the method of Earp *et al* (1984) was used to phosphorylate plasma membrane proteins. Phosphorylation assays, total volume 100 µl, contained 50 µg of plasma membrane, 30 mM $MgCl_2$ or 5 mM $MnCl_2$, 10 µM sodium orthovanadate, 0.02% (v/v) Triton-X-100 in 20 mM PIPES pH 7.0. The reaction was carried out on ice and initiated by the addition of 10 µCi [^{32}P-γ] ATP (2 µM, specific activity 5000 Ci/mmol). The samples for SDS gel electrophoresis were terminated after 5 minutes by adding 25 µl of hot (55°C) sample buffer and boiling for 5 minutes. Prior to SDS gel electrophoresis NaH_2PO_4 was added to a final concentration of 1 mM. The sample was then loaded onto a 10% polyacrylamide gel. The phosphorylation assay was carried out in the absence of ligand or in the presence of 10 units IL-4 (mouse) or 100 units (human), either recombinant or purified, or 100 units of recombinant Ifn-γ where indicated.

2.13. Incorporation of [^{32}P]-Phosphate into Plasma Membrane Proteins

The amount of radioactivity incorporated into acid insoluble material was estimated by taking 3 x 5 µl samples from the terminated reaction above (2.12.) and spotting them onto Whatman No.3 filter paper discs. The discs were washed 4 times over a period of 40 minutes in 10% TCA, 10 mM sodium pyrophosphate, then for 5 minutes in ethanol. The discs were then dried and counted in 3 ml ecoscint on a Beckman LS 6800 scintillation counter.

2.14. ATP Binding Capacity of Plasma Membrane Proteins

To determine which, if any, proteins could bind ATP the photoaffinity label γ-[^{32}P]-8-Azido ATP was used in phosphorylation assays similar to those described in 2.12. 8-Azido ATP binds irreversibly to ATP binding proteins in the presence of UV light. Phosphorylation assays contained 50 µg of membrane, 30 mM $MgCl_2$, 10 µM Na_3VO_4, 0.02% Triton-X-100 and 3 µCi [^{32}P-γ] 8-Azido ATP in 20 mM PIPES. The assays were carried out at room temperature on glass slides and the reaction was initiated by UV irradiation for one minute. The reaction was terminated by the addition of 25 µl of hot (55°C) sample buffer and the samples were boiled for 5 minutes. A final concentration of 1mM NaH_2PO_4 was added before loading the sample onto a 10% acrylamide gel for electrophoresis. The assays were carried out in the presence and absence of 10 units rmIL-4. To ensure labelling was specific assays were performed in the absence of UV light and also in the presence of excess unlabelled ATP (final concentration 50 mM).

2.15. Phosphorylation of Peptides by Plasma Membrane Protein Kinases

It has been shown that cAMP dependent protein kinases and casein protein kinases recognise substrates at least in part by the primary amino acid sequence around target residues. There is also evidence of homology between tyosine protein kinase sites. Short peptides (see Fig4.), with one potential phosphorylation site surrounded by the amino acids thought to be needed for recognition of the site, have been used as exogenous substrates for plasma membrane protein kinases in the phosphorylation reactions (2.12). Peptide phosphorylation assays contained 10 µg of membrane, 30 mM $MgCl_2$ or 5 mM $MnCl_2$, 10 µM Na_3VO_4, 0.02% (v/v) Triton-X-100 and 3 units IL-4 in 20 mM PIPES pH 7.0 to a total volume of 30 µl. Phosphorylation assays were carried out at 30°C in the presence of 2mM Substrate Protein Kinase (RRSRC), 0.2 mM Phosphate Acceptor Peptide (PAP) or 2 mM [Val^5] Angiotensin II and were initiated by addition of 3 µCi [^{32}P-γ] ATP (1Ci/mMol). The reactions were terminated after 3 minutes by the addition of 50 µl of 5% TCA. The samples were spun in a microfuge and

Figure 4 Amino Acid Sequence of Peptide Substrates

Figure 4 shows the amino acid sequence of the three peptides used as exogenous substrates in the phosphorylation assays (section 2.15). Each peptide as one potential phosphate acceptor site (in bold) and positively charged amino acids (in italics) at the N terminal to allow binding to the negatively charged paper.

FIGURE 4. Amino Acid Sequence of Peptide Substrates.

RR-SRC.

Arg-*Arg*-Leu-Ile-Glu-Asp-Ala-Glu-**Tyr**-Ala-Ala-Arg-Gly

[Val^5]-Angiotensin II.

Asp-*Arg*-Val-**Tyr**-Val-His-Pro-Phe

Phosphate Acceptor Peptide.

Arg-*Arg*-Lys-Ala-**Ser**-Gly-Pro-Pro-Val

three 20 µl aliquots of supernatant were spotted onto 2 x 2cm squares of Whatman P81 ion exchange chromatography paper. The peptides can bind to this negatively charged paper by virtue of two positively charged terminal amino acids. The papers are washed at room temperature for 40 minutes with four changes of 30% acetic acid, which removes excess [^{32}P-γ]ATP. The papers were dried and counted in 3 ml of ecoscint in a Beckman LS 6800 scintillation counter.

2.16. Analysis of Total Phosphoamino Acid Content by Two Dimensional Thin Layer Electrophoresis

Membrane protein phosphorylation was carried out exactly as in 2.12. except the reaction was stopped by adding BSA to a final concentration of 50 µg/ml. After a few minutes trichloroacetic acid was added to a final concentration of 10% (V/V). Samples were left on ice for 1 hour then proteins were pelleted in a microfuge for 5 minutes. The pellet was washed 2 times with ethanol and left to air dry. The pellet was resuspended in 200 µl of 5.7 N HCl and incubated for 1.5 hours at 110°C. Any remaining liquid was lyophilised.

2.16.1. *Analysis of the Phosphoamino Acid Content of Proteins Eluted from SDS-PAGE Gels*

Proteins identified from autoradiographs were excised from SDS-PAGE gels. The paper was removed and the gel ground in 50 µl 50 mM NH_4HCO_3, 0.1% SDS and 5% 2 mercaptoethanol. After grinding the volume is made up to 500 µl and the sample is shaken continually for 1 hour at room temperature. The acrylamide is pelleted by centrifuging for 5 minutes in a microfuge. A final concentration of 50 µg/ml BSA and 10% (V/V) TCA is added to the supernatant as described in 2.16.

To obtain proteins from gels that have been previously treated with KOH the protocol is followed exactly as above except the proteins are not precipitated with BSA/TCA since during KOH treatment peptide bonds as well as the phosphodiester bonds have been hydrolysed.

2.17. Two Dimensional Thin Layer Electrophoresis

Buffers

First dimension buffer, pH 1.9

78 ml glacial acetic acid

25 ml 88% (v/v) formic acid

897 ml H_2O

Second dimension buffer, pH 3.5

50 ml glacial acetic acid

5 ml pyridine

945 ml H_2O

Dye

1 mg/ml xylene cyanol

5 mg/ml ε-DNP lysine

Two dimensional thin layer electrophoresis was carried out according to the method of Cooper *et al* (1983a). The samples from 2.16 and 2.16.1. were dissolved in 10 µl of pH 1.9 buffer and 10 µl of a standard mix of phosphoamino acids (1 mg/ml of each of tyrosine, threonine and serine phosphate). 5 µl of the sample was carefully spotted onto 10x10 cm cellulose thin layer plates. A small amount of dye was run parallel to the sample to allow progress of electrophoresis to be monitored. The cellulose sheet was homogeneously wet with pH 1.9 buffer using Whatman 3mm filter paper to concentrate the sample. The thin layer plate was placed into an electrophoresis tank and electrophoresis was performed at a constant voltage of 500 volts. The sample was allowed to migrate halfway across the cellulose sheet towards the anode (20 minutes). The cellulose sheet was then dried, wet with pH 3.5 buffer, rotated through 90^{o} and again allowed to migrate halfway towards the positive pole (20 minutes). When the cellulose sheet was removed from the tank it was dried and sprayed with 0.1% (w/v) ninhydrin in acetone and developed in a 60^{o}C oven until the standard phosphoamino acids could be identified. The plate was then subjected to

autoradiography to visualise the radioactive phosphoamino acids.

After autoradiography each phosphoamino acid was cut out and counted in 3ml of ecoscint to determine [^{32}P]-phosphate incorporation into each phosphoamino acid.

2.18. Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to method of Laemmli (1970).

Stock Solutions

Unless otherwise indicated all solutions were stored at 4^0C.

Solution A

45% (W/V) Acrylamide

1.2% (W/V) N,N'-methylene *bis* acrylamide

Solution was deionized with amberlite monobed resin (MB-3), filtered and stored in the dark.

Solution B

1.5 M Tris-HCl, pH 8.8

0.13% (V/V) TEMED

Solution C

12% (W/V) SDS

Stored at room temperature.

Solution D

10% (W/V) Ammonium persulphate

Solution E

0.625 M Tris-HCl, pH 6.8

Reducing Loading Buffer (x5)

65 mM Tris-HCl, pH 6.8

2% (W/V) SDS

10% (V/V) Glycerol

5% (V/V) 2-Mercaptoethanol

0.001% (W/V) Bromophenol blue

Stored at -20^{0}C

Resevoir Buffer (x5)

125 mM Tris

660 mM Glycine

1% (W/V) SDS

Stored at room temperature.

2.18.1.*Separating Gel Preparation*

10% separating gels were prepared from stock solutions as follows;

Solution A	13.3 ml
Solution B	15.0 ml
Solution C	0.5 ml
Solution D	0.5 ml
Deionized water	30.7 ml
TEMED	50.0 µl

The solution was poured into casting apparatus and allowed to polymerise.

2.18.2.*Stacking Gel Preparation*

Stacking gels were prepared from stock solutions as follows;

Solution A	2.64 ml
Solution C	0.20 ml
Solution D	0.20 ml
Solution E	2.40 ml
Deionized water	18.67 ml
TEMED	24 µl

The solution was poured on top of the separating gel and allowed to polymerise around a 10 or 20 well teflon comb.

2.18.3. *Molecular Weight Markers*

In each gel 5 µl of low molecular weight markers were run parallel to the samples.

	Subunit molecular weight	Source
Phosphorylase b	94,000	rabbit muscle
Albumin	67,000	bovine serum
Ovalbumin	43,000	egg white
Carbonic anhydrase	30,000	bovine erythrocyte
Trypsin inhibitor	20,100	soyabean
α-lactalbumin	14,400	bovine milk

2.18.4.*Electrophoresis*

Samples in reducing loading buffer were loaded into individual wells in the stacking gel. Electrophoresis was performed at a constant current of 45 mA for 3-4 hours or 10 mA for 17-18 hours at room temperature or until the dye front reached the bottom.

2.18.5.*Coomassie Blue Staining*

After electrophoresis gels were stained with 0.25% (w/v) coomassie

blue in destain (4.5 vols. ethanol, 4.5 vols. deionized water and 1 vol. glacial acetic acid) for 1 hour then in destain until protein bands were revealed. Gels were then dried onto Whatman 3mm chromatography paper under vacuum at 80°C and autoradiographed at -70°C.

2.18.6.*Silver Staining*

SOLUTIONS

All solutions were freshly prepared and used within 5 minutes of preparation.

Solution A

0.8 g of silver nitrate in 4 ml of dH_2O.

Solution B

21 ml of 0.36% (w/v) NaOH, 1.4 ml of 14.8 M ammonia.

Solution C

Solution A was added dropwise to solution B while stirring vigorously and then made to 100 ml with dH_2O.

Solution D

2.5 ml 1% (v/v) citric acid mixed with 0.25 ml 38% (v/v) formaldehyde and made up to 500 ml with dH_2O.

Silver staining of gels was carried out according to the method of Wray *et al* (1981). After SDS-PAGE gels were soaked in 50% methanol overnight to remove SDS. The gel was stained in solution C for 15 minutes with gentle agitation and then washed in deionised water. The gel was developed using solution D and proteins appeared within 15 minutes. The reaction was terminated by rinsing the gel in deionised water.

2.18.7.*Alkali Treatment of SDS-PAGE Gel*

Two identical gels were stained and destained as in 2.18.5. then incubated, for 90 minutes at 55°C, in two changes of 5 mM sodium dihydrogen phosphate either with or without 1 M potasium hydroxide. Gels were rinsed in deionized water then placed in destain for 30 minutes before being dried under vacuum at 80°C onto Whatman 3mm filter paper and autoradiographed.

2.18.8.*Processing of Autoradiographs*

X-ray film was sandwiched between dried gels and a Dupont "Cronex" intensifier screen in a light proof cassette. Films were stored at -70°C until appropriate exposure.

2.18.9.*Development of Films*

After exposure, autoradiographic images were visualised by processing of films in a Kodak X-OMAT processor.

2.18.10.*Densitometry*

Autoradiographs were analysed by linear laser densitometry using a Bio-Rad densitometer. Data were collected without electronic boosting or enhancement, and the data were analysed on an Ollivetti M24 microcomputer system.

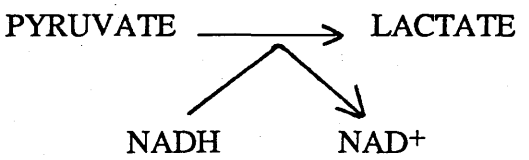
2.19. Estimation of Purification of Plasma Membranes

To ensure that the method used for preparation of membranes had enriched plasma membrane and there was minimal contamination from components of other cellular compartments, enzyme activities which could be assigned to one, or at most two, sub-cellular compartments were assayed in homogenised cells and membranes prepared as described in 2.11. The cells used were resting B cells from BALB/c mouse spleen and cells from a human B lymphoma cell line, Daudi. The marker enzymes assayed were 5' nucleotidase and leucylaminopeptidase for plasma membrane, fumarase as a marker of mitochondrial contamination as well as lactate dehydrogenase,

which is also a marker of cytosolic contamination. Catalase was used as a marker for peroxisomes, acid β galactosidase and β N acetylglucosaminidase as markers for lysosomes, galactosyl transferase for the golgi and NADPH-cytochrome C reductase as a marker for endoplasmic reticulum.

2.19.1.*Lactate Dehydrogenase*

Lactate dehydrogenase was assayed at 37°C by following the decrease in absorbance at 340 nm due to the formation of NAD^+ from NADH in the reaction (Houslay and Tipton, 1973).



The reaction cocktail, 1 ml, consisted of 0.05 M Tris HCl pH 7.4, 0.32 M sucrose, 0.36 mM pyruvate and 100 mM NADH. 10 µl of 1:1000 dilution of commercial lactate dehydrogenase (as a positive control) or 10 µl of whole cell homogenate or membrane was added to start the reaction. The reaction was carried out in the presence and absence of 0.1% (v/v) Triton-X-100. The change in absorbance was followed using a Cecil spectrophotometer coupled to an SE 120 BBC chart recorder. The specific activity of the enzyme (units/mg protein) was calculated. The molar extinction coefficient for NAD, $\varepsilon = 6.22 \times 10^3$ l mol^{-1} cm^{-1}.

2.19.2.*Catalase*

Catalase activity was measured by the evolution of oxygen from hydrogen peroxide at 240 nm (Darnell *et al* 1986). The reaction cocktail, 1 ml, consisted of 0.1% H_2O_2 in 100 mM potassium phosphate buffer pH 7.4. The reaction was carried out at 37°C in the presence of 0.1% (v/v)Triton-X-100 and was initiated by the addition of 10 µl of either commercial catalase (1:1000), whole cell homogenate or membrane. Measurements were made as in 2.19.1. The molar extinction coefficient for catalase was $\varepsilon = 312$ l mol^{-1} cm^{-1}.

2.19.3.*Fumarase*

Fumarase is a Krebs cycle enzyme responsible for the conversion of malate to fumarate. Fumarase activity was determined by the method of Hill and Bradshaw (1969) by following the appearance of fumarate as detected by an increase in absorbance at 240 nm. The reaction was performed at 25°C and consisted of 0.05 M malic acid in 0.1 M potassium phosphate buffer pH 7.6 and 10 µl of a 1:1000 dilution of commercial fumarase or 10 µl of whole cell homogenate or membrane to initiate the reaction. The reaction was carried out in the presence of 0.1% Triton-X-100 to disrupt the mitochondria. The assay was recorded on the equipment used in 2.19.1. The molar extinction coefficient for fumarase was $\varepsilon = 2.11\ l\ mmol^{-1}\ cm^{-1}$.

2.19.4*NADPH-Cytochrome C Reductase*

NADPH-cytochrome C reductase was assayed by the method of Williams and Kamin (1962). Cytochrome C acts as an electron acceptor when NADPH is oxidised to $NADP^+$. The reaction cocktail consisted of 0.9 ml of 50 mM sodium phosphate buffer pH 7.2, 50 µl of a 25 mg/ml solution of cytochrome c, 10 µl of 10 mM EDTA and 10 µl of whole cell homogenate or membrane. To initiate the reaction 100 ml of a 2 mg/ml solution of NADPH was added and the change in absorbance at 552 nm was followed using the same equipment as in 2.19.1. The molar extinction coefficient for cytochrome C was, $\varepsilon = 27{,}000\ l\ mol^{-1}\ cm^{-1}$.

2.19.5.*5' Nucleotidase*

5' nucleotidase was assayed by monitoring the release of ^3H-adenosine from ^3H-adenosine 5' monophosphate (AMP) (Newby *et al.*, 1975). 10 µl of commercial 5' nucleotidase (0-0.2 units) or 10 µl of whole cell homogenate or membrane was incubated with 190 µl of a solution containing 50 mM triethanolamine hydrochloride pH 7.6, 10 mM magniesium sulphate, 1 mM AMP and 2-[^3H]-AMP (105cpm/190ml) for 10 minutes at 30°C. The reaction was terminated by the addition of 40 µl of 0.15 M zinc sulphate which precipitates unreacted AMP. The solution was kept on ice for 3 hours when 40 µl of 0.3 N barium hydroxide was added and the samples

were incubated for a further 15 minutes on ice. After incubation the samples were centrifuged for 3 minutes at 14000g and a 150 µl aliquot of supernatant was removed and counted in 3ml of ecoscint. The activity of 5' nucleotidase in the whole cell homogenate and membrane was determined from a standard curve of commercial 5' nucleotidase.

2.19.6.*Leucylaminopeptidase*

Leucylaminopeptidase was assayed as leucyl-2-napthylamidase using leucyl-2-napthylamide as a substrate (Wachsmith *et al* 1966). For the reaction 10 µl of whole cell homogenate or membrane was incubated for 10 minutes at 37^0C in 50 µl of 80 mM sodium phosphate buffer pH 7.25, 0.17 mM leucyl-2-napthylamide and 0.3% (w/v) Triton-X-100. The reaction was terminated by the addition of 3 ml 0.1 M 2-amino-2-methyl propan-1-ol HCl buffer pH 10.4. The 2-napthylamide liberated was measured fluorimetrically in a LS-5 luminescence spectrophotometer using an excitation wavelength of 340 nm and monitoring emmision at 410 nm. The fluorimeter was calibrated by measuring the flouresence of 4 methylumbelliferone between 0 and 0.5 nmoles to form a standard curve. 1 nmole of 4 methylumbelliferone is the fluorescent equivalent of 36 pmole of 2-napthylamide. 4 methylumbelliferone (10mg) was dissolved in 1 ml of 1-methyl-2-pyrollidone then diluted to 10 mg/ml in water.

2.19.7. *Acid β Galactosidase and β N Acetylglucosaminidase*

β galactosidase and β N acetylglucosaminidase were assayed by the method of Peters *et al* (1972). In the assay 4-methylumbelliferyl-α-D-galactoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide were hydrolysed by β galactosidase and β N acetylglucosaminidase respectively to leave 4-methylumbelliferone which can be measured fluorimetrically. In the reaction 20 µl of whole cell homogenate or membrane was incubated with 40 ml of buffer pH 4.5, containing 50 mM sodium acetate, 100 mM sodium chloride and 0.8% (w/v) Triton-X-100, on ice for 20 minutes to disrupt the lysosomes. A further 80 µl of acetate buffer containing 100 mM sodium chloride and 1 mM 4 methlyumbelliferyl β galactoside or 0.4 mM 4

methylumbelliferyl β N acetylglucosaminidase was added and the reaction proceeded at 37^0C for 10 minutes (β galactosidase) or 5 minutes (β N acetylglucosaminidase). The reaction was terminated by the addition of 3 ml of 0.1 M 2-amino-2-methyl propan-1-ol HCl pH 10.4. The fluoresence of the 4 methylumbelliferone liberated was measured at 444 nm with an excitation of 364 nm on equipment as in 2.19.6. A standard curve of 4 methylumbelliferone (0-0.5 nmoles) was constructed as in 2.19.6.

2.19.8.*Galactosyl Transferase*

Galactosyl transferase was measured by its ability to transfer galactose from UDP-galactose to ovalbumin (Beaufay *et al* 1974). The assay cocktail, final volume 100 µl, consisted of 0.1 M sodium cacodylate pH 6.2, 1.25 mM UDP [U-^{14}C] galactose diluted to a specific activity of 0.4 mCi/mmol with unlabelled UDP-galactose, 10 mM ATP, 10 mM $MnCl_2$, 30 mM 2-mercaptoethanol, 5% ovalbumin and 0.2% Triton-X-100. The reaction was initiated by the addition of 20 µl of commercial galactosyl transferase (0-0.02 units) or 20 µl of cell homogenate or membrane. The reaction proceeded at 37°C for 60 minutes when 0.2 ml of 8% trichloroacetic acid (TCA) was added to terminate the reaction. The samples were centrifuged and the supernatant was discarded and the pellet washed three times with 8% TCA. After washing the pellet was dissolved in 0.8 ml 1N NaOH by heating at 80°C for 20 minutes. This solution was neutralised with 1 ml of 1N acetic acid. The [^{14} C]-galactose bound to ovalbumin was determined by counting 1.5 ml of the neutralised solution in 10 ml of ecoscint in a Beckman LS 6800 scintillation counter.

3. RESULTS.

CHARACTERISATION OF B LYMPHOCYTES AND PLASMA MEMBRANE.

CHAPTER 3

CHARACTERISATION OF B LYMPHOCYTES AND PLASMA MEMBRANE.

3.1. T CELL DEPLETION

There are two types of lymphocytes, B lymphocytes, distinguished by surface immunoglobulin, and T lymphocytes. This study has centered on the B lymphocytes. B lymphocytes were isolated from BALB/c mouse spleen, human peripheral blood and tonsils, all secondary lymphoid tissues. In the small lymphocyte population, both T and B lymphocytes are similar in size and are morphologically identical. T and B lymphocytes can only be distinguished phenotypically by the proteins on their cell surface.

3.1.1. *Murine T cell Depletion*

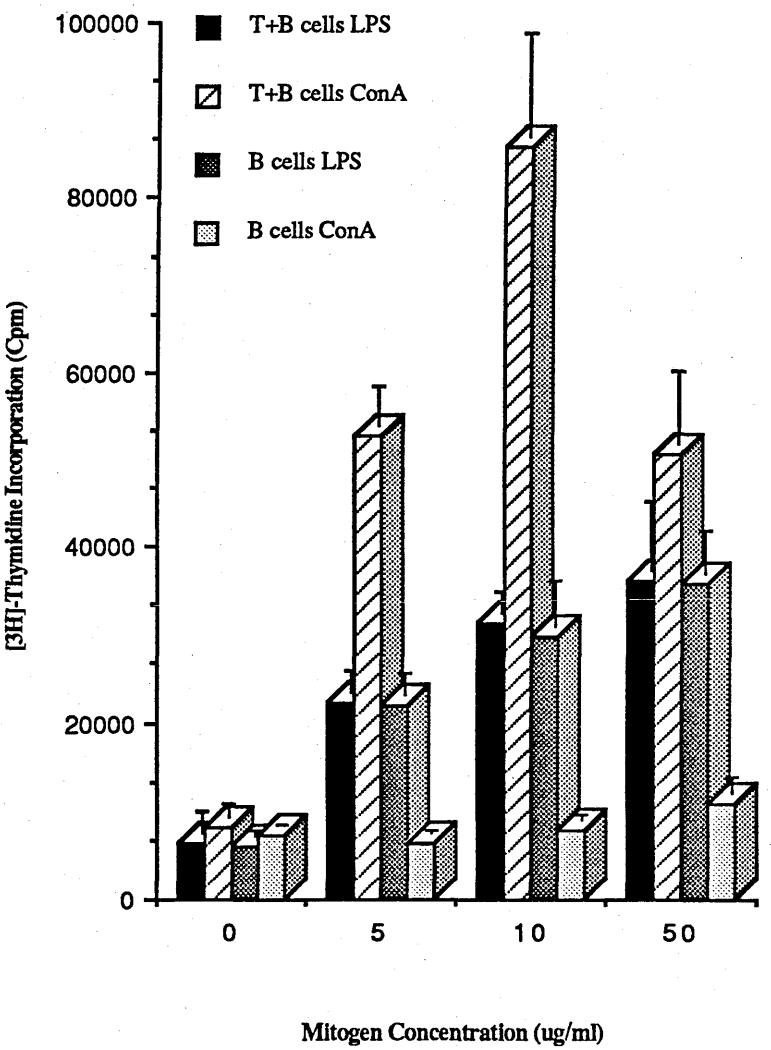
Murine T cell depletion was accomplished by the use of four monoclonal antibodies which are directed against antigens on T cell membranes, Thy1, Lyt1, Lyt2 and L3T4, and complement.
The number of lymphocytes before T cell depletion was on average $1x10^8$ cells/spleen and after T cell depletion and percoll separation the number of resting lymphocytes was $2x10^7$ cells/spleen as determined by simple cell counting. To ensure that there was selective depletion of T cells, two methods were used; responsiveness to mitogens and flow cytometry.

3.1.2. *Determination of Lymphocyte Population by Response to Mitogens*

Concanavalin A (Con A) and lipopolysaccharide (LPS) are polyclonal activators of T and B cells respectively (Melchers & Anderson 1984). When Con A and LPS are added to lymphocyte cultures T lymphocytes proliferate in response to Con A and B lymphocytes to LPS. Aliquots of lymphocytes were taken before and after T cell depletion. Both populations of cells were cultured with various concentrations of either Con

Figure 5 Responsiveness of Purified B Lymphocytes to Concanavalin A and Lipopolysaccharide

Aliquots of lymphocytes from BALB/c spleen before and after T cell depletion were cultured in complete medium plus 50 μM mercaptoethanol in the presence of 0, 5, 10 and 50 μg/ml of LPS or Con A for 24 hours. 1 μCi per well of [^{3}H]-methyl thymidine was added in the last 6 hours of culture before harvesting. Cells were harvested onto nitrocellulose filters and cpm were determined by liquid scintillation counting (materials & methods 2.7.). Incorporation is represented as counts per minute and is the average of three determinations (x +/- SD, n=3).


100000
80000
60000
40000
20000
0
T+B cells LPS
T+B cells ConA
B cells LPS
B cells ConA
[3H]-Thymidine Incorporation (Cpm)
0
5
10
50
Mitogen Concentration (ug/ml)

A or LPS for 24 hours as described in section 2.7. Proliferation of the cells in response to the mitogens was determined by measuring the incorporation of [^{3}H] methyl thymidine into the cells in the last 6 hours of culture. Figure 5 shows the results from such an experiment.

Lymphocytes from either population cultured in the absence of either mitogen showed very little incorporation of thymidine. This would be expected as the cells are in a quiescent state with no stimulus to drive them into the cell cycle. The cells before T cell depletion, which would consist of a mixture of T and B cells, responded to both mitogens at all concentrations showing a large increase in thymidine incorporation above the resting cells. The cell population present after T cell depletion, which should consist mostly of B cells, were stimulated to proliferate in response to LPS, a B cell mitogen. Incorporation of thymidine by the 'purified B' cells was similar to that seen in lymphocytes before T cell depletion at all the concentrations of LPS. The T cell mitogen, Con A, did not stimulate the B cell enriched population to proliferate. Incorporation of thymidine into the 'purified B' cell poulation in response to Con A was at the level of unstimulated cells at all concentrations of Con A.

These results demonstrate that before T cell depletion both T and B cells are present but after T cell depletion the cells are mostly B with very few T cells present, as demonstrated by their lack of response to Con A.

3.1.3. *Estimation of T Cell Depletion by Flow Cytometry*

The efficiency of T cell depletion was also estimated by the use of flow cytometry. Lymphocytes before and after T cell depletion were incubated with each of four rat IgG2b monoclonal antibodies specific to T cells, Thy1, Lyt1, Lyt2 and L3T4. A second antibody conjugated to fluorescein isothiocyanate (FITC) was then used to detect the T cell specific antibodies. The fluorescence intensity of the cells was then determined by

flow cytometry, which gave an indication of the number of T cells present in each population. Figure 6 shows plots of fluorescence intensity versus cell number for both cell populations stained with each of anti-L3T4, anti-Lyt1 and anti-Lyt2.

L3T4 is a protein found on all helper T cells. Figure 6 panel A shows the intensity of fluorescence of lymphocytes before and after T cell depletion stained for L3T4. The mean fluorescence of cells before T cell depletion was 250 and after 100. There has therefore been a reduction in the number of $L3T4^+$ cells on T cell depletion. Autofluorescence of the cells has been accounted for but the 'purified B' cells are still quite heavily stained. Control experiments with second antibody alone showed that there was substantial non specific staining due to the second antibody.

Staining for T cells with anti-Lyt1, found on all helper T cells, did not show a significant decrease in fluorescence on T cell depletion, figure 6 panel B. This suggests that there has been virtually no depletion of T cells but this is contradictory to the experiments with anti-L3T4. Again the apparent staining of the 'purified B' cell population is probably due to the non specific binding of the second antibody to the cells.

Lyt2 is a marker for cytotoxic and suppressor T cells. Staining for cells with anti-Lyt2, figure 6 panel C, showed a mean fluorescence intensity for cells before T cell depletion of 300 and after 100. Therefore there was less binding of anti-Lyt2 after T cell depletion suggesting that there has been a successful depletion of cytotoxic and suppressor T cells.

The results for the binding of anti-Thy1 are displayed differently, figure 7, panel B, represents the fluorescence intensity of cells before T cell depletion minus the fluorescence due to non specific binding of the second antibody. Panel A is a similar representation of the population of cells after T

Figure 6 Analysis of Murine T Cell Depletion by Flow Cytometry

Aliquots of BALB/c spleen cells were taken before and after T cell depletion and analysed for the presence of T cells by the use of T cell specific antibodies and flow cytometry. The cells were prepared for flow cytometry as described in materials & methods 2.10.

The first antibody was 25 µl of 1:100 dilution of

A anti-L3T4

B anti-Lyt1

C anti-Lyt2

The second antibody was 50 µl of a 1:50 dilution of anti-rat IgG FITC.

Each figure represents a plot of fluorescence intensity against cell number. Panel A, B and C show the traces for cells before and after T cell depletion, as indicated in the key beside each panel, minus autofluorescence of the cells.

Panel A

. . . . L3T4 on 'purified B cells'

____ L3T4 on 'T & B cells'

Panel B

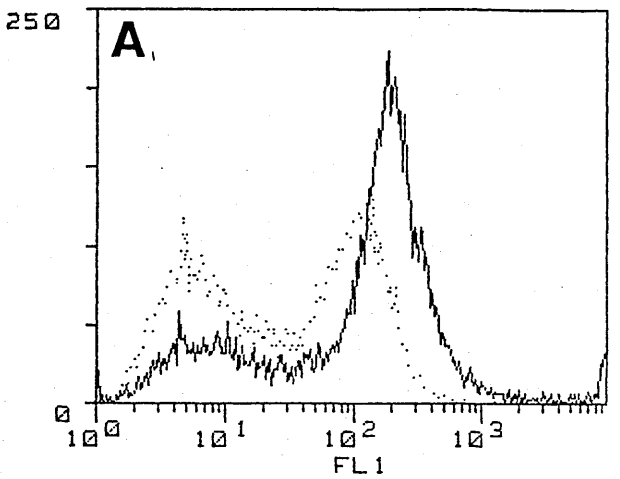
. . . . LyT1 on 'purified B cells'

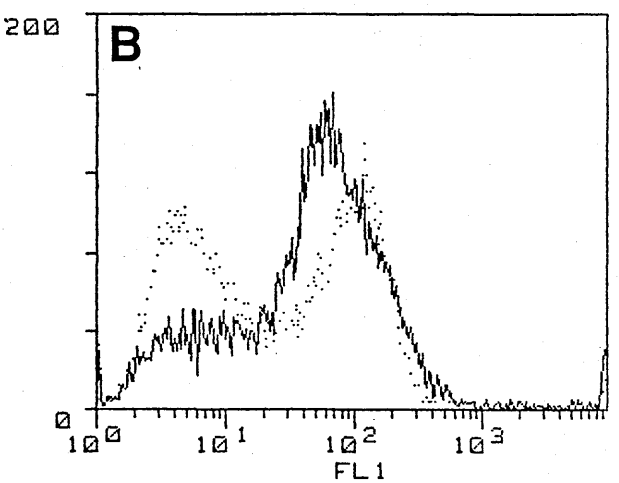
____ LyT1 on 'T & B cells'

Panel C

____ LyT2 on 'purified B cells'

. . . . LyT2 on 'T & B cells'


250
A
0
10^0
10^1
10^2
10^3
FL1


200
B
0
10^0
10^1
10^2
10^3
FL1

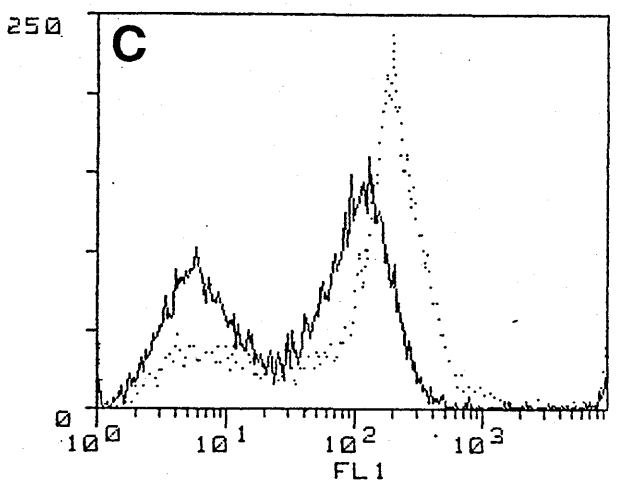

250
C
0
10^0
10^1
10^2
10^3
FL1

Figure 7 Analysis of Thy1 on Murine Spleen Cells Before and After T Cell Depletion

Aliquots of BALB/c spleen cells were taken before and after T cell depletion and analysed for the presence of T cells by the anti-Thy1 and flow cytometry. The cells were prepared for flow cytometry as described in materials & methods 2.10.

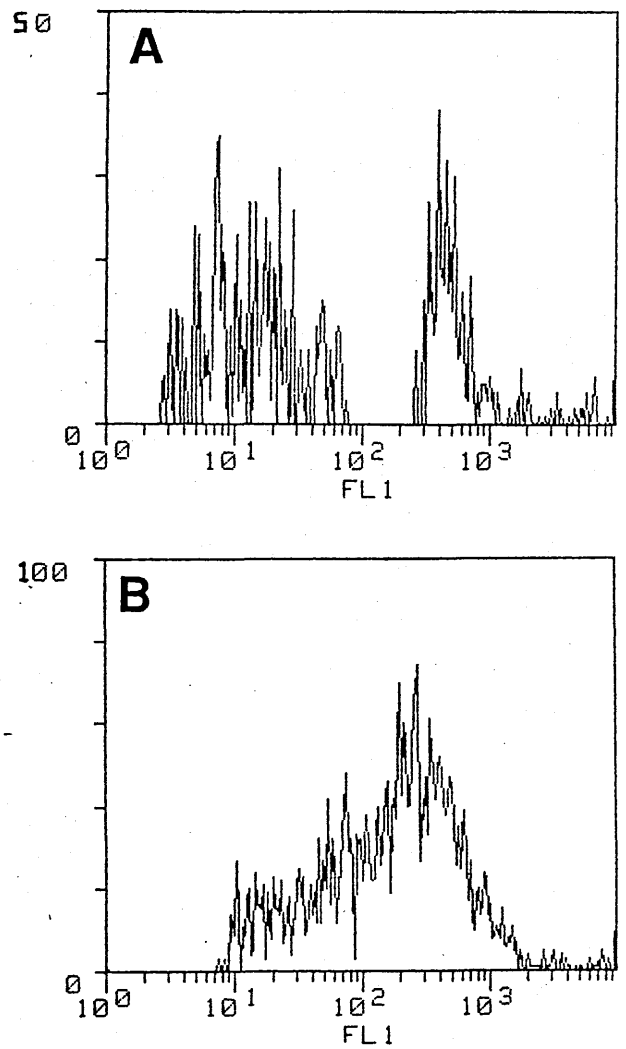
The first antibody was 25 µl of 1:100 dilution of anti-Thy1 and the second antibody was 50 µl of a 1:50 dilution of anti-rat IgG FITC. Panel B is the trace for anti-Thy1 staining of T cells before depletion minus autofluorescence and non specific staining due to the second antibody. Panel A is in the same format representing cells after T cell depletion.

Panel A

____ Thy1 on 'purified B cells' - non specific staining

Panel B

____ Thy1 on 'T & B cells' - non specific staining


50
A
0
10^0
10^1
10^2
10^3
FL1
100
B
0
10^0
10^1
10^2
10^3
FL1

cell depletion. There has been a decrease in the number of cells at the higher fluorescence intensity, 300, in the 'purified B' cell population but there is still binding of the anti-Thy1 to the 'purified B' cells. Recent reports have indicated that Thy1 is expressed on some murine B cells in response to LPS and IL-4 (Snapper *et al.*, 1988a). If this is the case then some fluorescence might be expected from purified B cells if any of the cells have previously seen antigen. However, similar experiments using NIMR1, which recognises the Thy1.2 determinant, showed a significant decrease in binding after T cell depletion suggesting that the T cells had been successfully depleted from the cell population (R. Butcher and W. Cushley personal communication). This suggests that it is non specific binding of the antibodies that is responsible for the observed binding in the 'purified' B cell population.

Identification of T cells by antibodies against cell surface proteins and analysis by flow cytometry has indicated a decrease in the number of T cells in the 'purified B' cell population. This depletion is not conclusive with all the antibodies used, mainly due to the non specific binding of the second antibody. If these results are taken together with the results from the mitogen responsiveness experiments there is evidence that the T cell depletion has been successful for murine spleen cells.

3.1.4. *Human T cell Depletion*

T lymphocytes from human peripheral blood and tonsils were separated by their ability to bind sheep red blood cells by a specific receptor, the CD2 molecule (section 2.3.1.). The efficiency of this T cell depletion was determined by flow cytometry. T and B lymphocytes were identified by simultaneous two-colour immunofluorescence using antibodies against CD3 on T cells (which are FITC conjugated) and against CD19 on B cells (which are PE conjugated).

Figure 8 Analysis of T and B Lymphocytes from Human Tonsils by Flow Cytometry

Aliqouts of lymphocytes from human tonsils were taken before (A &B) and after (C &D) T cell depletion and analysed for the presence of T and B lymphocytes by simultaneous two colour immunofluorescence (CD3-FITC for T cells and CD19-PE for B cells). The cells were prepared for flow cytometry as described in materials and methods 2.10. The cells were incubated on ice for 20 minutes with 5 µl of simultest T and B cell test from Becton Dickinson. Lymphocytes were selected on the basis of forward and side scatter. The T cells are represented by green fluorescence on the x axis (FITC-anti-CD3) and the B cells by red fluoresence on the y axis (PE-anti-CD19).

A nonspecific staining of 'T and B' cells

B 'T and B' cells

C non specific staining of 'purified B' cells

D 'purified B' cells

The lymphocytes were gated into quadrants on the basis of fluorescence and statistics were performed.

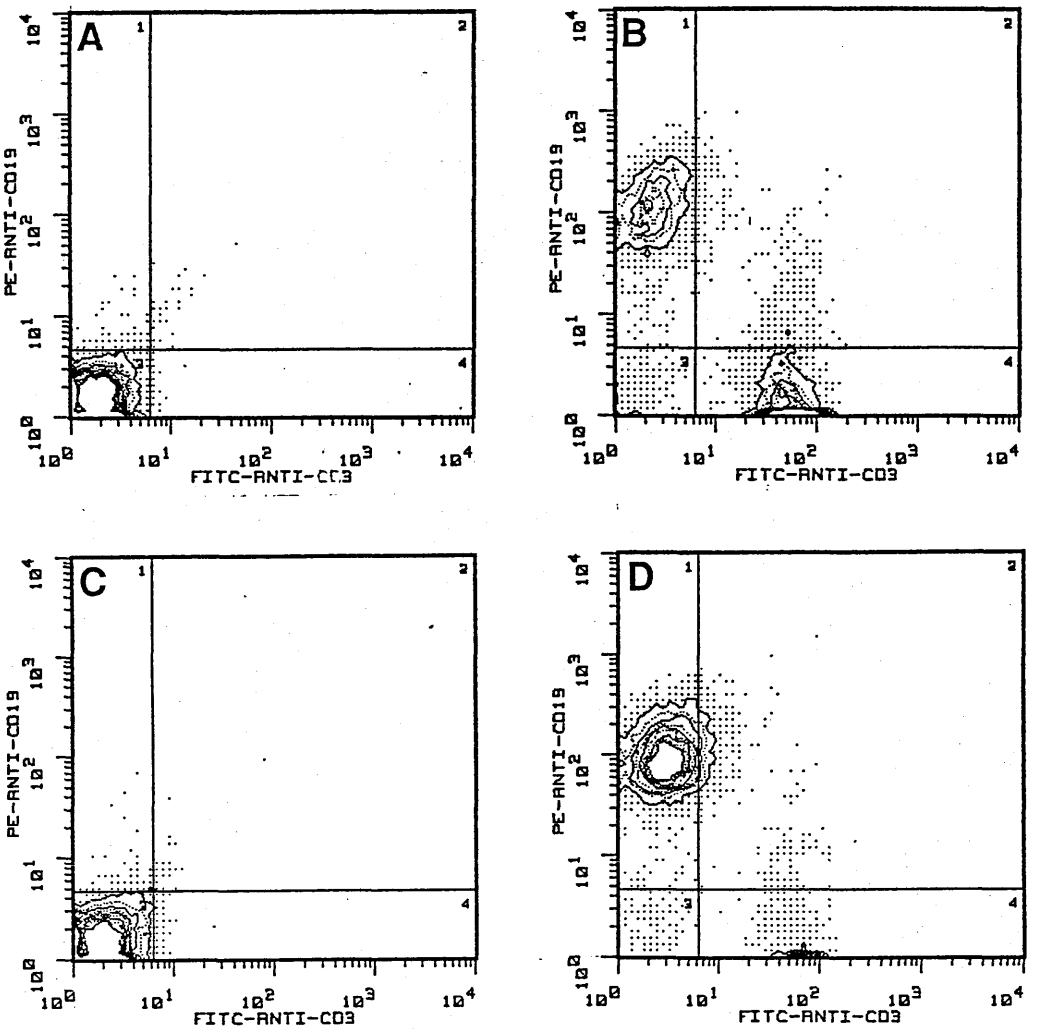

A
B
C
D
PE-ANTI-CD19
FITC-ANTI-CD3
1
2
3
4

Figure 9 Analysis of T and B Lymphocytes from Human Peripheral Blood by Flow Cytometry

Aliqouts of lymphocytes from human peripheral blood were taken before (A &B) and after (C &D) and analysed for the presence of T and B lymphocytes by simultaneous two colour immunofluorescence (CD3-FITC for T cells and CD19-PE for B cells). The cells were prepared for flow cytometry as described in materials and methods 2.10. The cells were incubated on ice for 20 minutes with 5 µl of simultest T and B cell test from Becton Dickinson. Lymphocytes were selected on the basis of forward and side scatter. The T cells are represented by green fluoresence on the x axis (FITC-anti-CD3) and the B cells by red fluoresence on the y axis (PE-anti-CD19).

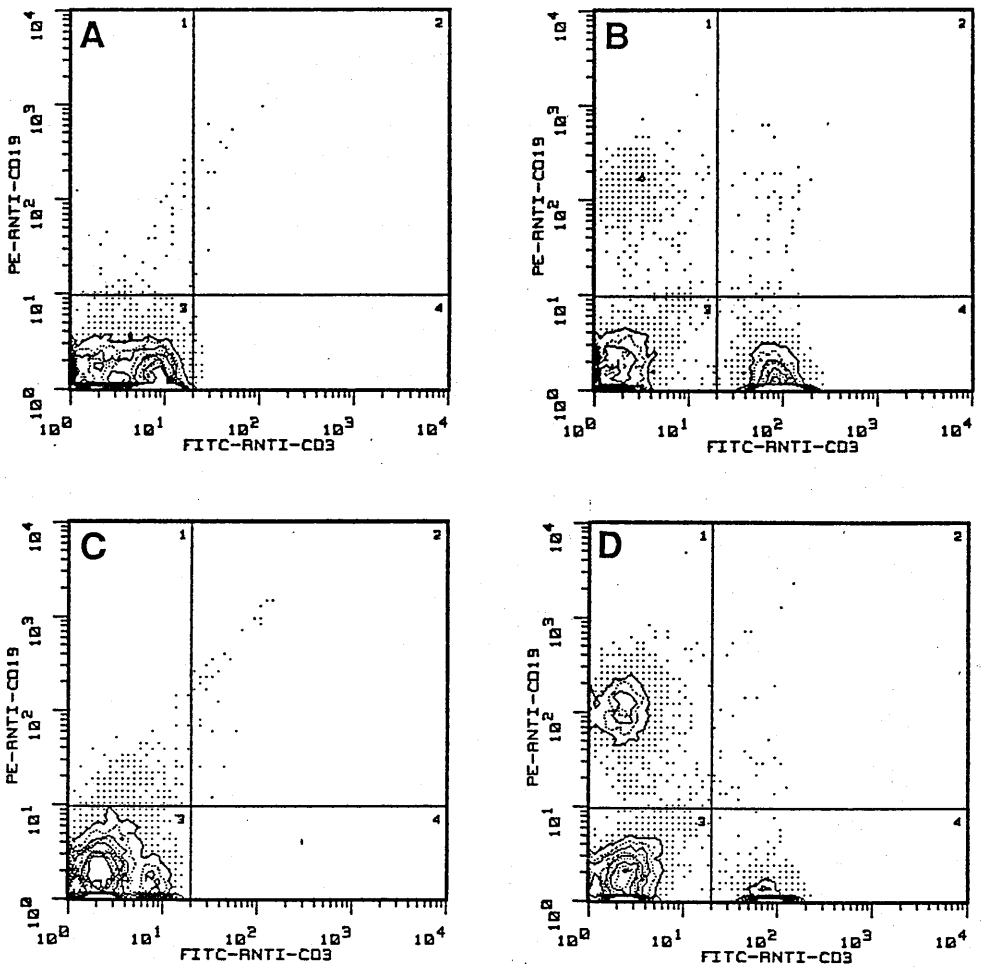
A nonspecific staining of 'T and B' cells

B 'T and B' cells

C non specific staining of 'purified B' cells

D 'purified B' cells

The lymphocytes were gated into quadrants on the basis of fluorescence and statistics performed.


A
B
C
D
PE-ANTI-CD19
FITC-ANTI-CD3
1
2
3
4

Figures 8 and 9 show the results obtained for tonsillar and peripheral blood lymphocytes respectively. Panels A and C represent non specific staining of the cells in each population. Panels B and D show the cells stained for T and B cells before and after T cell depletion respectively. Controls showed that there was very little non specific staining. In tonsillar cells 99% of cells (figure 8, panels A &C) were unstained and in peripheral blood greater than 90% of the cells were unstained in controls (figure 9, panels A &C). In lymphocytes before T cell depletion, as would be expected, both T and B cells could be detected . Peripheral blood comprised 59% T cells and 7% B cells and 32% neither B or T cells (figure 9, panel B). After T cell depletion 27% B cells and 13% T cells could be detected (figure 9, panel D). A large population of cells was unstained, 58%, which suggests that there are cells other than T and B lymphocytes in the preparation.

Cells from tonsils before T cell depletion consisted of 38% B cells and 56% T cells (figure 8, panel B). After T cell depletion the percentage of B cells was increased to 91% and T cells were reduced to 7% (figure 8, panel D). In tonsillar cells there were less than 5% of unstained cells suggesting that most of the cells in tonsils are T and B lymphocytes, which is consistent with it being a lymphoid tissue. In both tonsils and peripheral blood there has been a significant depletion of T cells and enrichment of B cells on T cell depletion. The results indicate that a purified B cell population was obtained.

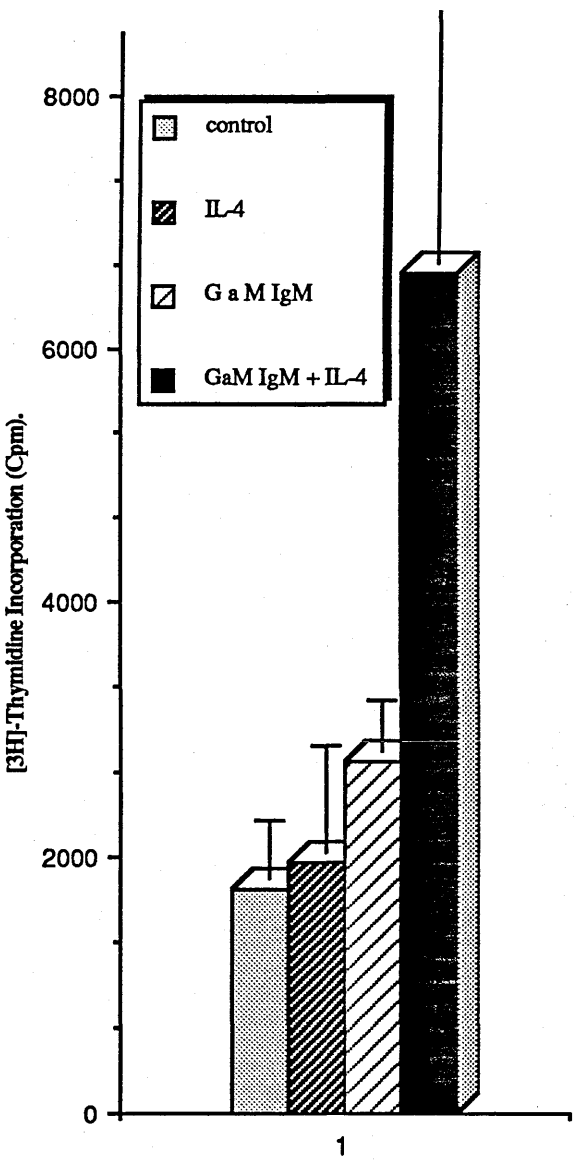
3.2. Response of Resting B Lymphocytes to IL-4

3.2.1. *Costimulation assay*

In the mouse interleukin 4 has been shown to exert its effect primarily on small resting B cells or B cells in the early G_1 phase of the cell cycle. One of the properties of murine IL-4 is to act as a costimulant with anti Ig antibodies to cause entry of the B cells into the cell cycle (Howard *et al.*, 1982). A bioassay has been developed using this costimulator activity of IL-

Figure 10 Costimulation Assay of Murine B Cells

High density resting B cells from BALB/c spleen (sections 2.3.1. & 2.3.3.) were cultured in complete medium plus 50 μM mercaptoethanol for 48 hours in the presence of ligands as indicated in the key. [^{3}H] methly thymidine was added in the final 16 hours before harvesting the cells onto nitrocellulose filters (materials and methods 2.9.). Thymidine incorporated into the cells was determined as counts per minute (CPM). (x +/- SD, n=6).


8000
6000
4000
2000
0
control
IL-4
G a M IgM
GaM IgM + IL-4
[3H]-Thymidine Incorporation (Cpm).
1

4 on DBA/2 mice (Ohara *et al* 1985). This assay was used to ensure that the resting B cells prepared from BALB/c mice were responding to IL-4. Bioassays were performed (as described in 2.9.) where high density resting B cells from mouse spleen were cultured in medium alone or in the presence of anti IgM, IL-4 or anti IgM + IL-4 for 56 hours and [^{3}H] methyl thymidine was added in the last 16 hours. The results from such an experiment are displayed in figure 10.

Cells cultured in medium alone exhibit only a low level of [^{3}H] methyl thymidine incorporation as would be expected for resting B cells. There was little incorporation of [^{3}H] methyl thymidine above control of the cells cultured with anti Ig. This observation is consistent with the work of Noelle *et al* (1983) and Cambier *et al* (1982), which demonstrate that anti IgM alone does not drive B cells into the cell cycle unless at concentrations of greater than 50 μg/ml. B cells cultured with IL-4 alone also exhibited little proliferation. When B cells were cultured with both anti IgM and IL-4 there was an increase in [^{3}H] methyl thymidine incorporation from 2000 to 7000 cpm indicating that the cells were being driven into the cell cycle. These results demonstrated that the resting B cells prepared from BALB/c mouse spleen were responsive to IL-4 in a anti-IgM costimulator assay.

3.2.2. *Detection of MHC Class II Antigens by Flow Cytometry*

One of the most dramatic effects of IL-4 on resting murine B lymphocytes is the 10-15 fold increase in expression of MHC class II antigens (Roehm *et al.*, 1984; Noelle *et al.*, 1984). Resting B lymphocytes and lymphocytes cultured in the presence of IL-4 for 24 hours were analysed for their expression of MHC class II antigens. MHC class II antigens were detected with an anti-Ia antibody and a second antibody conjugated to FITC and analysed by flow cytometry (section 2.10.). Figure 11 compares the intensity of fluorescence, which is a measure of MHC class II expression, in

Figure 11 Induction of MHC Class II Antigens by IL-4 on Murine B Cells

High density resting B cells and resting B cells cultured for 24 hours with IL-4 were prepared for flow cytometry (2.10.). The cells were stained with 25 µl of a 1:10 dilution of anti-mouse (I-A) followed by 50 µl of 1:50 dilution of anti-rat IgG FITC and then subjected to flow cytometry. The results are displayed as fluorescence intensity against cell number.

Panel A

. . . . MHC class II expression in Go B cells

____ MHC class II expression in IL-4 cultured B cells

Panel B

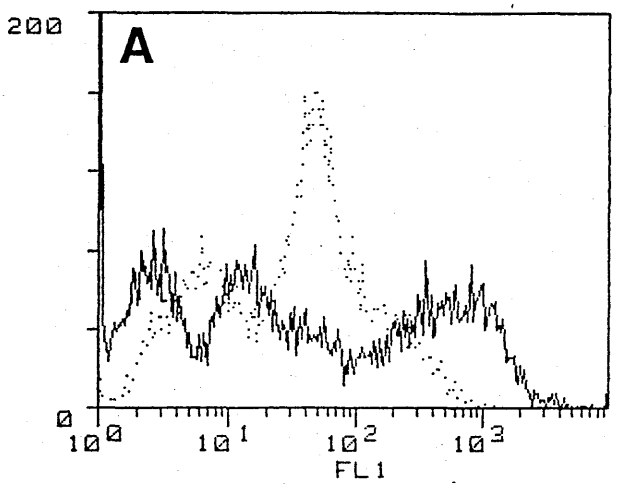
. . . . MHC class II expression in Go B cells

____ MHC class II expression in IL-4 cultured B cells

..... MHC class II expression due to IL-4 (by deducting the fluorescence due to MHC class II expression on resting from B cells cultured with IL-4)

Panel C

____ MHC class II expression due to IL-4 (by deducting the fluorescence due to MHC class II expression on resting from B cells cultured with IL-4)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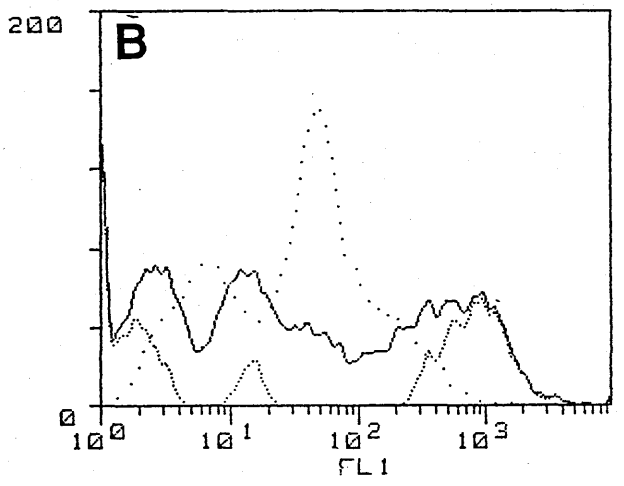
A
0
10^0
10^1
10^2
10^3
FL1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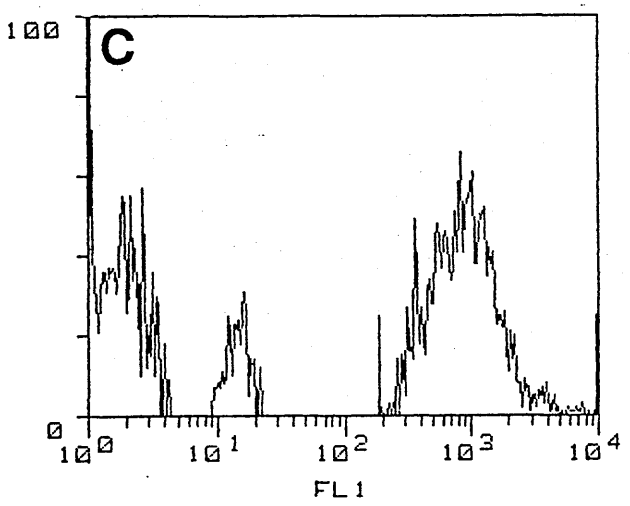
B
0
10^0
10^1
10^2
10^3
FL1


100
C
0
10^0
10^1
10^2
10^3
10^4
FL1

resting cells and cells cultured with IL-4 (panel A). Resting B cells showed some expression of MHC class II antigens with a peak fluorescence of 40. Cells cultured with IL-4 showed a shift in fluorescence intensity with a peak fluorescence of 700. Panel B compares the fluorescence intensity of resting B cells and B cells cultured with IL-4 and shows the fluorescence that is due to culturing the cells with IL-4. This is more clearly demonstrated in panel C where the fluorescence observed represents the increase in MHC class II which is due to culturing the B cells with IL-4. Culturing B cells with IL-4 has led to a 17 fold increase in MHC class II expression which is consistent with that observed by others. (Roehm *et al.*, 1984; Noelle *et al.*, 1984). This provides further evidence that the B cells prepared from BALB/c spleen can respond to IL-4.

3.2.3. *Response of Human B Lymphocytes to Human Interleukin 4*

The responsiveness of the human B lymphocytes from peripheral blood and tonsils to IL-4 was determined by the increase in expression of CD23, the low affinity receptor for IgE. Culturing B lymphocytes with IL-4 increases the expression of CD23 (Defrance *et al.*, 1987a). CD23 expression was detected by using a monoclonal antibody against CD23 followed by a secondary FITC conjugated antibody. CD23 expression was measured in B cells from both peripheral blood (figure 12) and tonsils (figure 13).

Figure 12, panel A represents the autofluorescence of B cells from peripheral blood cultured for 24 hours in the absence of ligand and the equivalent cells stained with second antibody only and stained for CD23 expression. The autofluorescence of the B cells falls below an intensity of 10. The was a little non specific staining of the cells with the second antibody but fluorescence due to the binding of the monoclonal antibody against CD23 could be clearly identified. Figure 12, panel B compares the

expression of CD23 in B cells cultured for 24 hours in the presence and absence of IL-4. In both cases the non specific fluorescence due to the second antibody has been deducted. The mean peak fluorescence of B cells cultured in the absence of ligand is 11 whereas in the presence of ligand it has increased to 200. The fluorescence due to CD23 on IL-4 cultured B cells shows a wide spread suggesting that the amount of CD23 expressed per B cell is variable. It does demonstrate, however, that B cells isolated fom peripheral blood are responding to human IL-4 by increasing the expression of CD23.

The expression of CD23 on B cells isolated from tonsils was also determined. Figure 13 compares CD23 on B cells cultured in the absence of ligand, the presence of IL-4 and the presence of PMA and calcium ionophore for 24 hours. The mean peak fluorescence for cells cultured in the absence of ligand was 10 in tonsillar B cells. Culturing the B cells in the presence of IL-4 caused a shift in the fluorescence intensity to 50 although the peak was wide (panel A). B cells that were cultured in the presence of PMA and calcium ionophore showed levels of CD23 similar to the cells cultured in the absence of ligand (panel B). This indicated that the B cells were responding to IL-4 by increasing CD23 expression but this effect was not mimiced by PMA and calcium ionophore. PMA and calcium ionophore mimic the effects of growth factors that activate the hydrolysis of $PtdInsP_2$. The results from expression of CD23 would suggest that IL-4 does not act through $PtdInsP_2$ hydrolysis to increase CD23 expression. These results are substantiated by Finney *et al* (1989) who demonstrated that phorbol ester and calcium ionophore alone could not mimic the effect of IL-4 in inducing CD23 expression. Exposure of the B cells to dibutyryl cAMP, which mimics cAMP elevation, phorbol ester and calcium ionophore did induce CD23 expression. This suggests that the action of IL-4 on B cells may involve $PtdInsP_2$ hydrolysis leading to the production of DAG and $InsP_3$ but it also involves an intracellular messenger that elevates cAMP levels.

Figure 12 Expression of CD23 on Human B Lymphocytes from Peripheral Blood

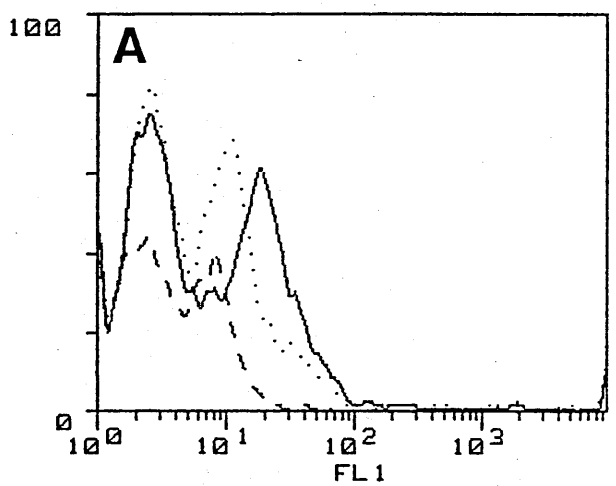
High density resting B cells separated from peripheral blood were cultured in complete medium plus 50 μM 2 mercaptoethanol for 24 hours with no additions or with 1000 units/ml rhIL-4. Cells were prepared for flow cytometry (section 2.10.) and stained with 25 μl of a 1:10 dilution of anti-CD23 antibody followed by 50 μl of anti-mouse Fc-FITC (1:50). Lymphocytes were selected by their forward and side scatter. Dead cells were gated out by counter staining with propidium iodide. Non specific staining of the second antibody is shown on graph A and is deducted in graph B.

Panel A

- - - - autofluorescence
. . . . control cells stained with second antibody only
____ control cells stained for CD23

Panel B

- - - - autofluorescence
..... control cells stained for CD23 - second antibody
____ IL-4 treated cells stained for CD23 - second antibody


100
A
0
10⁰
10¹
10²
10³
FL1

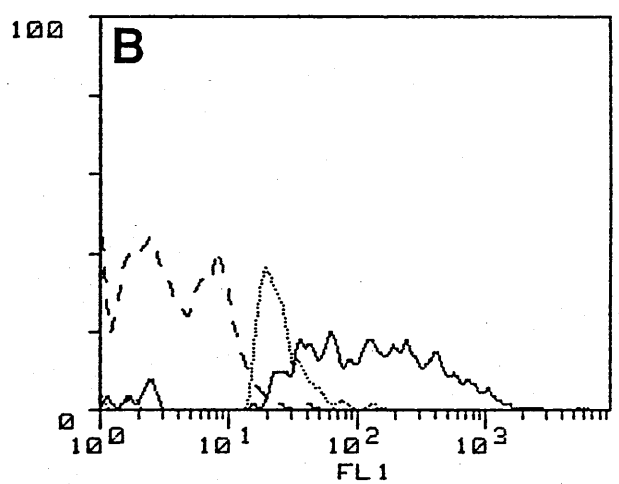

100
B
0
10⁰
10¹
10²
10³
FL1

Figure 13 Expression of CD23 on Human B Lymphocytes from Tonsils

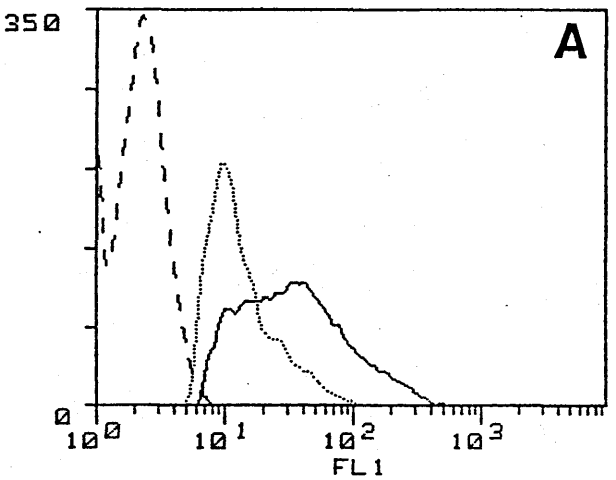
High density resting B cells separated from tonsils were cultured in complete medium plus 50 μM 2 mercaptoethanol for 24 hours with no additions or with 1000 units/ml rhIL-4 or with 10 ng/ml PMA + 1 μM colimycin. Cells were prepared for flow cytometry (section 2.10.) and stained with 25 μl of a 1:10 dilution of anti-CD23 antibody followed by 50 μl of anti-mouse Fc-FITC (1:50). Lymphocytes were selected by their forward and side scatter. Dead cells were gated out by counter staining with propidium iodide. Non specific staining of the second antibody was deducted in each case.

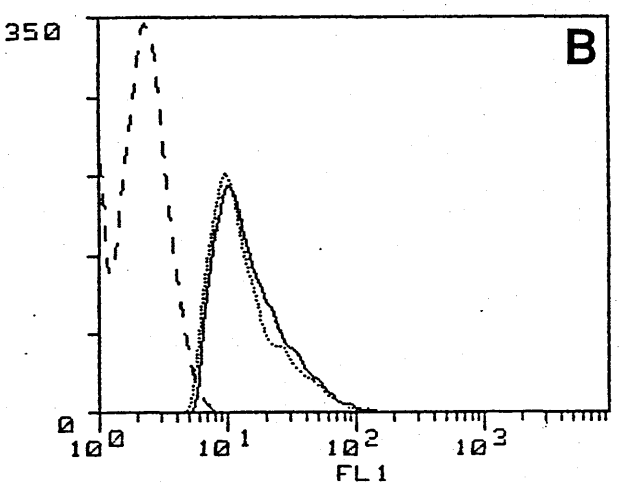
Panel A

- - - -	autofluorescence
.....	control cells stained with second antibody only
____	IL-4 treated cells stained for CD23 - second antibody

Panel B

- - - -	autofluorescence
.....	control cells stained for CD23 - second antibody
____	PMA + colimycin treated cells stained for CD23 - second antibody


350
A
0
10⁰
10¹
10²
10³
FL1


350
B
0
10⁰
10¹
10²
10³
FL1

The B cells isolated from both peripheral blood and tonsils can respond to IL-4 by increasing the expression of CD23.

3.3. Expression of Cell Surface Receptors on B Lymphocytes as Determined by Flow Cytometry

3.3.1. *Expression of Immunoglobulin on Murine and Human B Lymphocytes*

B cells are distinguished by their expression of immunoglobulin on the membrane which is similar to secreted immunoglobulin except for an extra transmembrane portion. B cells initially express IgM and IgD at the cell surface. The expression of immunoglobulin on B cells isolated from BALB/c spleen was observed by staining the cells with an antibody against IgG (H+L) conjugated to FITC and analysis by flow cytometry. Resting cells, cells cultured for 24 hours in the absence or presence of IL-4 and cells cultured for 24 and 48 hours with LPS were analysed (figure14, A & B). Resting cells had a high level of Ig as determined by fluorescence intensity with a mean peak of 250. The mean fluorescence peak was increased on culture to 500, which represents a two fold increase in Ig expression. The expression of Ig was not altered above 500 in cells cultured with IL-4 or LPS.

The expression of IgM was analysed on human tonsillar B cells by staining the cells with an antibody against human IgM conjugated to FITC and analysed by flow cytometry. Figure 15A shows the traces obtained for resting cells and cells cultured for 24 hours in the presence and absence of IL-4. Two peaks of fluorescence can be seen with mean peak fluorescence intensities of 7 and 100 in each case. Autofluorescence of the cells does not interfere with the fluorescence due to the anti-IgM FITC binding. This suggests that there might be two populations of cells one expressing more

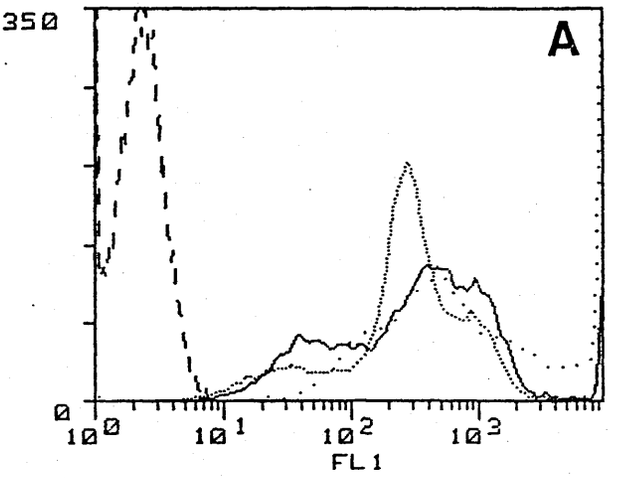
Figure 14 Expression of Immunoglobulin on Murine B Lymphocytes
High density resting B lymphocytes from BALB/c spleen were uncultured, cultured in the absence of ligand, the presence of 100 units/ml rmIL-4 or 10 μg/ml LPS for 24 hours or 10 μg/ml LPS for 48 hours. Aliquots of cells from each of these conditions was analysed for the expression of Ig. Cells were prepared for flow cytometry (section 2.10.) and stained with 5 μl anti-mouse IgG (H+L) FITC. The cells were then analysed by flow cytometry gating out dead cells by counter staining with propidium iodide.

Panel A

- - - - autofluorescence
..... resting B cells (uncultured)
. . . . B cells cultured in the absence of ligand
____ B cells cultured with 100 units/ml rmIL-4

Panel B

- - - - autofluorescence
..... resting B cells (uncultured)
____ B cells cultured for 24 hours with 10 μg/ml LPS
. . . . B cells cultured for 48 hours with 10 μg/ml LPS


350
A
0
10^0
10^1
10^2
10^3
FL1

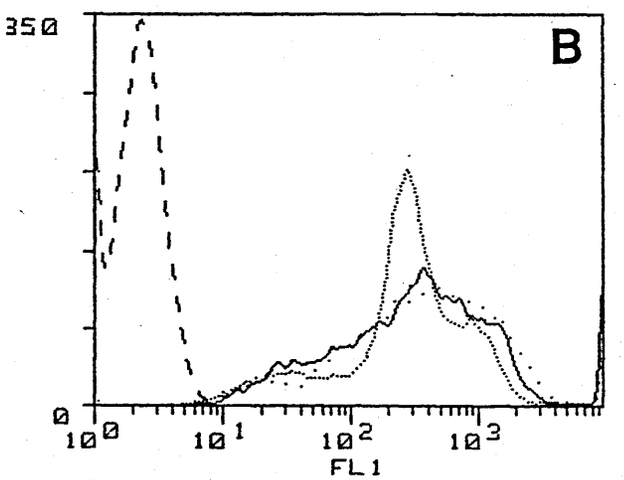

350
B
0
10^0
10^1
10^2
10^3
FL1

Figure 15 Expression of Immunoglobulin on Human B Lymphocytes

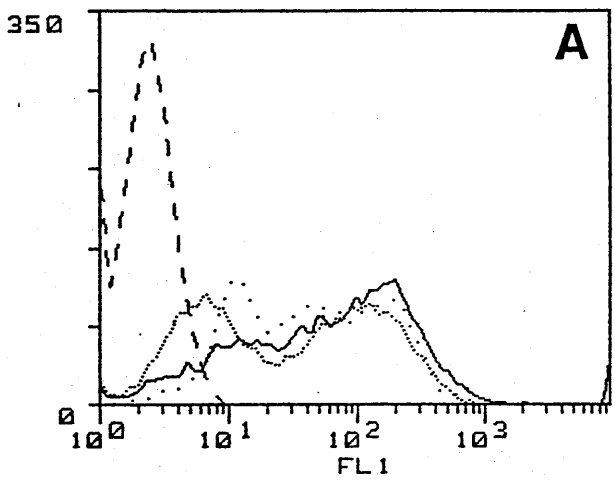
High density resting B cells were isolated from tonsils and analysed for expression of IgM. Resting B cells were also cultured for 24 hours in the absence of ligand, the presence of 1000 units/ml rhIL-4 or with 10 ng/ml PMA + 1 μM colimycin and analysed for IgM. The cells were prepared for flow cytometry as described in materials and methods 2.10. and stained with 5 μl 1:50 dilution of anti human IgM-FITC. Lymphocytes were selected by their forward and side scatter and dead cells were gated by counter staining with propidium iodide.

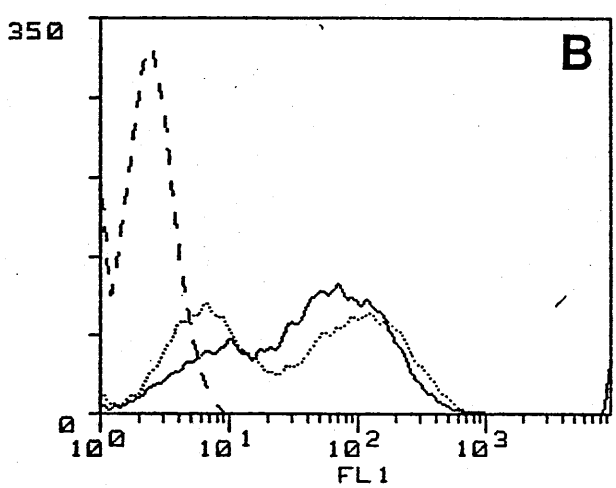
Panel A

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured in the absence of ligand
____	B cells cultured with 1000 units/ml rhIL-4

Panel B

- - - -	autofluorescence
.....	resting B cells (uncultured)
____	B cells cultured with 10 ng/ml PMA + 1 μM colimycin


350
A
0
10^0
10^1
10^2
10^3
FL1


350
B
0
10^0
10^1
10^2
10^3
FL1

IgM than the other. Alternatively the less bright peak may be due to non specific binding of the antibody. Figure 15B compares expression of IgM in resting cells and cells cultured for 24 hours with PMA and calcium ionophore. Again the cultured cells show the same pattern of IgM expression as the resting B cells.

The fact that all cells analysed in both mouse and human samples appear to express immunoglobulin suggests that they are B cells which is further evidence of the efficiency of T cell depletion. In both cases the expression of immunoglobulin does not appear to increase significantly on activation.

3.3.2. *Expression of Insulin Receptors on B Lymphocytes*

Resting B lymphocytes from BALB/c spleen, lymphocytes cultured in the absence of ligand for 24 hours, the presence of IL-4 for 24 hours, the presence of LPS for 24 hours and the presence of LPS for 48 hours were analysed for the expression of insulin receptors. The cells were incubated with insulin conjugated to FITC and then analysed by flow cytometry. Figure 16 shows the results obtained from such an analysis. Freshly isolated resting B cells demonstrated a peak fluorescence of 40. On culturing the cells in the presence or absence of IL-4 two peaks of fluorescence were observed; one at 40, equivalent to the uncultured cells, and a new brighter peak at 2000 (panel A). The increased expression of the insulin receptor was observed on all cultured cells even in the absence of ligand, and culturing with LPS (panel B) or IL-4 did not increase expression any further. This means that culturing the B cells increases the expression of insulin receptors and this is probably due to factors that are present in the foetal calf serum. The less bright peak may be due to non specific binding of the label as the ligand was used to detect the receptor rather than an antibody against the receptor itself. Alternatively the two distinct fluorescent peaks observed on culturing may represent two different affinities of insulin receptor on B cells.

Figure 16 Expression of Insulin Receptors on Murine B Lymphocytes

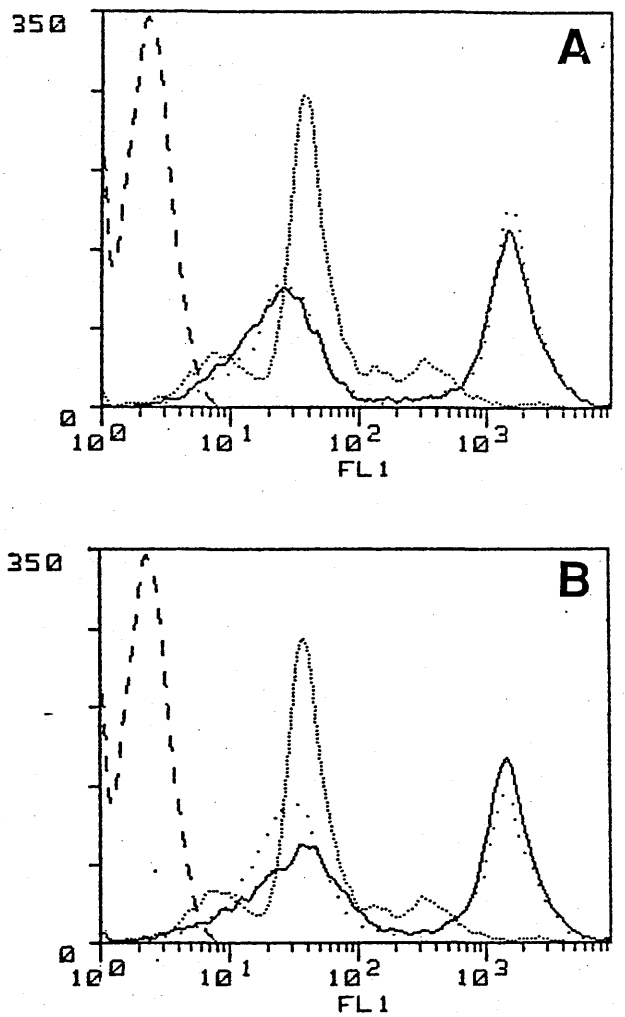
Resting B cells from BALB/c mouse spleen and B cells cultured in the absence of ligand, the presence of 100 units/ml rmIL-4, the presence of 10 μg/ml LPS all for 24 hours or with 10 μg/ml LPS for 48 hours were analysed by flow cytometry for the expression of insulin receptors. The cells were stained with 25 μl of a 1:10 dilution of FITC-insulin following the protocol for preparation of cells for flow cytometry (section 2.10.). Dead cells were gated by counter staining with propidium iodide.

Panel A

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured in the absence of ligand
____	B cells cultured in the presence of 100 units/ml rmIL-4

Panel B

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured for 24 hours with 10 μg/ml LPS
____	B cells cultured for 48 hours with 10μg/ml LPS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10^0
10^1
10^2
10^3
FL1
350
B
0
10^0
10^1
10^2
10^3
FL1

Figure 17 Expression of Insulin Receptors on Human B Lymphocytes

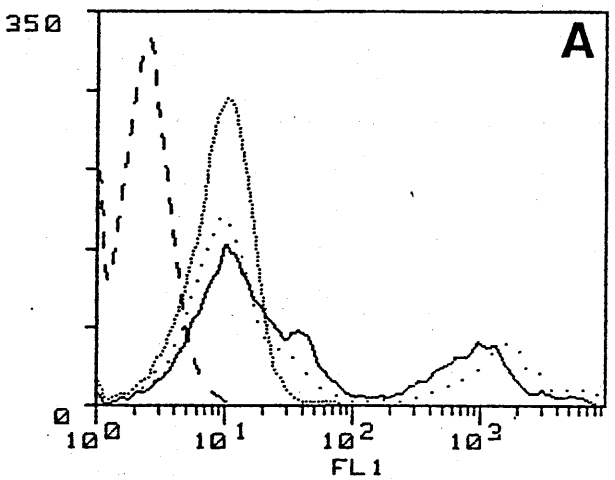
High density resting B cells were isolated from human tonsils and analysed for the expression of insulin receptors. B cells were also cultured in the absence of ligand, the presence of 1000 units/ml rhIL-4 or with 10 ng/ml PMA + 1μM colimycin for 24 hours and similarly analysed for expression of insulin receptors. The cells were prepared (section 2.10.), stained with 25 μl of insulin-FITC (1:10) and subjected to flow cytometry.

Panel A

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured in the absence of ligand
____	B cells cultured in the presence of 1000 units/ml rhIL-4

Panel B

- - - -	autofluorescence
.....	resting B cells (uncultured)
____	B cells cultured with 10 ng/ml PMA + 1μM colimycin


350
A
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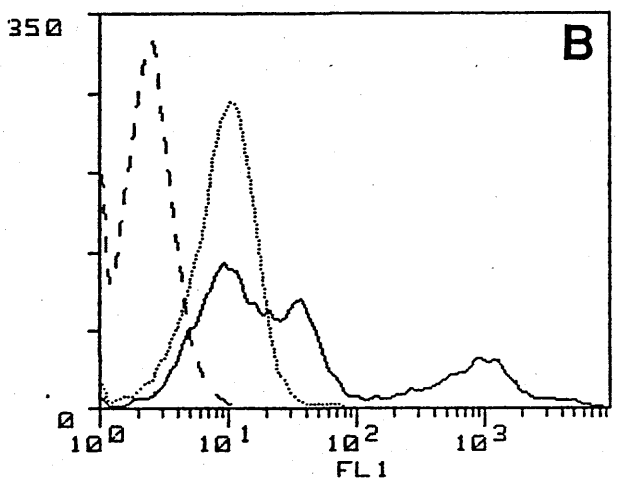
10^0
10^1
10^2
10^3
FL1


350
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FL1

B lymphocytes from human tonsils showed a similar pattern of expression of insulin receptors to the murine B cells, figure 17. The resting B cells demonstrated one peak of fluorescence which had a mean value of 10. On culturing the cells two distinct peaks were again observed with a mean fluorescence intensity of 10 and 1000. Neither the presence of IL-4 or PMA and calcium ionophore increased the fluorescence above that observed with cells cultured in the absence of ligand.

3.3.3. *Expression of Transferrin Receptors on B Lymphocytes*

Both murine and human B cells were analysed for the expression of transferrin receptors by the ability of the cells to bind transferrin labelled with FITC, figures 18 and 19 respectively. Murine and human B cells showed similar results. Freshly prepared resting mouse B cells had a mean peak fluorescence of 3 which was increased to 6 on culturing the cells either in the absence of ligand or the presence of IL-4 or LPS for 24 hours or LPS for 48 hours. Resting uncultured human tonsillar B cells had a mean fluorescence intensity of 4 and showed no increase in fluorescence intensity on culture either in the absence of ligand or the presence of IL-4 or PMA and calcium ionophore for 24 hours. In both murine and human cells the level of fluorescence detected in the presence of FITC-transferrin was above the autofluorescence of the B cells. In cells that have entered the cell cycle, eg. LPS treated cells, the expression of transferrin receptors should have increased as the cells are synthesising DNA and some of the enzymes eg. ribonucleotide reductase require iron which enters the cell via the transferrin receptors. The failure to observe this increase in receptor expression may be explained by the fact that transferrin itself was used to detect the receptor. The transferrin was labelled with FITC and this may have altered the specificity of the interaction of transferrin with the receptor and hence the failure to detect binding.

Figure 18 Expression of Transferrin Receptors on Murine B Lymphocytes

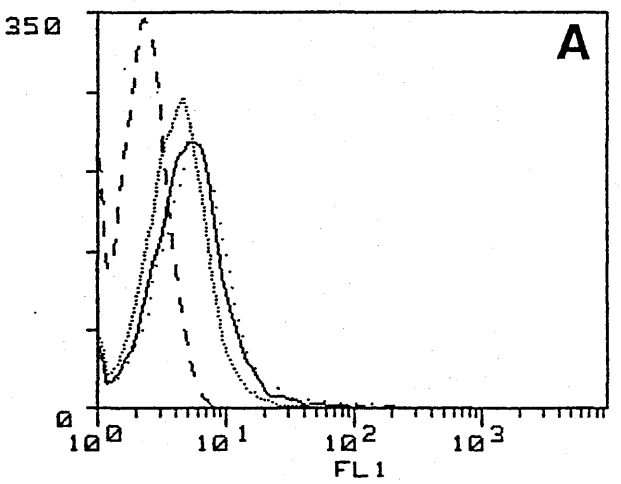
Resting B cells from BALB/c mouse spleen and B cells cultured in the absence of ligand, the presence of 100 units/ml rmIL-4, the presence of 10 μg/ml LPS all for 24 hours or with 10 μg/ml LPS for 48 hours were analysed by flow cytometry for the expression of transferrin receptors. The cells were stained with 25 μl of a 1:10 dilution of FITC-transferrin following the protocol for preparation of cells for flow cytometry (section 2.10.). Dead cells were gated by counter staining with propidium iodide.

Panel A

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured in the absence of ligand
____	B cells cultured in the presence of 100 units/ml rmIL-4

Panel B

- - - -	autofluorescence
.....	resting B cells (uncultured)
____	B cells cultured for 24 hours with 10 μg/ml LPS
. . . .	B cells cultured for 48 hours with 10μg/ml LPS


350
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10^0
10^1
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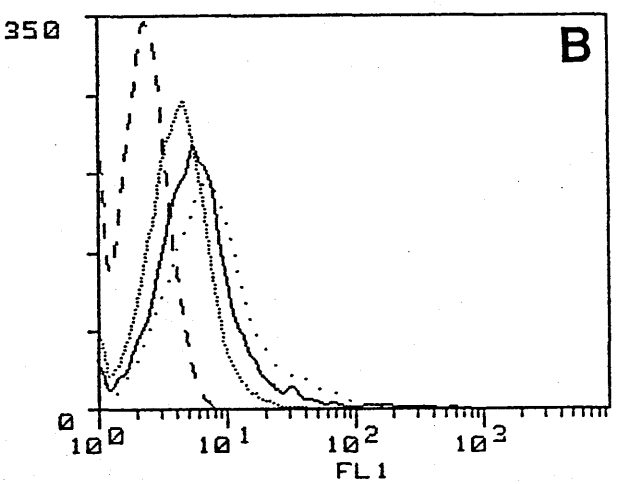
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10^0
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FL1

Figure 19 Expression of Transferrin Receptors on Human B Lymphocytes

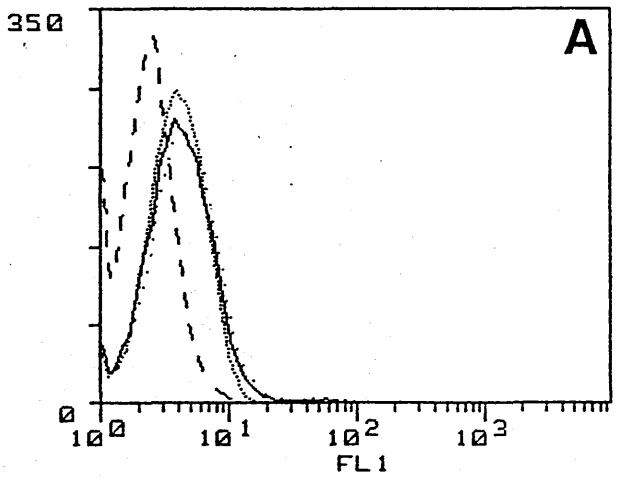
High density resting B cells were isolated from human tonsils and analysed for the expression of transferrin receptors. B cells were also cultured in the absence of ligand, the presence of 1000 units/ml rhIL-4 and with 10 ng/ml PMA + 1μM colimycin for 24 hours and similarly analysed for expression of transferrin receptors. The cells were prepared (section 2.10.), stained with 25 μl of transferrin-FITC (1:10) and subjected to flow cytometry.

Panel A

- - - -	autofluorescence
.....	resting B cells (uncultured)
. . . .	B cells cultured in the absence of ligand
____	B cells cultured in the presence of 1000 units/ml rhIL-4

Panel B

- - - -	autofluorescence
.....	resting B cells (uncultured)
____	B cells cultured with 10 ng/ml PMA + 1μM colimycin


350
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10^0
10^1
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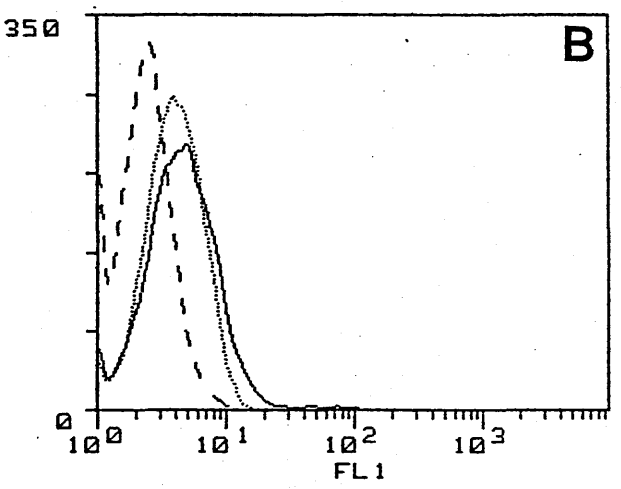
FL1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10^3
FL1

3.3.4. *Expression of IL-2 Receptors on Human B Lymphocytes*

The expression of the receptor for IL-2 on human tonsillar B cells was determined in cells cultured in the presence and absence of IL-4 by using a FITC labelled antibody against the IL-2 receptor, figure 20, panel A. In both cases the fluorescence detected was above the autofluorescence of the B cells. The mean fluorescence intensity of B cells cultured for 24 hours in the absence of ligand was 3 and in the presence the value was 8. The peak for the IL-4 cells showed a increase in fluorescence by the presence of a shoulder on the control peak. These results indicate that B cells that are cultured in the absence of ligand have some receptors for IL-2 on the surface which is increased when the cells are cultured with IL-4. These preliminary results have been further substantiated by simultaneous two and three colour experiments by others in the laboratory (R. Butcher and W. Cushley, personal communication).

3.3.5. *Expression of Ia on Human B Lymphocytes*

B cells isolated from human tonsils were cultured in the presence and absence of IL-4 for 24 hours and analysed for the expression of Ia. Cells were stained with an antibody against Ia labelled with FITC then subjected to flow cytometry, figure 20, panel B. The results show that the B cells express high levels of Ia with a mean peak fluoresence of 1500. The presence of IL-4 does not appear to affect the expression of Ia above that of the control cells. The possibility that there is non specific binding cannot be ruled out. It has been reported that human B cells endogenously express high levels of MHC class II antigens and therefore an increase is more difficult to detect (O'Garra *et al.*, 1988). Although, human IL-4 was observed to increase MHC class II expression on Burkitt lymphoma cell lines this has not been detected on normal human B cells (Rousset *et al.*, 1988).

Figure 20 Analysis of IL-2 Receptors and Ia Expression on Human B Lymphocytes

High density resting B cells were separated from human tonsils and cultured for 24 hours either in the absence (control) or presence of 1000 units/ml rhIL-4. Expression of IL-2 receptors and Ia on the B cells was then determined. B cells were stained with 5 µl anti-IL-2 receptor (FITC labelled), panel A or 5 µl anti Ia (FITC labelled), panel B and prepared for flow cytometry as described in section 2.10. Lymphocytes were selected by forward and side scatter and dead cells were gated by counter staining with propidium iodide.

Panel A

. . . . autofluorescence

..... control B cells stained for IL-2 Receptors

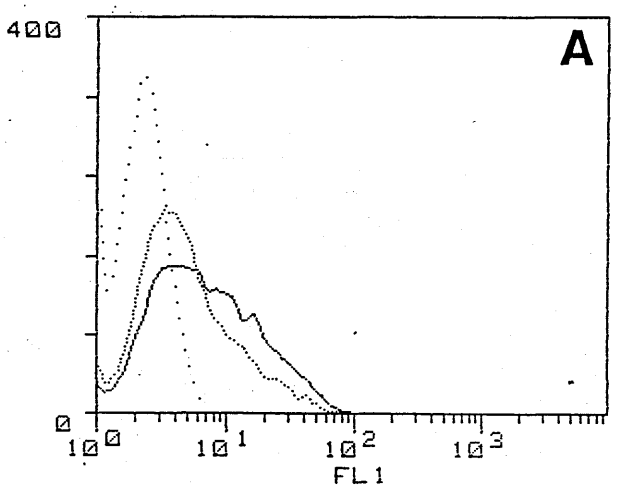
____ IL-4 B cells stained for IL-2 Receptors

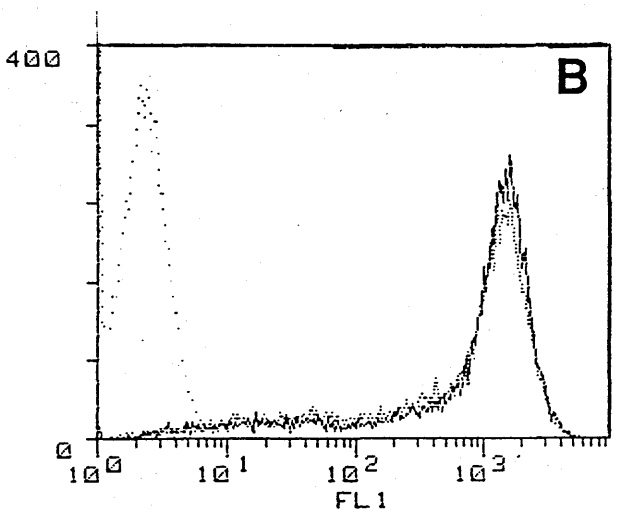
Panel B

. . . . autofluorescence

..... control B cells stained for Ia

____ IL-4 B cells stained for Ia


400
A
0
10^0
10^1
10^2
10^3
FL1


400
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FL1

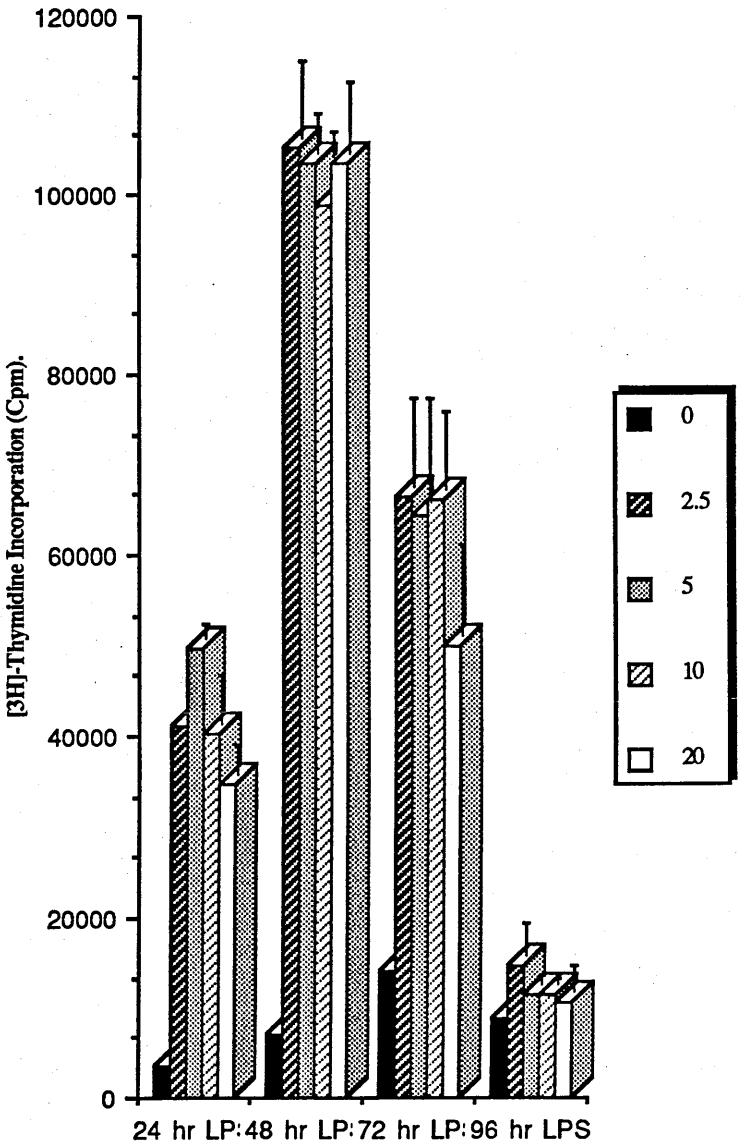
3.4. Lipopolysaccharide as a Mitogen for Murine B Lymphocytes

LPS is a polyclonal activator of murine resting B lymphocytes driving them into the cell cycle (Andersson *et al.*, 1972). Polyclonal activators excite a large proportion of all resting B cells irrespective of their MHC haplotype. LPS drives about 25% of resting B cells to enter the cell cycle. To determine the responsiveness of B cells to LPS at various times after addition and the most effective concentration of LPS, resting B lymphocytes were cultured with 0, 2.5, 5, 10 and 20 μg/ml LPS for 24, 48, 72 and 96 hours. Proliferation was measured by the uptake of [^{3}H] methyl thymidine which was added in the last 6 hours of culture (described in section 2.8.). The cells were harvested onto nitrocellulose filters and counted. Figure 21 is a representation of this experiment.

Resting B cells that were cultured in the absence of LPS show a very low incorporation of [^{3}H] methyl thymidine which indicates virtually no proliferation. At 24 hours the level of [^{3}H] methyl thymidine incorporation is low in response to LPS; the cells are just beginning to enter the cell cycle. LPS activates B cells asynchronously and therefore at early stages after addition of LPS only a small number of cells may show signs of activation which would account for the low levels of proliferation observed at 24 hours. Wetzel & Kettman (1981) observed a lag in the response of cells to LPS of up to 24 hours. Beyond 24 hours, all the cells that can be activated by LPS should have entered the cell cycle and this was the case after 48, 72 and 96 hours of culture with LPS. The cells showed the greatest response to LPS after 48 hours of culture and this was beginning to fall after 72 hours and was reduced to nearly background after 96 hours. After several cell divisions the LPS may become rate limiting and the cells can fall back to resting state. LPS also induces some B cells to become secrete immunoglobulin which would reduce proliferation (Andersson *et al.*, 1972).

Figure 21 The Mitogenic Effect of Lipopolysaccharide on B Lymphocytes Measured by the Uptake of [^{3}H]-methyl Thymidine

High density resting B cells isolated from murine spleen were cultured in complete medium including 50 μM 2-mercaptoethanol. Lipopolysaccharide was added to the cultures at 0, 2.5, 5, 10 and 20 μg/ml. The B cells were cultured for 24, 48, 72 and 96 hours and 1 μCi of [^{3}H]-methyl thymidine was added to each well in the last six hours of culture (materials & methods 2.8.). The cells were harvested onto nitrocellulose filters and counted. The [^{3}H] methyl thymidine incorporated is presented as counts per minute and is the mean value of three determinations. (x = +/- SD, n=3)


120000
100000
80000
60000
40000
20000
0
[3H]-Thymidine Incorporation (Cpm).
0
2.5
5
10
20
24 hr LP:
48 hr LP:
72 hr LP:
96 hr LPS

It was observed that at all concentrations of LPS the incorporation of [^{3}H] methyl thymidine was similar. The LPS concentration between 2.5 and 20 μg/ml did not seem critical and a concentration of 10 μg/ml LPS was chosen for subsequent cultures.

3.5. Characterisation of Plasma Membrane

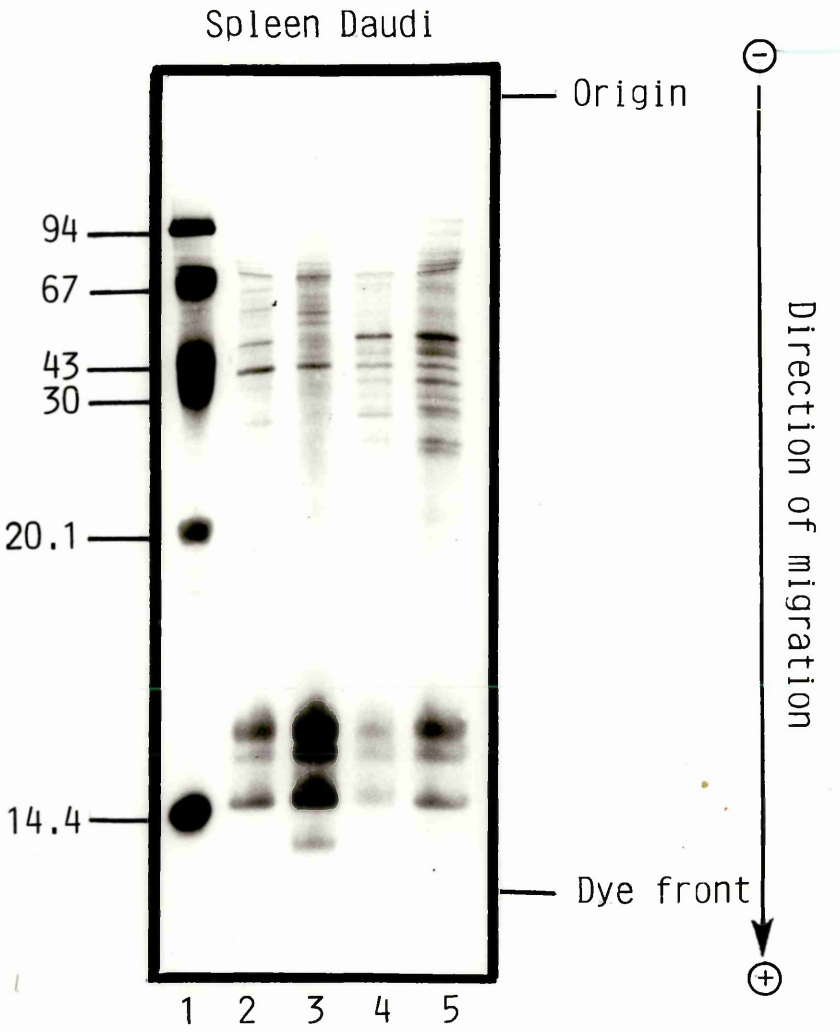
With the simple procedure for plasma membrane preparation that has been employed it was necessary to ensure that there was an enrichment in plasma membrane with minimal contamination from other cellular compartments. This was accomplished by comparing the SDS-PAGE profiles of homogenised whole cells and membranes by silver staining (Figure 22) and, more specifically, by evaluating enzyme activities that are characteristic of each cellular compartment in both crude homogenates and putative cell membrane fraction. Enzyme activities have been compared in B cells from BALB/c mouse spleen and cells from the Daudi cell line and are summarised in tables 2 and 3.

3.5.1. *Visualisation of Whole Cell and Membrane Profiles by Silver Staining*

Proteins from whole cells and the prepared membranes from both BALB/c spleen cells and Daudi cells were separated by gel electrophoresis. The proteins were visualised by silver staining described by Wray *et al* (1981). This technique for visualising proteins is very sensitive and can detect nanogram levels of proteins. Figure 22 shows a silver stained gel of proteins from the whole cell homogenate and plasma membrane from mouse B cells and Daudi cells. Comparison of cell homogenate and plasma membrane shows that several proteins that are present in the cell homogenate are missing or depleted in the plasma membrane. In membranes prepared from spleen proteins of molecular weights 94, 53, 51 and 46 kDa have been reduced. Daudi cells show several proteins between 64 and 78 kDa and also

Figure 22 Silver Stained SDS Gel of B Cell Homogenate and Plasma Membrane

25 μg of protein from mouse resting B cell homogenate (lane 2), plasma membrane (lane 3) and cell homogenate (lane 4) and plasma membrane (lane 5) from Daudi cells were separated on a SDS gel. The proteins were visualised by silver staining (section 2.22.). Lane 1 contains low molecular weight markers.


Spleen Daudi
Origin
94
67
43
30
20.1
14.4
Dye front
Direction of migration
1 2 3 4 5

proteins of 47, 42, 33 and 32 kDa which cannot be detected in the membranes prepared from these cells. This would be expected as any proteins representative of cellular compartments other than the plasma membrane eg. cytosol or golgi should have been removed. In some cases a depletion rather than a complete absence is observed which is due to the fact that the method of protein detection is very sensitive and therefore even the smallest amount of contamination can be detected. With other proteins there is an enrichment observed in the plasma membrane fraction rather than a depletion, for example in membranes from spleen cells there are proteins of molecular weight 42, 31, 27 and 24 kDa which present in a greater amount than is observed in whole cells. This enrichment is due to the proteins representative of the plasma membrane as there will be more of them per mg of protein. The differences observed between the whole cell homogenate and plasma membrane in both mouse B cells and Daudi cells suggest that a partial purification has been obtained. These differences cannot be assigned to the loss or gain of specific proteins only an overall increase or decrease in protein. A more specific method of ensuring purified plasma membrane is by using marker enzymes.

3.5.2. *Lactate Dehydrogenase*

Lactate dehydrogenase has been located in two subcellular compartments, the mitochondrion and the cytoplasm. The two activities can be separated by performing the assay (2.19.1.) in the absence and presence of Triton-X-100, a detergent which is needed to disrupt the mitochondria. In the absence of Triton-X-100 the activity of the free, cytosolic, lactate dehydrogenase can be measured and in the presence of Triton-X-100 the latent activity is released and so total lactate dehydrogenase activity is measured. If the free activity is subtracted from the total activity a measure of the latent activity can be obtained which is mostly due to the mitochondrial lactate dehydrogenase.

The cytosolic lactate dehydrogenase was typically 0.127 units/mg in the whole cell homogenate compared to 0.022 units/mg in the membrane of splenic mouse B cells, a decrease of 83%, and the mitochondrial lactate dehydrogenase also showed a decrease from 0.082 units/mg to 0.046 units/mg. In Daudi cells there was a 89% decrease in the cytosolic lactate dehydrogenase from 0.890 units/mg to 0.097 units/mg and a 75% decrease in mitochondrial from 0.223 units/mg to 0.065 units/mg (table 3). These results indicate that there was substantially reduced lactate dehydrogenase activity in plasma membrane compared to whole cell homogenate in both mouse spleen cells and Daudi cells. The activities have been decreased between 70-80% and therefore this indicates that the plasma membrane fraction is significantly depleted of cytoplasm and mitochondria.

3.5.3. *Catalase*

Catalase is an enzyme which can be used as a cytoplasmic marker, since its activity is specifically found in peroxisomes (Darnell *et al.*, 1986). Catalase displays considerable latency and the assay was therefore performed in the presence of Triton-X-100 (described in section 2.19.2.). Tables 2 and 3 show the values obtained for catalase in Daudi and mouse B cells and membranes. Daudi cells showed levels of catalase activity of 30.08 units/mg which was reduced to 9.28 units/mg in the membranes, a decrease of 69%. Mouse B cells had an activity of 4.64 units/mg compared to 1.37 units/mg in membranes, a 70% reduction. These results are consistent with those obtained for lactate dehydrogenase in that there is minimal contamination in the membrane fraction from the cytoplasm.

3.5.4. *Fumarase*

Fumarase forms part of the Krebs cycle and is, therefore, located in the mitochondria. Fumarase activity was measured by following the production of fumarate from the dehydration of malate (Hill & Bradshaw 1969) (described in section 2.19.3.). The assay was performed in the

presence of Triton-X-100 to allow the release of fumarase from any unbroken mitochondria. In the mouse spleen (table 2) the whole cell homogenate had 0.020 units/mg of fumarase activity and the plasma membrane contained 0.002 units/mg, a reduction of 90%. Daudi cells (table 3) showed similar levels of fumarase activity with 0.015 units/mg in whole cell homogenates and 0.005 units/mg in membranes, a decrease of 66%. This showed a substantial reduction in mitochondria in the plasma membrane which was consistent with the findings for lactate dehydrogenase.

3.5.5. *NADPH-Cytochrome C Reductase*

NADPH-cytochrome C reductase is an enzyme found in the endoplasmic reticulum. Cytochrome C acts as an electron acceptor when NADPH is oxidised to NADP+. NADPH-cytochrome C reductase was assayed by the method of Williams and Kamin (1962) (described in section 2.19.4.). In mouse B cells (table 2) 1.54 x 10^{-2} units/mg of NADPH-cytochrome c activity was detected and 5.29 x 10^{-3} units/mg in the membrane fraction. This represented a 66% depletion of NADPH-cytochrome c reductase from the membrane fraction. In Daudi cells (table 3) there was a decrease of 100% from 6.97 x10^{-4} units/mg in whole cell homogenates to no detectable level in the membranes. From these results it was assumed that there was a significant depletion of endoplasmic reticulum in the membranes.

3.5.6. *5' nucleotidase*

5' nucleotidase was assayed by the method of Newby *et al* (1975) as described in materials and methods (2.19.5.). 5' nucleotidase has been confirmed to be an ectoenzyme present exclusively on the cell surface in mouse lymphocytes (Uusitalo & Karnovsky 1977a) and it can therefore be used as a marker for plasma membrane. The amount of 5' nucleotidase found in homogenate from mouse B cells was 0.013 units/mg and in Daudi cells 0.001 units/mg compared to 0.150 units/mg in membranes from mouse B cells and 0.008 units/mg in Daudi membranes (tables 2 and 3). The levels

of 5' nucleotidase indicated that in membrane prepared from mouse B cells there was a 12 fold enrichment, and in membranes prepared from Daudi cells, an 8 fold enrichment. These increases are consistent with the findings of other workers. Ferber *et al* (1972) saw an enrichment by a factor of 25 in lymphocyte plasma membranes, in human thymocyte plasma membrane a 16 fold increase was reported (Allan & Crumpton 1972) and in plasma membrane a 12-16 fold increase in 5' nucleotidase activity was observed (Avruch & Wallach 1971). Although the increases observed in 5' nucleotidase activity in plasma membranes are consistent the levels detected are variable. It has previously been reported that lymphocytes isolated from the spleen have six times the amount of 5' nucleotidase activity than lymphocytes isolated from the thymus (Uusitalo & Karnovsky 1977a). It has been suggested that there are two populations of lymphocytes with respect to 5' nucleotidase activity, one population with 5' nucleotidase on their surface and the other with none (Uusitalo & Karnovsky 1977b). The proportion of positive cells changes within a population and this suggests the reason why results are variable giving high standard deviations. The reason for the different populations is unknown.

From the results shown in tables 2 and 3 it is evident that there has been a 90% increase in the amount of 5' nucleotidase in the membranes compared to the whole cell homogenate thus suggesting that there has been a substantial enrichment in plasma membrane.

3.5.7. *Leucylaminopeptidase*

Leucylaminopeptidase is an enzyme located on the plasma membrane. It was measured as described in materials and methods (2.19.6.) by a modification of the method of Wachsmith *et al* (1966). The amount of pmoles of 2 napthylamide liberated was calibrated from a standard curve prepared by measuring the fluorescence of known amounts of 4 methylumbelliferone. In mouse B cells an average value of 128 pmoles/mg

of protein was obtained which was increased to 172 pmoles/mg in membranes. This only represents an increase of 25% which could be explained by the fact that small high density B cells already have a high plasma membrane to cytoplasmic ratio and therefore the increase may not be as significant as would be expected with cells that have a smaller plasma membrane to cytoplasmic ratio. In Daudi cells the amount of napthylamide liberated was 27 pmoles/mg in whole cell homogenate and 60 pmoles/mg in membranes which represents an increase of 55%. It has been reported by Evans (1978) that the amounts of leucylaminopeptidase vary in different plasma membranes which would explain the different amounts detected here. The results show a small but significant increase in leucylaminopeptidase in the membrane preparation, therefore, there is an enrichment for plasma membrane.

3.5.8. *β N Acetylglucosaminidase and β Galactosidase*

β N acetylglucosaminidase and β galactosidase are the most active of the β glycosidases. They are found in the lysosomes which are cytoplasmic particles involved in acid hydrolysis. β N acetylglucosaminidase and β galactosidase were assayed by the method of Peters *et al* (1972) (described in section 2.19.7.). In the assay 4-methylumbelliferyl-α-D-galactoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide are hydrolysed by β galactosidase and β N acetylglucosaminidase, respectively, to yield 4-methylumbelliferone which can be measured fluorimetrically. A standard curve of the fluorescence of 4-methylumbelliferone was prepared and the values for the whole cell homogenate and membrane were determined from this (tables 2 and 3). The amount of β galactosidase found in B cells from mouse spleen was 5053 pmoles/mg of protein and this was reduced to 4308 pmoles/mg in membranes. In Daudi cells 2875 pmoles/mg were found and 1532 pmoles/mg in membranes prepared from these cells. These values only represent depletions of 15% and 47% in mouse B cells and Daudi cells respectively. Looking at the levels of β N acetyl glucosamine, tables 2 and 3,

again the membranes still have a lot of activity. Mouse B cells recorded amounts of 5266 pmoles/mg in the whole cell homogenate and 5000 pmoles/mg in membranes and Daudi cells 2406 pmoles/mg and 1234 pmoles/mg in the membranes. This only represents depletions of 5% and 49%. There may therefore be some contamination in the membranes from lysosomes. The contamination is contradicatory to the results obtained from the other enzymes assayed. Lysosomes are cytoplasmic particles and lactate dehydrogenase and catalase indicated that there was minimal contamination from the cytoplasm. It could be that as the assay is sensitive and the β glycosidases are very active that the values indicated for β galactosidase and β N acetyl glucosamine are therefore, an over estimation.

3.5.9. *Galactosyl Transferase*

Galactosyl transferase has been found to be concentrated in the golgi (Fleischer *et al* 1969). Galactosyl transferase activity was measured by using ovalbumin as an acceptor for galactose (described in materials and methods 2.19.8.). The transfer of galactose from UDP-galactose to ovalbumin has been found to be proportional to the concentration of galactosyl transferase (Beaufay *et al* 1974). The plasma membrane has a galactosidase activity which can hydrolyse the galactose from UDP giving false results but the assay cocktail is such that it does not promote the activity of this enzyme. In membrane prepared from mouse B cells, the activity of galactosyl transferase was 0.09 units/mg, a decrease of 53%, compared to the levels in the whole cell homogenate, 0.19 units/mg. In Daudi cells a value of 0.032 units/mg was detected compared to 0.009 units/mg in the membrane, a decrease of 72%. From these results the putative plasma membrane is substantially free from golgi membrane. This is particuarly important as golgi and plasma membrane have similar densities in sucrose gradients which may have resulted in copurification (Evans 1978).

Table 2 Enzyme Marker Activities in Murine B Lymphocytes

Table 2 represents the amounts of each enzyme found in homogenates of mouse B cells and the putative plasma membranes (materials and methods 2.19.1.-2.19.8.). The values shown are averages of values obtained over several experiments. Values are expressed as specific activities (units/mg) except for leucylaminopeptidase, β N acetylglucosamine and β galactosidase which are expressed as pmoles product/mg protein.

TABLE 2 Enzyme Marker Activities in Murine B Lymphocytes

Enzyme	Specific Activities (units/mg protein)	
	Homogenate	Membrane
Lactate Dehydrogenase		
cytoplasmic	0.127	0.022
mitochondrial	0.082	0.046
Catalase	4.64	1.37
Fumarase	0.020	0.002
NADPH-Cytochrome C reductase	0.0154	0.00529
5'Nucleotidase	0.013	0.150
Galactosyl transferase	0.190	0.090
Leucylaminopeptidase (pmoles/mg)	128	172
β galactosidase (pmoles/mg)	5053	4308
β N acetylglucosaminidase (pmoles/mg)	5266	5000

Table 3 Enzyme Marker Activities in Daudi Cells

Table 2 represents the amounts of each enzyme found in homogenates of Daudi cells and the putative plasma membranes (materials and methods 2.19.1.-2.19.8.). The values shown are averages of values obtained over several experiments. Values are expressed as specific activities (units/mg) except for leucylaminopeptidase, β N acetylglucosamine and β galactosidase which are expressed as pmoles product/mg protein.

TABLE 3 Enzyme Marker Activities in Daudi Cells

Enzyme	Specific Activities (units/mg protein)	
	Homogenate	Membrane
Lactate Dehydrogenase		
cytoplasmic	0.890	0.097
mitochondrial	0.223	0.065
Catalase	30.08	9.28
Fumarase	0.015	0.005
NADPH-Cytochrome C reductase	0.000697	0
5'Nucleotidase	0.001	0.008
Galactosyl transferase	0.032	0.009
Leucylaminopeptidase (pmoles/mg)	27	60
β galactosidase (pmoles/mg)	2875	1532
β N acetylglucosaminidase (pmoles/mg)	2406	1234

3.5.10.*Evaluation of Plasma Membrane*

Comparison of enzyme activities in cellular homogenate and the putative plasma membrane fraction indicate that a high purity of plasma membrane has been attained. In both Daudi and mouse spleen cells a minimum of 70% reduction in contaminating cellular compartments has been achieved whereas there has been a substantial enrichment in plasma membrane as determined by leucylaminopeptidase and 5' nucleotidase. Protein profiles of homogenised whole cells and plasma membranes indicated several differences with reductions of some proteins and enrichment of others.

The protein profiles of splenic B cells and Daudi cells show several differences as determined by SDS-PAGE. These differences are substantiated by the enzyme activities found in the different cell types. Daudi cells have higher levels of the cytosolic and mitochondrial enzymes and lower levels of plasma membrane enzymes. This is consistent with the fact that resting B cells, such as those from mouse spleen, are small and, therefore, have a high surface area to cell volume ratio. Resting B cells are therefore a good source of plasma membrane and contamination should be minimal because of the small amount of cytoplasm. Daudi cells because they are B cell lymphomas are continually proliferating and, therefore, contain more cytoplasm consequently more mitochondrial and cytoplasmic associated enzymes. The evidence however, suggests that in both Daudi and splenic resting B cells the plasma membrane has been substantially purified.

3.6. Discussion

The data from chapter three indicate that the methods employed to obtain a 'purified' B cell population from a 'mixed' lymphocyte population were sucessful. In the separation of B cells from murine spleen this was demonstrated by the use of antibodies against molecules on the T cell surface in conjunction with flow cytometry and secondly by the response of the cells

to T and B cell specific mitogens. The efficiency of human B cell separation from tonsils and peripheral blood was demonstrated by simultaneous two colour immunofluorescence using antibodies against CD3 on T cells and CD19 on B cells. This method indicated that a substantial enrichment in B cells had been obtained. Anti-immunoglobulin studies on both murine and human 'purified' B cells further demonstrated that the population of cells was greater than 90% B cells. Both murine and human B cells were assessed for their response to the lymphokine IL-4. Murine B cells demonstrated proliferation in a costimulator assay with anti-IgM and showed a 17 fold increase in MHC class II expression after 24 hours in culture with IL-4. The effect of IL-4 on human B cells was demonstrated by an increase in CD23 expression after culture with IL-4. Human B cells were also shown to express Ia but this was at a high level even in the absence of ligand in the culture medium. Culturing human B cells with IL-4 increased the expression of the IL-2 receptor.

Insulin receptors were expressed on both murine and human B cells. Transferrin receptor expression was low and this was possibly due to using FITC labelled transferrin to detect the receptor.

The purity of the plasma membrane prepared from the B cells was analysed by silver staining of SDS-PAGE gels showing a comparison of whole cell proteins and plasma membrane protein. This technique showed an enrichment in some proteins in the plasma membrane and a depletion in others. The plasma membrane was further analysed for purity by measuring enzyme activities that are specific to cellular compartments. These enzyme assays indicated that the 'putative' plasma membrane contained very little contamination from other cellular compartments.

4. RESULTS.

THE EFFECTS OF IL-4 ON PROTEIN KINASE ACTIVITIES IN RESTING AND MITOGEN-ACTIVATED B CELLS

CHAPTER 4

THE EFFECTS OF IL-4 ON PROTEIN KINASE ACTIVITIES IN RESTING AND MITOGEN ACTIVATED B CELLS

4.1. TCA Precipitation of Total Phosphorylated Membrane Protein from Resting Murine B Lymphocytes

Membranes prepared from high density resting murine B lymphocytes were phosphorylated with γ-[^{32}P] ATP in the presence or absence of IL-4. After termination of the kinase reaction, three 5 µl aliquots were removed from each reaction for TCA precipitation (described in 2.13.). A comparison of the total membrane protein phosphorylation in the presence and absence of IL-4 in seven independent experiments is given in table 4. In five out of seven of the experiments represented there is an increase in total membrane phosphorylation when IL-4 is present in the kinase assay. The increase in [^{32}P]-phosphate incorporation in the presence of IL-4 is variable ranging from 6-42% greater than when IL-4 was absent from the assay. In the other two experiments represented there is a decrease in total phosphate incorporation in the presence of IL-4. It appears therefore that there is some variability in the incorporation of phosphate into membrane proteins between experiments. In the majority of experiments an increase in phosphate incorporation is observed in the presence of IL-4 but this increase is variable. Technically, the efficiency in washing of the filter papers may account for part of the discrepancy. In this experiment the effect of IL-4 on total membrane protein is quantitated rather than the effect on individual proteins. It is therefore a combined effect that is being observed which may involve the phosphorylation of some proteins and the dephosphorylation of others. In individual experiments, this may cause a variation in total phosphorylation.

Table 4 Total Incorporation of Phosphate into Membrane Proteins

Membranes from high density resting murine B cells were phosphorylated in the absence and presence of 10 units of IL-4 (section 2.12.). Three 5 µl samples were removed from the terminated reaction and spotted onto filter discs. The proteins were precipated with 10% ice cold TCA (section 2.13.) and the [^{32}P]-phosphate incorporated into the proteins determined by liquid scintillation counting. Table 4 displays the results of seven representative experiments expressed in cpm (x +/- SD, n=3).

TABLE 4:- Total incorporation of phosphate into membrane proteins.

No IL-4	± IL-4
8047 ± 720	13950 ± 3299
5898 ± 1313	7244 ± 1378
9259 ± 126	9850 ± 51
15516 ± 2241	15248 ± 2633
2413 ± 52	3164 ± 725
7265 ± 1135	6572 ± 617
24064 ± 1636	28715 ± 3707

4.2. Thin layer electrophoresis of total phosphorylated membrane protein

To try and identify if phosphorylation of a particular amino acid was responsible for the changes in total phosphorylation of membrane proteins observed in the presence of IL-4, phosphorylated membrane proteins from resting murine B cells were subjected to acid hydrolysis (section 2.16.) and the resulting phosphoamino acids separated by two dimensional thin layer electrophoresis (section 2.17.) (figure 23). Phosphorylation of the membrane proteins were performed in the presence (B) and absence (A) of IL-4. Three phosphoamino acids could be identified by the use of standard phosphoamino acids, phosphoserine, phosphothreonine and phosphotyrosine. From the autoradiograph of the thin layer plates it appears that incorporation into all three phosphoamino acids is increased in the presence of IL-4. The phosphoamino acids were quantified by scraping each amino acid from the thin layer plate and counting the radioactivity. Table 5 shows the results from three experiments. This confimed that there was an increase in all three phosphoamino acids in the presence of IL-4. In the first of the experiments it appears that phosphorylation of tyrosine increased 75% compared to increases of 44% and 33% for serine and threonine respectively. This would suggest that IL-4 causes the greatest increase in tyrosine phosphorylation. This observation is not supported by the second two experiments where the phosphate incorporation into all three amino acids is approximately equivalent. Phosphotyrosine, however, accounts for the smallest percentage of total acid stable phosphate in proteins, only 0.05%, whereas phosphoserine and phosphothreonine account for 90% and 10%, respectively (Sefton *et al.*, 1980). An increase in phosphotyrosine is therefore more significant as this phosphoamino acid is less abundant in cells.

Figure 23 Incorporation of Phosphate into Phosphoamino Acids

Membrane proteins from resting murine B cells were phosphorylated in the absence (A) and presence (B) of 10 units of IL-4 (section 2.12.). The reaction was terminated by protein precipitation and the proteins were then acid hydrolysed (materials & methods 2.16.). The hydrolysed proteins were mixed with a mixture of three standard phosphoamino acids and subjected to two dimensional thin layer electrophoresis (section 2.17.) to allow separation of phosphoamino acids. The standard phosphoamino acids were visualised by ninhydrin staining and the labelled phosphoamino acids were visualised by autoradiography.

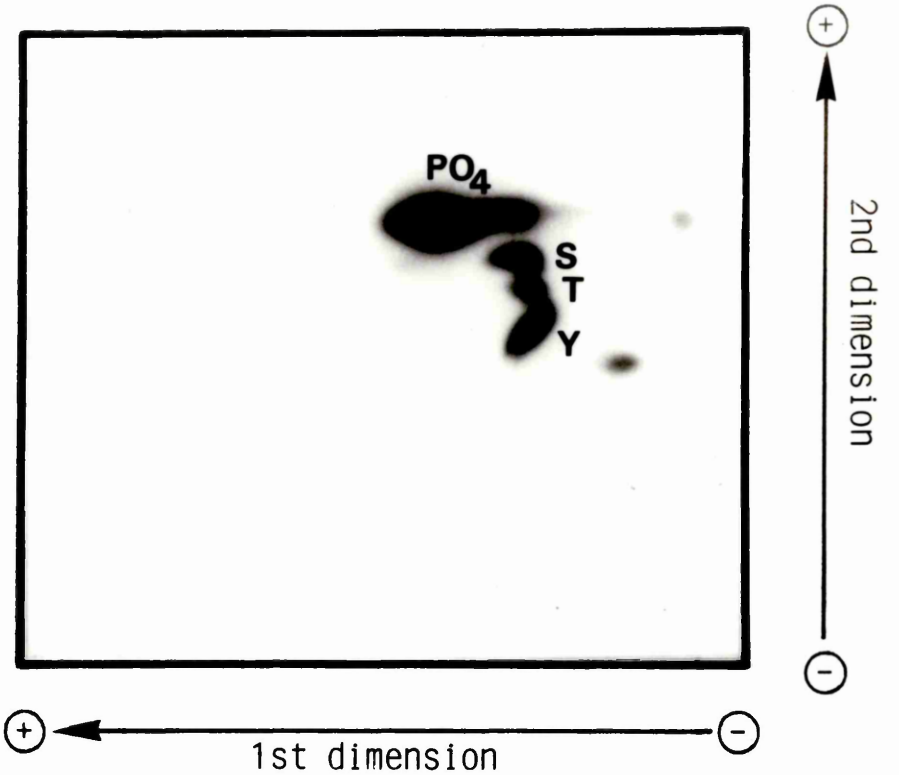
Y = phosphotyrosine

T = phosphothreonine

S = phosphoserine

P = free phosphate

A. Resting B cell membrane



B. Resting B cell membrane + IL-4

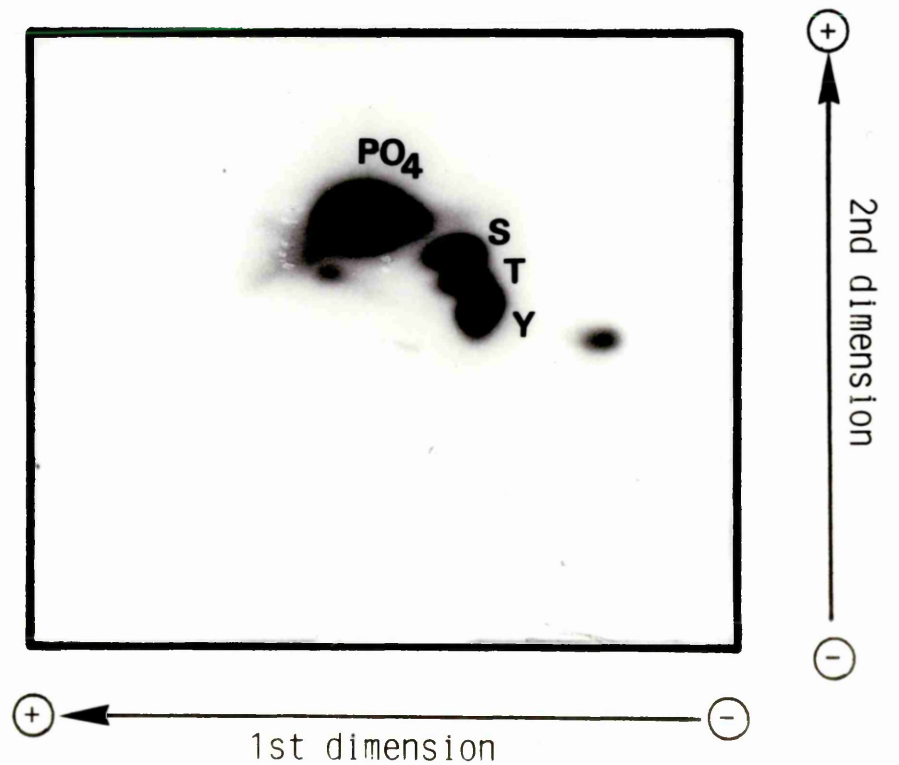


Table 5 Incorporation of Phosphate into Phospho Serine, Threonine and Tyrosine

The incorporation of phosphate into serine, threonine and tyrosine was determined by excising the separated phosphoamino acids from TLE plates such as that described in figure 23. Each individual phosphoamino acid was then counted in 3 ml ecoscint in a Beckman scintillation counter. Table 5 represents the results of three individual experiments expressed in cpm.

TABLE 5. Incorporation of phosphate into phospho-serine, threonine and tyrosine.

Experiment.	Amino Acid	Counts per minute. - IL-4	+ IL-4	Increase (%)
	Ser	145	216	44
1	Thr	54	96	33
	Tyr	155	632	75
	Ser	226	492	54
2	Thr	95	242	61
	Tyr	139	386	64
	Ser	240	510	53
3	Thr	142	412	65
	Tyr	86	176	77

4.3. Effect of Interleukin 4 on Membrane Phosphorylation in Resting Murine B Lymphocytes

High density resting B cells from BALB/c mouse splenocytes were isolated from the 1.08/1.09 g/ml interface of a percoll gradient (described in materials & methods 2.3.1.& 2.3.3.). Membranes were prepared from these cells and the proteins were phosphorylated in kinase assays (Figure 24) in the presence (track b) and absence (track a) of recombinant murine IL-4 (10 units/assay). The membrane phosphoproteins were separated by SDS PAGE and visualised by autoradiography.

An overall enhancement of phosphate incorporation was observed in the presence of IL-4. This enhancement is consistent with the increase in phosphate incorporation observed in TCA insoluble material in the presence of IL-4 and in the increase in [^{32}P]-phosphate uptake into serine, threonine and tyrosine observed with thin layer electrophoresis. There is however a specific increase in phosphorylation of a protein marked by the arrow on figure 24 in the presence of IL-4. In a number of independent experiments the molecular weight of this protein consistently fell within the range of 40-44 kDa. It is thought that this phosphoprotein is identical to the 42 kDa protein previously described by Justement *et al* (1986) and was therefore termed 42 kDa. To determine the increase in intensity of phosphorylation of the 42 kDa protein the autoradiograph was analysed by linear laser densitometry. Figure 25 illustrates the intensity of the phosphorylated proteins in the presence and absence of IL-4 as determined by densitometry of the autoradiograph. The intensity of three proteins, 62 kDa, 51 kDa and 42 kDa observed on the autoradiograph have increased in the presence of IL-4. There is a low level of phosphorylation of the 42 kDa protein in the absence of IL-4 constituting 3.75% of the total area under the graph. This was increased to 7.15% in the presence of IL-4. The two higher molecular weight proteins constitute 7.8% and 9.4% respectively of the total area under the graph in the absence of IL-4.

Figure 24 Membrane Phosphoprotein profiles from Murine Resting B Lymphocytes

Aliquots of 50 μg of membrane protein prepared from high density resting B cells were incubated on ice for 3 minutes with 10 μCi γ-[^{32}P]-ATP in the absence (track a) or the presence (track b) of 10 units rmIL-4 (described in materials & methods 2.12.). The reactions were terminated by the addition of SDS-PAGE sample buffer and the proteins separated by gel electrophoresis and visualised by autoradiography. Low molecular weight markers were run on the gel and visualised by coomassie blue staining. The arrow indicates a phosphoprotein of 42 kDa.

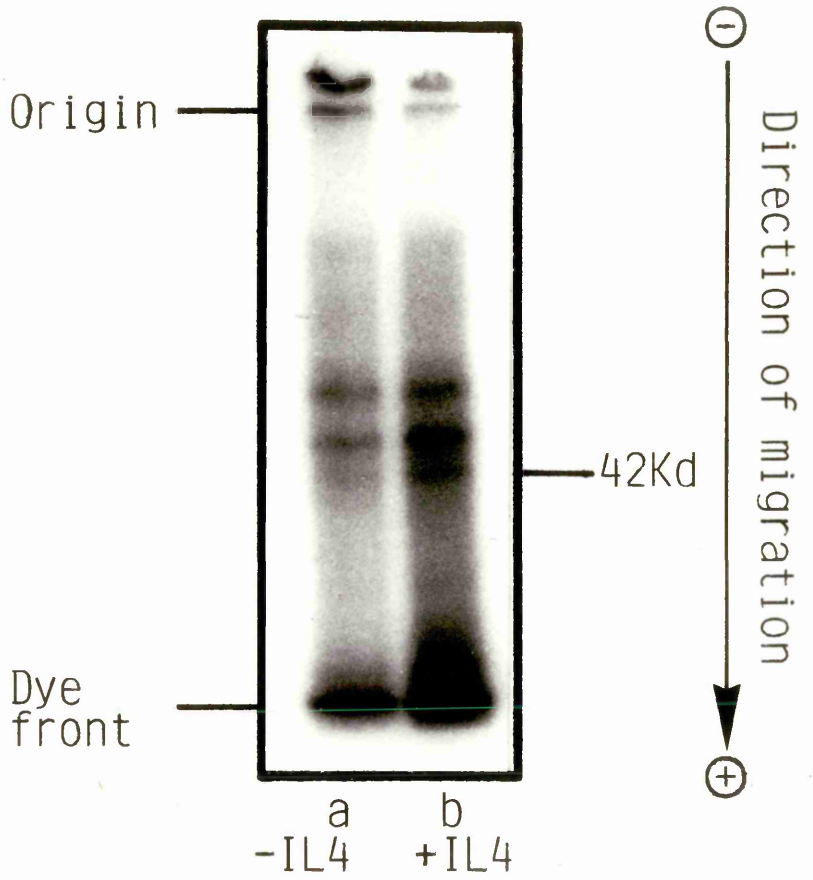
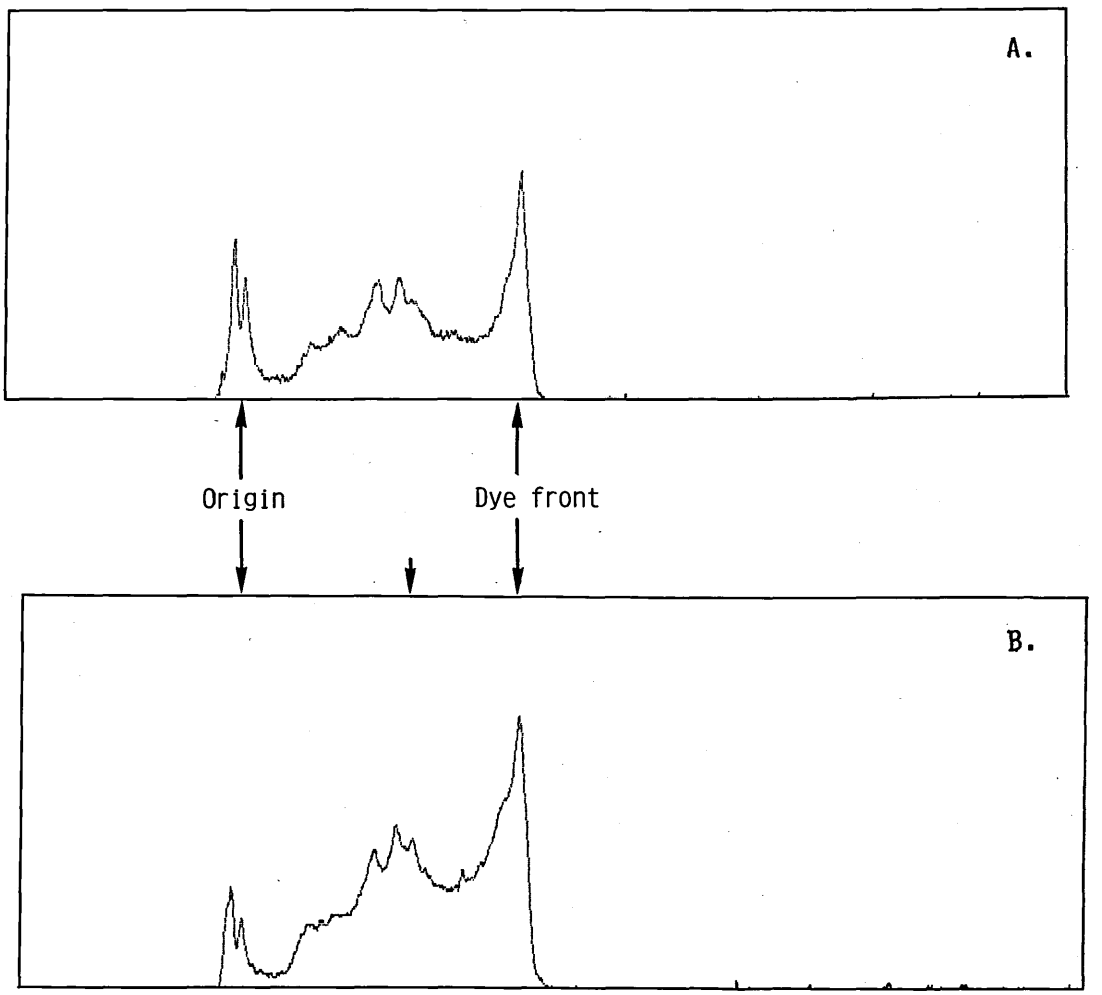

Origin
42Kd
Dye
front
a
-IL4
b
+IL4
Direction of migration

Figure 25 Laser Densitometry of Autoradiographs of Phosphoprotein Profiles

The autoradiograph of figure 24 was subjected to densitometry using the Bio-Rad 620 system. The profiles shown were obtained with background subtraction but without filtering, enhancement or boosting. The data were resolved by automatic integration on the Olivetti M24 system. The top panel illustrates the trace for unstimulated membrane phosphoproteins and the lower panel shows the equivalent data for the IL-4 stimulated samples. The positions of the origins and the dye front correspond to those indicated on the original autoradiograph (figure 23), and the position of the 42 kDa protein is also indicated. The x axis of the figures represent migration (mm), while the y axis represent optical density.


A.
Origin
Dye front
B.

In the presence of IL-4 this is increased to 10.8% and 10.7% respectively. The results from densitometry are consistent with the fact that IL-4 is inducing protein phosphorylation in high density resting murine B lymphocytes. An attempt was made to determine which amino acid was phosphorylated on the 42 kDa protein. This was performed by eluting the 42 kDa protein from the gel, hydrolysing it and separating the phosphoamino acids by thin layer electrophoresis, as described in materials and methods 2.16.1.-2.17. Due to the low incorporation of [^{32}P]-phosphate into the 42 kDa protein, possibly due to the low numbers of IL-4 receptors on resting B cells, no phosphoamino acids were visualised on autoradiography of the TLE plate. The phosphoamino acid(s) in the 42 kDa protein, therefore, remain(s) unidentified.

4.4. Effect of Purified and Recombinant IL-4 on Membrane Phosphoproteins on LPS Activated Murine B Cells

Resting murine B cells express only low numbers of receptors for IL-4 (Park *et al.*, 1987a; Ohara & Paul, 1987). The number of receptors for IL-4 can be increased 5-10 fold on activation of the B cells with LPS. To determine if an increase in the number of IL-4 receptors per B cell increased the intensity of phosphorylation of the 42 kDa protein membranes were prepared from low density 'activated' cells after 24 and 48 hours of culture with LPS. The membrane proteins were phosphorylated with γ-[^{32}P]-ATP in the absence of ligand or the presence of purified IL-4 or recombinant IL-4. Three 5 µl aliquots of terminated reaction mix were taken for determination of total protein phosphorylation by TCA precipitation (table 6).

Phosphoproteins were separated by gel electrophoresis and visualised by autoradiography (figure 26).

Table 6 represents the total incorporation of [^{32}P]-phosphate into

Table 6 Total [^{32}P]-Phosphate Incorporation into Membranes from Mitogen Activated B Cells

Membranes were prepared from high density resting murine B cells and low density 'activated' B cells after culture with LPS for 24 or 48 hours. Each type of membrane was incubated with 10 μCi γ-[^{32}P]-ATP in the presence or absence of 10 units of IL-4 and the reaction was terminated by the addition of 55°C sample buffer. Three 5 μl aliquots were removed from the terminated reaction mix and spotted onto filter discs. The discs were washed four times in 10% TCA and 10 mM sodium pyrophosphate and then counted in 3 ml ecoscint in a Beckman LS 6800 scintillation counter. Table 6 illustrates the results of three experiments expressed in cpm (x +/- SD, n = 3)

TABLE 6. Total [^{32}P]-phosphate incorporation into membranes from mitogen activated B cells.

Resting Cells

-IL-4	+IL-4
19367 ± 1541	25459 ± 1893
3638 ± 197	4175 ± 415
8470 ± 1470	9792 ± 1222

LPS (24Hrs)

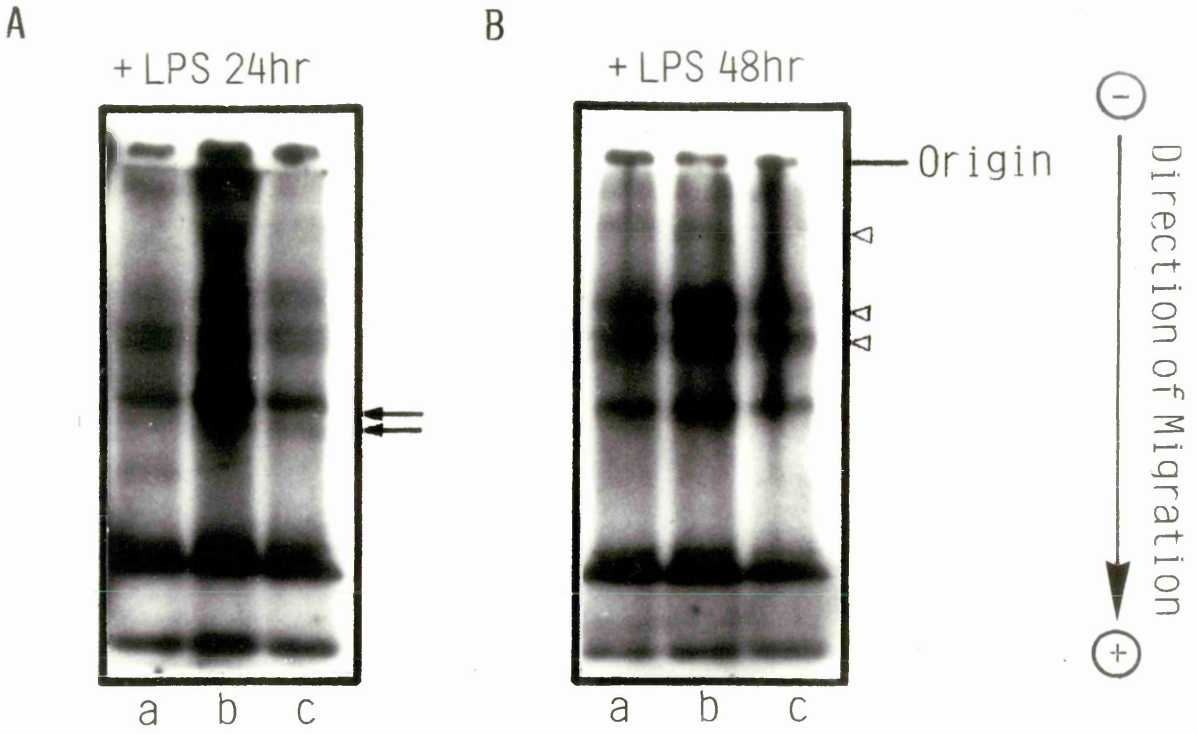
-IL-4	+IL-4
22979 ± 494	17422 ± 1059
3476 ± 147	3755 ± 397
5925 ± 1219	8341 ± 1183

LPS (48Hrs)

-IL-4	+IL-4
22131 ± 2936	26022 ± 2095
3843 ± 507	3687 ± 385
9600 ± 3913	7814 ± 718

Figure 26 Phosphoprotein Profiles of LPS Activated Murine B Lymphocytes

High density resting B cells were prepared as described in materials and methods 2.3.1. & 2.3.3. and cultured for 24 hours, panel A, or 48 hours, panel B, with 10 μg/ml LPS. After culture the B cells were separated on percoll gradients and membranes were prepared from the low density 'activated' cells (1.065/1.055 g/ml percoll interface). Membrane proteins were exposed to 10 μCi γ-[^{32}P]-ATP in the absence of ligand (lane a) the presence of 10 units affinity purified IL-4 (lane b) or 10 units recombinant IL-4 (lane c). The proteins were separated by SDS-PAGE and visualised by autoradiography. Low molecular weight markers were run on the gel and visualised by coomassie blue staining.


A
+ LPS 24hr
a b c
B
+ LPS 48hr
Origin
a b c
Direction of Migration

membrane proteins from resting cells, 24 hour LPS cultured cells and 48 hour LPS cultured cells in the presence or absence of rmIL-4. In resting cells, in all three experiments shown, there is an increase in phosphate incorporation in the presence of IL-4. This is the same in two out of the three cases for the membranes prepared from B cells cultured with LPS for 24 hours. Membranes prepared from B cells cultured for 48 hours show a decrease in phosphate incorporation when IL-4 is included in the phosphorylation assay in two out of the three examples given. As before these results illustrate that the total phosphate incorporation into membrane proteins is variable. There does not appear to be a consistent pattern for the incorporation of [^{32}P]-phosphate into membrane prepared from each type of B cell in fact the [^{32}P]-phosphate into all three appears very similar within each experiment.

The profiles of membrane phosphoproteins are identical with the purified and the recombinant form of the IL-4 (Figure 26, tracks b and c). The purified IL-4 was isolated from supernatants of EL4 cells, which had been stimulated with PMA, using immobilised IIBII, an antibody to IL-4. Residual PMA present in the purified IL-4 could be increasing protein phosphorylation via protein kinase C. This possibility can be excluded as the recombinant IL-4 is produced by microbial expression of plasmid vectors containing the gene for murine IL-4 and, therefore, has no PMA present.

In membranes prepared from low density "activated" cells after culture with LPS for 24 hours there was an increase in phosphorylation of the 42 kDa protein in the presence of IL-4 (figure 26, panel A). Phosphoproteins of higher molecular weights, 105, 90,77 and 71 kDa were also starting to appear in the membranes from cells cultured with LPS that were absent in the phosphoprotein profiles resting B cell membranes (figure 24). These higher molecular weight phosphoproteins were present in the presence and absence of IL-4 and, therefore, were not a specific effect of the

IL-4. Membranes prepared from low density cells after 48 hours of culture with LPS indicated an increase in intensity of these higher molecular weight phosphoproteins (figure 26, panel B). Phosphorylation of these proteins is probably a reflection of activation of the B cells by LPS. LPS has been shown to activate protein kinase C (Chen *et al.*, 1986) which phosphorylates proteins on serine and threonine residues.

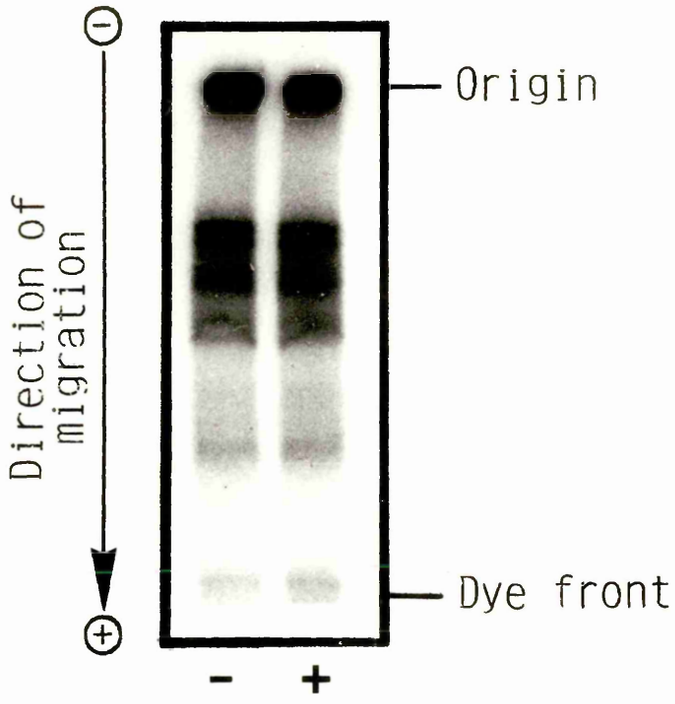
There was no evidence of phosphorylation of the 42 kDa protein in the membranes of cells cultured with LPS for 48 hours in the presence of either purified (track b) or recombinant (track c) IL-4. Lipopolysaccharide is a mitogen for B cells driving them into the cell cycle. Figure 21 illustrates the effect of LPS on B cells by measuring the uptake of [^{3}H]-methyl thymidine. At 24 hours after addition of LPS the cells are just beginning to enter the cell cycle as B cells are activated asynchronously from resting and show a lag in the response to LPS of up to 24 hours (Wetzel & Kettman, 1981). By 48 hours all LPS responsive cells have entered the cell cycle and are proliferating as determined by the uptake of [^{3}H]-methyl thymidine. The IL-4 stimulated phosphorylation of the 42 kDa protein is observed only in membranes prepared from high density resting B cells and "activated" B cells at early stages of the cell cycle but is not present in B cells at later stages in the cell cycle even though there are more IL-4 receptors on these cells. This suggests that the IL-4 induced phosphorylation of the 42 kDa protein is an early event in B cell activation.

4.5. Membrane Phosphoprotein Profiles of a Murine Cell Line

Membranes were prepared from the cell line, P388D$_1$. P388D$_1$ is a mouse macrophage-like cell line, derived from DBA/2 mice, possessing greater than 1100 IL-4 receptors/cell (Ohara & Paul, 1987; Lowenthal *et al.*, 1988). The membrane proteins from this cell line were phosphorylated by γ-[^{32}P]-ATP in the presence and absence of IL-4. The phosphoproteins were

Figure 27 Phosphoprotein Profiles of Membranes from P388D$_1$ Cells

Membranes were prepared from P388D$_1$ cells and subjected to γ-[^{32}p]-ATP in the presence (track b) or the absence (track a) of 10 units rmIL-4. The proteins were separated by SDS-PAGE and the phosphoproteins were visualised by autoradiography.


Origin
Direction of migration
Dye front
-
+

separated by SDS-PAGE and visualised by autoradiography.

Figure 27 shows the phosphoprotein profiles of P388D_1 in the presence and absence of IL-4. The profile shows that there has been incorporation of [^{32}P]-phosphate into several proteins. There does not, however, appear to be any difference in the phosphoprotein profiles in P388D_1 cells with and without IL-4 as a stimulatory ligand. P388D_1 cells are continuously proliferating and, therefore, the need for the 42 kDa phosphorylation event may have been passed. This is consistent with observations in LPS activated cells where 42kDa phosphorylation was shown to be an early event in signal transduction.

The phosphoprotein profiles of P388D_1 are quite different from the profiles observed from BALB/c spleen cells. There are several higher molecular weight proteins in membranes from P388D_1 cells, as was seen in the LPS cultured cells, which are probably a reflection of cell activation.

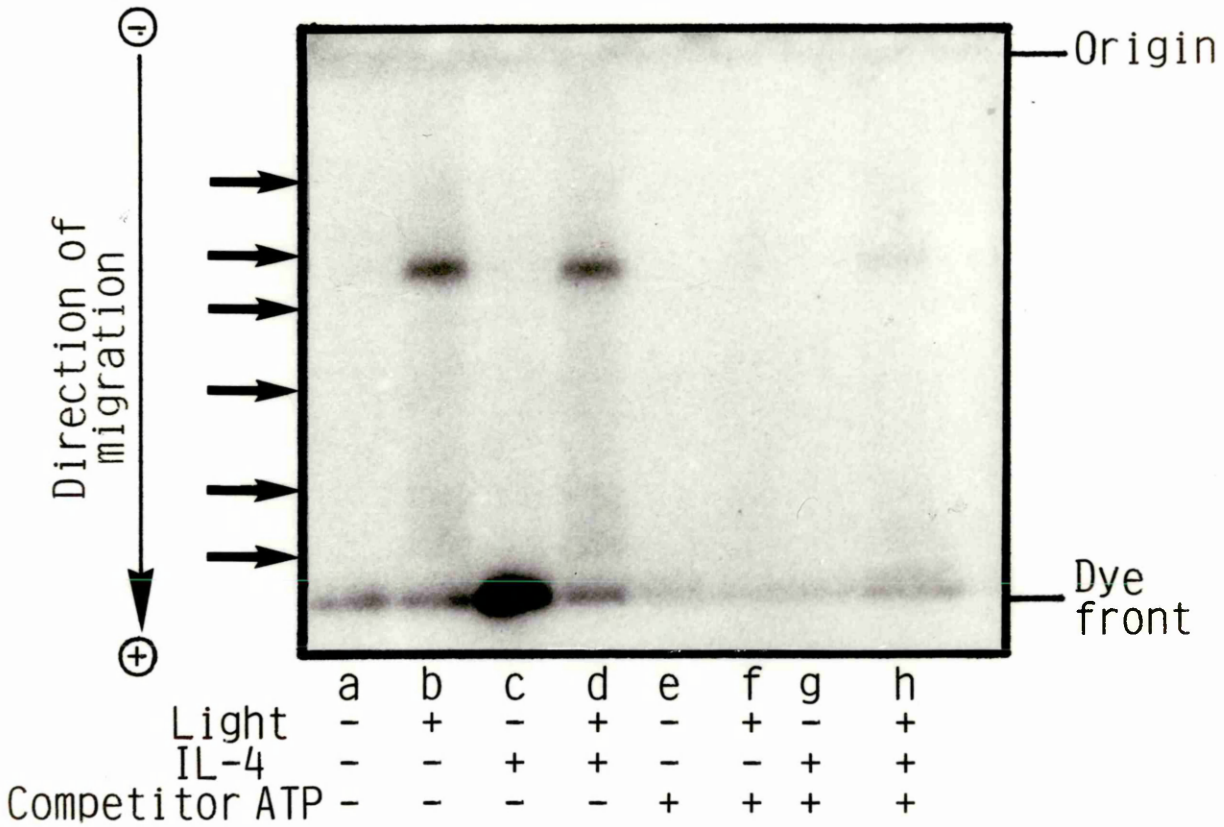
4.6. ATP Binding Proteins in Resting Murine B Lymphocytes

Several receptors for mitogenic growth factors possess protein kinases which are capable of autophosphorylation. The possibility that the 42 kDa protein possessed intrinsic phosphotransferase activity was investigated. Membranes were prepared from high density resting B cells and binding of ATP to the membrane proteins was estimated by using the photoaffinity label 8-azido-γ-[^{32}P]-ATP, which binds to ATP binding proteins irreversibly in the presence of UV light. ATP binding assays were performed as described in materials and methods 2.14. and labelled proteins were separated by gel electrophoresis and visualised by autoradiography.

In the presence of UV light only one major band was seen on the autoradiograph (Figure 28, track b). This ATP binding protein had a molecular weight of 50 kDa averaged over several experiments. In the

Figure 28 ATP Binding Proteins in Resting B Lymphocytes Plasma Membranes

Membrane proteins from resting murine B lymphocytes were incubated with 3 μCi of the photoaffinity label 8-azido-γ-[^{32}P]-ATP (materials and methods section 2.14.). The assay was performed on microscope slides and illuminated with UV light for 60 seconds at room temperature. The reaction was terminated by the addition of SDS-PAGE sample buffer and the proteins separated by SDS-PAGE and visualised by autoradiography. Where indicated rmIL-4 was present at 10 units per assay and unlabelled ATP at a concentration of 50 mM. Low molecular weight markers were run on the gel and visualised by coomassie blue staining. Arrows represent molecular weights of 94, 67, 43, 30, 20.1 and 14.4 kDa.


Origin
Dye front
Direction of migration
⊖
⊕
a b c d e f g h
Light - + - + - + - +
IL-4 - - + + - - + +
Competitor ATP - - - - + + + +

absence of UV light, there were no ATP binding proteins identified (tracks a and c) indicating that binding of 8-azido γ-[^{32}P]-ATP is dependent on the presence of UV light. There were no other ATP binding proteins identified when the experiment was carried out in the presence of IL-4, in fact, there was no change in the labelling of the 50 kDa protein (track d) . IL-4 does not appear to affect the binding of ATP to the 50 kDa protein. In the presence of excess unlabelled ATP the majority of photoaffinity labelling could be abolished indicating that the label was specifically identifying ATP binding proteins (tracks e-h).

With longer exposures of the autoradiograph there was evidence of some labelled proteins of higher molecular weights which were also present in the absence of light. These proteins may be phosphorylated by protein kinases using the 8-azido γ-[^{32}P]-ATP as a substrate as it is the γ-phosphate that is radioactive. This could have been overcome using 8-azido-α-[^{32}P]-ATP which could not have been used as a substrate for phosphorylation.

The only major ATP binding protein in high density resting B lymphocytes identified from this experiment was a protein with a molecular weight of 50 kDa. The phosphoprotein identified in membranes from resting B cells in the presence of IL-4 was 42 kDa. The molecular weights of both proteins have been determined over several experiments and the difference is consistently observed. Membrane proteins phosphorylated by γ-[^{32}P]-ATP have been run on SDS gels parallel to 8-azido γ-[^{32}P]-ATP labelled proteins and the proteins have different mobilities (chapter 5, figure 38). Other evidence for the proteins being different is that phosphorylation of the 42 kDa protein is enhanced in the presence of IL-4 whereas IL-4 does not appear to affect the labelling of the 50 kDa protein. This suggests that the 42 kDa phosphoprotein is not a protein kinase itself but is a protein kinase substrate.

4.7. Effect of Interleukin 4 on Membrane Protein Phosphorylation in Resting Human Tonsillar B Lymphocytes

Membrane phosphoproteins from human tonsillar B cells were analysed to determine if murine and human B cells had similar protein kinase mediated signal transduction pathways for IL-4. Membranes were prepared from high density resting B cells isolated from human tonsils (section 2.3.2. & 2.3.3.) and phosphorylated using γ-[^{32}P]-ATP. The membrane proteins were separated by SDS-PAGE and visualised by autoradiography (figure 29).

Membrane proteins were phosphorylated in the presence (tracks b & d) and absence (tracks a & c) of rhIL-4 (100 units/assay). Although several phosphoproteins are evident there are no apparent differences in the profiles in the presence of IL-4. Figure 29, panel B represents gels which have been soaked in 1M KOH for 90 minutes at 55^{o}C. This procedure hydrolyses aliphatic but not aromatic phosphate ester bonds and therefore the alkali resistant phosphoproteins indicate the presence of phosphotyrosine (Cooper and Hunter., 1982). This gives a good preliminary estimate of the phosphotyrosine containing proteins, but is not unequivocal since some serine and threonine phosphoproteins may be alkali resistant. Treatment of the gels with alkali has greatly reduced the number of phosphoproteins detectable but again no differences can be observed between proteins phosphorylated in the presence and absence of IL-4. This suggests that the binding of IL-4 to its complementary receptor in the human system does not involve the phosphorylation of a 42 kDa protein. It must of course be noted that the murine and human B lymphocytes come from different anatomical sources, spleen and tonsils, respectively.

Figure 29 Membrane Phosphoprotein Profiles of Human Tonsillar B Lymphocytes

Membrane from high density resting human tonsillar B lymphocytes were subjected to *in vitro* phosphorylation as described in materials and methods 2.12. IL-4 where indicated was present at 100 units per assay of rhIL-4. The assays were performed in the presence of 30 mM $MgCl_2$ or 5 mM $MnCl_2$ as indicated on the gel. The proteins were separated by SDS gel electrophoresis and phosphoproteins visualised by autoradiography (panel A). An identical gel was run and soaked in alkali at 55°C for 90 minutes prior to autoradiography (panel B). Molecular weight markers are indicated on the gel.

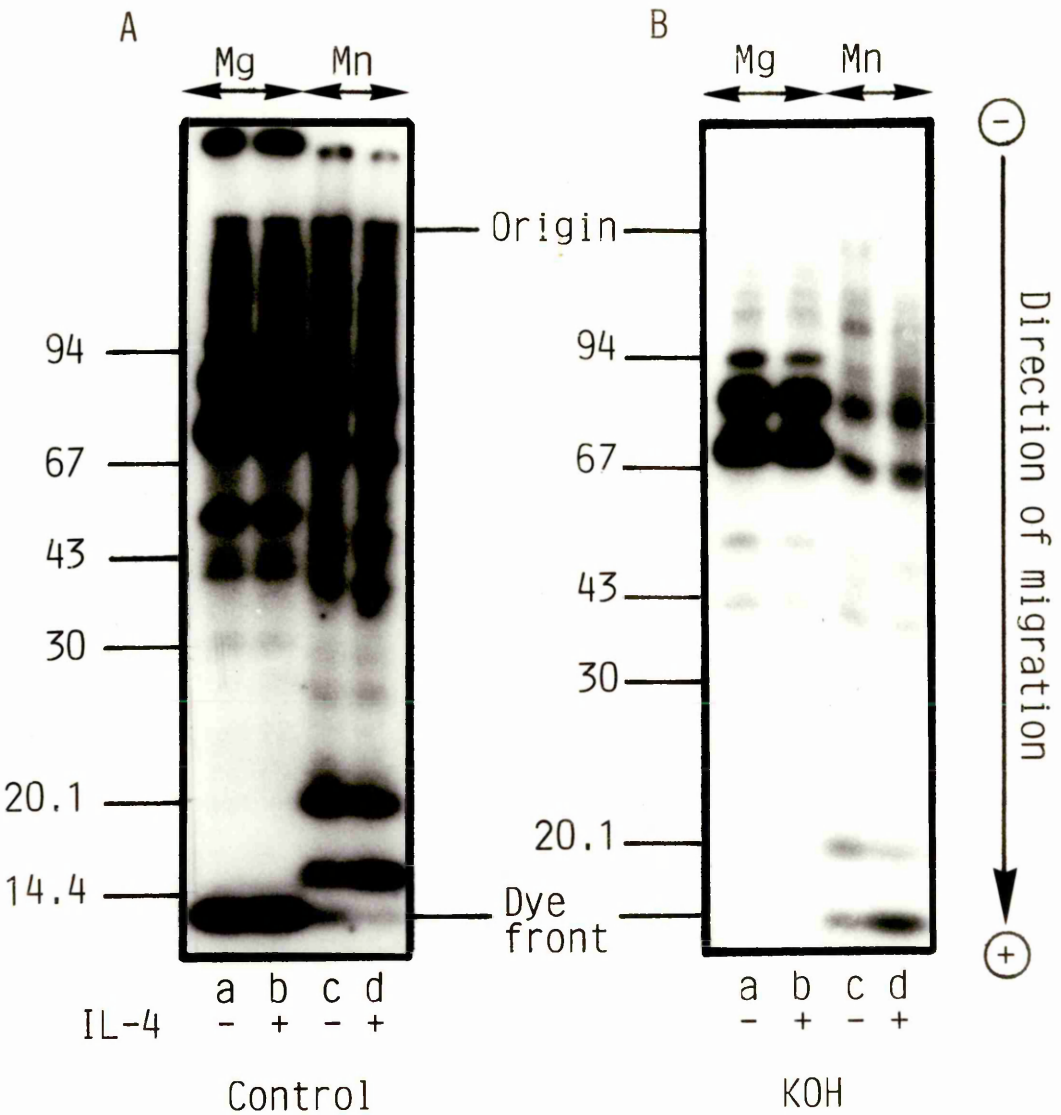
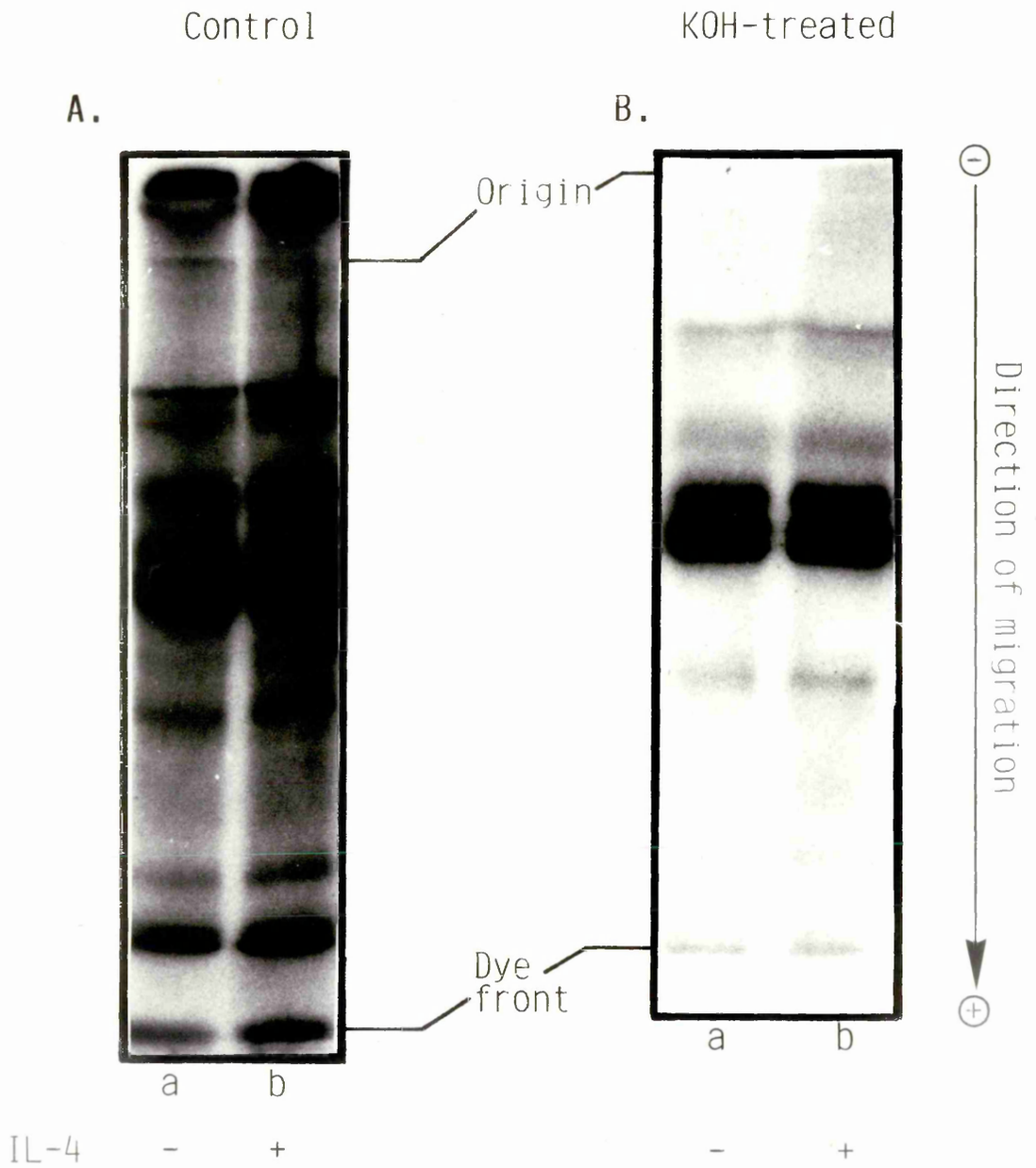

A
Mg
Mn
Origin
94
67
43
30
20.1
14.4
Dye
front
a b c d
IL-4 - + - +
Control
B
Mg
Mn
94
67
43
30
20.1
a b c d
- + - +
KOH
Direction of migration

Figure 30 Membrane Phosphoprotein Profiles of Human B Cells from Peripheral Blood

Membranes were prepared from high density resting B cells isolated from peripheral blood(section 2.3.2. & 2.3.3.). The membrane proteins were incubated with γ-[^{32}P]-ATP in the presence (track a) or absence (track b) of 100 units of rhIL-4. The phosphoproteins were separated by SDS-PAGE and visualised by autoradiography (panel A). An identical gel was run and treated with 1M KOH at 55^{o}C for 90 minutes prior to autoradiography (panel B). This experiment was performed in conjunction with Miss A. Wood in the laboratory.


Control
KOH-treated
A.
B.
Origin
Dye front
Direction of migration
a
b
a
b
IL-4
-
+
-
+

4.8. Membrane Phosphoprotein Profiles of Resting Human B Lymphocytes Isolated from Peripheral Blood

As a comparison to tonsillar B cells, membranes were also prepared from human B cells isolated from peripheral blood. These membranes were exposed to γ-[^{32}P]-ATP in the presence or absence of IL-4 and the phosphoproteins separated by gel electrophoresis and visualised by autoradiography (figure 30).

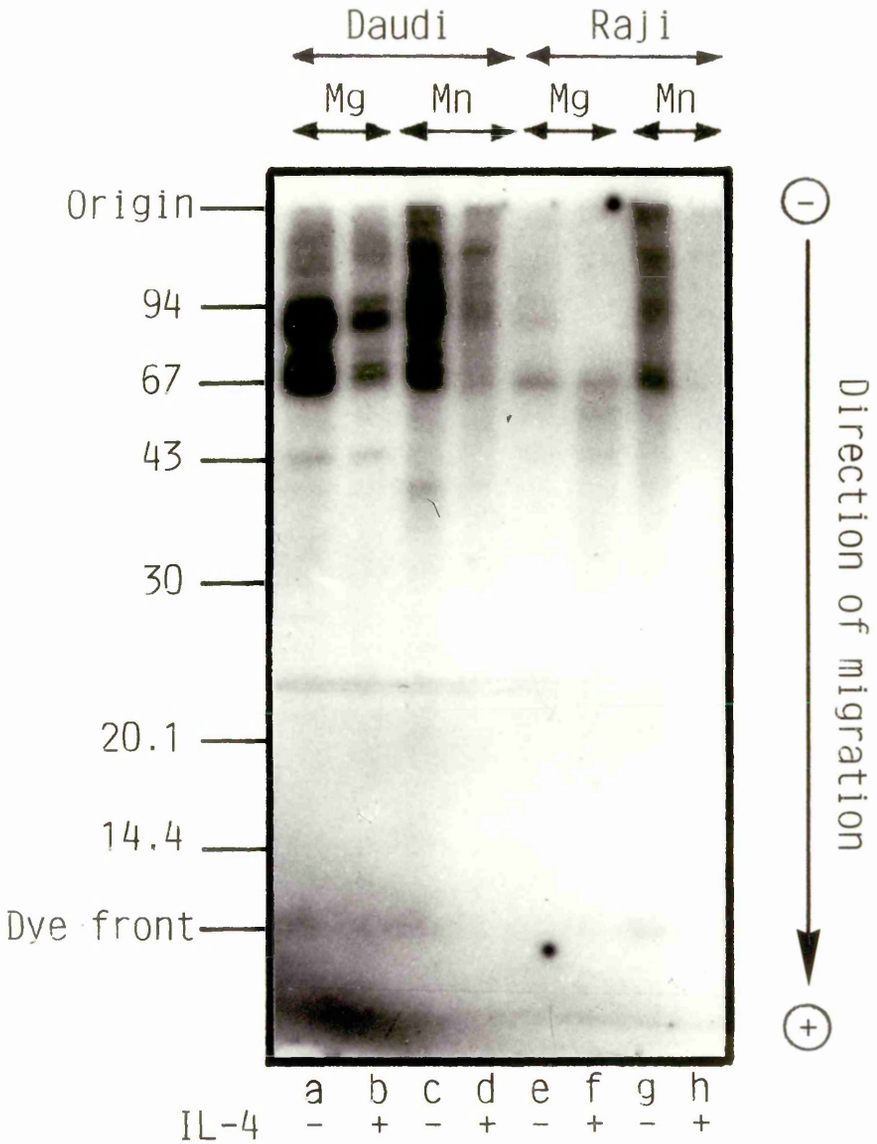
Figure 30 illustrates that, again, although several phosphoproteins were evident there was no difference in the profiles when IL-4 was present in the kinase assay. Panel B represents the equivalent gel treated with KOH and this shows that the profiles in the presence and absence of IL-4 are similar. This experiment substantiates the finding in human tonsillar B cells that the signals transduced when IL-4 binds to its receptor are different in the mouse and human system.

4.9. Membrane Phosphoproteins in Human B cell lines

Membranes were prepared from cells of two human B lymphoma cell lines, Daudi and Raji. Both cell lines have been reported to express high numbers of IL-4 receptors (Park *et al.*, 1987b). The phosphoprotein profiles from both cell lines are complex, exhibiting a large number of phosphoproteins, particularly at higher molecular weights (figure 31). Phosphorylation of these proteins is possibly due to the activity of protein kinase C as these cells are continuously proliferating. There does not appear to be any differences in the phosphoprotein profiles of samples treated with (track a) or without (track b) IL-4. Gels treated with KOH (panel B) indicate a reduction in the the number of phosphoproteins but no differences are observed between membranes phosphorylated in the presence or absence of IL-4. This again illustrates the absence of enhanced phosphorylation of the 42 kDa protein in human cells and in continuously proliferating cells.

Figure 31 Membrane Phosphoprotein Profiles of Human B Cell Lines

Membranes prepared from Daudi and Raji cells were subjected to *in vitro* phosphorylation in the presence or absence of 100 units per assay of rhIL-4 (described in materials & methods 2.12.). *In vitro* phosphorylation was performed in the presence of either 30 mM $MgCl_2$ or 5 mM $MnCl_2$ as indicated on the gel. Molecular weight markers are indicated on the gel.


Daudi
Raji
Mg
Mn
Mg
Mn
Origin
94
67
43
30
20.1
14.4
Dye front
a b c d e f g h
IL-4 − + − + − + − +
Direction of migration

4.10. Discussion

The work detailed in this chapter suggests that the binding of both purified and recombinant IL-4 to isolated murine B cell membranes enhances the phosphorylation of a protein with a molecular weight of 42 kDa. It is thought that this protein is identical to that previously described (Justement *et al.*, 1986). The phosphorylation of this protein appears to be restricted to membranes prepared from resting B cells and B cells that are in early stages of activation. Phosphorylation of the protein was not observed in membranes from B cells which had entered the cell cycle due to stimulation with LPS for 48 hours or in cell lines which were continuously proliferating even though these cells possess 5 to 10 times more IL-4 receptors per cell than resting cells (Park *et al.*, 1987a; Ohara & Paul, 1987). This may mean that phosphorylation of the 42 kDa protein is an early event in B cell activation and B cells that are in the cell cycle do not require this event. The signals transduced by LPS to activate the B cell may be through a different pathway which by-passes phosphorylation of the 42 kDa protein and the later action of IL-4 does not require it. Indeed LPS has been reported to activate protein kinase C whereas in the mouse the activation of PKC has been shown not to be involved in the signals transduced by IL-4 (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986). Alternatively the 42 kDa protein may already be phosphorylated and is therefore unable to be labelled with γ-[^{32}P]-ATP.

IL-4 has several different biological effects on B cells and appears to act differently on resting and activated cells, in one case as a competence factor and in the other as a differentiation factor. IL-4 acts as a competence factor on resting B cells to enhance their response to antigen (Howard *et al.*, 1982). B cells that have been treated with LPS are committed to the cell cycle and therefore do not require a competence signal. The presence of IL-4 in this case promotes immunoglobulin isotype switching to IgG1 and IgE and is, therefore, acting as a differentiation factor (Vitteta *et al.*, 1985; Sideras *et*

al., 1985; Noma *et al.*, 1986; Isakson, 1986). It may be, therefore, that the signal IL-4 gives to the cell when acting as a competence factor differs to the signal given when it is acting as a differentiation factor. In this respect a second set of IL-4 receptors may exist that are of lower affinity than those described previously (Lowenthal *et al.*, 1988; Park *et al.*, 1987a; Ohara & Paul, 1987). The competence signal may be delivered to resting B cells through the high affinity receptors involving the phosphorylation of a 42 kDa membrane protein as part of the signal transduction pathway. The later differentiation signal may be delivered to the B cells through lower affinity receptors whose signal transduction pathway is different or the phosphorylation of the 42kDa protein is by-passed.

No equivalent phosphoprotein was detected in human B cells either from peripheral blood, tonsils or cell lines. This may be due to the different anatomical sources of the B cells in mouse and human ie. spleen compared to tonsils or peripheral blood. The human B cells may be in a pre-activated state having already encountered antigen *in vivo*. The other explanation could be that the signals transduced when IL-4 binds to its receptor are distinct in the mouse and human. Several differences have been observed in the biological activities of IL-4 between species and the lymphokine does not act across the species barrier (Mosmann *et al.*, 1986). The amino acid sequence of mouse and human IL-4 is 50% homologous and therefore, the differences between species may exist in the IL-4 receptors. The published estimated molecular weight for the binding components of the IL-4 receptor are 75 kDa in the mouse (Park *et al.*, 1987a; Ohara & Paul, 1987) and 139 kDa in the human (Park *et al.*, 1987b). A second IL-4 binding component has now been identified in human burkitt lymphoma Jijoye cells with a molecular weight of 70 kDa (Galizzi *et al.*, 1988). Recently the murine IL-4 receptor has been cloned and sequenced and the mature receptor estimated to have a molecular weight of some 140 kDa (Drs D. Cosman and S. Gillis, Immunex Corporation, personal communication to W. Cushley). The molecular

weights for human and murine IL-4 receptors may not therefore be so different although the human receptor still remains to be sequenced. Recent studies with human B cells have shown that the signals transduced when human IL-4 binds to its receptor include phosphoinositide hydrolysis, elevation of cAMP levels and the activation of protein kinase C (Finney *et al.*, 1989). In the mouse, it has been reported that binding of murine IL-4 does not involve any of these events (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986).

The phosphoamino acids in the 42 kDa protein are so far undefined. The use of thin layer electrophoresis and other biochemical procedures has failed to yield any definitive data. The question of the identity of the 42 kDa protein remains unclear although several candidates may be proposed. In several systems ligand binding to plasma membrane associated receptors stimulates an intrinsic tyrosine kinase activity which undergoes autophosphorylation. Experiments with the photoaffinity label 8-azido-γ-[^{32}P]-ATP suggested that the 42 kDa protein did not bind ATP and was therefore unlikely to possess intrinsic phosphotransferase activity. This does not prevent the 42 kDa protein being a substrate for a tyrosine kinase that is associated with the receptor. A tyrosine kinase substrate of between 40 kDa and 45 kDa, now termed 42 kDa, has been found with various mitogens, including EGF, TPA, PDGF, and IGF-1, in a number of cells (Hunter & Cooper 1985). This protein is highly conserved and represents only 0.001% of total cell protein which would account for the low level of phosphate incorporation observed.

A second possibility is that the 42 kDa phosphoprotein represents the low affinity receptor for IgE. One of the biological effects of IL-4 is to increase the expression of the low affinity receptor for IgE, FcRε in the mouse (Hudak *et al.*, 1987) and CD23 in the human (Defrance *et al.*, 1987a). IL-4 also induces the release of the soluble form of this molecule

from the membrane bound form (Bonnefoy *et al.*, 1988). The molecular weight of the membrane form of FcRε/CD23 is 45 kDa and is a single glycosylated polypeptide. This molecule is expressed at low levels in resting B cells which would be consistent with the weak phosphorylation signal observed on the 42 kDa phosphoprotein. Prediction of the primary protein structure of human FcRε/CD23 from the nucleotide sequence reveals that a sequence exists in the cytoplasmic tail region of the molecule that may serve as a potential phosphate acceptor site (Ludin *et al.*, 1987). The arguement against this molecule being a candidate for the 42 kDa phosphoprotein is that it is found in both mouse and human and as yet the phosphorylation of the 42 kDa protein has not been observed in human B cell plasma membranes.

A further possibility is that the the 42 kDa phosphoprotein is the heavy chain of the major histocompatibility complex class 1. This molecule is expressed on the surface of all nucleated cells and erythrocytes and the heavy chain is a 45 kDa glycosylated polypeptide (Benacerraf, 1981). The arguements against this protein being the 42 kDa phosphoprotein are that there has been no reported effect of a direct action of IL-4 on MHC class 1. The MHC class I antigen has also been reported to be phosphorylated on ser 335 by protein kinase C (Shackelford & Trowbridge, 1986) and binding of IL-4 to its receptor on murine resting B cells does not appear to activate PKC or effect its translocation to the plasma membrane (Justement *et al.*, 1986). This does not rule out the possibility that the MHC class 1 could be a substrate for other protein kinases.

Another candidate for the 42 kDa phosphoprotein is a guanine nucleotide binding (G) protein. These are a family of proteins which are similar in structure and are essential to a variety of signal transducing reactions that occur at cell membranes (for a review see Neer and Clapham, 1988). Many cell surface recptors which act through adenylate cyclase have

been shown to do so through two G proteins. One of these G proteins has a stimulatory effect and the other an inhibitory. A second family of receptors which transmit messages by activating a phosphoinositidase that hydrolysis polyphosphoinositides producing two second messengers, DAG and IP_3, has been reported to act via a G protein. G proteins have also been found associated with the insulin receptor tyrosine kinase (O'Brien *et al.*, 1987). The β subunit of the insulin receptor has been shown to possess an intrinsic tyrosine kinase activity (Kasuga *et al.*, 1983). It has now been reported that this kinase activity is capable of phosphorylating the α subunits of Gi and Go when they are in a holomeric form (O'Brien *et al.*, 1987). This suggests that when insulin binds to its receptor signals may be transduced through G proteins which are activated by the phosphorylation of a tyrosine residue on the α subunit.

G proteins are characterised by a heterotrimeric structure consisting of α, β and γ subunits (Neer and Clapham, 1988). The β and γ subunits are 35-36 kDa and 8 kDa respectively and are highly conserved. The α subunit with a molecular weight between 39-52 kDa has some highly conserved regions (regions for the binding and hydrolysis of GTP) and others that are more diverse (the receptor and effector recognition sites). Interaction of GDP-αβγ with an activated receptor results in the binding of GTP to the α subunit. This GTP-α subunit then dissociates from the β and γ and binds to the effector molecule resulting in a modification in the activity of this molecule. Activation is terminated by the endogenous GTPase activity of the α subunit which converts the GTP to GDP (Harnett and Klaus, 1988a). The GDP-α can then recombine with the β and γ subunits and then interaction of GDP-αβγ with the receptor initiates another round of G protein activation.

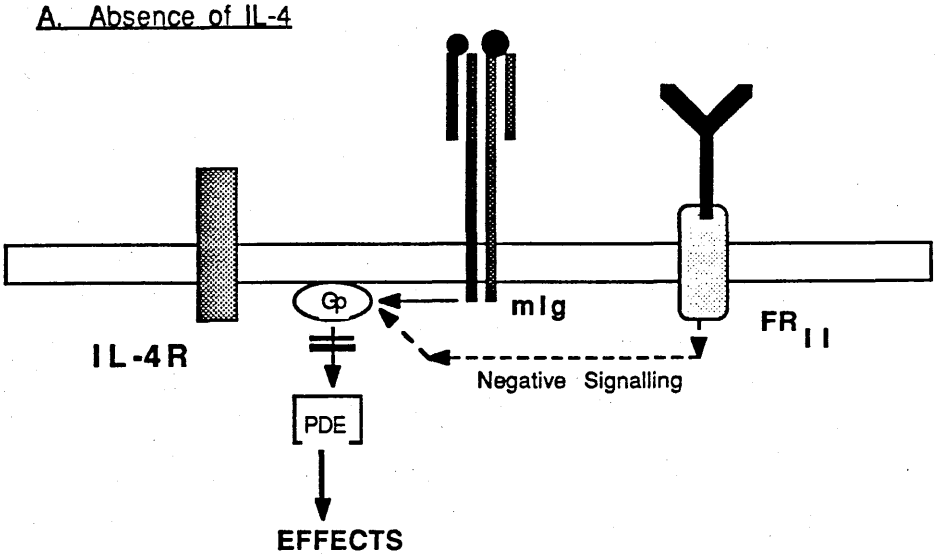
G proteins have been found in a wide variety of systems and there have been reports of the involvement of G proteins in the regulation of B lymphocytes. As discussed in the introduction (section 1.12.) binding of

antigen to sIg results in the generation of the second messengers IP_3 and DAG and it has been shown that both sIgM and sIgD receptors are coupled to the phosphoinositide system by a G protein (Gold *et al.*, 1987; Harnett & Klaus, 1988a). Recently it has been shown that the inhibition of proliferation observed when intact Ig binds to Fc receptors is due to the uncoupling of the antigen receptor from the G protein (Harnett & Klaus, 1988b; Rigley *et al.*, 1989). This negative signal can be overcome by IL-4 allowing B cells to proliferate (O'Garra *et al.*, 1987). It is interesting therefore to speculate that IL-4 may be overcoming the uncoupling of the antigen receptor from the G protein by either activating its own G protein or the uncoupled G protein eg. by phosphorylation of the α subunit. Figure 32 shows a schematic representation of how this might occur.

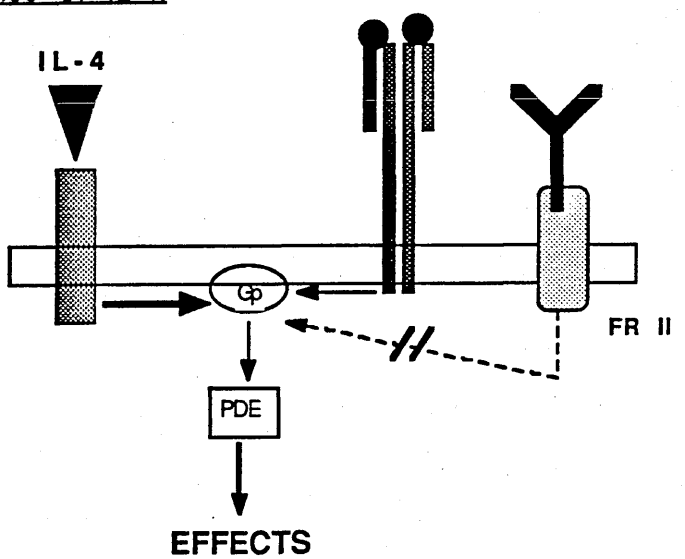
In order to determine if a G protein was involved in the signal transduction of IL-4 on binding to its receptor preliminary experiments were performed assessing the GTPase activity in membrane from resting B cells in the presence and absence of IL-4 (in conjunction with Dr G. Milligan, Department of Biochemistry, University of Glasgow). The results indicated that there was no significant increase in GTPase activity in the presence of IL-4. This does not eliminate the possibility of the involvement of a G protein in IL-4 signalling as only a small change in the GTPase activity may occur in the presence of IL-4 due to the low number of IL-4 receptors on resting cells. There are several other methods for identifying a G protein which include the use of antibodies raised against G proteins either to individual subunits or synthetic peptides. Sensitivity to cholera and pertussis toxin has also been used to identify G proteins. Cholera toxin results in ADP ribosylation of the G protein and therefore if ADP ribosylation and phosphorylation could be correlated on the 42 kDa protein this would provide evidence for the 42 kDa phosphoprotein belonging to the family of G proteins. The observation that the 42 kDa protein is not phosphorylated in human B cells does not eliminate the possibility that it is a G protein as this

Figure 32 Interplay of Three Cell Surface Receptors in Murine B Cells
The signals transduced when antigen binds to its receptor, membrane immunoglobulin, involve the activation of a G proten, Gp. When intact immunoglobulin G binds to FR_{II} this results in a negative signal which prevents the signals transduced by the antigren receptor. This negative signalling is accomplished by the uncoupling of Gp from membrane immunoglobulin (Rigley *et al.*, 1989), panel A. Interleukin 4 has been shown to be capable of overcoming this negative signal (O'Garra *et al.*, 1987). The results from this thesis suggest that part of the signalling when murine IL-4 binds to its receptor involve the phosphorylation of a 42 kDa membrane protein. A candidate for this phosphoprotein is the α subunit of a G protein. It is, therefore, tempting to speculate that IL-4 overcomes the negative effect of FR_{II} on antigen receptor by phosphorylating the antigen receptor associated G protein and thereby activating it and allowing subsequent PtdInsP2 hydrolysis to ocurr, panel B.

A. Absence of IL-4



B. Presence of IL-4.



would be consistent with the existence of distinct second messenger systems activated by IL-4 in the two species.

The final possibility is that the 42 kDa phosphoprotein represents a unique component of the murine IL-4 receptor which is distinct from the 75 kDa protein, which has been shown to bind IL-4, and the sequenced 140 kDa protein.

5. RESULTS.

INDUCTION OF A NOVEL PLASMA MEMBRANE PHOSPHOPROTEIN FOLLOWING CULTURE OF RESTING B CELLS WITH INTERLEUKIN 4

CHAPTER 5

INDUCTION OF A NOVEL PLASMA MEMBRANE PHOSPHOPROTEIN FOLLOWING CULTURE OF RESTING B CELLS WITH IL-4.

5.1. Membrane Phosphoprotein Profiles from Murine B Cells Cultured with LPS or IL-4

The data of chapter 4 suggest that exposure of membranes from high density resting murine B lymphocytes to IL-4 in the presence of γ-[^{32}P]-ATP promotes the phosphorylation of a 42 kDa protein. Phosphorylation of this protein could only be detected in membranes from resting murine B cells and B cells in early stages of activation but not in cycling cells or cell lines, even though these latter cells possess more IL-4 receptors per cell (Ohara and Paul, 1987). Culturing B cells with IL-4 has been shown to increase the expression of the IL-4 receptor (Ohara & Paul, 1987). B cells that have been cultured with IL-4 have a greater number of receptors per cell, but the cells are not in the cell cycle as IL-4 alone is an insufficient stimulus to drive resting B cells into S phase. The membrane phosphoprotein profiles from B cells Cultured in the presence of IL-4 for 24 hours were analysed for enhanced phosphorylation of the 42 kDa protein.

Figure 33 represents the membrane phosphoprotein profiles from freshly prepared resting murine B cells (lanes a & b), low density B cells after culture with LPS for 24 hours (lanes c & d) or 48 hours (lanes e & f) and resting B cells cultured with 100 units/ml rmIL-4 for 24 hours (lanes g & h). *In vitro* phosphorylation assays were performed in the presence and absence of 10 units/assay rmIL-4 (section 2.12.). The phosphoprotein profiles of membranes prepared from freshly isolated high density resting B cells (lanes a and b) indicated that the presence of IL-4 in the kinase assay promotes the phosphorylation of a protein in the 42 kDa molecular weight range; this is consistent with the data of figure 24. The enhanced phosphorylation of the

42 kDa protein was also evident in membranes prepared from low density B cells cultured with LPS for 24 hours (lanes c and d). The phosphoprotein profiles of membranes from low density B cells cultured for 48 hours with LPS (lanes e and f) were faint but on prolonged exposures of the gel there was no evidence of phosphorylation of a 42 kDa protein. Phosphorylation of a 42 kDa protein was evident in the profiles of membranes from B cells that had been cultured with IL-4 for 24 hours (lanes g and h). In this case phosphorylation of the protein did not depend on the presence of IL-4 in the phosphorylation assay as the intensity of the band appeared equivalent in the absence (lane g) or presence (lane h) of IL-4 in the assay. The membrane phosphoprotein profiles from B cells cultured in the presence of IL-4 also showed phosphorylation of a novel protein. This protein represented the major band in the profiles and had a molecular weight in the range 72-76 kDa, as determined over several experiments. The 75 kDa protein was phosphorylated to the same extent whether IL-4 was included in the phosphorylation assay or not.

Figure 34 represents the autoradiographs of two gels, one gel was treated with 1M KOH in phosphate buffer at 55°C for 90 minutes (panel A) while the other was in phosphate buffer alone (panel B) prior to autoradiography. The phosphate ester linkage of phosphotyrosine is more stable to alkaline hydrolysis than those of phosphoserine and phosphothreonine (Cooper and Hunter, 1981b). This means that treatment of SDS gels with alkali allows preliminary estimation of the number of phosphoproteins which contain phosphotyrosine. In this case, treatment of the SDS gel with alkali substantially reduced the number of phosphoproteins visible. The majority of high molecular weight membrane phosphoproteins from LPS cultured cells have been removed which is consistent with the idea that they may be protein kinase C substrates. The 75 kDa protein, however, remains as a major autoradiographic signal in alkali treated gels (panel A, lanes g and h) which suggests that this protein may contain phosphotyrosine.

Figure 33 Membrane Phosphoprotein Profiles of B Lymphocytes After Culture with LPS or IL-4

High density resting B cells were isolated from BALB/c mouse spleen (materials and methods 2.3.1. and 2.3.3.). Membranes were prepared from an aliquot of resting cells (tracks a and b) and the remaining cells were divided into three aliquots and either cultured with 10 μg/ml LPS for 24 hours (tracks c and d) or 48 hours (tracks e and f) or with 100 units/ml IL-4 for 24 hours (tracks g and h) prior to membrane preparation. The cells cultured with LPS were separated on percoll gradients and low density 'activated' cells from the 1.065/1.055 g/ml were used for preparation of membranes. In vitro phosphorylation assays were performed using 10 μCi γ-[^{32}P]-ATP in the presence or absence of 10 units/assay of IL-4 as indicated on the figure. Proteins were separated by SDS-Page and phosphoproteins were visualised by autoradiography.

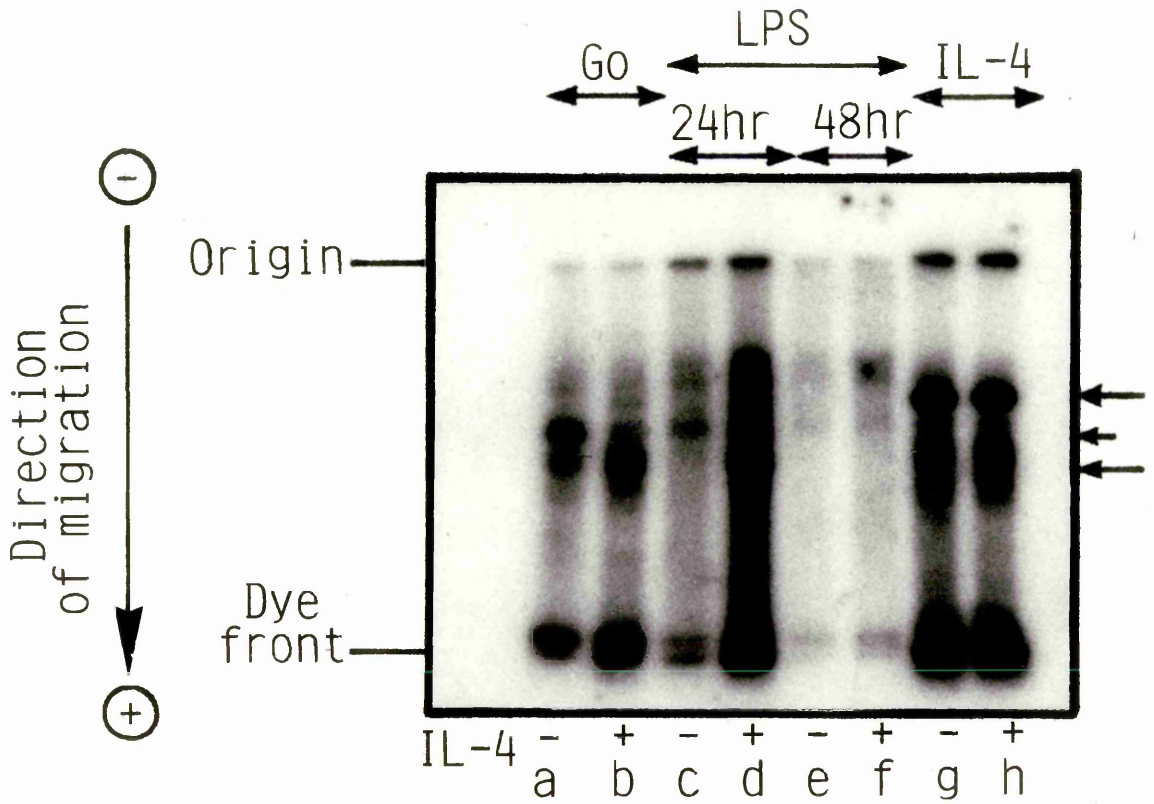
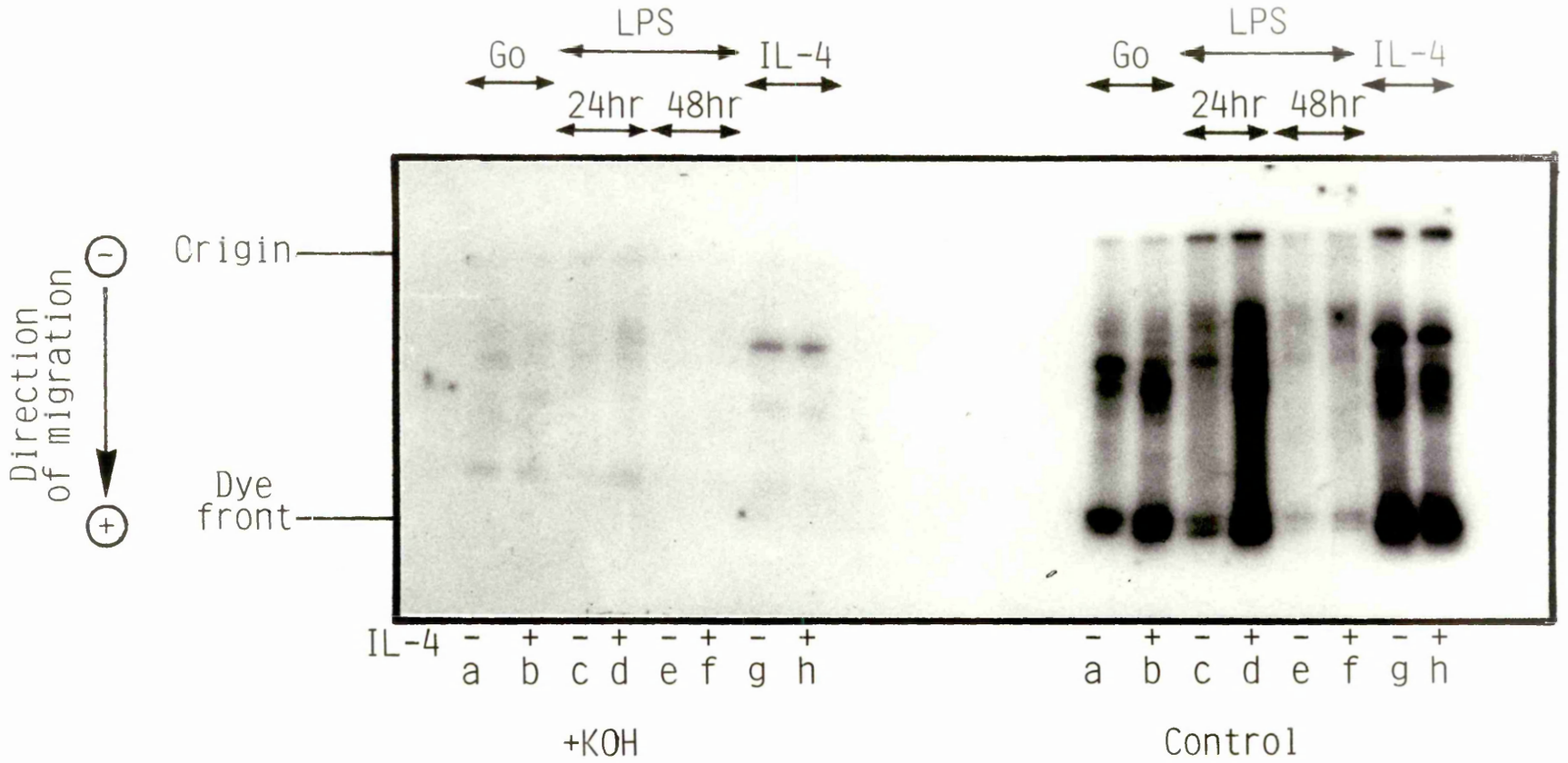

LPS
Go
IL-4
24hr
48hr
Origin
Dye
front
Direction
of migration
IL-4 - + - + - + - +
a b c d e f g h

Figure 34 Total and Alkali Resistant Phosphoprotein Profiles

This experiment was identical to that described in figure 33. Proteins were separated on two identical gels. After electrophoresis one gel was soaked in phosphate buffer (RHS) and the other in phosphate buffer containing 1M KOH (LHS). Both gels were incubated at 55^{o}C for 90 minutes before being dried and autoradiographed (materials and methods 2.18.7.).


LPS
Go
IL-4
24hr
48hr
LPS
Go
IL-4
24hr
48hr
Crigin
Dye
front
Direction
of migration
−
+
IL-4 − + − + − + − +
a b c d e f g h
+KOH
− + − + − + − +
a b c d e f g h
Control

5.2. The Effect of Anti-IL-4 Antibody on the 75 kDa Phosphoprotein

Figure 33 suggests that the phosphorylation of the 75 kDa protein is specific to the presence of IL-4 in the culture. Culturing the B cells with LPS fails to induce the phosphorylation of this protein which may be due to the fact that LPS drives the B cells directly into the cell cycle, by-passing the activated state which IL-4 alone induces. To ensure that the induction of phosphorylation of the 75 kDa protein was a specific effect of culturing the B cells with IL-4 and not an effect induced solely by culturing the cells, particularly in the presence of serum, two experiments were performed (Figure 35). High density murine B cells were cultured in medium alone (tracks a and b), in the presence of 100 units/ml rmIL-4 (tracks c and d) or in the presence of rmIL-4 and 11B11 (tracks e and f) and membrane proteins from these cells were subjected to *in vitro* phosphorylation in the presence or absence of 10 units/assay rmIL-4 (Figure 35). The total and alkali resistant phosphoprotein profiles are represented in panels A and B, respectively. 11B11 is an antibody which neutralises the effects of IL-4 (Ohara *et al.*, 1985). It has been shown to inhibit the biological effects of IL-4, and prevents the binding of IL-4 to its receptor (Park *et al.*, 1987a). Membrane phosphoprotein profiles for cells cultured in the absence of ligand show a major phosphorylated species of 82, 64, 53 and 19 kDa but there is no evidence of the 75 kDa protein either in the presence or absence of IL-4 in the phosphorylation assay. Figure 35, panel B, tracks a and b, indicated that only the 64 kDa phosphoprotein showed any alkali resistence. The membrane phosphoprotein profiles of cells cultured in the presence of IL-4 show similar phosphoproteins to those cultured in the absence of ligand with the exception of a phosphoprotein at 75 kDa, which was not present in the control membranes. The 75 kDa protein was alkali resistant (panel B, tracks c and d) and also a protein of slightly higher mobility corresponding to a molecular weight of 64 kDa. The 75 kDa phosphoprotein was observed in

Figure 35 Effect of Anti-IL-4 Antibody on Membrane Phosphoprotein Profiles

High density resting B cells from BALB/c mouse spleen were cultured (in a total volume of 10 ml) for 24 hours in complete medium in the absence of ligand (tracks a and b) or the presence of 100 units/ml rmIL-4 (tracks c and d) or in the presence of 100 units/ml rmIL-4 and 20% (v/v) supernatant of the 11B11 hybridoma. Membranes were prepared from these cells and they were subjected to *in vitro* phosphorylation with 10 μCi g-[^{32}P]-ATP in the presence and absence of 10 units/assay rmIL-4 as indicated on the figure. The proteins were separated by gel electrophoresis on two identical gels. One gel was soaked in the presence of alkali (panel B) and the other in the absence (panel A) and the gels then autoradiographed. The arrow indicates the 75 kDa phosphoprotein.

Direction of Migration
(-)
(+)
A
IL4
Con IL4 IIBII
IL4 + + + + + +
a b c d e f
-KOH
B
IL4
Con IL4 IIBII
IL4 + + + + + +
a b c d e f
+KOH

both the presence and absence of IL-4 in the *in vitro* assay although in this case there is evidence of a slight enhancement in phosphorylation when IL-4 is included in the *in vitro* phosphorylation assay. Membranes from cells cultured with IL-4 and 11B11 have similar phosphoprotein profiles to membranes from cells cultured in the absence of ligand. This is particularly noticable in the alkali-treated gel (panel B, tracks e and f) where there is no band at 75 kDa. The inclusion of IL-4 in the phosphorylation assay does not appear to influence, either positively or negatively, the phosphorylation of the 75 kDa protein.

The data are consistent with the hypothesis that the appearance of the 75 kDa membrane phosphoprotein is specifically induced by the presence of IL-4 in a 24 hour culture and that culture in serum-containing medium alone is insufficient to induce this event.

5.3. Effect of Interferon-γ on the Appearance of the 75 kDa Phosphoprotein

Culturing murine B cells with IL-4 for 24 hours induces the appearance of an alkali resistant 75 kDa phosphoprotein. Inclusion of the 11B11 antibody in the culture inhibits this effect. 11B11 prevents the binding of IL-4 to its receptor, which suggests, therefore, that binding of IL-4 to its receptor results in induction of the 75 kDa protein. Ifn-γ is an inhibitor of the biological effects of IL-4 (Rabin *et al.*, 1986b; Mond *et al.*, 1986) but it does not bind to the IL-4 receptor or prevent binding of IL-4 itself to the receptor (Park *et al.*, 1987a; Park *et al.*, 1987b). It was, therefore, interesting to study the effects of Ifn-γ on the IL-4 induced appearance of the 75 kDa protein. Resting B cells were cultured in the absence of ligand, in the presence of 100 units/ml rmIL-4 alone, 1000 units/ml rmIfn-γ alone or with 1000 units/ml rmIfn-γ and 100 units/ml rmIL-4 together, *in vitro* phosphorylation assays were then performed on the membranes from these cells in the presence and absence of 10 units/assay rmIL-4 or 100 units/assay

rmIfn-γ as stimulatory ligands. Figure 36 is a representation of such an experiment.

The phosphoprotein profiles of membranes from cells cultured for 24 hours in medium alone (tracks a, b and c) show phosphorylation of proteins at 84, 70, 64, 59, 55, 49, 45 and 22 kDa. There was no evidence of the 75 kDa protein, which is consistent with the data of figure 35. The membrane phosphoprotein profiles from B cells cultured with IL-4 show similar phosphoproteins to control membranes (tracks d, e and f) with the exception of a protein of 55 kDa, which is not phosphorylated in membranes from B cells cultured with IL-4. Two additional phosphoproteins are present in membranes from B cells cultured in the presence of IL-4 and these are a 75 kDa protein and a 30 kDa protein. The membrane phosphoprotein profiles from B cells cultured in the presence of Ifn-γ (tracks g,h and i) appeared to be identical to the profiles from B cells cultured in the presence of IL-4.

Phosphorylation of both the 75 and 30 kDa phosphoproteins were evident in the profiles from B cells cultured in the presence of Ifn-γ. The profiles were not altered by inclusion of either cytokine as stimulatory ligand in the *in vitro* phosphorylation assay. The membrane phosphoprotein profiles of cells cultured in the presence of both IL-4 and Ifn-γ (tracks j, k and l) were very similar to that of control cells (tracks a, b and c). There was no evidence of phosphorylation of the 75 kDa protein or the 30 kDa protein in these tracks. As in the profiles of control cells, a 55 kDa protein was phosphorylated. An equivalent gel to figure 36 was treated with 1 M KOH prior to autoradiography to determine which of the phosphoproteins might contain phosphotyrosine (figure 37). The major alkali resistant phosphoproteins were a 84 kDa prtotein, present in all tracks, and a 75 kDa protein, present only in the profiles from B cells cultured in the presence of either IL-4 (tracks d, e and f) or Ifn-γ (tracks g, h and j). The phosphorylation of the 75 kDa protein is partially obscured by the heavy

Figure 36 Effect of Ifn-γ on Membrane Phosphoprotein Profiles

Resting B cells were cultured for 24 hours in complete medium alone (tracks a, b and c) or with 100 units/ml rmIL-4 (tracks d, e and f) or with 1000 units/ml rmIfn-γ (tracks g, h and i) or with both rmIL-4 (100 units/ml) and rmIfn-γ (1000 units/ml) together (tracks j, k and l). Membranes were prepared from these cells and they were subjected to *in vitro* phosphorylation assays as described in materials and methods 2.12. in the absence of ligand or the presence of 10 units/assay rmIL-4 or 100 units/assay rmIfn-γ (as indicated under each track). The membrane proteins were separated by SDS-PAGE and the phosphoproteins visualised by autoradiography. The molecular weight markers indicated on the figure represent 94, 67, 43, 30, 20.1 and 14.4 kDa.

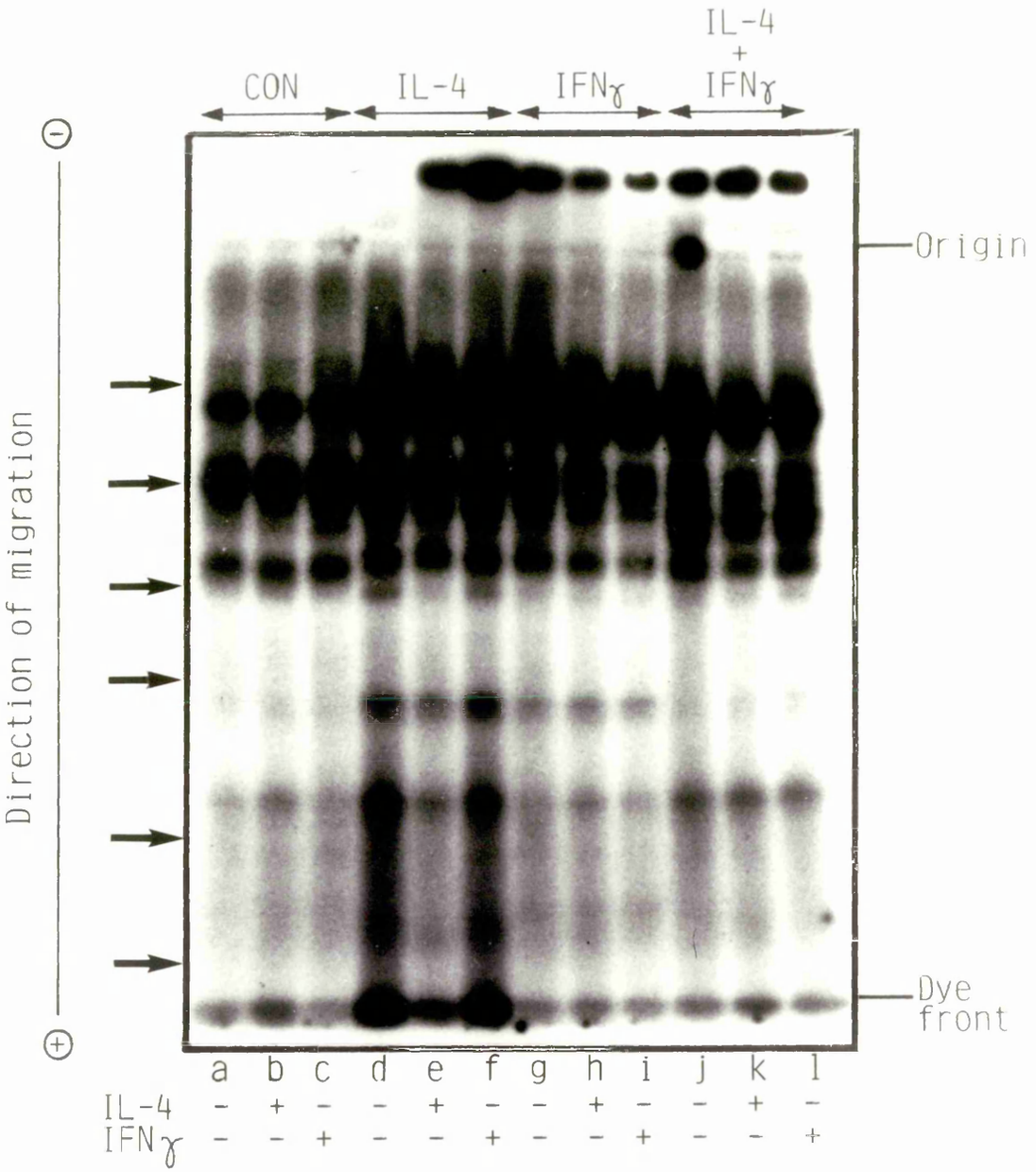
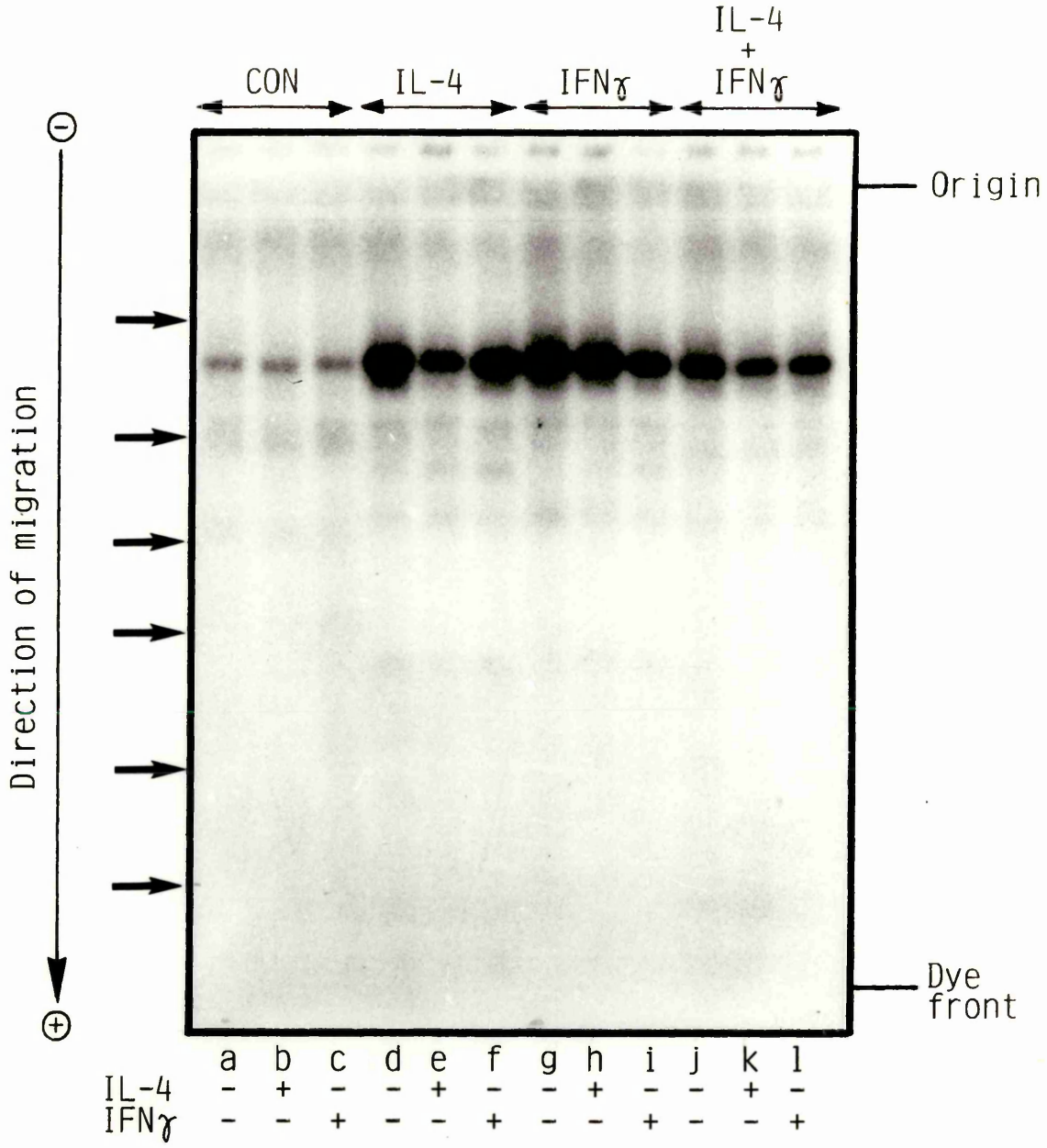

CON
IL-4
IFNγ
IL-4 + IFNγ
Origin
Dye front
Direction of migration
⊖
⊕
a b c d e f g h i j k l
IL-4 - + - - + - - + - - + -
IFNγ - - + - - + - - + - - +

Figure 37 Alkali Resistant Phosphoproteins

The samples for gel electrophoresis were prepared exactly as described in the legend to figure 36. The gel was soaked in 1M KOH at 55°C for 90 minutes prior to autoradiography. The arrows represent molecular weight markers of 94, 67, 43, 30, 20.1 and 14.4 kDa.


IL-4
+
IFNγ
CON
IL-4
IFNγ
Origin
Dye
front
Direction of migration
⊖
⊕
a b c d e f g h i j k l
IL-4 - + - - + - - + - - + -
IFNγ - - + - - + - - + - - +

phosphorylation of the 84 kDa protein and appears as a shadow in figure 37, but comparison with control cells (figure 37, tracks a, b and c) provides support for its presence.

It appears, therefore, that the binding of IL-4 or Ifn-γ to their respective receptors can induce the appearance of a 75 kDa and a 30 kDa phosphoprotein. The fact that both lymphokines together abolish this effect is indicative of their ability to mutually inhibit each others activity.

5.4. Determination of the Ability of the 75 kDa Phosphoprotein to Bind ATP

Membranes were prepared from high density resting murine B cells and from B cells that had been cultured with IL-4 for 24 hours. Aliquots of these membranes were exposed to the photoaffinity label 8-azido-γ-[^{32}P]-ATP. The reaction was initiated by illumination with UV light for one minute (section 2.14.). After termination of the reaction, sample buffer was added and the proteins were separated by SDS-PAGE. The proteins capable of binding ATP were visualised by autoradiography (figure 38). ATP binding proteins were determined in membranes from both freshly prepared resting B cells and B cells cultured with IL-4 in the presence or absence of IL-4 (as indicated on figure 38). To determine the specificity of binding, excess unlabelled ATP was added to equivalent samples (as indicated on figure 38). Aliquots of membrane from both resting B cells and B cells cultured in the presence of IL-4 were also subjected to kinase assays (lanes 1, 6, 7 and 12 from the left). The membrane proteins were incubated with γ-[^{32}P]-ATP in the presence or absence of 10 units/assay rmIL-4 and the proteins separated by gel electrophoresis and visualised by autoradiography (section 2.12).

An ATP binding protein with a molecular weight of approximately 50 kDa was observed in membranes from both resting B cells and B cells that had been cultured with IL-4. The intensity of labelling of this protein was

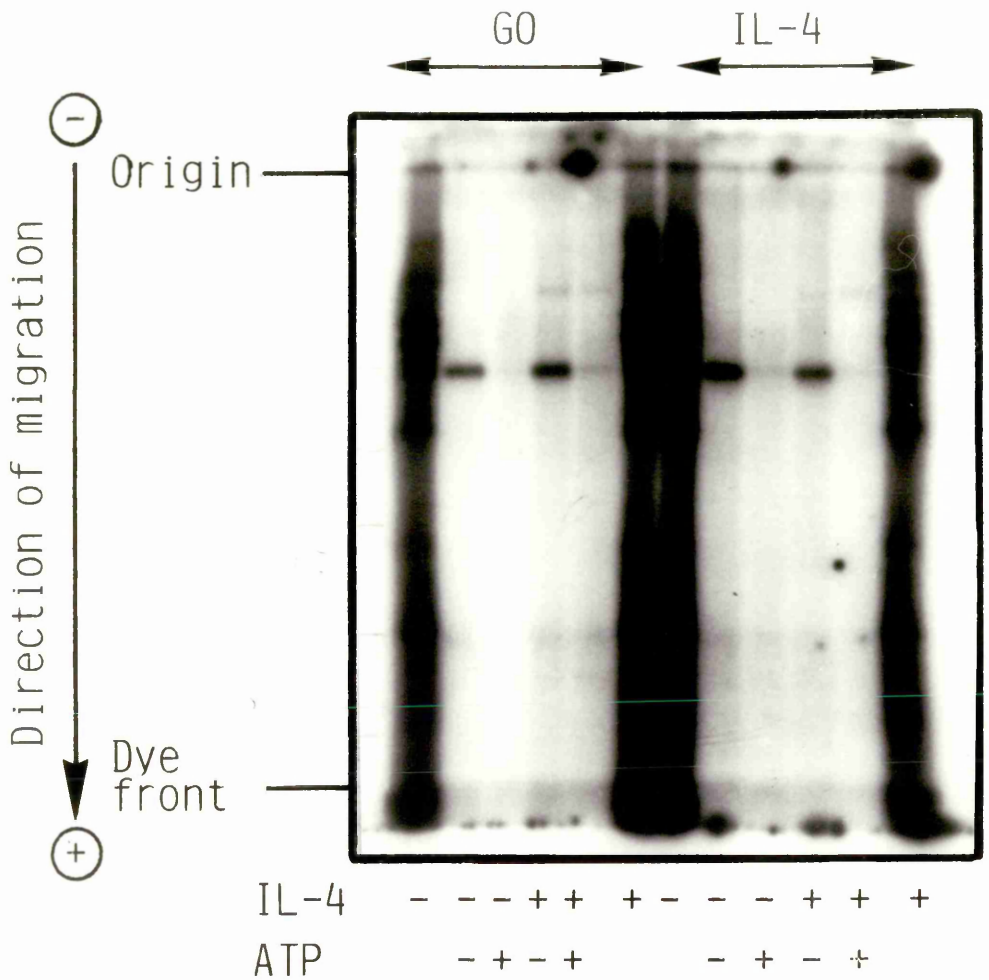
similar in the presence and absence of IL-4 in the assay, and the majority of binding of the labelled ATP was abolished in the presence of unlabelled ATP. This protein is likely to be identical to that observed in resting murine B cell membranes (chapter 4, figure 28).

There was also evidence of another ATP binding protein of a lower intensity on the autoradiograph. This protein was only evident when IL-4 was present in the assay and had a molecular weight of 78 kDa. The 78 kDa ATP binding protein was found in membranes from resting B cells and B cells that had been cultured with IL-4. The presence of unlabelled ATP reduced the binding of labelled ATP to the protein but did not appear to totally abolish it. It may be that the higher affinity or more abundant 50 kDa protein is binding the cold ATP, thereby permitting continued labelling of the lower affinity 78 kDa protein. Comparison of the membrane phosphoprotein profiles from B cells cultured in the presence of IL-4 shows that the 75 kDa phosphoprotein identified in figures 33 and 35 has a slightly higher mobility to the 78 kDa ATP binding protein. It is most tempting to speculate that these two proteins may be identical and that the observed difference in molecular weight is due to the binding of ATP. However, the arguement against the two proteins being identical is that the 78 kDa ATP binding protein is also observed in membranes from high density resting B cells and the 75 kDa phosphoprotein cannot be detected in these membranes. It may be that in resting membranes the 78 kDa protein can bind ATP, but the substrate phosphorylation site or protein is not available until culture with IL-4. More probably, the 75 kDa protein may be present in low amounts in membranes from resting cells and although the assay for the detection of ATP binding proteins is sensitive enough to detect these low amounts, the assay for the detection of protein kinase substrates may have a higher threshold which results in an apparently negative result.

The data from figure 38 suggest that two B lymphocyte plasma

Figure 38 ATP Binding Proteins in Membranes from Resting B Cells and B Cells Cultured with IL-4

Membranes were prepared from resting murine B cells and B cells cultured for 24 hours with 100 units/ml rmIL-4. Membrane proteins were incubated with 3 μCi of the photoaffinity label 8-azido-γ-[^{32}P]-ATP (materials and methods section 2.14.). The assay was performed on microscope slides and illuminated with UV light for 60 seconds at room temperature. The reaction was terminated by the addition of SDS-PAGE sample buffer and the proteins separated by SDS-PAGE and visualised by autoradiography. Where indicated rmIL-4 was present at 10 units per assay and unlabelled ATP at a concentration of 50 mM. Membrane proteins were also subjected to *in vitro* phosphorylation (2.12) in the presence or absence of 10 units/assay rmIL-4. The proteins were separated on the same gel as the ATP binding proteins (lane 1, 6,7 and 12 from the left).


GO
IL-4
Origin
Direction of migration
Dye front
IL-4 - - - + + + - - - + + +
ATP - + - + - + - +

membrane proteins are capable of binding ATP, a 50 kDa protein and a 78 kDa protein. The 50 kDa ATP binding protein has an equivalent molecular weight to a phosphoprotein observed in membranes from resting B cells, and the level of labelling of this protein does not appear to be affected by the presence or absence of IL-4 as a stimulatory ligand. The 78 kDa ATP binding protein may be equivalent to the 75 kDa phosphoprotein uniquely observed in B cells cultured in the presence of IL-4. If these proteins are indeed equivalent it may be that IL-4 activates a putative protein tyrosine kinase which undergoes an autophosphorylation reaction.

5.5. Synthetic Peptide Substrates for Murine B Cell Membrane Kinases

A large number of protein kinases have now been identified and an important factor has been the striking sequence similarity in their catalytic domains. There are several short, highly conserved sequences (Hunter, 1987). At the N terminal the sequence Gly - X - Gly - X - Gly. followed by Lys, 15-20 residues downstream are conserved and both form part of the binding site. At a distance of between 80-180 residues there is a sixty amino acid region containing the conserved sequences Arg - Asp - Leu., Asp - Phe-Gly , and Ala - Pro - Glu (Hunter and Cooper, 1985).

Cyclic AMP-dependent protein kinases recognise substrates partly on the basis of their primary amino acid sequence. The serine residue phosphorylated by the kinase usually has one or two basic amino acids on the N-terminal side (Hunter, 1982). Tyrosine protein kinases are characterised by a lysine or arginine seven residues to the N-terminal side of the phosphorylated tyrosine and one or more acidic residue around the tyrosine (Hunter, 1982; Hunter, 1987).

These conserved sequences in the catalytic site of protein kinases allow short peptide sequences to be constructed that can act as exogenous

substrates for protein kinases. Short peptides, RR-src, [Val^5]-angiotensin II and phosphate acceptor peptide (figure 4), were used as exogenous substrates in *in vitro* phosphorylation assays to determine if the protein kinases in resting B cell membranes were capable of phosphorylation of exogenous substrates (section 2.15.). The phosphorylation assays were carried out in the presence or absence of 3 units/assay rmIL-4. The reactions were terminated by the addition of TCA at 0, 1, 2, 5 and 10 minutes, and three 20 µl aliquots were spotted onto 2 x 2cm squaes of Whatman P81 paper. This paper has negative charges and, therefore, the positively charged amino acids at the N terminal of the peptides can bind to the paper. The papers were washed throughly and then counted by liquid scintillation.

The results from this experiment were plotted on graphs of assay time versus incorporation of phosphate into the peptides (figure 39). In each case the incorporation in the absence of peptide, the presence of peptide and the presence of peptide plus IL-4 are shown. In each case it can be seen that there are counts bound to the paper even in the absence of peptide. This can either be due to phosphorylated membrane proteins binding to the papers or perhaps free [^{32}P]-phosphate or γ-[^{32}P]-ATP. In this system it is a range of protein kinases that are present in the membrane rather than a single purified kinase and, secondly, membrane proteins that act as protein kinase substrates will compete with the peptide substrates for phosphorylation. Panel A represents the incorporation of phosphate into RR-src. In the presence of RR-src there is an increase in phosphate incorporation above that measured with membrane alone. This suggests that phosphorylation of RR-src is being detected. The presence of IL-4 enhances incorporation of [^{32}P] into RR-src, showing an increase in phosphate incorporation up to 5 minutes and then a slight decrease. Panel B represents the incorporation of phosphate into [Val^5]-Angiotensin II. The data showed a similar general trend to that reported for RR-src. In the presence of the peptide there was phosphorylation above that detected with membrane alone. The addition of

Figure 39 Incorporation of [^{32}P]-Phosphate into Peptide Subtrates

Membranes were prepared from resting B lymphocytes and *in vitro* phosphorylation assays were performed using RRsrc (2mM), [Val^{5}]-angiotensin II (2mM) and Phosphate acceptor peptide (0.2mM) as substrates. The assays were performed as described in materials and methods 2.15. and were terminated with TCA at 0, 1, 2, 5 and 10 minutes. Figure 39 shows the results from these experiments

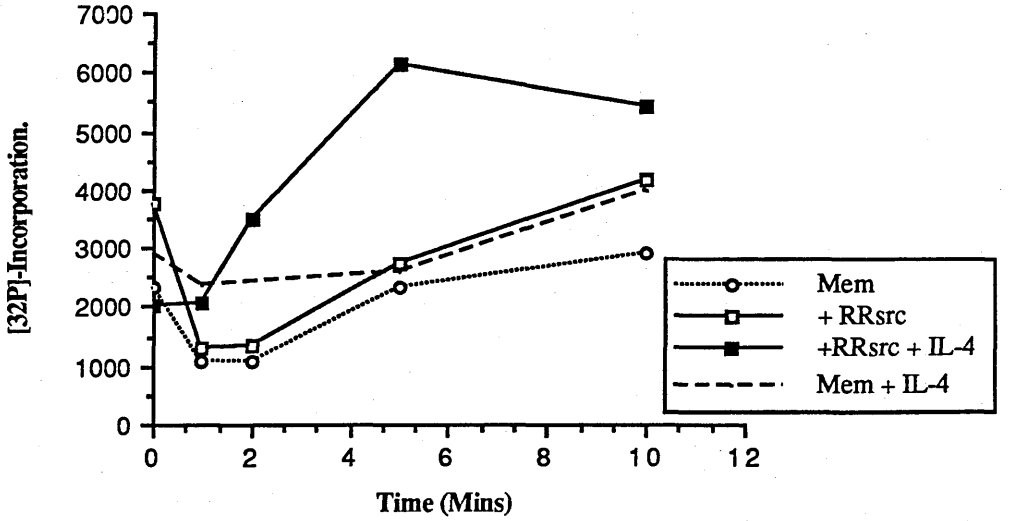
Panel A RRsrc

Panel B Ang

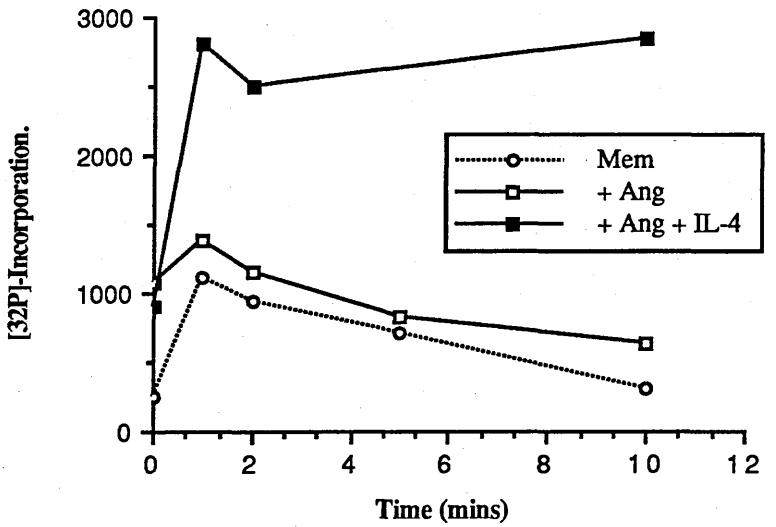
Panel C PAP

In each case the assay was performed with B cell plasma membrane alone, B cell plasma membrane in the presence of 3 units/assay rmIL-4, B cell plasma membrane and peptide and B cell plasma membrane, peptide and 3 units/assay rmIL-4 as indicated on each graph.

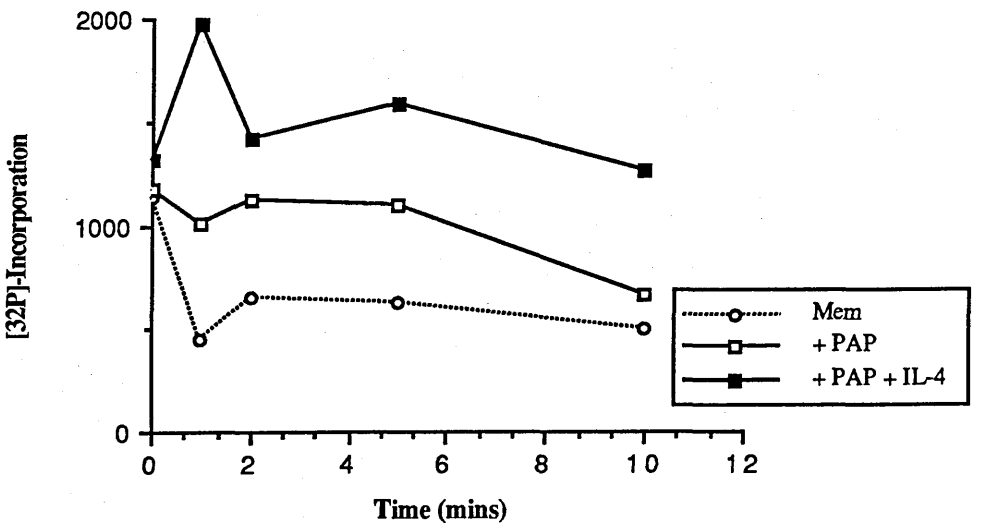
A. RR-src.



B. Angiotensin II



C. Phosphate Acceptor Peptide.



IL-4 to the assay resulted in a sharp increase in incorporation suggesting that the presence of IL-4 is activating one or more kinases. Panel C represents phosphate incorporation into phosphate acceptor peptide (PAP). In the absence of peptide some background phosphorylation can be detected. When PAP is added there is a higher level of incorporation, suggesting that PAP is acting as a protein kinase substrate. As previously, in the presence of IL-4, there is an increase in [^{32}P]-phosphate incorporation over time which is above the level in the absence of IL-4. In each case, to determine whether IL-4 was enhancing peptide phosphorylation or background membrane protein phosphorylation, assays were performed with membrane and IL-4 alone in the absence of peptide. The increase in incorporation was not significant, suggesting that a specific increase in phosphorylation of the peptide was being detected in the presence of IL-4.

It appears that all three peptides can act as exogenous substrates for B cell plasma membrane protein kinases and that the activity of these protein kinases is increased when IL-4 is present. It cannot, however, be determined which kinase is responsible for the phosphorylation as several kinases may exist in the plasma membrane. The fact that both tyrosine (RR-src and Angiotensin) and serine (PAP) residues were phosphorylated suggests that at least two different classes of protein kinase are present.

5.6. The Affinity for ATP of B Lymphocyte Plasma Membrane Protein Kinase(s)

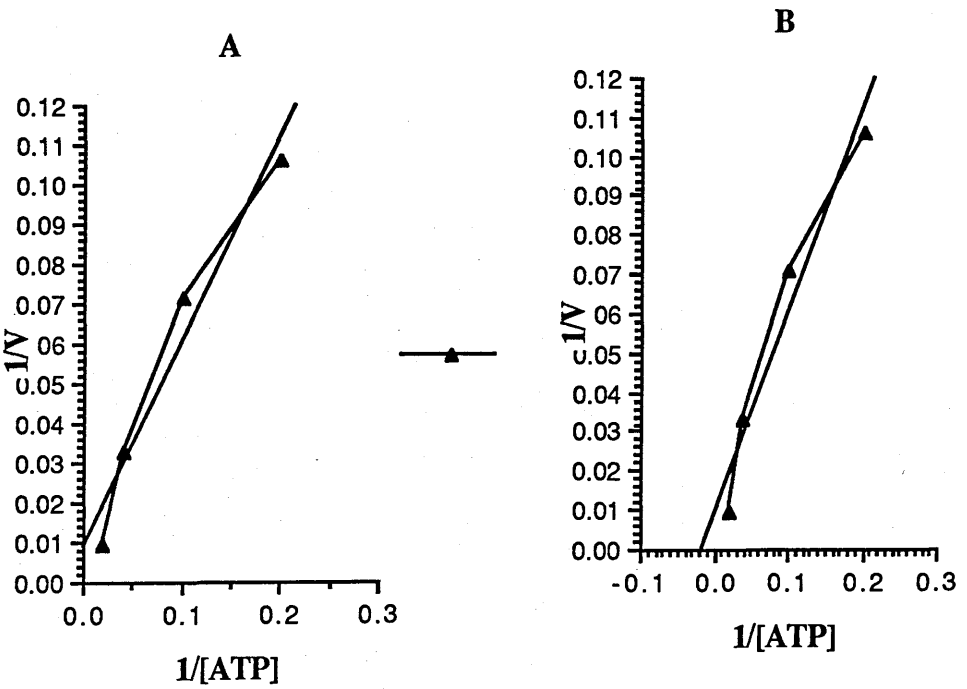
The use of peptides as exogenous substrates allows the determination of some kinetic parameters. In this case, definitive values cannot be obtained as it is not a purified kinase preparation which is being assayed, but rather an effect of multiple protein kinases. The affinity of the plasma membrane protein kinases from resting B cells and B cells cultured in the presence of 100 units/ml rmIL-4 for ATP was determined in the presence and absence of IL-4 as a stimulatory ligand. The assays illustrated were

performed in the presence of 30 mM $MgCl_2$. *In vitro* kinase assays were performed using RR-src as an exogenous substrate at various concentrations of γ-[^{32}P]-ATP (0, 2.5, 5, 10, 25 and 50 μM). Three 20 μl aliquots from the terminated reaction were spotted onto P81 paper which was washed and counted. The results obtained were converted into pmoles/min/mg and graphs of 1/V (pmoles/min/mg) against 1/[ATP] were plotted (figures 40 and 41), and are summarised in table 7.

The K_m for ATP and V_{max} parameters for IL-4-stimulated and control assays were estimated from the appropriate intercepts (Table 7). The data suggest that, in the case of resting cell membranes, addition of IL-4 as stimulatory ligand causes a small decrease in V_{max}, and also altered the K_m value for ATP from 50 μM to 25 μM. A similar trend of data was obtained when assays were performed using membranes prepared from B lymphocytes which had been cultured for 24hrs with IL-4. In this instance, the V_{max} value was again slightly reduced (by an almost identical magnitude to that observed with control membranes), but the K_m value was altered some 25 fold, from 100 μM to 4 μM. Thus, addition of IL-4 to B cell membranes has very little effect upon the rate of phosphorylation of the peptide substrate (consistently in the range of 100 pmoles / minute/ mg membrane protein), but had the effect of increasing the affinity of IL-4-driven protein kinases for ATP in the presence of Mg^{2+} as cation. This effect is most striking in the membranes which have been prepared from cells cultured with the lymphokine for 24hrs. This would not be inconsistent with a long-term mode outcome of IL-4 action being increased levels of expression of its receptor and associated protein kinases. Indeed, given the low level of receptor present at resting cell membranes, it may be that the data obtained from the membranes derived from cells cultured overnight with the cytokine are a more accurate reflection of the kinetic parameters relating to IL-4-driven phosphorylation of the RR-src peptide substrate. Overall, however, the data are consistent with the hypothesis that IL-4 does influence the activities of

Figure 40 Phosphorylation of RRsrc by Resting B Cell Plasma Membrane Protein Kinases

In vitro phosphorylation assays were performed with plasma membrane from resting B cells in the presence of RRsrc (2mM) as described in section 2.15. The assays were performed in the presence or absence of 3 units/assay rmIL-4 at γ-[^{32}P]-ATP concentrations of 0, 2.5, 5, 10, 25 and 50 μM. The reactions were terminated by the addition of TCA and three 20 μl aliquots were spotted onto P81 paper. The papers were washed and counted and the counts incorporated into RRsrc determined. The pmoles of [^{32}P]-phosphate/min/mg protein were calculated and graphs of 1/pmoles/min/mg against 1/[γ-[^{32}P]-ATP] (μM^{-1}). The data points were fitted to a simple linear curve, and *x* and *y* axis intercepts extrapolated from this line.


A
B
1/V
1/[ATP]
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.0
0.1
0.2
0.3
-0.1

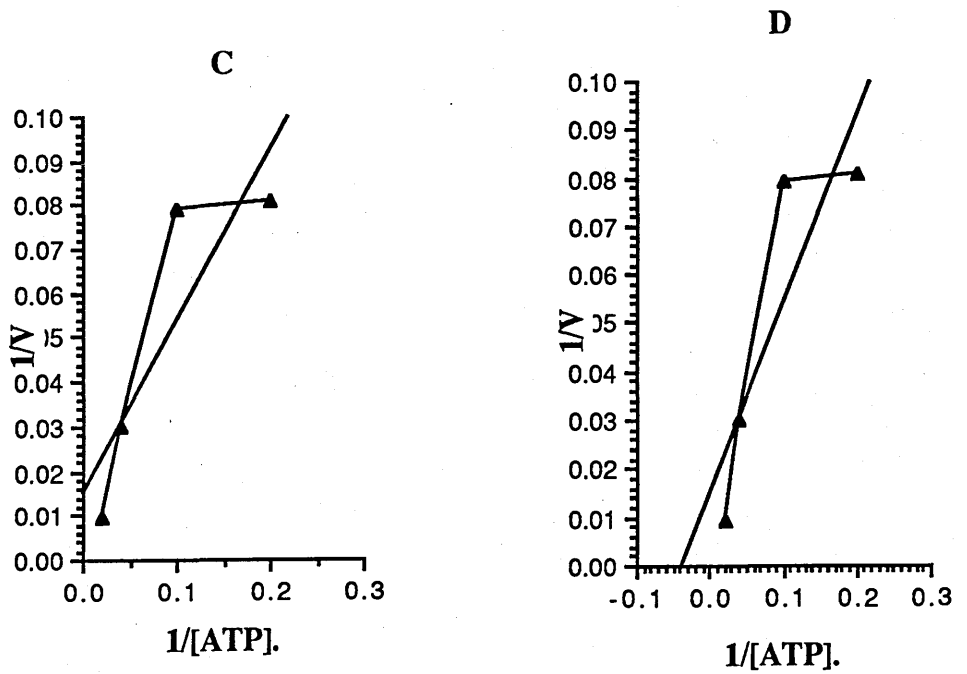
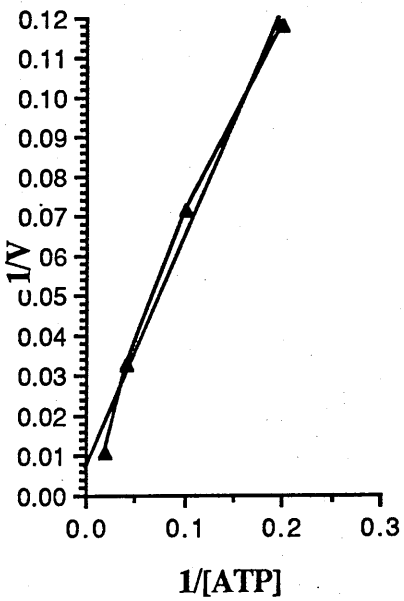

C
D
1/V
1/[ATP].
0.10
0.09
0.08
0.07
0.06
0.04
0.03
0.02
0.01
0.00
0.0
0.1
0.2
0.3
-0.1

Figure 41 Phosphorylation of RRsrc by Plasma Membrane Protein Kinases from B cells cultured with IL-4.

In vitro phosphorylation assays were performed with plasma membrane from B cells culutred overnight with IL-4. Assays were performed in the presence of RRsrc (2mM) as described in section 2.15, andthe data plotted and handled as detailed in the legend to figure 40.

A


0.12
0.11
0.10
0.09
0.08
0.07
1/V 06
0.05
0.04
0.03
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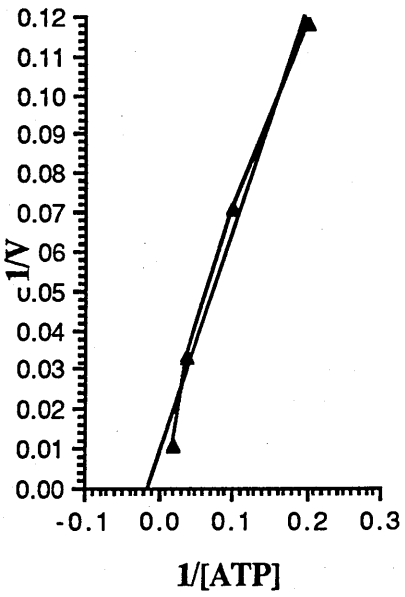
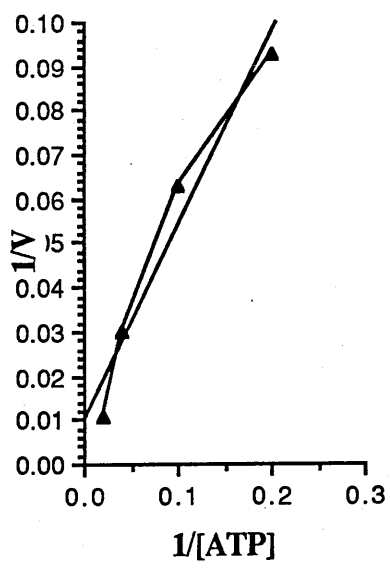
0.01
0.00
0.0
0.1
0.2
0.3
1/[ATP]

B


0.12
0.11
0.10
0.09
0.08
0.07
1/V 06
0.05
0.04
0.03
0.02
0.01
0.00
-0.1
0.0
0.1
0.2
0.3
1/[ATP]

C


0.10
0.09
0.08
0.07
0.06
1/V 05
0.04
0.03
0.02
0.01
0.00
0.0
0.1
0.2
0.3
1/[ATP]

D

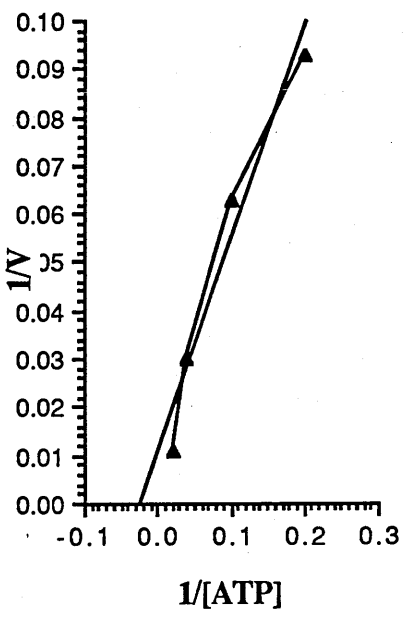

0.10
0.09
0.08
0.07
0.06
1/V 05
0.04
0.03
0.02
0.01
0.00
-0.1
0.0
0.1
0.2
0.3
1/[ATP]

Table 7 Km and V_{max} values for B Lymphocyte Plasma Membrane Protein Kinases

The Km and Vmax values for B lymphocyte plasma membrane kinases were calculated from the intercepts on the appropriate axis from the graphs in figures 40 and 41.

TABLE 7. Km and Vmax Values for B Lymphocyte Plasma Membrane Protein Kinases.

Resting Cells

	Vmax (pmol/min/mg)	Km (μM)
-IL-4	100	50
+IL-4	67	25

Cells after Overnight Culture with IL-4

	Vmax (pmol/min/mg)	Km (μM)
-IL-4	125	100
+IL-4	100	4

protein kinases associated with the B cell plasma membrane, and that it mediates its effect by increasing the affinities of such kinases for the Mg^{2+}-ATP substrate complex. Whether such kinases are part of the IL-4 receptor itself or are associated molecules activiated at a later stage following the initial ligand binding event remains to be clarified.

5.7. Membrane Phosphoprotein Profiles of Human Tonsillar B Cells After Culture

The question as to whether the 75 kDa phosphoprotein was unique to the murine system was answered by performing similar experiments with membranes prepared from B cells isolated from human peripheral blood and tonsils. High density resting B cells were isolated from human tonsils and cultured in medium alone, in the presence of 1000 units/ml rhIL-4 or in the presence of 10 ng/ml PMA and 1 μM calcium ionophore. PMA activates protein kinase C and calcium ionophore increases intracellular calcium mimicing the two second messengers, DAG and IP_3, respectively. Membrane proteins were phosphorylated in the presence of γ-[^{32}P]-ATP, and the resulting phosphoproteins separated by gel electrophoresis and visualised by autoradiography. The membrane phosphoprotein profiles from these cells are shown in figure 42.

The profiles from control cells (figure 42, tracks a-d) are similar whether IL-4 is included as stimulatory ligand in the assay or not. The sole exception is that of an enhancement in phosphorylation of a 72kDa protein when IL-4 is included in the *in vitro* assay. The autoradiographic signal from this phosphoprotein is resistant to alkali (figure 43, tracks a-d). The major band in the membrane phosphoprotein profiles of control cells is at 64 kDa with a faint band below this at 60 kDa, and the phosphorylation of both is independent of the inclusion of IL-4 in the assay. The membrane profiles from cells cultured with IL-4 (figure 42, tracks e-h) and PMA plus calcium

Figure 42 Membrane Phosphoprotein Profiles of Human Tonsil B Lymphocytes After Culture

Resting B cells were isolated from human tonsils and cultured for 24 hours in complete medium (tracks a-d), or with 1000 units/ml rhIL-4 (tracks e-h) or PMA (10 ng/ml) and calcium ionophore (1 μM) (tracks i-l). After culture membranes were prepared from the cells and subjected to *in vitro* phosphorylation with 10 μCi γ-[^{32}P]-ATP in the presence or absence of 100 units/assay rhIL-4. The assay was carried out in the presence of either 30mM $MgCl_2$ (tracks a, b, e, f, i and j) or 5mM $MnCl_2$ (tracks c, d, g, h, k and l). Proteins were separated by gel electrophoresis and phosphoproteins were visualised by autoradiography.

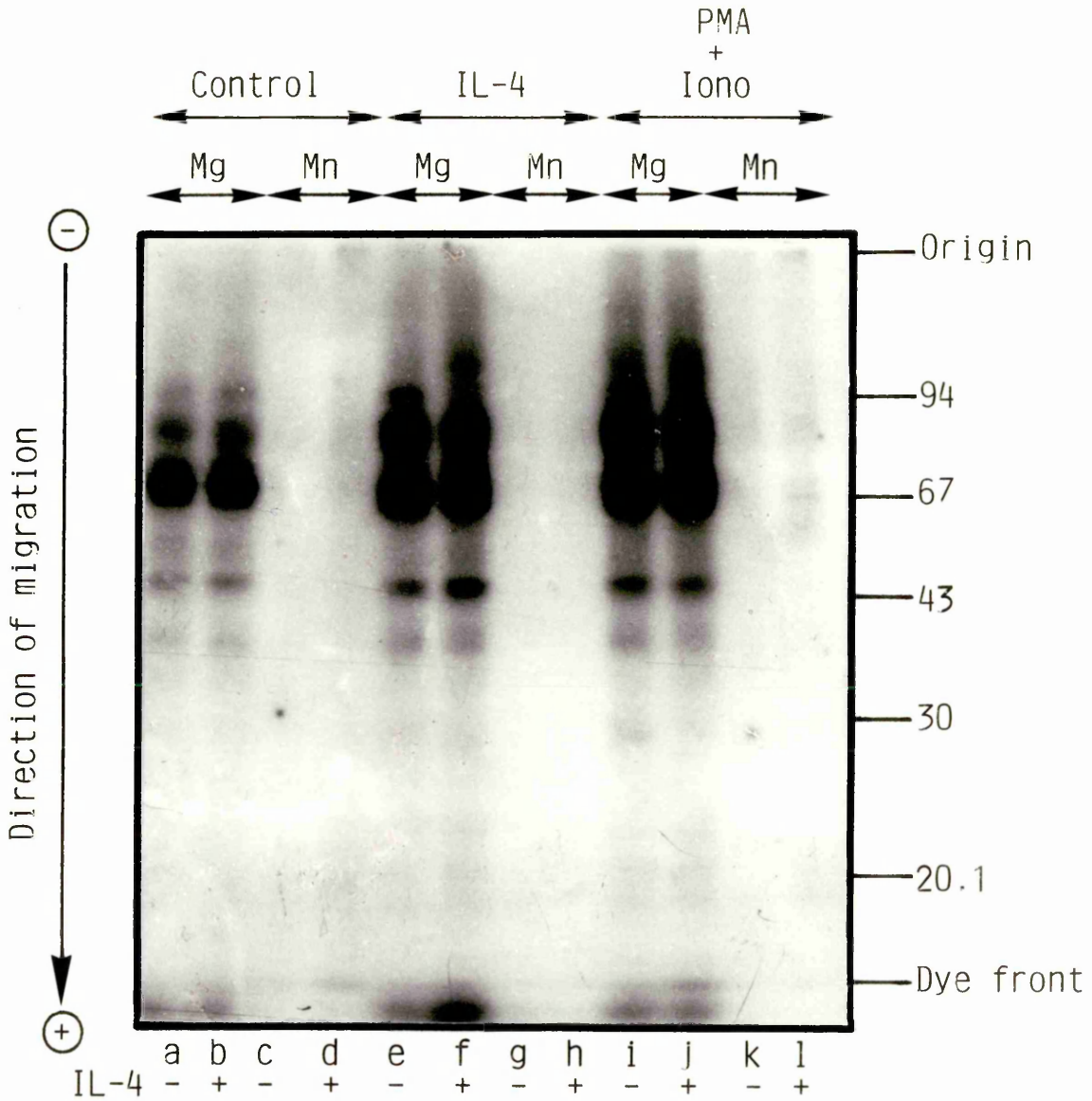
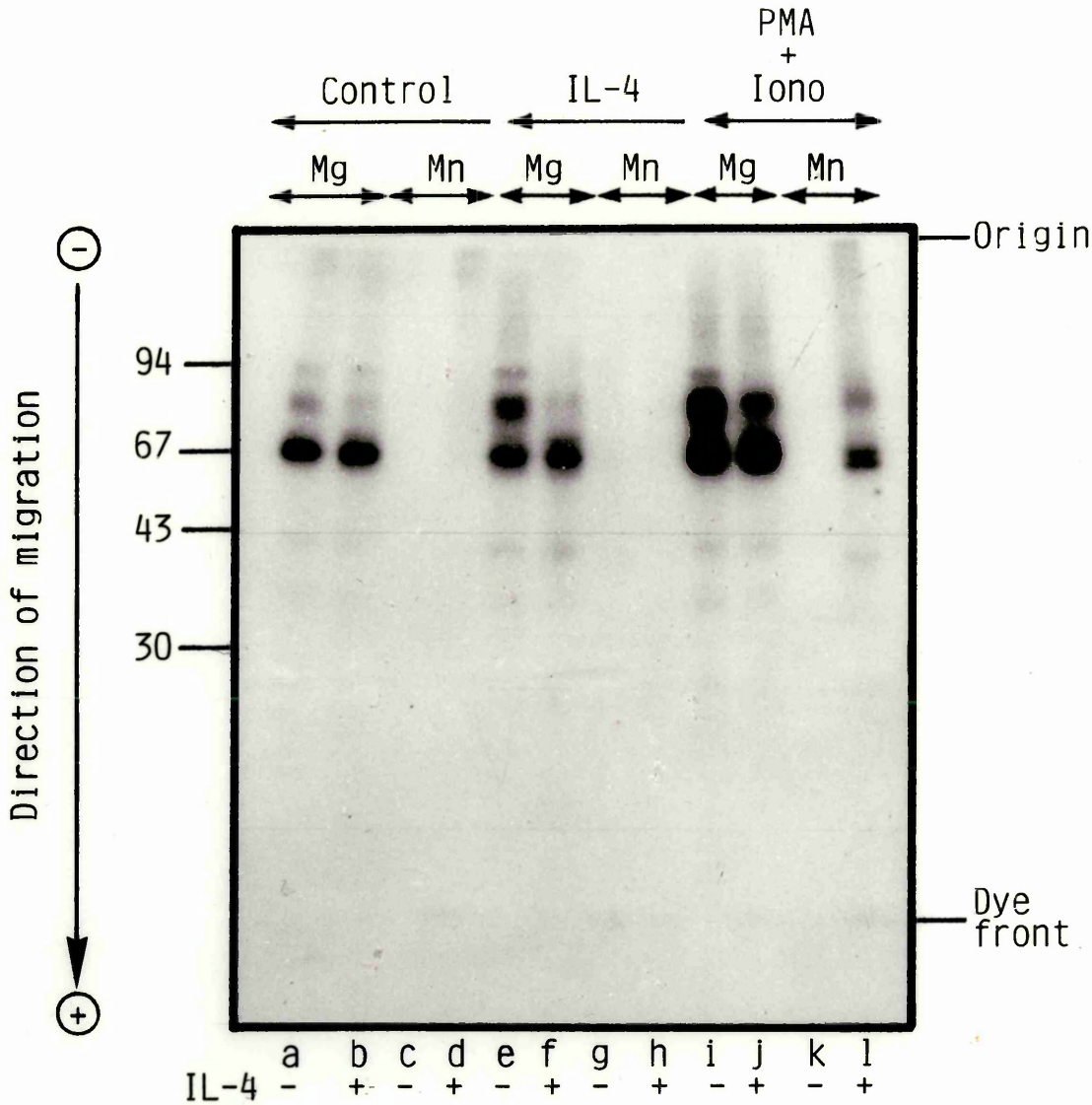

PMA
+
Control
IL-4
Iono
Mg
Mn
Mg
Mn
Mg
Mn
Origin
94
67
43
30
20.1
Dye front
Direction of migration
a b c d e f g h i j k l
IL-4 - + - + - + - + - + - +

Figure 43 Alkali Resistant Membrane Phosphoproteins From Human B Lymphocytes After Culture

This experiment was identical to that described in the legend to figure 39. The gel was treated with 1M KOH at 55°C for 90 minutes prior to autoradiography.


PMA
+
Iono
Control
IL-4
Mg
Mn
Mg
Mn
Mg
Mn
Origin
94
67
43
30
Dye
front
Direction of migration
a b c d e f g h i j k l
IL-4 - + - + - + - + - + - +

ionophore (figure 42, tracks i-l) show an enhancement in phosphorylation of this doublet, with an apparent preference for the lower of the two bands, i.e., the 60 kDa species. From figure 43, the data suggest that only the higher, 64 kDa, band of this doublet is resistant to alkali.

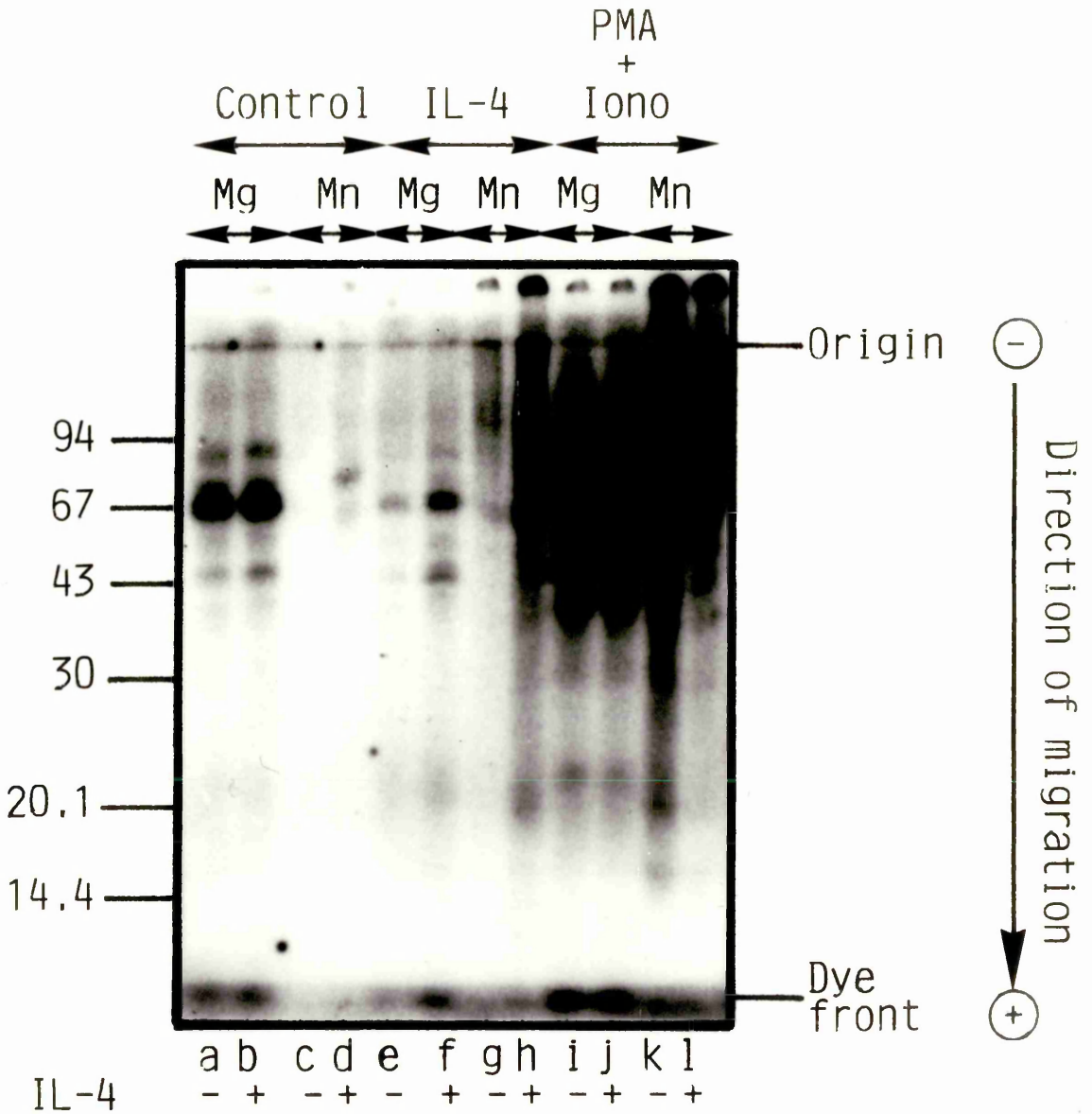
The membrane phosphoprotein profiles from B cells cultured with IL-4 show two distinct changes when IL-4 is included in the phosphorylation assay. Phosphorylation of a protein of 88 kDa disappears when IL-4 is included in the assay (figure 42, tracks e and f). This 88 kDa phosphoprotein was present in the membrane phosphoprotein profiles of control cells whether IL-4 was included in the assay or not, but the effect of culturing the cells with IL-4 then exposing the membranes from these cells to IL-4 as stimulatory ligand results in a loss of this phosphorylation event. Membrane phosphoprotein profiles from B cells cultured with PMA and calcium ionophore also show a disappearence of the 88 kDa band when IL-4 is included in the assay. The 88 kDa protein was alkali resistant (figure 43). Secondly, in the membrane phosphoprotein profiles from cells which have been cultured with IL-4 there is phosphorylation of a protein of apparent molecular weight 72 kDa which is considerably enhanced when IL-4 is included in the assay. In the membrane phosphoprotein profiles from cells that have been cultured with PMA plus calcium ionophore, the 72 kDa protein appears to be present whether IL-4 is included in the assay or not. The 72 kDa protein is also resistant to alkali (figure 43).

5.8. Membrane Phosphoprotein Profiles of B cells from Human Peripheral Blood After Culture

Resting B cells were isolated from human peripheral blood and cultured for 24 hours in medium alone, in the presence of 1000 units/ml rhIL-4 or in the presence of 10 ng/ml PMA and 1 μM calcium ionophore. The membrane proteins from these cells were phosphorylated with γ-[^{32}P]-ATP

Figure 44 Membrane Phosphoprotein Profiles of Human Peripheral Blood B Lymphocytes After Culture

Resting B cells were isolated from human peripheral blood and cultured for 24 hours in complete medium (tracks a-d) or with 1000 units/ml rhIL-4 (tracks e-h) or PMA (10 ng/ml) and calcium ionophore (1 μM) (tracks i-l). After culture membranes were prepared from the cells and subjected to *in vitro* phosphorylation with 10 μCi γ-[^{32}P]-ATP in the presence or absence of 100 units/assay rhIL-4. The assay was carried out in the presence of either 30mM $MgCl_2$ (tracks a, b, e, f, i and j) or 5mM $MnCl_2$ (tracks c, d, g, h, k and l). Proteins were separated by gel electrophoresis and phosphoproteins were visualised by autoradiography.


PMA
+
Control
IL-4
Iono
Mg
Mn
Mg
Mn
Mg
Mn
Origin
94
67
43
30
20.1
14.4
Dye
front
Direction of migration
a b c d e f g h i j k l
IL-4
- + - + - + - + - + - +

in the presence or absence of 100 units/assay rhIL-4 (section 2.12.). The membrane phosphoprotein profiles of B cells from peripheral blood were similar to those isolated from tonsils. Phosphoprotein profiles of membranes from control cells (figure 44, tracks a-d) showed no differences, regardless of whether IL-4 was included in the kinase assay or not. The membrane phosphoprotein profiles of B cells cultured in the presence of IL-4 were faint and, as a result, little information could be deduced from them. The membrane phosphoprotein profiles from B cells cultured with PMA plus calcium ionophore showed an enhancement in phosphorylation of a protein of 72 kDa relative to control cells. The intensity of labelling of this phosphoprotein was independent of the inclusion of IL-4 in the *in vitro* phosphorylation assay.

The membrane phosphoprotein profiles of human B cells suggest that culture with IL-4 or PMA plus calcium ionophore induces the appearance of a 72 kDa phosphoprotein which is alkali resistant. A low level of phosphorylation of this protein is also observed in the membrane profiles from control cells, but only when IL-4 is included in the *in vitro* kinase assay. It is possible that the 72 kDa phosphoprotein observed in membranes from human B cells is similar to the 75 kDa protein found in membranes from murine B cells. This question could be addressed, in the first instance, by two-dimensional gel electrophoretic analyses or by high resolution tryptic phosphopeptide mapping.

5.9. The Requirement for Metal Ions in the Phosphorylation Assay

The hydrolysis of ATP requires the presence of metal ions, usually Mg^{2+} or Mn^{2+}. The metal ion can exist at high concentrations in neutral solutions and acts as a superacid, complexing with ATP to weaken the bond of the leaving group. The literature documents the use of both Mg^{2+} and

Mn^{2+} as divalent cations in phosphorylation assays. Carpenter *et al* (1979) studied the effect of EGF on phosphorylation of A431 membranes by determining the incorporation of [^{32}P]-phosphate into total membrane protein over various concentrations of Mg^{2+} and Mn^{2+}. The requirement for metal ions was obligatory and EGF-stimulated protein phosphorylation was detected at all concentrations of metal ions. The half maximal incorporation of [^{32}P] into protein was achieved at a Mg^{2+} concentration of 50 mM, whereas a lower concentration of Mn^{2+}, 10 mM, was required. A higher incorporation of [^{32}P]-phosphate was obtained with 50 mM Mg^{2+} than 10 mM Mn^{2+}. A tyrosine protein kinase activity in LSTRA cells phosphorylates a protein of molecular weight 58 kDa, p58 (Casnellie *et al.*, 1982). This protein was heavily labelled when either Mn^{2+} or Mg^{2+} were employed as divalent cation cofactor. However, approximately 5 times more radioactivity was incorporated into the 58 kDa protein in the presence of Mn^{2+} than Mg^{2+} when both were used at 10 mM. In phosphorylation reactions with higher initial levels of ATP (28 μM) it was found that the initial velocity of phosphorylation was higher in the presence of $MgCl_2$ than $MnCl_2$, but as the ATP was depleted dephosphorylation was more prominent in the presence of $MgCl_2$. This indicated that the tyrosine kinase either had a lower Km for ATP in the presence of $MnCl_2$ or that $MnCl_2$ was inhibiting an endogenous phosphoprotein phosphatase. A tyrosine kinase in the detergent extract of the LSTRA cell line was assesed for its ability to phosphorylate exogenous peptides and it was determined that the kinase had a five-fold greater activity (V_{max}) in the presence of Mg^{2+} than Mn^{2+} (Casnellie *et al.*, 1982). These experiments indicate that the preferred metal ion is dependent on the kinase under study. The protein kinase activities associated with human B cell plasma membranes were investigated in the presence of $MgCl_2$ or $MnCl_2$ (figures 29, 31, 42, 43 and 44). Phosphorylation assays (section 2.12.) were performed in the presence of 30 mM $MgCl_2$ or 5 mM $MnCl_2$ and the phosphoproteins were separated by SDS-PAGE and visualised by autoradiography. In figure 29 the proteins in tracks a and b were

phosphorylated in the presence of 30 mM $MgCl_2$ and, in tracks c and d, in the presence of 5 mM $MnCl_2$. The phosphoprotein profiles are similar in all four tracks but greater [^{32}P]-phosphate incorporation has been achieved in the presence of $MgCl_2$ with the exception of one protein of 19 kDa which is only present with $MnCl_2$ as cation. The phosphoprotein profiles in figure 44 also indicate a greater [^{32}P]-phosphate incorporation in the presence of $MgCl_2$. In figures 42 and 43 in the presence of $MnCl_2$ no phosphoproteins can be detected at this autoradiographic exposure. The phosphoprotein profiles from Daudi and Raji cells (figure 31) appear to be approximately equivalent in the presence of $MgCl_2$ or $MnCl_2$. Comparison of the membrane phosphoprotein profiles in the presence of Mg^{2+} or Mn^{2+} indicated that Mg^{2+} was the preferred metal ion of kinases associated with human B cell membranes at the concentrations used.

5.10. DISCUSSION

The main finding from the experiments described in this chapter is that culturing resting murine and human B cells with recombinant IL-4 induces the phosphorylation of a 75 kDa membrane protein. This protein is resistant to alkali suggesting that it may be phosphorylated on tyrosine residues. Ligand binding appears to be essential for the appearance of the phosphoprotein. Culture in complete, serum-containing medium in the absence of IL-4 did not induce this event. Furthermore, the inclusion of 11B11, anti-IL-4 antibody, in the culture prevented phosphorylation of the 75 kDa protein presumably by binding to IL-4 and hence preventing the binding of the cytokine to its receptor. Interestingly, the 75 kDa phosphoprotein appears in the profiles of membranes phosphorylated in the presence or absence of IL-4 in the assay. An explanation for this may come from the fact that IL-4 binds to its receptor with high affinity and, even after washing the cells, some IL-4 from the culture medium may still remain bound to the plasma membrane receptor (Ohara and Paul, 1987; Park *et al.*, 1987a; Park

et al., 1987b).

Phosphorylation of the 75 kDa protein is not evident in the membrane profiles from resting cells even when IL-4 is included in the phosphorylation assay. This observation is consistent with the presence of IL-4 in the culture medium being essential for the appearance of this phosphoprotein. This suggests that culturing the B cells with IL-4 either induces the appearance of the 75 kDa protein at the plasma membrane or causes a conformational change of an existing 75 kDa membrane protein which allows the protein to be phosphorylated on subsequent exposure to γ-[^{32}P]-ATP. Perhaps the requirement for culture in the presence of IL-4 reflects the capacity of the lymphokine to up-regulate the expression of its own receptor (Ohara and Paul, 1987). In contradiction to this, culturing murine B cells with LPS has also been shown to increase expression of IL-4 receptors and yet there is no there is no evidence of a 75 kDa protein in the membrane phosphoprotein profiles from such cells. There are, however, several differences in the action of IL-4 and LPS on B cells. Culturing the B cells with LPS drives them into the cell cycle and to antibody secretion (Andersson *et al.*, 1972) whereas culturing with IL-4 alone induces only an activated state and the cells do not enter the cell cycle (Howard *et al.*, 1982). Regarding the signalling mechanisms of the two ligands, LPS has been shown to activate protein kinase C (Chen *et al.*, 1986) whereas other reports indicate that the signals transduced when IL-4 binds to its receptor do not include the activation of protein kinase C, at least in the murine system (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986). Further evidence for their different actions on B cells comes from their regulation of immunoglobulin isotype switching. IL-4 promotes the secretion of IgG1 from LPS treated B cells (Vitetta *et al.*, 1985; Sideras *et al.*, 1985; Noma *et al.*, 1986; Isakson, 1986). Although both ligands are required to enhance IgG1 secretion, the two ligands act independently of each (Snapper *et al.*, 1987). When resting murine B cells were cultured with IL-4 for 48 hours then washed and LPS

added, IgG1 secretion was enhanced. This suggests, then, that LPS is acting on B cells by a different mechanism from IL-4 and that this LPS-activated pathway does not involve the phosphorylation of a 75 kDa protein, even when IL-4 is included in the phosphorylation assay.

In contrast to the murine system, the 75 kDa phosphoprotein is present in membrane profiles from human B cells cultured with PMA plus calcium ionophore and its phosphorylation is independent of IL-4 in the *in vitro* phosphorylation assay. PMA and calcium ionophore mimic DAG and IP_3, respectively, to activate protein kinase C. Recent reports on human B cells indicated that binding of IL-4 to its receptor induced an increase in IP_3 followed by an increase in calcium (Finney *et al.*, 1989). It was suggested that the signals transduced by IL-4 in the human system may involve $PtdInsP_2$ hydrolysis. This signal alone is insufficient to induce CD23 expression on B cells, an event which also requires an increase in cAMP (Finney, *et.al.*, 1989). The results suggest that the activation of the B cells by PMA plus calcium ionophore, which induce protein kinase C, result in phosphorylation of a 72 kDa membrane protein. In contradiction to this is the fact that protein kinase C phosphorylates proteins on serine and threonine residues and preliminary results suggest that the 75 kDa protein is phosphorylated on tyrosine residues. This does not rule out the possibility that the 75 kDa protein can be phosphorylated on more than one amino acid. In the insulin and the EGF systems binding of ligand to the homologous receptor activates a receptor associated tyrosine kinase which promotes an autophosphorylation reaction (Cohen *et al.*, 1982; Kasuga *et al.*, 1983). It has, however, been demonstrated that the receptor can also be phosphorylated on threonine residues by protein kinase C (Hunter *et al.*, 1984). The binding of the ligand to the receptor activates $PtdInsP_2$ hydrolysis which results in the activation of PKC (Sawyer and Cohen, 1981; Takayama, 1984). There is also evidence that that the tyrosine kinases encoded by src, $pp60^{v\text{-}src}$ (Sugimoto *et al.*, 1984) and ros, $pp68^{v\text{-}ros}$ (Macara *et al.*, 1984) can

phosphorylate PI, PIP and DAG. The fact that activation of two distinct kinases has been shown in other systems which involve cell growth, suggests perhaps that two kinases may be involved in the signals transduced by IL-4 in the human system.

The finding that interferon-γ causes similar, if not identical, alterations in the membrane phosphoprotein profiles of murine B lymphocytes to those observed with the same cells cultured with IL-4 is particularly interesting in view of the antagonistic effects of the lymphokines in many biological assays (Rabin *et al.*, 1986b; Mond *et al.*, 1986). Although the lymphokines are antagonistic towards each other, they have many similar effects on B cells. Both lymphokines promote directed immunoglobulin isotype switching in B cells in the presence of LPS: IL-4 drives switching to IgG1 or IgE (Vitetta *et al.*, 1985; Sideras *et al.*, 1985; Noma *et al.*, 1986; Isakson, 1986) and Ifn-γ to IgG2a (Snapper *et al.*, 1988c). This has been shown to be accomplished by a similar molecular mechanism in both cases. Hence, the induction of similar protein kinases by IL-4 and Ifn-γ may be accounted for in terms of their effect on similar genes required for isotype switching. IL-4 and Ifn-γ again have similar effects on MHC class II expression. Ifn-γ is inhibitory to the increase in MHC class II antigens seen on culture of B cells with IL-4, but Ifn-γ itself increases MHC class II antigens on macrophages, B cell tumours and immature B cells (Mond *et al.*, 1986). IL-4 has been shown to influence the level of a DNA binding protein which binds to a sequence in the MHC class II genes resulting in enhanced gene transcription (Boothby *et al.*, 1988). Ifn-γ has also been shown to exert its effect at the level of gene transcription by a trans acting protein (Amaldi *et al.*, 1989). This is further evidence of the two ligands using a similar protein kinase in order to activate similar genes.

The simultaneous exposure of B cells to IL-4 and Ifn-γ abolishes the effects on membrane phosphoprotein profiles observed with either ligand

alone. This is consistent with the capacity of the lymphokines to mutually inhibit each others biological effects.

The molecular identity of the 75 kDa phosphoprotein remains undefined. One possibilty is that the phosphoprotein represents the binding component of the IL-4 receptor which has been previously identified as having a molecular weight of 75 kDa in the mouse (Park *et al.*, 1987a) and 139 kDa and 70 kDa in the human (Park *et al.*, 1987b; Galizzi *et al.*, 1988). In this respect the IL-4 receptor may be similar to other ligand receptor systems ie. insulin, EGF, PDGF etc. whose receptors possess an intrinsic tyrosine protein kinase activity which result in an autophosphorylation. The results of experiments employing 8-azido-γ-[^{32}P]-ATP are not entirely inconsistent with this possibility as a protein of 75 kDa can be identified as binding ATP although not as strongly as the 50 kDa protein, whether this protein is identical to the 75 KDa phosphoprotein remains to be unequivocally defined. The alkali resistance of the 75 kDa phosphoprotein suggests that it may be phosphorylated on tyrosine but the use of other biochemical techniques have been unable to confirm this. Antibodies raised against tyrosine phosphate or azobenzylphosphonate, an analogue of tyrosine phosphate, although specific for tyrosine residues, did not bind to any membrane phosphoproteins as analysed by immunoblotting. It may be that the membrane proteins were of insufficient quantities and the antibodies of such low affinity that tyrosine phosphate was unable to be detected. Techniques employing two dimensional thin layer electrophoresis showed an increase in [^{32}P]-phosphate incorporation into serine, threonine and tyrosine phosphate in total membrane protein analyses (figure 23). The phosphoamino acids present in the 75 kDa protein could not be detected by this method due to the low incorporation of [^{32}P]-phosphate. The protein kinase activity of membrane proteins from resting B cells and B cells cultured with IL-4 was also analaysed by the use of exogenous peptide substrates containing one potential phosphorylation site but the results were

inconclusive. The most compelling data against the hypothesis that the 75 kDa phosphoprotein is part of the IL-4 receptor is that the murine IL-4 receptor has now been identified by cloning and nucleotide sequencing to be 140 kDa in the mature, fully glycosylated form (Drs D. Cosman and S. Gillis, Immunex Corporation, personal communication to Dr. W. Cushley). This does not rule out the possibility that the 75 kDa protein is a result of protease activity as protease inhibitors were not included in the phosphorylation experiments. Secondly, Ifn-γ promotes the appearance of the 75 kDa protein and reports suggest that it does not bind to the IL-4 receptor. Ifn-γ binding to its receptor may activate a protein kinase which phosphorylates the IL-4 receptor preventing signal transduction perhaps by decreasing the affinity of the receptor for ligand. Although binding studies suggest that Ifn-g has no effect on the binding of IL-4 to its receptor. Alternatively the 75 kDa phosphoprotein induced by Ifn-γ may be of a similar molecular weight but a different phosphoprotein to that induced by IL-4. The fact that IL-4 and Ifn-γ together inhibit the appearance of the 75 kDa protein suggests that the inhibition occurs before the phosphorylation event. It is possible that Ifn-γ inhibits the induction of the 75 kDa to the membrane.

The 75 kDa phosphoprotein may be a component of the IL-4 receptor distinct from the binding component or a protein distinct from but associated with the receptor.

Another candidate structure for the 75 kDa phosphoprotein based on the molecular weight is the β subunit of the high affinity receptor for IL-2 (Smith, 1988). This structure is particuarly interesting in light of the data in chapter 3 and also subsequent two-colour flow cytometric studies which have been performed (R. Butcher and W. Cushley, Department of Biochemistry, University of Glasgow) indicating that culturing human B cells with IL-4 increases the expression of the CD25 antigen. The high affinity IL-2 receptor consists of a 55 kDa protein, CD25 and a 75 kDa protein. The IL-2 receptor

is also found to be similar on both human and mouse B cells.

It is also interesting to consider the mechanism by which the 75 kDa protein appears in the membrane as it is not detected in membranes from resting B cells. The 75 kDa protein may be a result of *de novo* gene expression stimulated by culturing the cells with IL-4 or alternatively it may exist in a cytoplasmic location and be translocated to the plasma membrane. The 75 kDa protein may, of course, already exist in the plasma membrane and the phosphorylation event may represent the translocation of a protein kinase of which the 75 kDa protein is a substrate. A final possibility is that the 75 kDa protein or its associated kinase activity are present in resting cell membranes but are either not active or are below the sensitivity limits of the *in vitro* kinase assay.

6. GENERAL CONCLUSIONS.

6. GENERAL CONCLUSIONS

Control of B lymphocyte activation and differentiation by lymphokines involves the interplay of several molecules. The interaction of these molecules with their receptors induces a variety of biological effects in the cell. Numerous biological effects of IL-4 have been reported, but the signals transduced when IL-4 binds to its receptor to elicit these effects are poorly understood. Comparison with other systems shows that protein phosphorylation and dephosphorylation is frequently involved in the control of cell function. Experiments in this thesis, employing plasma membranes isolated from B , have identified two membrane associated phosphoproteins which respond to the binding of IL-4 to its receptor.

The 42 kDa phosphoprotein is specific to the murine system. Phosphorylation of this protein was only evident in membranes from resting B cells and B cells at early stages in the cell cycle. Phosphorylation of the 42 kDa protein is perhaps a signal transduced by the interaction of IL-4 with its receptor on resting B cells to potentiate their subsequent response antigen. The 42 kDa phosphoprotein was not evident in membranes isolated from human B cells. The human B cells were isolated from peripheral blood and tonsils whereas the mouse B cells were from spleen. The distinct anatomical sources of the B cells may be an explanation for the differences in protein phosphorylation observed between species. It may be that the human B cells, although in the resting state, had already encountered antigen *in vivo* and, hence, were in an activated state that did not require the 42 kDa phosphorylation signal. Alternatively the biochemical mechanism of action of IL-4 may vary between the two species. ATP binding experiments have indicated that the 42 kDa phosphoprotein does not bind ATP, which suggests that it is a protein kinase substrate.

Culturing high density resting murine B cells in the presence of IL-4 results in the appearance of a 75 kDa signal in the membrane phosphoprotein profiles of these cells. Ligand binding is apparently required for the appearance of this phosphoprotein since culture in medium alone or in the presence of 11B11, anti IL-4, fails to induce the alteration in membrane phosphoprotein profile. The 75 kDa phosphoprotein was not evident in the membrane profiles from B cells cultured with LPS. The 75 kDa signal was present in the membrane profiles of B cells cultured in the presence of Ifn-γ, which inhibits many of the biological effects of IL-4. However, the 75 kDa signal was not present in the membrane phosphoprotein profiles of B cells cultured with IL-4 and Ifn-γ simultaneously. The 75 kDa protein was alkali resistant and ATP binding experiments suggested that it may bind ATP. The membrane phosphoprotein profiles from human B cells cultured in the presence of human IL-4 indicated the phosphorylation of a 72 kDa protein which was alkali resistant. Culturing B cells in the presence of PMA plus calcium ionophore also induced phosphorylation of the 72 kDa protein in human B cell plasma membranes. Whether the murine and human proteins are identical remains to be unequivocally determined.

The data from this study suggest that in the murine system IL-4 influences the phosphorylation of two membrane-associated proteins, a 42 kDa protein and a 75 kDa protein. The 42 kDa signal can be detected in membranes from resting B cells and this suggests that the location of the protein is in the plasma membrane and it is the interaction of IL-4 with its receptor that results in phosphorylation of the protein. The murine 75 kDa phosphoprotein and the human 72 kDa phosphoprotein cannot be detected in membranes prepared from resting B cells but only in membranes prepared from B cells that have been cultured with IL-4 or, in the human system, PMA plus calcium ionophore. This suggests that perhaps the 75 kDa protein is not present in the plasma membrane of resting B cells but that

culturing with IL-4 either induces the translocation of this protein from another location to the plasma membrane or induces the transcription of the gene encoding this protein. These competing possibilities could be resolved by culturing B cells with IL-4 for different times and subjecting the membranes to *in vitro* phosphorylation to determine the appearance of the 75 kDa phosphoprotein. Rapid appearance would indicate a translocation event whereas a longer lag would suggest that gene transcription was involved. It is also possible that the 75 kDa is located in the plasma membrane of resting cells, but at levels below the sensitivity of the kinase assay. The presence of IL-4 may increase the amount of the 75 kDa protein available for phosphorylation. This is consistent with the fact that IL-4 increases expression of the IL-4 and IL-2 receptors, both of which are candidates for the 75 kDa phosphoprotein.

The question as to the identity of these proteins remains to be answered although several candidate structures have been suggested. One suggestion is that the 75 kDa protein is an IL-4 receptor associated tyrosine kinase which is activated upon IL-4 binding resulting in an autophosphorylation reaction. The action of IL-4 would therefore be analogous to other growth factor receptor systems. The binding of PDGF (Frackelton *et al.*, 1983), EGF (Cohen *et al.*, 1982) and insulin (Kasuga *et al.*, 1983) to their respective receptors has been shown to induce a receptor associated tyrosine kinase which results in autophosphorylation of the receptor. Phosphorylation of proteins on tyrosine residues has previously been reported for both T and B lymphocytes (Earp *et al.*, 1984; Casnellie *et al.*, 1982) including the T cell antigen receptor (Klausner *et al.*, 19867; Samelson *et al.*, 1986). It has been hypothesised that the 42 kDa phosphoprotein may be the α subunit of a guanine nucleotide (G) binding protein. If this were the case then the IL-4 receptor system may be analogous to the insulin receptor system. The β subunit of the insulin receptor has been shown to possess an intrinsic tyrosine kinase activity

which is capable of phosphorylating the α subunits of Gi and Go (O'Brien *et al.*, 1987). Binding of IL-4 to its receptor may, therefore, activate a 75 kDa receptor associated tyrosine kinase, resulting in both autophosphorylation and phosphorylation the α subunit of a 42 kDa G protein. Methods for identifying the involvement of a G protein in signal transduction have already been discussed in chapter four. If the 75 kDa phosphoprotein is part of the IL-4 receptor then antibodies which have been raised against the receptor may be able to immunoblot and/or immunoprecipitate the protein. The 75 kDa protein is the same molecular weight as the IL-4 binding component identified in the murine system (Park *et al.*, 1987a). If the 75 kDa phosphoprotein does indeed represent the binding component of the IL-4 receptor, then crosslinking of [^{125}I]-IL-4 to IL-4 receptors present on plasma membranes isolated from B cells cultured with IL-4 should isolate a 75 kDa IL-4 binding component which is capable of autophosphorylation in an *in vitro* kinase assay.

Alternatively the 42 kDa phosphoprotein may be a protein kinase C substrate. There have been reports of the activation of two independent kinases in other ligand receptor systems. Both EGF and insulin binding to their respective receptors have been shown to enhance phosphatidylinositol turnover which leads to the activation of protein kinase C (Sawyer and Cohen, 1981; Takayama, 1984). Further evidence of the involvement of two independent kinases in signal transduction comes from RNA tumour viruses. *In vitro*, the tyrosine kinases encoded by *src*, pp60$^{v\text{-}src}$ (Sugimoto *et al.*, 1984) and *ros*, pp68$^{v\text{-}ros}$ (Macara *et al.*, 1984) have been shown to phosphorylate phosphatidyl inositol, phosphatidyl inositol 4 phosphate and 1,2 diacylglycerol which results in the activation of protein kinase C. Binding of IL-4 to its receptor may, therefore, activate a 75 kDa receptor associated tyrosine kinase which is capable of enhancing polyphosphoinositol hydrolysis resulting in the activation of protein kinase

C of which the 42 kDa protein is a substrate. The involvement of protein kinase C in IL-4 signal transduction could be confirmed or eliminated by performing the *in vitro* phosphorylation assay in the presence of protein kinase C inhibitors. Data from two groups suggest that the 42 kDa protein is not a protein kinase C substrate (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986).

An alternative possibity is that IL-4 binding to its receptor results in the activation of one or more protein kinases of which the 42 kDa and 75 kDa proteins are substrates. The 42 kDa and 75 kDa phosphoproteins, although both involved in the signal transduction by IL-4, may be functionally unrelated.

The other candidate structure for the 75 kDa phosphoprotein based on molecular weight is the β subunit of the IL-2 receptor (Smith, 1988). The expression of CD25 of human B cells has been shown to be enhanced after culture of the cells with IL-4. This could, potentially, enhance the ability of the B cell to respond to IL-2. IL-2 has been shown to be capable of stimulating the proliferation and differentiation of large bouyant B cells (Mond *et al.*, 1985) and can also act as a costimulant with antigen in the activation of resting B cells (Pike *et al.*, 1984). Moreover, IL-2 has been shown to increase tyrosine phosphorylation (Salzman *et al.*, 1988). The question as to whether the 75 kDa phosphoprotein is the IL-2 receptor could be investigated by immunoblotting membrane phosphoprotein profiles from IL-4 cultured B cells with antibody specific for the β subunit. Alternatively cross-linking experiments could be performed using [^{125}I]-IL-2, similar to those detailed above for IL-4.

The membrane phosphoprotein profiles from B cells cultured with IL-4 and Ifn-γ simultaneously do not show the 75 kDa phosphoprotein,

which is consistent with the ability of Ifn-γ to inhibit the activity of IL-4. Interestingly, membrane phosphoprotein profiles from B cells cultured with Ifn-γ alone did show a 75 kDa signal. It is tempting to speculate that this 75 kDa phosphoprotein is identical to that induced by IL-4. If IL-4 and Ifn-γ induce phosphorylation of the same protein this could suggest two possibilities. If the 75 kDa protein is a component of the IL-4 receptor then phosphorylation of the protein by Ifn-γ may be on a different phosphoamino acid, or on the same type of phosphoamino acid at a different site in the protein. Phosphorylation of the 75 kDa protein by Ifn-γ may, therefore, result in a reduced or abolished protein kinase activity. Phosphorylation of the EGF receptor tyrosine kinase on serine and threonine was shown to result in a loss of EGF binding, which was due to a decrease in the affinity of the receptor for EGF (Hunter *et al.*, 1984). The arguement against this situation for Ifn-γ is that Ifn-γ does not inhibit the binding of IL-4 to its receptor, at least to the high affinity receptor identified by Park *et al* (1987a) and Ohara and Paul (1987). If the 75 kDa phosphoprotein is a component of the IL-2 receptor, phosphorylation of it by Ifn-γ would not account for the inhibitory effects of the action of IL-4 on resting B cells.

It could be that IL-4 and Ifn-γ inhibit each others effects at the level of gene transcription. In this case, if culturing B cells with IL-4 induces transcription of the 75 kDa phosphoprotein Ifn-γ may inhibit this transcription event. This hypothesis would also hold for the inhibition of Ifn-γ induced gene transcription by IL-4. The exact nature of the interaction of IL-4 and Ifn-γ, with regard to the 75 kDa phosphoprotein, cannot be established until the identity of the 75 kDa phosphoprotein is determined.

In the human system the 72 kda phosphoprotein may be identical to the murine 75 kDa phosphoprotein. In this case, all the candidates for the 75 kDa phosphoprotein are relevant for the human 72 kDa phosphoprotein.

In the human system, the appearance and phosphorylation of the 72 kDa phosphoprotein can be induced by PMA plus calcium ionophore which suggests that PMA plus calcium ionophore are mimicing the effect of culturing B cells with IL-4. In the murine system, culturing B cells with LPS does not result in the appearance of the 75 kDa phosphoprotein. This is perhaps an arguement for suggesting that the phosphoproteins are different in the two systems. The signals transduced by IL-4 binding to its receptor on human B cells do not appear to include phosphorylation of a 42 kDa protein. It is, therefore, tempting to speculate that the biochemical mechanism of action is distinct in the two species.

The two IL-4 induced phosphoproteins identified in this study were from *in vitro* phosphorylation of B lymphocyte plasma membranes. The effects of IL-4 on phosphorylation should also be demonstrated in whole B cells or permeabilised B cells using [^{32}P]-orthophosphate in order to correlate these results with relevance *in vivo*. The difficulties with this is that a very small signal is involved due to the low numbers of IL-4 receptors per cell and, therefore, it is difficult to detect a phosphorylation signal. Analysis of protein kinase events in detegent-permeabilised cells may provide an useful experimental system for *in vivo* resolution of the questions posed by the data of this thesis.

7. REFERENCES.

7. REFERENCES

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