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THE ROLE OF MEMBRANE PROTEIN PHOSPHORYLATION IN MITOGEN AND INTERLEUKIN FOUR MEDIATED ACTIVATION OF MURINE B LYMPHOCYTES

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SUMMARY

Activation of lymphocytes by the mitogens phytohaemagglutinin (PHA), 12-o-tetradecanoylphorbol-13-acetate (TPA) and anti-Ig has been observed to result in phosphorylation of a number of cellular proteins.

Lymphocyte activation by anti-Ig or TPA results in an increase in tyrosine phosphorylation of human B cell membrane proteins, thus implicating tyrosine kinase activities in the transmission of lymphocyte activation signals. In addition to the increase in phosphotyrosine levels upon activation by TPA, there is a concomitant increase in phosphoserine content due to the activation of protein kinase C. Lipopolysaccharide (LPS) causes translocation of protein kinase C from the cytoplasm to the plasma membrane and as lipid A, the free lipid portion of LPS, activates protein kinase C the possibilty arises that LPS, like TPA, activates protein kinase C directly.

Plasma membrane was prepared from resting and LPS activated murine splenic B cells and enzyme marker assays indicated that the isolation procedure adopted was successfully enriching for plasma membrane.

A comparision of total membrane protein phosphorylation in resting and LPS activated murine splenic B cells was carried out to determine if activation of these cells by LPS involved protein phosphorylation. Of the five samples examined, only 2 exhibited an increase in total membrane protein phosphorylation after a 24 hour incubation, in the presence of LPS, relative to resting B cell membranes. However, after a 48 hour incubation all five samples exhibited elevated levels of total membrane protein phosphorylation. Thus, activation of murine splenic B cells by LPS does appear to involve protein kinase activities.

Phosphoamino acid analysis of resting and LPS activated B cell plasma membrane proteins was carried out in an attempt to identify the

phosphoamino acid(s) involved in this increase in total membrane protein phosphorylation. The results of this phosphoamino acid analysis were variable and so it was not possible to determine clearly which phosphoamino acids were responsible for the increase observed.

A 47Kd protein was identified by SDS-PAGE analysis of resting and LPS activated membrane protein profiles and this protein appeared to be more heavily phosphorylated upon LPS activation. Although both phosphoserine and phosphotyrosine were identified in this protein, it was not possible to accurately quantitate the individual phosphoamino acids and therefore determine which phosphoamino acid was responsible for the apparent increase in phosphorylation.

Antigen specific activation of lymphocytes involves the action of T cell derived growth and differentiation factors, lymphokines. As the interleukin-2 receptor has been demonstrated to be phosphorylated by protein kinase C the possible involvement of protein phosphorylation in the action of interleukin-4 was investigated. As LPS activated B cells have been reported to express elevated numbers of interleukin-4 receptors the effect of interleukin-4 on membrane protein phosphorylation was investigated for both resting and LPS activated B cell membranes.

No consistent alteration in the total membrane protein phosphorylation was observed for either resting or LPS activated B cell membrane, in response to IL-4. This observation did not eliminate the possibility that there were alterations in phosphorylation occurring at the level of individual proteins. In view of this membrane protein phosphorylated in the presence of IL-4 was examined by SDS-PAGE and compared to the results obtained from membrane protein phosphorylated in the absence of IL-4. While no alteration in phosphoprotein profile was observed, the possibilty remains that the level of individual phosphoamino acids within a given protein is changing although the total protein phosphorylation levels

apparently remain unchanged. The small number of interleukin-4 receptors estimated to occur on a B cell may explain the lack of detectable response, at the biochemical level, in this system and more sensitive detection methods may be required to identify any changes occurring.

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Abbreviations.

The following abbreviations are used in this thesis.

BCGF	B cell growth factor
BCDF	B cell differentiation factor
CDP-DAG	cytidine diphosphatidyl diacylglycerol
Con A	concanavalin A
DAG	diacylglycerol
DNP	dinitrophenyl
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
G-anti-M	goat anti-mouse
G×M	goat anti-mouse
Ig	immunoglobulin
Ig M, G	immunoglobulin classes M, G
IGF	insulin-like growth factor
IL-4	interleukin-4
IP_3	inositol-1,4,5-trisphosphate
KOH	potassium hydroxide
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MHC	major histocompatibility complex
PA	phosphatidic acid
PDGF	platelet derived growth factor
PHA	phytohaemagglutinin
PI	phosphatidyl inositol
PIP_2	phosphatidyl inositol-bis-phosphate
PMA	phorbol 12-myristate 13-acetate
pNPP	p-nitrophenyl phosphate
$pp60^{v\text{-}src}$	the 60K phosphoprotein produced upon action of

the viral src gene product

$pp60^{c\text{-}src}$ the 60K phosphoprotein produced upon action of the cellular src gene product

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

TLE thin layer electorphoresis

TNP trinitrophenyl

TPA 12-O-tetradecanoyl phorbol 13-acetate

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CHAPTER 1 : INTRODUCTION

1. INTRODUCTION.

1.1. DISCOVERY OF PHOSPHOTYROSINE.

The importance of protein phosphorylation as a mechanism for the regulation of protein function is exemplified in the control of glycogen metabolism. In this system cAMP dependent protein kinase phosphorylates, and thereby inactivates glycogen synthase, thus inhibiting glycogen synthesis. cAMP-dependent kinase also phosphorylates and activates phosphorylase kinase which in turn phosphorylates, and activates, glycogen phosphorylase promoting glycogenolysis. Therefore, the level of phosphorylation of the proteins in the glycogen cascade regulates glycogen metabolism. However, phosphorylation in this system occurs solely on serine residues (Cohen, 1982).

Prior to 1979 the only acid stable phosphoaminoacids found in vivo were phosphoserine and phosphothreonine. In 1979 the existence of a new acid stable phosphoaminoacid, in the medium T antigen of polyoma virus, that was distinct from phosphoserine and phosphothreonine was reported (Eckhart et al., 1979). Incubation with 1 M KOH at 55^{o}C released more than 90% of the phosphoaminoacids of the large T antigen while releasing only 20% from the medium T antigen. The phosphoaminoacids in the large T antigen were identified as phosphoserine and phosphothreonine and so it was unlikely that the novel phosphoaminoacid of medium T antigen was either of these. It was known as early as 1941 that the phosphate ester bond of phosphotyrosine was relatively more stable than that of phosphoserine and phosphothreonine under alkaline conditions (Plimmer, 1941), and therefore this previously unidentified phosphoamino acid was identified as phosphotyrosine. Phosphotyrosine was also detected in complete pronase digests of the medium T antigen, indicating that

the phosphotyrosine detected was not an artifact of acid hydrolysis, but was a phosphoaminoacid distinct from phosphothreonine and phosphoserine (Eckhart et al., 1979).

1.2. DISCOVERY OF TYROSINE SPECIFIC PROTEIN KINASES.

Phosphotyrosine accounts for only approximately 0.05% of the total acid stable protein phosphate in eucaryotic cells. However, despite the relative paucity of phosphotyrosine, evidence is accumulating that tyrosine specific protein kinase activities may be an important class of regulatory enzymes.

1.2.1. Tyrosine Protein Kinases and Retroviruses.

The first description of a protein with tyrosyl protein kinase activity was provided by Hunter and Sefton (1980) from studies involving the immunoprecipitation of the src gene product of Rous Sarcoma Virus (RSV), $pp60^{v\text{-}src}$. The RSV transforming gene product, $pp60^{v\text{-}src}$, was identified as the factor responsible for phosphorylating the immunoglobulin heavy chain at tyrosine in immunoprecipitates from chicken cells infected with the virus. Chicken cells transformed by RSV demonstrated elevated levels of phosphotyrosine, compared to their non-transformed counterparts and, since phosphotyrosine was identified in $pp60^{v\text{-}src}$ itself, it was proposed that $pp60^{v\text{-}src}$ would phosphorylate tyrosine in vivo, ie. in the intact cell (Hunter & Sefton 1980). Persuasive evidence that $pp60^{v\text{-}src}$ had the inherent ability to transfer the γ-phosphate of ATP to tyrosine in protein was that Escherichia coli bearing a plasmid containing the RSV src gene synthesised a protein that phosphorylated proteins at tyrosine. The $pp60^{v\text{-}src}$ was identified as the protein responsible for this activity as E. coli containing similar plasmids which lacked only the src gene exhibited no tyrosine kinase function, suggesting that the activity must have been due to the presence of the src gene product. Therefore this activty must have been due to the

expression of the src gene product encoded by the transfected plasmid (Gilmer & Erikson, 1981, McGrath & Levinson, 1982).

The transforming proteins of other retroviruses have since been shown to exhibit tyrosine specific protein kinase activity (see Table 1). Most of these retroviral tyrosine kinase activities interact with the plasma membrane and this localisation at the plasma membrane may be an essential feature for cell transformation. The tyrosine protein kinases encoded by temperature sensitive mutant strains of RSV, Feline Sarcoma Virus (FeSV) and Avian Erythroblastosis Virus (AEV) have been demonstrated to dissociate from the membrane at their non-permissive temperature (Beung & Hayman, 1984). Since the transforming gene product of Abelson murine leukemia Virus shows a correlation between its dissociation from the plasma membrane and a loss of tyrosine kinase activity (Boss et al., 1981) there is a possible link between membrane tyrosine protein kinase activities and transformation.

The cellular homologue of $pp60^{v\text{-}src}$, $pp60^{c\text{-}src}$, was identified as being structurally similar, although not identical to, the viral homologue $pp60^{v\text{-}src}$ (Erikson et al., 1979). A comparison of the phosphorylation patterns produced by each gene product were similar as both had major phosphorylation sites at a serine residue on the amino terminal 60% of the polypeptide and on a threonine residue on the carboxy terminal 40%, and $pp60^{c\text{-}src}$, like $pp60^{v\text{-}src}$, would phosphorylate the heavy chain of immunoglobulin (Collet & Erikson, 1978, Erikson et al., 1979,1980). $pp60^{c\text{-}src}$, the cellular homologue, phosphorylated tyrosine residues in vivo, ie. in intact cells, (Hunter & Sefton, 1980), demonstrating that cells uninfected by retroviruses contain at least one kinase that specifically phosphorylates tyrosine.

Table 1

Tyrosine Protein Kinases as Retroviral Oncogene Products

Viral Source	Oncogene	Protein (m.w.)	Location
Rous Sarcoma Virus (RSV)	v-src	60 K	plasma membrane adhesion plaques
Avian erythroblastosis virus (AEV)	v-erb B	74 K	plasma membrane
Feline sarcoma virus (FeSV)	v-fes	95K	plasma membrane
Fujinami avian sarcoma virus (FSV)	v-fps	140 K	plasma membrane
Esh sarcoma virus	v-yes	80K	plasma membrane

1.3. PHOSPHOTYROSINE AND MITOGENIC HORMONES.

After the discovery that $pp60^{c-src}$ expresses tyrosine protein kinase activity in uninfected cells, tyrosine protein kinase activities were identified in a variety of cell types (Hunter & Sefton, 1980).

An area which has provided an insight into the possible funtions of cellular tyrosine protein kinase activities is the interaction between mitogenic hormones and their receptors.

1.3.1. Platelet Derived Growth Factor.

Platelet derived growth factor, PDGF, is a heterodimeric, disulphide-linked polypeptide released from the α granules of activated platelets and is the major growth promoting factor in serum for cells of connective tissue origin (Ek *et al.*, 1982, Ek & Heldin, 1982).

In vitro studies, ie. those involving broken cells or plasma membranes, indicate that binding of PDGF to its cell surface receptor leads to the activation of a receptor-associated tyrosine protein kinase. If human fibroblasts or glial cell plasma membranes are incubated in the presence of ^{32}P-γ-ATP a tyrosine protein kinase activity is stimulated upon PDGF addition, and the major endogenous phosphate acceptor appears to be the PDGF receptor itself (Ek *et al.*, Ek & Heldin 1982, Heldin *et al.*, 1983). *In vivo* studies utilising fibroblasts labelled with ^{32}P-orthophosphate stimulated with PDGF from which the cell extract was subjected to gel electrophoresis (Cooper *et al.*, 1982) or immunoprecipitation with an anti-phosphotyrosine antibody (Frackelton *et al.*, 1984) also suggested that the major substrate for PDGF-stimulated tyrosine phosphorylation is the PDGF receptor itself.

1.3.2. Insulin and Insulin Like Growth Factors.

Insulin and insulin like growth factors are mitogenic hormones that act on skeletal tissue and fibroblasts. The effects of insulin involve rapid metabolic changes and long term growth promotion, and both processes are initiated by insulin binding to high affinity cell surface glycoprotein receptors (Czech, 1977). Although insulin binds predominantly to the α subunit (Jacobs, 1979), phosphorylation occurs on the β subunit of the receptor (Kasuga et al., 1982a).

In intact rat hepatoma cells the initially low levels of both phosphoserine and phosphotyrosine in the β subunit were found to be increased by exposure to insulin (Kasuga et al. 1982a). In vitro, however, insulin binding induces phosphorylation of the receptor β sub-unit on tyrosine residues exclusively (Rosen et al., 1983, Kasuga et al. 1982b).

Subsequent experiments have demonstrated that the in vivo phosphorylation of the β subunit at tyrosine is an early and transient event in receptor activation and phosphotyrosine is rapidly replaced by phosphoserine and phosphothreonine (Pang et al., 1985). Studies with highly purified insulin receptor indicate that this phosphorylation is due to a kinase activity intrinsic to the receptor (Kasuga et al., 1983).

The insulin like growth factor-1 (IGF-1) receptor is structurally similar to the insulin receptor and immunoprecipitation studies indicate that the β subunit of the IGF-1 receptor is a target for IGF-1 stimulated phosphorylation (Rubin et al., 1983). Binding of insulin and IGF-1 to their respective receptors stimulates an increase in both autophosphorylation of the receptor and phosphorylation of exogenous substrates by these receptors (Rosen et al. 1983, Yu & Czech, 1984). In contrast, the purified insulin like growth factor-2

(IGF-2) receptor does not appear to be a tyrosine kinase capable of autophosphorylation although, in isolated adipoctye plasma membranes, the receptor appears to serve as a substrate for an endogenous tyrosine kinase (Corvera et al., 1986).

1.3.3 Epidermal Growth Factor.

Among the many effects, such as increased phosphatidyl inositol turnover (Moolenaar et al., 1984), that ensue upon EGF binding to its glycoprotein receptor is an autophosphorylation of the receptor at tyrosine (Carpenter et al., 1979, Hunter & Cooper, 1981). The major tyrosine phosphorylation site of the EGF receptor, in vivo, was identified as tyrosine 1173 (Hunter and Cooper, 1985). The addition of physiological concentrations of EGF to A_{431} cells, a human epithelioid tumour cell line rich in EGF receptors, causes an increase in phosphotyrosine in cellular protein. This phosphorylation occurs rapidly, within one minute, and is maintained for several hours (Hunter & Cooper, 1981). The EGF receptor will also phosphorylate exogenous substrates, such as histone and ribonuclease, and the kinase activity is EGF dependent (Carpenter et al., 1979).

In addition to phosphorylation on tyrosine residues, treatment of intact cells with EGF results in enhanced phosphorylation of serine and threonine residues of the receptor (Hunter & Cooper, 1981).

1.4. PHOSPHOTYROSINE IN CELL GROWTH REGULATION.

To determine if phosphotyrosine is involved in the regulation of cell growth it is necessary to correlate growth factor binding with receptor activation, kinase activation, substrate phosphorylation and biological response.

1.4.1. Platelet Derived Growth Factor.

There is a strong correlation between PDGF binding, PDGF stimulated phosphorylation and the biological response (Nishimura et al., 1982, Huang et al. 1982). For example, the PDGF stimulated

increase in phosphotyrosine in Swiss 3T3 cells exhibited the same dependence on PDGF concentration as the association of ^{125}I-PDGF with these cells, thus linking the binding of PDGF with tyrosine kinase stimulation (Cooper et al., 1982). Further evidence for the role of PDGF binding in cellular mitogenesis is provided by a study of Swiss 3T3 clones which have reduced binding of PDGF and a severely reduced mitogenic response (Bowen-Pope & Ross, 1982).

1.4.2. Insulin.

Many metabolic events linked to insulin binding reach half maximal stimulation at 0.2-0.5 nM insulin (Kahn, 1976), a concentration within the range reported to activate the receptor kinase activity in vitro (Haring et al., 1984). Further evidence suggesting a role for tyrosine phosphorylation in vivo is the absence of receptor phosphorylation in a mutant melanoma cell line which fails to respond to insulin (Haring et al., 1984). Point mutations at the insulin receptor tyrosine residues normally phosphorylated, results in a decrease in insulin stimulated tyrosine autophosphorylation in parallel with a decrease in insulin stimulated 2-deoxyglucose uptake (Ellis et al., 1986). Additionally, tumour promoters which inhibit insulin stimulated tyrosine protein kinase activity suppress a number of insulin mediated effects, such as the stimulation of glycogen synthase and tyrosine aminotransferase activities (Takayama et al., 1984) thus suggesting a correlation between receptor kinase and other enzyme activities. However antibodies to the insulin receptor, which mimic the physiological action of insulin, induce neither phosphorylation of the receptor nor activation of the receptor tyrosine protein kinase in vitro (Zick et al., 1983, Simpson & Hedo, 1984).

1.4.3. Epidermal Growth Factor.

The relationship between receptor occupancy and biological response has been studied extensively for EGF but the relationship between receptor kinase activity and mitogenesis remains unclear. For example, the reported half maximal binding for EGF to its receptor ranges from 2-10 fold higher than that determined for stimulation of mitogenesis (Aharonov et al., 1978, Cohen et al., 1975). This has led to the suggestion that, like the insulin system, only a fraction of the receptors need to be occupied to elicit a maximal physiological signal.

1.5. RECEPTOR PHOSPHORYLATION IN REGULATION OF HORMONE RECEPTOR SYSTEMS.

Despite the evidence supporting a role for tyrosine phosphorylation in the action of the mitogenic hormones there is, no definitive correlation between tyrosine phosphorylation and hormone action. An alternative role for phosphorylation at tyrosine in the mitogenic hormone receptor system is as a mechansim of regulating receptor function. Supporting this hypothesis is the finding that autophosphorylation of the insulin receptor has been shown to activate the insulin receptor tyrosine protein kinase activity of the β subunit in vitro (Rosen et al., 1983, Yu & Czech, 1984) thereby acting to overcome the requirement for the continued presence of insulin.

Modulation of growth factor action by other hormones may be regulated by receptor phosphorylation, resulting in alterations in both the receptor affinity and the receptor tyrosine kinase activity. The tyrosine kinase activity of one hormone receptor may be involved in regulating the activty of another as it has been reported that the EGF and IGF-1 receptors will phosphorylate the insulin receptor (Pike et al., 1984, Perisic & Traugh 1983). Thus, there may be regulatory

processes by which a specific hormone receptor activity is controlled by the presence of ligands other than its own.

1.6. PHOSPHOTYROSINE IN CELL PROLIFERATION AND TRANSFORMATION.

The action of both mitogenic hormone receptors and retroviruses have been shown to involve tyrosine protein kinase activities. As both these systems are involved in cell growth and proliferation, the retroviruses causing uncontrolled growth, the tyrosine protein kinase activities may have a central role in regulation of cellular proliferation and transformation. This hypothesis has been strengthened by the relationships between the retroviral transforming products and components of the mitogenic hormones and their receptors.

The product of the oncogene of the avian erythroblastosis virus, erb-B, has been identified as being homologous to the kinase domain of the EGF receptor (Downward et al., 1984). The viral transforming protein contained only the transmembrane and tyrosine protein kinase domains of the EGF receptor and lacked most of the extracellular domain responsible for EGF binding. As the v-erb oncogene product represents the truncated domain of the EGF receptor it may be that retroviral tyrosine kinases are analogous to permanently activated receptors which neither respond to external signals nor to down regulation. This would suggest that increased tyrosine kinase activity is a central feature of cellular transformation.

The tyrosine protein kinase domain of the insulin receptor has the closest homology with the v-ros gene of the VR-2 sarcoma virus but this oncogene product is not apparently membrane associated nor is the homology extensive enough to allow the interpretation that v-ros represents a truncated insulin receptor (Neckameyer & Wang, 1985).

A region of partial identity has been identified between a 90 residue stretch of PDGF and the predicted sequence of $p28^{sis}$, the putative transforming protein of simian sarcoma virus (SSV)

(Waterfield et al., 1983). This striking homology suggests that the virus has acquired cellular sequences which encode a growth factor identical, or very similar, to PDGF. Abnormal expression of PDGF, or a related protein, through transformation by SSV could lead to uncontrolled growth by transformed cells via secretion of the growth factor, thus maintaining the receptor in an activated state. As the PDGF receptor exhibits a tyrosine specific protein kinase activity upon PDGF binding the maintained activation of the receptor could result in increased tyrosine kinase activity.

1.7 CELL CYCLE, AN OVERVIEW.

Resting B cells are stimulated from a resting B, Go, state to an activated, Go*, state by the action of antigen binding in the presence of IL-4 (Oliver et al.,1985). B cells in the Go* state are described as competent as they are able to respond to lymphokines, resting B cells are not competent and are unable to respond to lymphokine signals.

In the presence of IL-4 (Ohara and Paul, 1985) and BCGF II (Foulkes and Rosner, 1985) the Go* B cells are driven on into the G1 phase of the cell cycle and onto DNA synthesis (S phase). After reaching the S phase of the cell cycle the B cells are committed to the cell cycle. Figure 1 summaries the cell cycle.

1.8. B CELL ACTIVATION.

The cellular basis of the humoral response of the immune system to antigen is proliferation of antigen specific B lymphocytes and their maturation to antibody secreting cells (Burnet, 1957).

When the immune system recognizes foreign antigens three different cell types collaborate in the induction of antibody formation. However, only the B lymphocytes synthesise antibody while helper T lymphocytes and macrophages cooperate in antibody production.

The activation of resting B cells occurs as a consequence of the

delivery of a number of external signals to the cell. There are at least three possible regulatory points during the activation process at which potentially important signals can be delivered to resting B cells;

i) direct contact between antigen and the specific surface receptor for antigen, ie. surface immunoglobulin;

ii) direct T cell - B cell interaction via the binding of the T cell antigen receptor to B cell surface Ia antigen in association with processed antigen; and

iii) the binding of non-specific T cell derived lymphokines to appropriate receptors on the B cell membrane.

1.8.1. Antigen-Surface Immunoglobulin Interaction.

Signal transduction across resting B cell plasma membranes results from crosslinking of surface immunoglobulin by anti-immunoglobulin reagents (Parker, 1975) (see section 1.10). These early signals have been found not to be sufficient to drive the cells into the cell cycle (Cambier et al., 1982) or to generate competency for the cells to respond to soluble growth and differentiation factors (Snow et al., 1983). This led to the proposal that the biochemical consequences which occur following T cell dependent antigen binding to B cells prepare the cell for a major growth stimulus, provided directly by the helper T cell.

1.8.2. T cell - B cell Interaction.

Since the binding of antigen to the surface immunoglobulin receptor, in the absence of T cell participation, does not result in the movement of B cells into the cell cycle, direct contact between B cell and helper T cell appears to play a critical role during the activation of resting B cells.

Studies by Mitchison (1971) demonstrated direct participation of T cells in the stimulation of B cells by T dependent antigens, and the

carrier effect he demonstrated required antigen-mediated linked recognition between the B and T cells.

The dependence of B cells on the presence of helper T cells to elicit a response to certain antigens has led to these antigens being described as T dependent antigens to distinguish them from antigens which can elicit a B cell response in the absence of T cells, these being described as T independent antigens.

The cellular interaction between T and B cells has been shown both in vitro (Andersson et al., 1980, Jones & Janeway, 1981) and in vivo (Sprent, 1978) to be MHC restricted by class II MHC (Ia) molecules, expressed on the B cell surface. Since the T cell antigen receptor binds both antigen and Ia molecules, the possibility exists that a signal is delivered to the B cell as a consequence of the T cell antigen receptor interacting with the B cell surface Ia molecules.

The uptake, processing and expression of antigen, by B cells, in the context of surface class II MHC molecules has recently been demonstrated (Lanzavecchia, 1985). B cells apparently process and express antigen in association with class II MHC antigen through a mechanism similar to that operative in macrophages (Lanzavecchia, 1985), except that at low antigen concentration there is a selective uptake of the antigen for which the B cell is specific (Rock et al., 1984). Mond et al. (1981) demonstrated that anti-Ig stimulation could induce hyper Ia expression on B cells and such B cells are more effective T cell activators than unstimulated B cells. This demonstration that B cells could function as antigen presenting cells during the process of T cell activation provided additional support for the concept that antigen and MHC class II molecules promote B cell-T cell interactions.

Baluyut and Subbarao (1986) have observed a synergistic

interaction between antibodies specific for Ia and surface IgM in the induction of murine B cell proliferation, thus providing evidence that a signal may be transmitted through surface MHC class II antigens. Cambier et al. (1986) observed that ligand binding to membrane Ia antigen caused rapid protein kinase C translocation, from cytoplasm to nucleus, suggesting that membrane MHC class II molecules can act as signal transducing molecules. However, it has been demonstrated that anti-Ia antibodies suppress mitogen-induced B cell expansion (Forsgren et al., 1984) thus suggesting the possibility of a different mechanism of signal transduction in mitogen-activated B cells.

Although T cell dependent MHC class II restricted stimulation of B cells is possibly the most common mechanism for B cell activation, other mechanisms have been documented.

1.8.3. Alloreactive T Cells.

It is possible to excite B cells to competence in a non-MHC restricted manner using alloreactive helper T cells. Two broad classes of alloreactive helper T cells can be found:

i) those that excite resting B cells expressing class II MHC (Ia) antigen only when specific antigen also binds to that cell (Schrier et al., 1980); and

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

1.8.4. T Independent Antigens.

Type II T cell independent antigens, eg. ficoll, present their antigenic determinants in a repetitive form to B cells and crosslinking of surface immunoglobulin induced by such repetitive determinants may be one prerequisite for excitation. Type II T cell independent antigens are not polyclonal activators of B cells even at high concentrations (Melchers & Andersson, 1984). This contrasts

with type I T independent antigens (eg. antigen coupled to LPS) which are polyclonal activators. At low concentrations type I antigens bind to selected antigen specific B cells via antigen specific immunoglobulin receptors; this concentrates the polyclonal activator on the antigen specific resting B cell and so excites it (Coutinho & Moller, 1975). However, at high concentrations polyclonal activators can circumvent the binding to membrane immunoglobulin and may use other thus far undefined receptor sites.

1.8.5. Excitation By Polyclonal Activators.

Mature small resting murine B lymphocytes are activated asynchronously from the resting state by polyclonal activators, such as lipopolysaccharide (Andersson et al., 1972) and lipoprotein (Melchers et al., 1975), irrespective of their MHC haplotype. These agents may crosslink putative complementary receptors on the surface membrane since they are known to exist as large polymeric structures in solution (Galanos & Luderitz, 1975). However no such receptors have yet been identified but as LPS has been demonstrated as being able to insert itself directly into the membrane it may be that LPS does not act via a specific receptor at all (Raetz et al., 1983).

Most mitogen activated B cells are reactive to more than one mitogen suggesting that multiple mitogen receptors might exist on single B cells (Andersson et al., 1979).

In summary, several studies in the murine system have demonstrated that the activation signals required to trigger antibody production by B cells vary considerably depending on the nature of the B cell population under investigation. Andersson et al. (1980) demonstrated that T cell derived helper factors could induce polyclonal immunoglobulin secretion in B cell blasts, whereas H-2 restricted T cell - B cell interactions were required for the activation of small resting B cells. Noelle et al. (1983)

demonstrated that TNP-KLH together with T cell derived growth and differentiation factors did not induce proliferation or differentiation of highly enriched TNP-binding B cells. However, purified TNP-binding cells did proliferate and differentiate when cultured with TNP-KLH in the presence of carrier primed T cells. This indicated that antigen specific resting B cells require activation by T cells to become responsive to growth and differentiation factors.

1.8.6. Macrophage - T Cell Interactions.

Macrophages and factors released by them can induce proliferation of spleen cells and cause production of polyclonal immunoglobulins (Lemke & Opitz, 1976, Moller et al., 1976) However, foetal calf serum, together with 2-mercaptoethanol, can substitute for macrophages in this reaction, indicating a non-specific function (Lemke & Opitz, 1976, Bevan et al., 1974). Macrophages, however, have a more specific function in lymphocyte activation as it has been noted that there is a physical association between lymphocytes and macrophages. Rosenthal et al. (1977) demonstrated that antigen pulsed guinea pig macrophages have a much greater potential for binding syngeneic lymphocytes than do non-pulsed macrophages. This association is longer lasting than the normally rapidly changing association between lymphocytes and macrophages.

An early event after antigen administration is its uptake by macrophages. These cells endocytose, digest and present on their surface the antigen, or fragments thereof, to the helper T cells that recognise the antigen or antigen fragments, in the context of class II MHC antigens expressed on the surface of the macrophages (Rosenthal & Shevach, 1973). This interaction between macrophages and T cells is MHC restricted in that a macrophage of a given haplotype can only present antigen to, and so activate, a helper T cell of the same

haplotype (Rosenthal & Shevach, 1973).

Ashwell et al. (1984) demonstrated that resting B cells can also act as antigen presenting cells in T cell activation. It has been determined that anti-immunoglobulin stimulation can induce hyper Ia expression on B cells and such activated B cells are more effective activators of T cells (Mond et al., 1981).

One result of the interaction between macrophages and T cells is the production of soluble products, lymphokines, by T cells which act on B cells to stimulate proliferation and maturation.

1.9. LYMPHOKINES PRODUCED BY HELPER T CELLS.

1.9.1. Interleukin 2.

Interleukin 2 (IL-2), formerly called T cell growth factor, is synthesised and secreted by T cells activated by antigen or mitogen and, upon binding to its glycoprotein receptor, IL-2 induces proliferation of thymocytes (Morgan et al., 1976, DiSabato et al., 1975). Additionally, IL-2 has been demonstrated to stimulate proliferation of cytotoxic T cells and in doing so enhance production of δ-interferon (Farrar et al., 1981). Although murine and human IL-2 have been identified as distinct molecules (Gillis et al., 1982) they do cross react in that murine IL-2 will stimulate human T cells and vice versa (Mosmann et al., 1987).

The murine monoclonal antibody, anti-Tac, was shown to block high affinity IL-2 binding to activated T cells and the T cell responses attributed to the interaction of IL-2 with these cells, thus suggesting that the Tac protein may correspond to the IL-2 receptor (Robb & Greene, 1983). Two classes of IL-2 receptor have now been identified (Robb et al., 1981 & 1984); a high affinity and a low affinity type, however the molecular changes in the Tac protein, the proposed IL-2 receptor, that would give rise to these affinity differences have not been elucidated.

Low affinity type IL-2 receptors have been identified on

activated T cells, human and murine T cell lines and on Tac positive B cell lines (Robb et al., 1984). The presence of IL-2 receptors on B cells (Robb et al., 1984, Waldman et al., 1984) suggested that IL-2 may play a role in the differentiation of activated B cells into immunoglobulin secreting cells.

1.9.2. T Cell Replacing Factor(s).

For the initiation of most B cell responses, binding of antigen to immunoglobulin on the cell surface is a necessary first step to activation. Exceptions to this rule are the polyclonal responses to lipopolysaccharides (Andersson et al., 1972) which circumvent the binding to surface immunoglobulin (Coutinho & Moller, 1975).

The observation that for B cells to respond to most antigens the presence of helper T cells was required suggested the possibility that the helper functions of T cells may be mediated by soluble products released from T cells. Schimpl and Wecker (1972) demonstrated that cell free supernatants from allo-antigen stimulated or Con A stimulated T cells could fully substitute for T cells in the in vitro response to unrelated antigens such as sheep red blood cells. They postulated that T cells stimulated by their specific antigen released a substance, T cell replacing factor, which was able to stimulate B cells to proliferate only when B cells carried antigens on their receptors.

On the basis of the experiments it was speculated that B cells could be activated into antibody secreting cells by two signals:

i) binding of antigen to specific immunoglobulin receptors on the B cell surface and,

ii) T cell derived antigen non-specific helper factors.

Kishimoto et al. (1975a & 1975b) attempted to induce an anti-DNP antibody response in DNP-primed rabbit B cells with a combination of antigen non-specific signals, ie. crosslinking of immunoglobulin

receptors by anti-immunoglobulin (anti-Ig), and cell free supernatants of antigen stimulated T cells. Anti-DNP antibody response or polyclonal immunoglobulin production was stimulated by anti-Ig and T cell derived factors. B cells stimulated by anti-Ig could absorb the activity of T cell derived helper factor(s), and cell division was not required for the anti-Ig activation.

This study could, therefore, dissect the activation process of B cells into two phases. The first phase was the anti-Ig dependent activation to a stage at which the B cells become responsive to T cell derived helper factors while the second stage was the factor dependent proliferation and differentiation of B cells into immunoglobulin secreting cells.

Similar attempts were made with murine B cells but purified soluble rabbit anti-Ig reagents failed to activate murine lymphocytes (Sieckman et al., 1978) However, the anti-Ig antibody although not mitogenic in soluble form, becomes a powerful mitogen for murine B cells when made insoluble by covalent attachment to sephadex beads (Parker, 1975) An interesting aspect of B lymphocyte activation by anti-Ig beads was that, unlike LPS, they failed completely to induce the differentiation of B cells to high rate immunoglobulin synthesis and secretion. This failure to induce the secretion of immunoglobulin was overcome by the addition of a cell free supernatant of spleen cells activated by Con A (Parker et al., 1979).

These results suggested that a T cell replacing factor, or factors, substituted for the helper function of T cells in the antibody response of B cells to T dependent antigens.

1.9.3. B Cell Specific Growth Factors.

The presence of B cell specific growth factor(s) was originally suggested by Howard et al. (1982). In their study, to measure B cell growth factor (BCGF) activity, proliferation of anti-μ-stimulated

murine B cells was augmented by the addition of a culture supernatant of phorbol myristate acetate (PMA) stimulated EL-4 thymoma cells. Adsorption of the culture supernatnant with an IL-2 dependent cytotoxic T cell line reduced IL-2 acivity but caused little or no reduction in B cell growth factor activity, suggesting the presence of a B cell specific growth factor distinct from IL-2.

The presence of BCGF distinct from IL-2 has been confirmed by the establishment of T cell - T cell hybridomas secreting BCGF molecules. Murine T cell hybrids between Con A stimulated splenic T cells and the thymoma BW5147 produced a B cell specific replication factor without any activity to induce immunoglobulin secretion in B cells or to induce proliferation of T cells. B cell specific replication factor (BRF) from this hybridoma, A32-26, induced replication of LPS stimulated B cell blasts but not immunoglobulin secretion, indicating that the factors involved in replication and maturation were distict molecules (Lernhardt et al., 1982, Leanderson et al., 1982) BRF did not, however, stimulate resting B cells.

The results obtained with conventional and hybridoma derived factors clearly demonstrate the presence of B cell specific growth factor(s) involved in the induction of proliferation of B cells.

Swain and Dutton (1982) reported the presence of two distinct kinds of murine B cell growth factors. In contrast to the 20K BCGF reported by Howard et al.(1982) which does not induce proliferation of the BCL_1 cell line the (DL)BCGF identified by Swain et al. (1983) did, thus it appeared that the 20K BCGF and (DL)BCGF were distinct molecules.

1.9.4. Factors With Both Growth and Differentiation Activities.

Although it was determined that the growth and differentiation activities were mediated by distinct molecules, for example BCGF has growth but not maturation activity, factors with both growth and differentiation activities have been identified. The myelomonocytic cell line, WEHI-3, was demonstrated to constitutively produce a factor that stimulated growth and differentiation of murine B cells (Booth et al., 1983). A similar activity with both growth and differentiation activities was identified in the superna tants of EL-4 cell cultures (Pike et al., 1982). Another factor with both growth and differentiation activities was factor B151-TRF, identified by Takatsu et al. (1980) which indicated that these activities act on normal murine B cells as well as on transformed cells such as the BCL_1 cell line.

1.9.5. B Cell Differentiation Factors.

Factors involved in the final differentiation process of B cells, B cell differentiation factors (BCDF) were identified by their ability to induce high rate immunoglobulin secretion (Schimpl and Wecker, 1972, 1975).

A BCDF, BCDF , was detected using BCDF-reactive cell lines (Pure et al., 1981) which induced IgM secretion in those BCL_1 cells incubated with supernatants from T cell lines, clones or hybridomas. BCDF lacked IL-2, -interferon or BCGF activities and so was a distinct differentiation factor.

1.9.6. Interleukin 4.

Murine interleukin 4, IL-4, formerly designated B cell growth factor 1 or B cell stimulatory factor 1, was originally described (Howard et al., 1982) on the basis of its ability to co-stimulate, with anti-IgM, the entry of resting murine B cells into S phase of the cell cycle. It was anticipated that IL-4 would act on B cells in a manner analogous to the action of IL-2 on T cells (Cantrell & Smith, 1983) in that resting B cells would be unresponsive to IL-4 until activation with anti-IgM induced IL-4 sensitivity. Addition of IL-4 would then determine that the activated B cell would enter S phase of the cell cycle. It was demonstrated (Oliver et al., 1985) that if B cells were treated with anti-immunoglobulin prior to IL-4 they did not enter S phase of the cell cycle. However, if the IL-4 incubation preceded the anti-immunoglobulin the IL-4 rendered the cells susceptible to anti-immunoglobulin mediated entry into S phase. These results suggested that IL-4 acts as a lymphokine that prepares B cells for anti-immunoglobulin mediated activation rather than as a growth factor, ie. it is a competence factor.

This ability of IL-4 to prepare B cells for more prompt entry into the S phase in response to subsequent culture with anti-IgM stimulants requires extended contact with B cells. It demonstrated that delaying the addition of IL-4 for 4 to 8 hours in a 24 hour preculture diminishes the degree of preparation of B cells, as did removal of IL-4 using the anti-IL-4 monoclonal antibody, 11B11 (Rabin et al., 1986). If cells are not pre-exposed to IL-4, anti-IgM will only cause B cells to enter the S phase after 20-30 hours of contact (DeFranco et al., 1982).

IL-4 has been demonstrated as having an effect on B cells stimulated by LPS. Not only has IL-4 been demonstrated to prepare B

cells from DBA/2 and BDF_1 mice to respond more promptly to LPS (Rabin et al., 1986) but it was demonstrated by Layton et al. (1984) that B cells stimulated with LPS secreted little, or no, IgG_1 unless IL-4 was present. The precursors of IgG_1 secreting cells lacked membrane IgG_1 at the onset of culture and so heavy chain switching must occur in response to IL-4. IL-4 has also been identified as causing secretion of IgE in B cells stimulated with LPS (Coffman et al., 1986).

In addition to this costimulatory effect IL-4 has been demonstrated to enhance expression of class II MHC molecules (Noelle et al., 1984) and to increase cell volume (Rabin et al., 1985).

Demonstrations that low concentrations of anti-Lyb 2.1 were stimulatory with anti-IgM, but not with crude preparations of IL-4, (Yakura et al., 1984) suggested that anti-Lyb 2.1 blocks absorption of IL-4 activity by B cells and that Lyb 2 was possibly the cellular receptor for IL-4. However, as anti-Lyb 2.1 stimulates B cell entry into S phase in the absence of co-stimulators, while IL-4 alone fails to do this, suggests that anti-Lyb 2.1 may not bind to the IL-4 receptor (Subbarao & Mosier, 1983).

As IL-4 has been purified to apparent homogeneity (Ohara et al., 1985) Ohara and Paul (1987) were able to carry out Scatchard analysis of the binding of ^{125}I-labelled IL-4 to various cell types, and so estimate the IL-4 receptor number per cell. They demonstrated that IL-4 receptors are located on resting murine splenic B cells, and that activation of these B cells, by stimulation with anti-IgM or LPS, caused a large increase in receptor number. Activation of T cells with Con A also caused an increase in IL-4 receptor number relative to that detected on resting T cells. IL-4 receptors were also detected in high numbers on macrophage cell lines, P388D1 and IC21, and on mast cells. This binding of ^{125}I-labelled IL-4 was inhibited by excess

Figure 1: Summary of the Proposed Site of Action of B Cell Factors Within the Cell Cycle.

B cells may be stimulated from a quiescent, G_0, state by IL-4 and antigen to an activated, G_0*, state (Howard et al., 1982, Oliver et al., 1985). Activated, G_0*, B cells enter the G_1 phase of the cell cycle under the influence of an early acting BCGF, possibly IL-4 (Ohara & Paul, 1985). Therefore it appears IL-4 acts both as a synergistic activator of B cells (Oliver et al., 1985) and as a BCGF (Ohara & Paul, 1985) that drives the B cells on into G_1 of the cell cycle. B cells activated to G_1 of the cell cycle only enter S phase under the influence of a late acting BCGF, $BCGF_{II}$ (Foulkes & Rosner, 1985).

Differentiation of B cells, to plasma cells, apparently occurs under the influence of a number of lymphokines including IL-2 (Swain et al., 1983) and BCDF (Pure et al., 1981), however it is not clear at which stage of the cell cycle these lymphokines act.

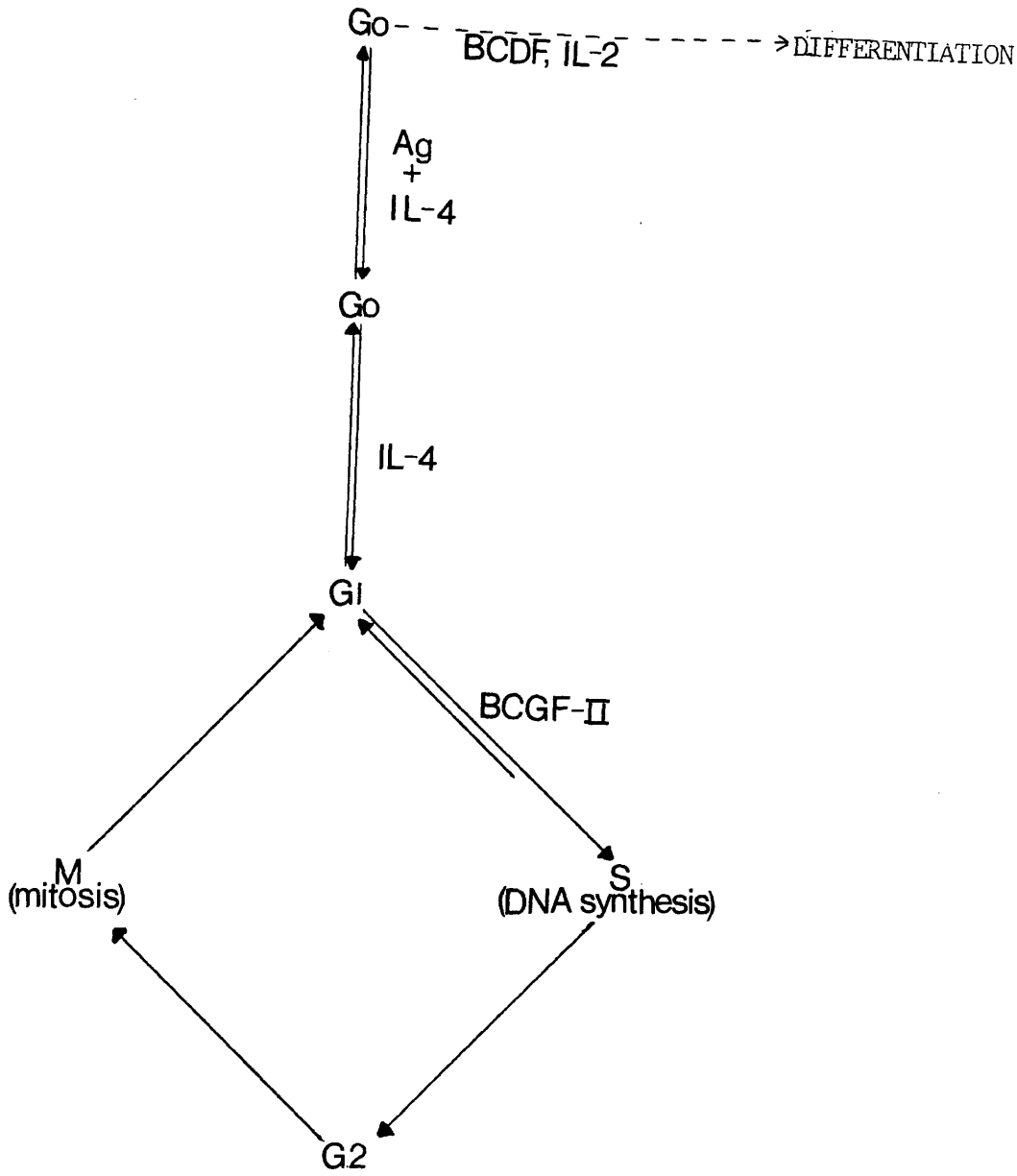


Figure 1 Summary of the proposed sites of action of B cell factors within the cell cycle.

non-radioactive IL-4 but not by recombinant mouse γ-interferon, although γ-interferon has been shown to block the effect of IL-4 on resting B cells (Mond et al.,1986) and so the action of interferon is not mediated via its binding to the IL-4 receptor. The monoclonal antibody anti-Lyb 2.1 also failed to inhibit ^{125}I-IL-4 binding. This failure together with the finding that T cells (Lyb 2 negative cells) express IL-4 receptor argues against a role for Lyb 2 as a receptor for IL-4. As yet the IL-4 receptor remains to be positively identified.

Thus it has been determined that there are muliple factors controlling B cell growth and differentiation and that these may also have mulitple actions within the immune system. Figure 1 summarises the proposed sites of action within the cell cycle of the different factors mentioned.

1.10. IMMUNOGLOBULIN RECEPTOR TRANSMEMBRANE SIGNALLING.

Investigations into the mechanism of transmembrane signalling in lymphocytes have indicated that signal transduction via surface immunoglobulin appears similar to thrombin receptors, muscarinic receptors and α_1 adrenergic receptors which transduce signals via initiation of phosphoinositide hydrolysis, yielding diacylglycerol and inositol phosphates which in turn appear to activate protein kinase C and calcium mobilisation respectively.

1.10.1. Membrane Depolarisation.

Monroe and Cambier (1983b) reported that B cell plasma membrane depolarisation resulted from surface immunoglobulin crosslinking by anti-Ig. Decreased uptake of the carbocyanine dye, 3,3'-dipentyloxacarbocyanine iodide (DiOC5(3)), upon membrane immunoglobulin receptor crosslinking was demonstrated to occur within the first minutes of stimulation, was apparently indicative of B cell plasma membrane depolarisation (Cambier & Monroe, 1983a & b). Supporting this interpretation was the observation that equivalent

changes in dye uptake occured after membrane depolarisation in response to elevation of extracellular potassium (Monroe & Cambier, 1983a). Also, anti-immunoglobulin antibody induction of this response was blocked in the presence of the potassium ionophore valinomycin (Monroe & Cambier, 1983a). Monroe and Cambier (1983a & b) demonstrated correlations between stimuli and conditions that induced decreased plasma membrane potential, and those that stimulated hyper Ia antigen expression. They also demonstrated a correlation between the stimuli and conditions that inhibited ligand induced plasma membrane depolarisation and inhibited hyper Ia expression.

Based upon these results Monroe and Cambier hypothesized that antigen-mediated immunoglobulin crosslinking resulted in signal transduction via membrane depolarisation, which resulted in increased Ia synthesis and cell surface expression. However, although there was a strong correlation between depolarisation and entry into the cell cycle by B lymphocytes in response to anti-Ig, plasma membrane depolarisation was itself an insufficient signal to support the transition of B cells from G_0 to G_1 (Cambier *et al.*, 1985).

1.10.2. Protein Kinase C.

Having defined membrane depolarisation as an early indicator of membrane immunoglobulin signal transduction, which appeared to be related to increased Ia expression the next area of investigation was the mechanism of coupling between immunoglobulin crosslinking and the changes in ion flux manifested by membrane depolarisation.

Protein kinase C was described by Ogawa *et al.* (1981) and shown to occur in high concentrations in lymphoid tissues. Protein kinase C has been demonstrated as being stimulated by biologically active phorbol diesters (Castagna, 1982) and phorbol diester has also been documented to cause induction of increased expression of Ia by normal B cells (Monroe *et al.*, 1984). A correlation was demonstrated

between the ability of phorbol diester analogues to activate murine B lymphocyte derived protein kinase C in cell free systems and the stimulation of membrane depolarisation and increased Ia antigen expression by intact B cells. This led to the hypothesis that protein kinase C might be the coupling element between surface immunoglobulin crosslinking and membrane depolarisation.

Although the molecular basis of the proposed protein kinase C - ion flux coupling is not well defined, K^+, Na^+ and H^+ fluxes have been demonstrated to be regulated by phosphorylation in other tissues (O'Brien & Krzeminiski, 1983, Moolenaar *et al.*, 1983).

The primarily cytosolic nature of protein kinase C was seemingly inconsistent with its proposed role as a coupler of surface immunoglobulin crosslinking and membrane depolarisation. However a translocation of protein kinase C from cytosol to membrane in PMA stimulated EL-4 thymoma cells was demonstrated (Kraft *et al.*, 1982), this was later observed with the physiological ligand IL-2 (Farrar & Anderson, 1985). This cytoplasm to membrane translocation supported the role of protein kinase C as a coupler of surface immunoglobulin crosslinking and membrane depolarisation.

1.10.3. Substrates For Protein Kinase C.

Although protein kinase C has been identified as being involved in signal transduction in lymphocyte activation little is known about which lymphocyte cell proteins are substrates for the enzyme.

Shackelford and Trowbridge (1984) demonstrated that the IL-2 membrane receptor was rapidly phosphorylated *in vivo* in normal T lymphocytes and in the leukemic T cell line, HUT 102B2, in response to TPA stimulation. That protein kinase C was directly responsible for this phosphorylation was determined by in vitro studies in which incubation with protein kinase C directly phosphorylated the IL-2

receptor (Shackelford & Trowbridge, 1986). Similar studies involving the heavy chain of the class II MHC antigens in place of the IL-2 receptor indicated that this protein was also a substrate, both in vitro and in vivo, for protein kinase C (Shackelford & Trowbridge, 1986).

A comparison of the protein phosphorylation induced by anti-IgM and phorbol 12-myristate-13-acetate (PMA) activation of murine B lymphocytes identified six prominent plasma membrane associated phosphoproteins, four of which were also associated with the cytoskeleton (Hornbeck & Paul, 1986). Examination of protein phosphorylation in cell lines derived from different tissues identified one major B cell phosphoprotein (M.W. 65-70 x 10^3daltons) which was absent in T cells (Hornbeck and Paul, 1986). Thus it was demonstrated that the major substrates which were phosphorylated following surface immunoglobulin crosslinking in lymphocytes are both transmembrane and cytoskeletal proteins thus adding further support for the proposed role of protein kinase C in lymphocyte transmembrane signal transduction.

1.10.4. Phosphoinositide Metabolism and Surface-Immunoglobulin Signalling.

The demonstration that protein kinase C was activated in vitro by phorbol esters, eg. PMA, and that this activation correlated with membrane depolarisation and increased Ia expression led to the proposal that protein kinase C was the factor responsible for coupling surface immunoglobulin crosslinking and membrane depolarisation. To prove that protein kinase C had such a role in vivo it was necessary to establish the physiological activator of protein kinase C and identify a link between surface immunoglobulin crosslinking, the physiological activator and protein kinase C.

Based on the finding that DAG, a product of phosphoinositide

hydrolysis which accompanies receptor binding in a number of systems, was a potent activator of protein kinase C it was proposed that DAG was the physiological activator of protein kinase C (Kishimoto et al., 1980). That DAG may be the physiological link between surface immunoglobulin crosslinking and protein kinase C was supported by the observation that exogenous DAG, either in the form of 1-oleyl-2-acetyl glycerol or diolein, induced both membrane depolarisation and increased Ia expression by normal B cells (Coggeshall and Cambier, 1985).

Kishimoto et al. (1980) have proposed that in situ, the activity of protein kinase C is regulated by phosphotidylinositol (PI) hydrolysis via the generation of DAG. If increased DAG generation from phosphotidylinositol is an important intermediary event in surface immunoglobulin mediated signal transduction, increased PI metabolism should be detectable soon after anti-immunoglobulin stimulation of B cells.

The first link between enhanced PI turnover and lymphocyte activation was demonstrated when phytohaemagglutinin was shown to stimulate incorporation of ^{32}P-orthophosphate into PI and phosphatidic acid (PA) in peripheral T cells (Fisher and Mueller, 1968). It was also observed that increased PI synthesis occurred after stimulating porcine B cells with anti-immunoglobulin antibodies (Maino et al., 1975).

Treatment of B cells with anti-immunoglobulins was demonstrated to result in activation of the PI cycle (see fig.2) (Coggeshall and Cambier, 1984). Using ^{32}P-orthophosphate labelled murine B cells it was determined that the increased phospholipid metabolism that occurred in response to anti-immunoglobulin involved only PI and PA and not phosphatidylcholine, ie. only those phospholipids involved in the PI cycle. Using cells double labelled with ^{32}P-orthophosphate and ^{3}H-glycerol it was determined that anti-immunoglobulin reagents

Figure 2: Phosphatidylinositol cycle.

Cross-linking of B cell surface immunoglobulin stimulates phospholipase C resulting in the breakdown of PIP_2 to produce DAG and IP_3. IP_3 stimulates the mobilisation of intracellular calcium stores which together with DAG activate protein kinase C. The DAG is recycled back to PIP_2 through PA, CDP-DAG, PI and PIP_2.

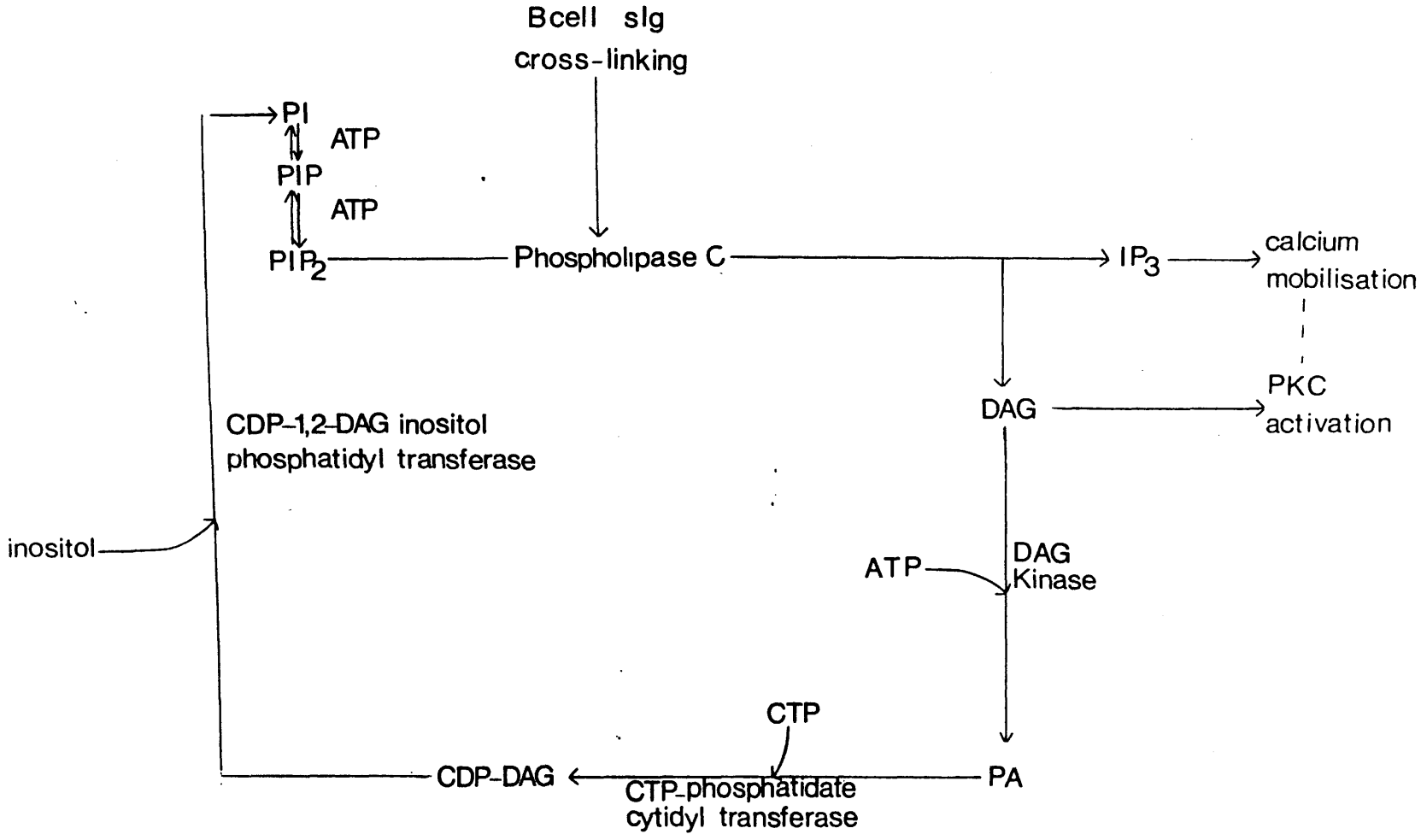


Figure 2 Phosphatidylinositol cycle.

only induced small increases in ^{3}H-glycerol incorporation compared to the large increases in ^{32}P-orthophosphate labelling (Coggeshall and Cambier, 1984). These results suggested that phospholipid turnover and not phospholipid synthesis accounted for the changes in PI and PA metabolism in B cells after treatment with anti-immunoglobulin.

That anti-immunglobulin transduces signals across the plasma membrane via the PI cycle was substantiated by Bjisterbosch et al. (1985) who demonstrated an early increase in the level of ^{3}H-inositol trisphosphate in ^{3}H-inositol labelled murine B cells, treated with this reagent. This suggested a breakdown of phosphoinositol-bis-phosphate (PIP_2) by phospholipase C. Bjisterbosch et al. (1985) also demonstrated an elevation in the level of ^{3}H-1,2-DAG after incubation of ^{3}H-arachidonic acid labelled cells with anti-immunoglobulin, also indicative of a breakdown of a phosphatidyl-bis-phosphate (PIP_2).

1.10.5. Calcium Mobilisation.

In addition to the release of DAG, due to phospholipase C action on PIP_2, there is a rapid concomintant release of inositol-1,4,5-trisphosphate, IP_3, in B cells (Bjisterbosch et al., 1985). Berridge (1983) suggested that the mobilisation of intracellular calcium was mediated by IP_3 released by the hydrolysis of PIP_2. Exogenous IP_3 has been shown to trigger mobilisation of intracelluar calcium in a number of tissues (Berridge & Irvine, 1984) so supporting this idea.

That calcium may be involved in lymphocyte signal transduction was determined when it was demonstrated that crosslinking of surface immunoglobulin triggered mobilisation of intracellular calcium in B lymphocytes (Braun et al., 1979), while anti-immunoglobulin was demonstrated to induce release of IP_3 which mobilised intracellular calcium in B lymphocytes (Ransom et al., 1986).

The role of this intracellular calcium in lymphocyte activation is still unclear although protein kinase C has been identified as being calcium dependent (Kishimoto et al., 1980) and so it may be that calcium acts synergistically with DAG to activate protein kinase C.

1.10.6. Model for Transmembrane Signalling in B Lymphocytes.

Cambier et al. (1985) have proposed a working model for transmembrane signalling by surface immunoglobulin (see fig.3). They hypothesize that crosslinking of surface immunoglobulin initiates, via phospholipase C, PIP_2 hydrolysis generating DAG and IP_3 which in turn activate protein kinase C and liberate intracellular calcium respectively. Protein kinase C then leads to a change in ion flux manifest by the membrane depolarisation and this altered ion flux mediates, by an unknown path, an increase in Ia expression on the cell surface. However, much remains to be elucidated before this model can be verified.

1.10.7. Alternative Mechanisms of Membrane Signal Transduction in B Lymphocytes.

Although the PI hydrolysis, calcium mobilisation, protein kinase C mechanism of membrane signal transduction is the most investigated mechanism, there does appear to be other mechanisms operative in B cells.

The first suggestion that increased PI metabolism was not essential for B cell activation demonstrated that there was no increase in PI metabolism in murine B cells after LPS or pokeweed mitogen stimulation (Betal et al., 1975). Further studies also indicated that although increased PI metabolism was an important early event in B lymphocyte activation it was not obligatory (Grupp and Harmony, 1985, Bjisterbosch et al., 1985). Anti-IgM unresponsive B cells from CBA/N mice failed to increase PI metabolism in response to anti-IgM and did not undergo DNA synthesis. When these deficient B

Figure 3: Model for Transmembrane Signalling via sIg.

Cross-linking of B cell surface immunoglobulin stimulates phospholipase C resulting in the breakdown of PIP_2 to IP_3 and DAG. IP_3 mobilises intracellular calcium stores which act with DAG to activate protein kinase C. Protein kinase C then mediates, via an unknown pathway, membrane depolarisation which is manifest in an increase by Ia expression.

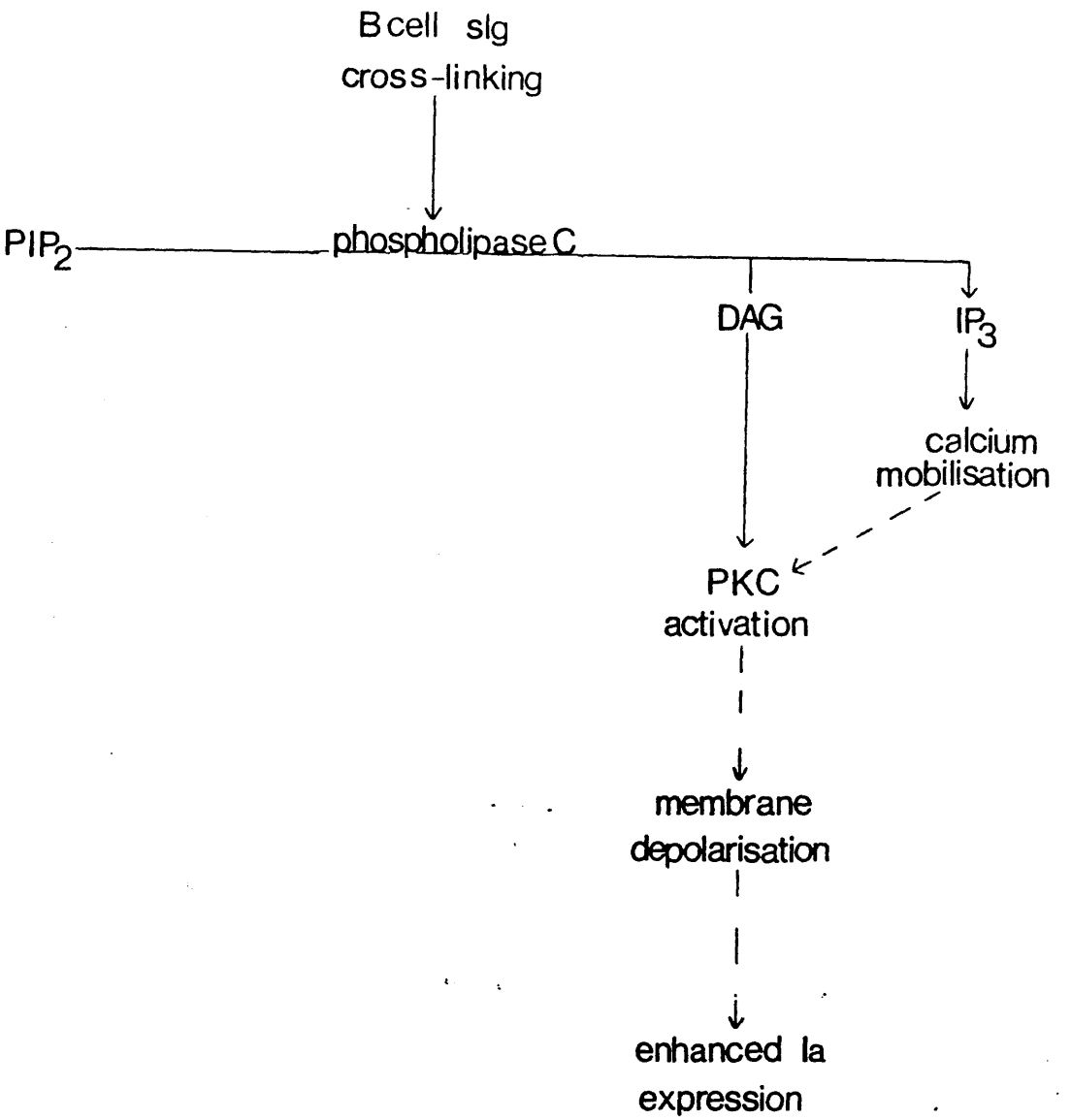


Figure 3 Model for transmembrane signalling via sIg.

cells were treated with LPS and anti-IgM, which drives them into S phase, there was still no activation of PI metabolism suggesting that LPS does not cause B cell activation via this mechanism (Grupp and Harmony, 1985). This finding was substantiated when it was observed that incubation of murine B cells with optimally mitogenic concentrations of LPS in the presence of lithium chloride, a known inhibitor of inositol-1-phophatase, did not result in the accumulation of inositol phosphates normally indicative of PI hydrolysis (Bjisterbosch et al., 1985).

In addition to antigens and polyclonal activators, B cells also require signal transduction in response to growth factor binding to their receptors. Farrar and Anderson (1985) demonstrated that IL-2 binding resulted in a translocation of protein kinase C, from the cytosol to the plasma membrane, thus suggesting that IL-2 signal transduction across the plasma membrane may be via the PI cycle as protein kinase C translocation is a prominent feature of this mechanism.

Recently, it has been demonstrated that IL-4 does not induce PI metabolism, calcium mobilisation, protein kinase C translocation or membrane depolarisation in resting murine B cells, but does appear to cause an increase in phosphorylation of a 44kD plasma membrane protein (Justement et al., 1986). As anti-Ig does not stimulate phosphorylation of this protein it has been suggested that IL-4 does not activate protein kinase C but may transduce signals across the plasma membrane via another membrane associated protein kinase.

1.11. TYROSINE SPECIFIC PROTEIN KINASE ACTIVITIES IN LYMPHOCYTES.

The development of synthetic peptides, with amino acid sequences similar to the tyrosine phosphorylation site of $pp60^{v\text{-}src}$, that acted as substrates for tyrosine specific protein kinases allowed the estimation of tyrosine protein kinase activity without interference by

other protein kinases. Such synthetic peptides were utilised by Swarup et al. (1983) to estimate the levels of tyrosine protein kinase activity in normal rat tissues. Spleen had the highest activity of the tissues tested and most of the activity was in the particulate fraction of the tissue. Swarup et al. (1983) identified two major bands, of molecular weight 53,000 and 56,000 daltons containing phosphotyrosine and it was suggested that the 56,000 dalton protein was similar, or identical, to that identified by Casnellie et al. (1982, 1984). Purified human peripheral T and B lymphocytes were demonstrated to have high tyrosine kinase activity comparable to that in rat spleen, therefore, suggesting that the type of cell in the rat spleen responsible for the high levels of tyrosine kinase activity may be the T and B lymphocytes (Swarup et al., 1983).

Investigations on tyrosine kinase activities in T and B lymphocytes have demonstrated that these cell types both contain tyrosine kinase activities but their patterns of tyrosine phosphorylation differ. Earp et al. (1984) compared the tyrosine phosphorylation patterns of membranes from normal splenic B cells, neoplastic B cells, normal thymic and splenic T cells and neoplastic T cells. Their results clearly indicated that T and B lymphocytes have distinct patterns of tyrosine phosphorylation. The major T cell band identified, p58, corresponded with that described by Casnellie et al. (1982) but in addition a phosphotyrosine containing band, p64, that was consistently observed in mouse thymocytes, splenic T cells and mouse and human neoplastic T cell lines was identified. B cells were also identified as having two major phosphotyrosine containing proteins. The B cell proteins were different from those identified in T cells as their molecular weights were 61,000 daltons (p61) and 55,000 daltons (p55). Both these B cell substrates were identified in human Raji cells, a human Burkitt lymphoma derived lymphoblastoid

cell line, and in mouse splenic B cells (Earp et al., 1984).

Casnellie et al. (1982) utilised a synthetic peptide to determine the tyrosine specific protein kinase activity in the particulate fraction of the lymphoma cell line, LSTRA. They demonstrated that this fraction had a high tyrosine protein kinase activity and identified an endogenous protein substrate with a molecular weight of 58,000 daltons, later amended to 56,000 daltons and referred to as $pp56^{LSTRA}$ (Casnellie et al., 1984). The LSTRA cell line was originally obtained from a mouse infected with Moloney murine leukaemia virus, MoMuLV, but as cells transformed with this virus do not have elevated levels of phosphotyrosine (Sefton et al., 1981) and as MoMuLV does not contain a kinase element (Shinnik et al., 1981) it was not possible to ascribe the high levels of phosphotyrosine in the $pp56^{LSTRA}$ to this agent. Therefore the protein, $pp56^{LSTRA}$ must be encoded by an endogenous cellular gene. Preincubation of mouse splenocyte membranes with N-p-tosyl-L-lysyl chloromethyl ketone, a known inhibitor of certain tyrosine kinases (Richert et al., 1979), markedly inhibited the phosphorylation of p64 and p58, the T cell proteins, while only minimally affecting the phosphorylation of the B cell proteins, p55 and p61 (Earp et al., 1984). The substrate specificities and cofactor requirements of tyrosine protein kinases identified in the particulate fractions of these two cell types have also been demonstrated to differ, thus suggesting that T and B lymphocytes may contain distinct tyrosyl kinases (Earp et al., 1985).

Marth et al. (1985) utilised a combination of olignucleotide and differential screening techniques to obtain cDNA clones that partially encode the $pp56^{LSTRA}$ identified by Casnellie et al. (1984). This gene was identified as being encoded by the genetic locus *IskT*, which encodes a protein of 509 residues distinct from, but related to, the products encoded by the avian *c-src* and *v-yes* genes. Transcription

of the IskT gene was shown to be restricted to tissues of lymphoid origin and transcripts were found in lymphocytes of both T and B lineages although the B lineage cells contained lower levels of transcripts than those of T lineage. The IskT gene has been demonstrated to be rearranged and over-expressed in the murine T cell lymphoma LSTRA (Marth et al., 1985). Therefore it was inferred that the IskT encoded tyrosine protein kinase was possibly involved in transducing proliferative or differentiative signals unique to lymphocytes.

Further evidence suggesting a role for tyrosine specific protein kinase activity in lymphocyte signal transduction was the demonstration that activation of freshly obtained human peripheral blood B lymphocytes by reaction with anti-IgM was associated with increased tyrosine phosphorylation relative to unstimulated levels (Nel et al., 1984).

1.11. Objectives.

As alterations in protein phosphorylation had been demonstrated to occur upon activation of lymphocytes by mitogens such as PHA, TPA and anti-Ig it was decided to investigate the possible involvement of membrane protein phosphorylation in murine B cell activation by LPS.

The report that IL-4 prepares B cells to respond more readily to LPS suggested that there was an relationship between interleukin and mitogenic activation of B cells. The additional report that B cell LPS blasts expressed more IL-4 receptors than resting B cells strengthened this hypothesis. Therefore a parallel study into changes in membrane protein phosphorylation upon IL-4 stimulation of LPS B cell blasts was undertaken.

CHAPTER 2 : MATERIALS

2. MATERIALS.

The following materials were used in this study and were provided by the suppliers listed.

2.1. ANIMALS.

BALB/c mice were supplied by the University of Glasgow biochemistry department breeding colony and were propagated by sibling mating. Both sexes were used at 8-12 weeks.

2.2. CELL CULTURE MATERIALS.

RPMI-1640 medium	) Northumbria Biologicals Ltd.
Glutamine	)
Penicillin-Streptomycin	) Gibco Ltd.
Foetal calf serum	)
Lipopolysaccharide (from E. coli 0127:B8)	) Sigma Chemical Co.
Goat anti-mouse IgM	)
2-mercaptoethanol	) Koch-Light Ltd.
Culture flasks	) Sterilin
96 well flat bottom culture plates	) Costar

2.3. B CELL SEPARATION MATERIALS.

Mouse T cell subset depletion kit	) Seralab
Guinea pig complement	)
Percoll	) Sigma Chemical Co.
Albumin	)

2.3.1 Phosphate Buffered Saline (PBS).

170 mM sodium chloride

3.4 mM potassium chloride

10 mM di-sodium hydrogen phosphate

1.8 mM potassium di-hydrogen phosphate

2.4. PHOSPHORYLATION ASSAY MATERIALS.

p-nitrophenyl phosphate) Sigma Chemical Co.

Sodium orthovanadate)

Piperazine-N,N'-bis[2-ethane sulphonic acid], PIPES)

Interleukin-4) courtesy of Dr. R.J. Noelle, Department of Biochemistry, Dartmouth Medical School.

2.5. RADIOCHEMICALS.

Methyl-^{3}H-Thymidine) Amersham International Inc.

specific activity 44 Ci/mmol)

UDP-[U-^{14}C] galactose)

specific activity >200 mCi/mmol)

Adenosine 5'[^{32}P-] triphosphate)

specific activity 3000 Ci/mmol)

[2-^{3}H] Adenosine 5'-monophosphate)

specific activity 14 Ci/mmol)

2.6. THIN LAYER ELECTROPHORESIS MATERIALS.

O-phospho-L-serine) Sigma Chemical Co.

O-phospho-L-threonine)

O-phospho-DL-tyrosine)

ε-DNP-lysine)

Ninhydrin)

Polyester sigmacell type 100 cellulose plates)

Xylene cyanol FF) Geo. T. Gurr Ltd.

2.7 PHOTOGRAPHIC MATERIALS.

X Omat-S Xray film) Kodak Ltd.

SX-80 developer)

FX-40 liquid fixer)

2.8 ENZYME ASSAY MATERIALS.

Catalase, bovine liver, EC 1.11.1.6)Sigma Chemical Co.

Fumarase, porcine heart, EC 4.2.1.2)

Lactate dehydrogenase, rabbit muscle type II, EC 1.1.1.27)

5'-Nucleotidase, Crotalus atrox venom, EC 3.1.3.5)

Galactosyl transferase, bovine milk, EC 2.4.1.22)

Hepatic cytochrome P-450) courtesy of N. Meftah,
Physiology & Pharmacology Dept.,
University of Glasgow.

Managanous chloride) BDH

Hydrogen peroxide)

UDP-galactose) Sigma Chemical Company.

Ethylenediaminetetraacetic acid, EDTA)

Sodium pyruvate)

Nicotinamide adenine dinucleotide, NAD^+)

Nicotinamide adenine dinucleotide, reduced form, NADH)

Triethanolamine)

Adenosine 5'-monophosphate)

Malic acid)

N-acetyl-D-glucosamine) Koch-Light Ltd.

Anion exchange resin, AG 1X-2, chloride form) Bio-Rad Labs

2.9 SDS-PAGE MATERIALS.

Acrylamide) BDH

N,N'-methylene-bis-acrylamide)

NNN'N'-tetramethylenediamine, TEMED)

SDS molecular weight markers) Sigma Chemical Co.

(MW-SDS-70 kit, range 14,000-70,000)

[lysozyme, egg white, 14.3K.

lactoglobulin, bovine milk, sub-unit, 18.4K.

trypsinogen, PMSF treated, bovine pancreas, 24K.

pepsin, porcine stomach mucosa, 34.7K.

ovalbumin, 45K.

albumin, bovine plasma, 66K.]

2.10. GEL STAIN MATERIALS.

Coomassie Brilliant Blue) Sigma Chemical Co.

Silver Nitrate) Johnson Matthey Chemicals Ltd.

2.11. MISCELLANEOUS MATERIALS.

Ecoscint) National Diagnostics

All other chemicals used were of "analar" grade and were supplied by BDH.

CHAPTER 3 : METHODS

3. METHODS.

3.1. PREPARATION OF A SINGLE CELL SUSPENSION FROM SPLEEN.

BALB/c mice were sacrificed by ether inhalation followed by cervical dislocation. Spleens were transferred to incomplete medium (RPMI-1640, 5 mg/ml penicillin/streptomycin) and teased into a single cell suspension, removing empty sheaths. Cells were then washed twice in incomplete medium.

3.2. CELL COUNTING.

All cell counting was carried out in the presence of 0.05% (w/v) trypan blue for determination of viability.

3.3. SEPARATION OF SPLENIC T CELLS AND B CELLS.

A single cell suspension of spleen cells was washed in incomplete medium, resuspended in erythrocte lysis buffer (17mM tris-HCl pH 7.2 containing 0.144 M NH_4Cl) and incubated at ambient temperature for 5 minutes. An equal volume of incomplete medium was added, the cells layered onto a half volume of 5% (w/v) albumin in incomplete medium and centrifuged at 1000 rpm for 7 minutes in a MSE Centaur 2 bench centrifuge. The erthrocyte depleted cells were then washed in incomplete medium. Equal volumes of each antibody from a Seralab T-cell depletion kit plus Guinea Pig complement was added to 2 x 10^9 cells and diluted, to 4 ml, with 1 x PBS (see Materials) to produce a 1/500 dilution of antibody and a 1/40 dilution of complement. Cells were then incubated at 37^{o}C for 40 minutes before washing in incomplete medium and resuspending in percoll of density 1.08 g/ml.

Figure 4: Separation of B Cell Populations on a Discontinuous Percoll Gradient.

A discontinuous percoll gradient with density layers of 1.09, 1.08, 1.075, 1.065 and 1.055 g/ml (the 1.08 g/ml layer containing the B cell population) was centrifuged at 1,400g for 30 minutes. The resting B cells were then collected from the 1.09 - 1.08 g/ml interface.

Separation of B Cell Populations on a Discontinuous Percoll Gradient

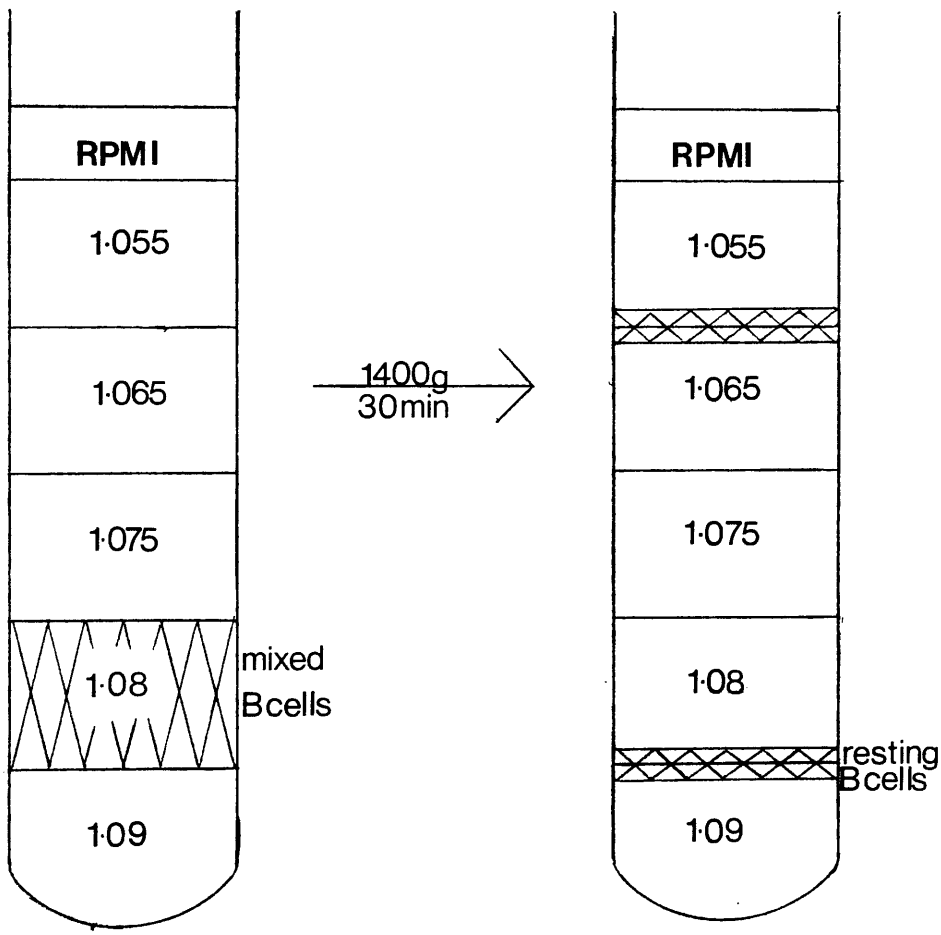


Figure 4

3.4. PERCOLL GRADIENT SEPARATION OF B CELL POPULATIONS.

Cells were separated on a percoll gradient as described by Ratcliffe and Julius (1982).

Percoll dilutions with densities of 1.09, 1.08, 1.075, 1.065 and 1.055 g/ml were prepared by diluting an iso-osmotic percoll stock (9 parts percoll: 1 part 10 x PBS) with 1 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 percoll density was layered into a 15 ml nitrocellulose tube, the 1.08 g/ml layer containing the cells, with a 1 ml top layer of incomplete medium (see figure 4). The gradient was centrifuged for 30 minutes, at 1400g in a SW27 Beckman rotor. Resting B-cells were collected at the 1.08-1.09 g/ml interface and washed in incomplete medium, while B-cell blasts collected at the 1.055-1.065 g/ml interface.

3.5. ^{3}H-THYMIDINE UPTAKE AS A MEASURE OF B CELL ACTIVATION BY LIPOPOLYSACCHARIDE.

To determine the optimal concentration of LPS for B-cell activation the following assay was carried out.

A single cell suspension was prepared, as in 3.1, 3.3 and 3.4, and the cells resuspended in complete medium +/- 50 uM 2-mercaptoethanol. 3.5×10^5 cells were plated into individual wells of a Costar 96 well flat bottom plate. To each alternate row

an equal volume of complete medium was added whilst to each remaining row an equal volume of LPS in complete medium was added to give triplicate wells containing concentrations of 2.5, 5, 10 or 20 μg/ml.

Cells were incubated for 16 hours at 37°C in 4% CO_2 after which 1 μCi of ^{3}H-methyl thymidine in incomplete medium was added to the rows 1 and 2. Cells were incubated for a further 6 hours before harvesting onto glass fibre filters. Rows 3 + 4, 5 + 6, 7 and 8 had 1 μCi of ^{3}H-methyl thymidine added to them at 42, 66, 88 and hours respectively and were harvested at 48, 72 and 96 hours respectively. The harvested cells were placed in 3 ml of Ecoscint and counted on a LKB 1211 Rackbeta liquid scintillation counter.

3.6. IL-4 BIOASSAY.

An IL-4 assay was carried out according to the method of Ohara et al. (1985).

A single cell suspension of BALB/c resting B cells was prepared as in 3.1, 3.3 and 3.4. Cells were then resuspended at 10^6 cells/ml and 10^5 cells added to each well of a Costar 96 well culture plate. The cells were then incubated with medium alone, 5 μg/ml goat anti-mouse IgM, 5 U/ml IL-4, 5 U/ml IL-4 plus 5 μg/ml goat anti-mouse IgM or 10 μg/ml LPS, for 56 hours before pulsing with 1 μCi ^{3}H-methyl thymidine for 16 hours. Cells were then harvested onto nitrocellulose filters and counted.

3.7. PREPARATION OF PLASMA MEMBRANE FROM B CELLS.

Plasma membrane was prepared by the method of Earp et al. (1984).

2-8 x 10^8 cells were centrifuged at 1000 rpm for 7 minutes in a MSE Centaur 2 and washed in 0.15 M NaCl. They were gently resuspended in 3.5 ml of homogenisation buffer (10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA) and homogenised at 4°C using 25 strokes of a glass hand pestle. 3.5 ml of homogenisation buffer containing 0.5 M sucrose was added and the homogenate centrifuged at

1000 rpm for 4 minutes to remove unbroken cells and nuclei. The resulting supernatant was aspirated and centrifuged for one hour at 105,000g in a SW65 Beckman rotor. The pellet was resuspended in 20 mM PIPES pH 7.0 and the protein concentration estimated by measuring absorbance at 280 nm.

3.8. IN VITRO PHOSPHORYLATION OF PLASMA MEMBRANES FOR SDS-PAGE.

Membrane phosphorylation using [^{32}P-α]-ATP was performed by a modification of the method of Earp et al. (1984).

100 µg of membrane was preincubated at 4°C for 10 min in the presence of 1 µM sodium orthovanadate, 30 mM $MgCl_2$, 1 mM pNPP, 20 mM PIPES pH 7.0. The addition of 5 µCi of [^{32}P-γ]-ATP, diluted to 10 µl in 20 mM PIPES pH 7.0, started the reaction. The reaction was allowed to proceed for 5 minutes before being terminated by the addition of 25 µl of hot (55°C) reducing loading buffer (see 3.10), the sample was then boiled for 5 minutes. Sodium dihydrogen phosphate was added to a concentration of 1 mM prior to electrophoresis on a 10% polyacrylamide gel (see 3.10).

3.8.1. Phosphorylation of Plasma Membranes in the Presence of IL-4.

This assay was carried out as in 3.8 except IL-4 was added to the incubation medium at a concentration of 50 U/ml.

3.9. TCA PRECIPITATION OF PHOSPHORYLATED MEMBRANE.

To quantitate the degree of total membrane phosphorylation samples of phosphorylated membrane were precipitated with trichloroacetic acid for scintillation counting.

Upon stopping the kinase reaction 3 x 10 µl samples were spotted onto Whatman filterpaper discs and dried. The discs were then washed, at 4°C, for 20 minutes in 20% TCA before washing twice in 10% TCA. A final 10 minute wash in ethanol was carried out and the discs dried and counted on a LKB 1211 Betarack liquid scintillation counter.

3.10. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

SDS-PAGE was carried out according to the method of Laemmli (1970) and was performed on 10% slab gels.

3.10.1. Stock Solutions.

Solution A.

45% (w/v) Acrylamide

1.2% (w/v) N,N'-methylene-bis-acrylamide. Deionized for 10 minutes with Amberlite MB-2 ion exchange resin. Solution A was filtered and stored at 4°C in the dark.

Solution B.

1.5 M Tris-HCl, pH 8.8

0.13% (v/v) TEMED

Stored at 4°C.

Solution C.

12% SDS

Stored at 4°C.

Solution D.

10% (w/v) Ammonium persulphate

Stored at 4°C.

Solution E.

0.65 M Tris-HCl pH 6.8

Stored at 4°C.

Reducing Loading Buffer.

65 mM Tris-HCl, pH 6.8

2% (w/v) SDS

10% Glycerol

5% (v/v) 2-Mercaptoethanol

0.01 mg/ml Bromophenol blue

Stored at -20°C

Reservoir Buffer.

25 mM Tris base, pH 8.0

132 mM Glycine

0.2% (w/v) SDS

Stored at room temperature.

3.10.2. Separating Gel Preparation.

10% separating gels were prepared as follows;

13.3 ml Solution A

15 ml Solution B

0.5 ml Solution C

0.5 ml Solution D

30.7 ml Deionized water

An additional 50 µl of TEMED was added to this solution before pouring into the casting apparatus and leaving to set.

3.10.3. Stacking Gel Preparation.

Stacking gels were prepared as follows;

3.64 ml Solution A

3.4 ml Solution E

0.2 ml Solution C

0.2 ml Solution D

18.67 ml Deionized water

24 µls of TEMED was added before pouring onto the top of the main gel and leaving to set around a 10 or 20 well teflon comb.

3.10.4. Electrophoresis Conditions.

Samples were loaded into individual wells in the stacking gel and electrophoresis performed at a constant current of 45 mA per gel for 4 to 5 hours at ambient temperature. 5 µl of low molecular weight standards (see Materials) were run next to the samples to facilitate molecular weight determinations.

3.11. TREATMENT OF GELS.

3.11.1. Coomassie Blue Staining.

After electrophoresis gels were stained with 0.5% (w/v) Coomassie blue (in destain solution) for one hour before placing in destain (4.5 vols ethanol, 4.5 vols deionized water, 1 vol glacial acetic acid) to reveal protein bands. Gels were then dried onto Whatman 3 mm filter paper for autoradiography and storage.

3.11.2. Silver Staining.

As an alternative to Coomassie blue staining some gels were silver stained according to the method of Wray et al. (1981).

All procedures were carried out using high purity water.

The SDS-PAGE gel was soaked overnight in 50% (v/v) methanol before staining. Within 5 minutes 0.8 g of silver nitrate was dissolved in 4 ml of water and 21 ml of 0.36% (w/v) NaOH mixed with 1.4 ml NH_4OH. The siver nitrate solution was added dropwise to the $NaOH/NH_4OH$ solution, adding extra NH_4OH dropwise to ensure the siver nitrate was properly dissolved. This solution was then made up to 100 ml. The gel was then stained in this solution, for 15 minutes with gentle agitation before washing in water. 3.5 ml of 1% (w/v) citric acid was mixed with 0.25 ml 38% (v/v) formaldehyde, made up to 500 ml with water, and poured onto the gel immediately to develop the bands. The reaction was stopped by rinsing in water and the gel stored in 50% (v/v) methanol.

3.11.3. KOH Treatment of SDS-PAGE Gels.

After staining, with Coomassie blue, and destaining, gels were incubated, for 90 minutes, in two washes of 5 mM sodium dihydrogen phosphate +/- 1 M KOH at 55°C. The gels were then rinsed in distilled water before placing in destain for 30 minutes to shrink the swollen gel. The gels were then dried onto Whatman 3mm filter paper and auotradiographed.

3.12. TWO DIMENSIONAL THIN LAYER ELECTROPHORESIS OF [^{32}P-γ]-ATP LABELLED MEMBRANES.

B-cell membranes labelled in vitro with [^{32}P-γ]-ATP were analysed by two dimensional TLE to determine the amounts of phosphoserine, phosphothreonine and phosphotyrosine. The method used was that developed by Cooper et al. (1983).

Solutions.

pH 1.9 Buffer.

50 ml 88% Formic acid

156 ml Glacial acetic acid

1794 ml Deionized water

pH 3.5 Buffer.

10 ml Pyridine

156 ml Glacial acetic acid

1890 ml Deionized water

Marker Dye.

1 mg/ml Xylene cyanol FF

5 mg/ml ε-DNP lysine

Phosphoaminoacid Standards.

1 mg/ml O-phospho-L-serine

1 mg/ml O-phospho-L-threonine

1 mg/ml O-phospho-DL-tyrosine

in pH 1.9 buffer.

3.12.1. Phosphorylation of Plasma Membrane for Thin Layer Electrophoresis.

The phosphorylation was carried out as in 3.8 except the reaction was stopped by the addition of an equal volume of 100% trichloroacetic acid. Samples were then left on ice for 20 minutes before centrifuging for 5 minutes at 13000g in a MSE Micro Centaur centrifuge. The resulting supernatant was discarded and the pellet

washed twice in acetone.

3.12.2. Thin Layer Electrophoresis.

A dry protein pellet was resuspended in 50 µl of 5.7 M HCl and incubated at 110°C for 2 hours. Any remaining liquid was lyophilised and the hydrolysed phosphoaminoacids resuspended in 10 µl of pH 1.9 buffer and 10 µl of phosphoaminoacid standard mix. 5 µl of this mix was spotted onto 10 cm x 10 cm cellulose coated plates as was 0.25 µl of marker dye. The cellulose plate was wetted using Whatman 3 mm filter paper wicks then transferred to the electrophoresis tank, containing pH 1.9 buffer, using the dampened wicks to provide electrical contact with the buffer.

Plates were electrophoresed at 50 V/cm for 20 minutes. The plates were dried and the wetting step repeated using pH 3.5 buffer after turning the plate through 90°. The second dimension was also run for 20 min at 50 V/cm. At the end of this run the plates were dried and sprayed with 0.2% (w/v) ninhydrin in acetone and placed in a 65°C oven to develop.

Plates were then autoradiographed and once an acceptable exposure was obtained the spots corresponding to phosphoserine, phosphothreonine and phosphotyrosine were scraped and placed in 3 ml of Ecoscint and counted.

3.13. ELUTION OF PROTEINS FROM SDS-PAGE GELS.

3.13.1. Non-KOH Treated Gels.

After examination of the autoradiographs which revealed bands of interest, the corresponding protein was cut from the dried SDS-PAGE gel and eluted by the following procedure.

The cut gel pieces were soaked for 3 hours in 10 ml of 30% (v/v) methanol, 10% (v/v) glacial acetic acid. The gel pieces were then chopped and incubated, with shaking, overnight in 500 µl of elution buffer (10 mM Tris-HCl pH 7.5, 1% SDS, 0.1 M Sodium Chloride, 2mM Magnesium Chloride and 5 mM 2-Mercaptoethanol). After removing the

gel pieces, 1 ml of 100% ethanol was added and the solution left at -20°C for 4 hours before centrifuging for 5 minutes at 13,000g in a MSE Micro Centaur to pellet the proteins. The pellet was then subjected to acid hydrolysis and thin layer electrophoresis as described in 3.12.2.

3.13.2. KOH Treated Gels.

As incubation of SDS-PAGE gels in 1 M KOH can result in the hydrolysis of peptide bonds, in addition to phosphate ester linkages, it was necessary to slightly adapt the elution procedure for proteins which had been subjected to this treatment.

Gel bands were excised into 30% (v/v) methanol, 10% (v/v) acetic acid and soaked for 3 hours before mincing into 500 µl of elution buffer and incubating overnight. The sample was then lyophilised before resuspending in pH 1.9 buffer for thin layer electrophoresis, as described in 3.12.

3.14. PROCESSING OF AUTORADIOGRAPHS.

X-ray film was sandwiched between dried gels, or TLE chromatograms, and a Dupont "Cronex" intensifier screen in a light proof cassette. Films were stored at -70°C during the exposure time.

3.15. DEVELOPMENT OF X-RAY FILM.

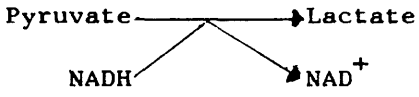
After exposure, autoradiographic images were visualized by immersing the film in SX-80 developer for 5 minutes, rinsing with water and then immersing in FX-40 fixer for 2 minutes. Films were finally rinsed in water before air drying.

3.16. ASSAY OF MARKER ENZYME ACTIVITIES.

To determine if the membrane isolation method employed was purifying plasma membrane and not another cellular membrane fraction, it was necessary to assay for the presence of enzyme activities which could be assigned to one, or at most two subcellular locations.

3.16.1. Lactate Dehydrogenase.

Lactate dehydrogenase was assayed by following a decrease in absorbance at 340 nm due to NADH being consumed in the reaction



The reaction cocktail, total volume 1 ml, consisted of a solution of 0.05 M Tris HCl pH 7.4 containing 0.32 M sucrose, 0.36 mM pyruvate, 100 µM NADH and 10 µl of a suitably diluted enzyme sample. The reaction was carried out at ambient temperature. The reaction was recorded using a Unicam spectrophotometer coupled to a Tarkan W + W Recorder 600 chart recorder.

Commercial lactate dehydrogenase (see Materials) was used as a positive control for the assay.

3.16.2. Fumarase.

Fumarase activity was determined by the method of Hill and Bradshaw (1969) by monitoring the appearance of fumarate, detected as an increase in absorbance at 240 nm.

The reaction cocktail, volume 1ml, contained 0.05 M Malic acid in 0.1 M potassium phosphate buffer pH 7.6 and 10 µl of suitably diluted enzyme plus Triton X-100, final concentration 0.05% (v/v), to disrupt the mitochondria. The reaction was carried out at 25°C and initiated by the additon of enzyme. The reaction was recorded on the equipment used in 3.16.1.

Commercial fumarase (see Materials) was used as a positive

Specific References for Methods of Assay of Marker Enzymes.

i) Lactate dehydrogenase:

Houslay, M.D. & Palmer, R.W. (1978) Changes in the form of Arrhenius plots of the activity of glucagon stimulated adenylate cyclase and other hamster plasma membrane enzymes upon hibernation: evidence consistent with a change in lipid bilayer assymetry. Biochem. J. 174, 909-919.

ii) Galactosyl transferase:

Heyworth, C.M., Wallace ,A.V. & Houslay, M.D. (1983) Insulin and glucagon regulate the activity of two distinct membrane-bound cAMP phosphodiesterases in hepatocytes. Biochem J. 214, 99-110.

iii) Cytochrome P420:

Omura, T. & Sato, R. (1964) The carbon monoxide binding pigment of liver microsomes. I. evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

iv) Catalase:

Evans, W.H. (1978) Preparation and characterisation of mammalian plasma membranes. Elsevier Biomedical Press, Amsterdam.

v) 5'-Nucleotidase:

Newby, A.C., Luzio, J.P & Hales, C.N. (1975) The properties and extracellular location of 5'-nucleotidase of the rat fat-cell plasma membrane. Biochem. J. 146, 625-633.

control for the assay.

3.16.3. 5'-Nucleotidase.

The activity of 5'-Nucleotidase was assayed by monitoring the release of ^{3}H-adenosine from ^{3}H-adenosine 5'-monophosphate (AMP).

A 10 µl sample containing the enzyme was mixed with 190 µl of a solution containing 50 mM triethanolamine hydrochloride pH 7.6, 10 mM magnesium sulphate and 1 mM AMP spiked with 2-[^{3}H]-AMP (100000 cpm/190 µl). The samples were incubated for 15 minutes at 30°C before the reaction was terminated by the addition of 40 µl of 0.15 M zinc sulphate. The samples were kept on ice for up to 3 hours before 40 µl of 0.3 N barium hydroxide was added. The incubation on ice was continued for a further 15 minutes after which the assays were centrifuged at 14000g for 3 minutes to remove the precipitate, and a 150 µl aliquot of the supernatant placed in 3 ml of Ecoscint and counted.

A standard curve of 5'-Nucleotidase activity was prepared using commercial enzyme (see Materials).

3.16.4. Catalase.

Catalase activity was measured by monitoring, at 240 nm, the evolution of oxygen upon hydrogen peroxide decomposition.

The reaction cocktail, volume 1 ml, contained 0.03% (v/v) hydrogen peroxide in 100 mM potassium phosphate buffer pH 7.4 and 10 µl enzyme. The reaction was carried out at ambient temperature and recorded using the same equipment as in 3.16.1.

The assay was tested using purified enzyme (see Materials).

3.16.5. Cytochrome P-420.

Cytochrome P-420, the inactive form of cytochrome P-450, is found within the microsomal fraction of cells and so was used as a marker for the presence of this subcellular fraction. The presence of cytochrome P-420 was determined through a carbon monoxide difference spectrum.

A reference and a sample tube containing 100 µg of sample, diluted to 1 ml, were reduced with sodium dithionite and the sample tube was bubbled with carbon monoxide and the absorbance monitored, over the range 400 to 500 nm, for the peak at 420 nm characteristic of this component.

The assay was tested using purified hepatic cytochrome P-450 (see Materials).

3.16.6. Galactosyl Transferase.

Galactosyl transferase was measured as UDP-galactose:N-acetyl-glucosamine transferase, which is responsible for the hydrolysis of UDP-galactose. The assay was carried out in the presence of N-acetyl-glucosamine which acts as an acceptor for the galactose released thus forming N-acetyl-aminolactose.

To disperse the golgi and release any enzyme latency 25 µl of sample in, 40 mM tris-HCl, pH 7.4, was mixed with 25 µl of 1% (v/v) Triton-X100, in 40 mM tris-HCl, pH 7.4, and incubated on ice for 10 minutes. 50 µl of assay cocktail (25 mM 2-mercaptoethanol, 4.25 mM magnesium chloride, 2.5 mM manganese chloride, 150 µM UDP-D-galactose and 2.25 mM N-acetyl-glucosamine) spiked with UDP-[U-^{14}C]-galactose (20,000 dpm/50 µl) was added and the reaction allowed to proceed for 15 minutes at 37^{o}C before quenching by the addition of 50 µl of 250 mM EDTA. The mix was then applied to 0.5 ml of a 1:2, resin:water, suspension of an anion exchange resin. The mix was incubated on ice, with occas ional vortexing, for 15 minutes. Finally, the samples were vortexed and centrifuged for 4 minutes in a MSE microcentaur and 250 µl of supernatant taken for scintillation counting.

The assay was tested and a standard curve prepared with purified enzyme (see Materials).

CHAPTER 4 : RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION.

4.1. EVALUATION OF RESTING B CELL ISOLATION.

4.1.1. T Cell Depletion.

A necessary step in the isolation of resting B cells from BALB/c splenocytes was the removal of T cells which account for approximately 50% of the lymphocyte population. T cells were depleted through the use of a Seralab T cell subset depletion kit comprising 4 different monoclonal antibodies directed against a variety of T cell specific membrane antigens.

Anti-mouse Thy-1 antibody binds to the Thy-1 antigen expressed on all murine T cells, while anti-mouse Lyt-1 antibody recognises the Lyt-1 antigen expressed on most murine T cells (Cobbold *et al.*, 1984, Aqel *et al.*, 1984). Anti-mouse Lyt-2 is an antibody directed against the Lyt-2 antigen expressed on cytotoxic T cells only (Ledbetter & Herzenberg, 1979), while the anti-mouse L3/T4 antibody recognises the L3/T4 antigen expressed on helper T cells (Dialynas *et al.*, 1983). Upon incubating a single cell suspension with these monoclonal antibodies and guinea pig complement, the antibodies bind to the relevant T cell antigens and the antibody-antigen complexes formed activate the classical complement pathway. Activation of this pathway leads to the formation of a C5-C9 membrane attack complex which disrupts the T cell membrane and so allows free exchange of electrolytes and water which leads to cell lysis (Roitt *et al.*, 1985).

As indicated in table 2 the lymphocyte number was typically reduced from 4.4×10^9 to 2.5×10^9, a depletion of 43%. As this figure correlates well with the percentage of T cells within the total lymphocyte population the depletion procedure was deemed to be successful.

Table 2: T Cell Depletion.

Red blood cell depleted lymphocytes were incubated with a 1/500 dilution of each antibody from a Seralab T cell subset depletion kit and a 1/40 dilution of Guinea pig complement for 40 minutes at 37°C. Cells were then harvested and the cell depletion determined.

Table 3: Recovery of Resting B Cells.

The T cell depleted lymphocyte population was added, in the 1.08 g/ml layer, to a percoll gradient (see figure 4). The gradient was centrifuged at 1400g for 30 minutes and the dense resting B cells collected from the 1.08-1.09 g/ml interface.

Table 2 T Cell Depletion

T Cell Number (before complement)	T Cell Number (after complement)	Percentage Depletion
4.4×10^9	2.5×10^9	43

Table 3 Recovery of Resting B Cells

Number of B Cells Added to Percoll Gradient	Number of B Cells Recovered at 1.08-1.09 Interface	Percentage Recovery
2.5×10^9	1.125×10^9	45

4.1.2. Separation of Resting and Large B Cells.

Percoll, a polyvinyl pyrollidone coated colloidal silica, is a useful gradient media for the separation of cell populations since, unlike other commonly used media, eg. sucrose or caesium chloride, it does not penetrate biological membranes and, therefore, does not affect the buoyant density of the cells being separated. It also does not exhibit the osmotic effects commonly seen with sucrose or caesium chloride and so more accurate buoyant densities may be determined.

Upon activation, B cells have been demonstrated to increase in cell volume (Rabin et al., 1985) and so their bouyant density falls. Therefore it is possible to separate small, dense, resting B cells from larger, less dense, activated B cell blasts by utilising a discontinuous percoll gradient.

It has been determined that the density of small resting B cells is such that upon centrifugation at 1400 g for 30 minutes in a discontious percoll gradient with density layers equivalent to 1.09, 1.08, 1.075, 1.065 and 1.055 g/ml, they will collect at the 1.08-1.09 g/ml interface (see fig.4). Larger activated B cells, because of their reduced density, collect at the 1.055-1.065 g/ml interface. Therefore, it is possible to separate the two populations of B cells (Ratcliffe and Julius, 1982).

Table 3 indicates the percentage of B cells recovered as small resting B cells and shows that the splenic B cell population was typically composed of 45% resting B cells.

4.2. OPTIMISATION OF B CELL ACTIVATION BY LPS AND THE EFFECT OF 2-MERCAPTOETHANOL.

4.2.1. Determination of LPS Concentration for Optimal B Cell Activation.

Lipopolysaccharide (LPS) has been reported to be a polyclonal activator of resting B cells and as such will drive these cells into the cell cycle resulting in cell proliferation and differentiation to antibody secreting cells (Andersson et al., 1972).

The optimal LPS concentration for B cell activation was determined by incubating resting B cells in the presence of different concentrations of LPS, for varying time periods, before harvesting onto nitrocellulose filters and counting the ^{3}H-methyl thymidine incorporated (see Methods). Table 4a shows the results from such an experiment and there are a number of interesting observations.

With each LPS concentration used, an increase in ^{3}H-methyl thymidine uptake and, therefore, cell activation was observed with time, up to 72 hours (see table 4a). This reflects the observation that B cells are activated asynchronously from resting (Melchers & Andersson, 1984), therefore as time proceeds there will be an increase in the number of cells entering the cell cycle which will result in an increase in the uptake of ^{3}H-methyl thymidine (see table 4a). However, by 96 hours the level of ^{3}H-methyl thymidine uptake had fallen. This fall may occur because by 96 hours the majority of B cells have been driven into the cell cycle and as LPS also drives B cells to differentiate into plasma secreting cells (Andersson et al., 1972), one would expect a decrease in the level of ^{3}H-methyl thymidine uptake as the primary function of plasma cells is the synthesis and secretion of immunoglobulin rather than proliferation to increase cell number. Alternatively, the fall could have resulted

Table 4a: 3H-Methyl Thymidine Uptake in Absence of 2-Mercaptoethanol.

Cells were incubated in complete medium in the presence of 0, 2.5, 5, 10 or 20 μg/ml LPS for 24, 48, 72 or 96 hours (the final 6 hours of culture was in the presence of 1 μCi/well), before harvesting. The data are presented as the mean of three determinations, and are given as counts per minute.

Table 4b: 3H-Methyl Thymidine Uptake in the Presence of 2-Mercaptoethanol.

Cell were treated as for 4a except the culture medium contained 50 μM 2-mercaptoethanol.

^{3}H - Methyl Thymidine Uptake in the Absence of 2 - Mercaptoethanol

Incubation Time (hr)	Concentration of LPS (µg/ml)				
	0	2.5	5	10	20
24	401	1,703	2,263	3,004	2,200
48	107	2,343	3,000	5,393	6,137
72	99	2,892	2,566	5,169	7,334
96	57	727	1,018	1,210	1,964

Table 4a

^{3}H - Methyl Thymidine Uptake in the Presence of 2 - Mercaptoethanol

Incubation Time (hr)	Concentration of LPS (µg/ml)				
	0	2.5	5	10	20
24	362	3,416	3,648	4,583	4,884
48	360	15,200	16,708	22,939	18,524
72	1,108	36,977	51,802	59,924	70,577
96	737	14,632	17,184	32,475	29,980

Table 4b

from nutrient depletion.

The optimal LPS concentration for activation with a 24 hour culture was observed to be 10 μg/ml, while 20 μg/ml was the optimal LPS concentration for activation at the longer incubations.

4.2.2. Effect of 2-Mercaptoethanol on LPS Activation.

B cells have been reported to require 2-mercaptoethanol for optimal growth in culture (Lemke & Opitz, 1976), therefore a second investigation into the optimal LPS concentration for B cell activation was carried out, this time in the presence of 2-mercaptoethanol (see Table 4b).

As with the previous experiment, ie. in the absence of 2-mercaptoethanol, there was an increase in the level of activation with time, up to 72 hours, for a given concentration of LPS. Therefore, as in the absence of 2-mercaptoethanol the B cells are activated asynchronously and activation reaches a maximum by 72 hours.

A comparison of tables 4a and 4b demonstrates that the degree of activation in the presence of 2-mercaptoethanol was greater than that observed in its absence, thus confirming the report that B cells, in culture, require 2-mercaptoethanol for maximal growth (Lemke & Opitz, 1976).

An interesting observation was that although the 72 hour culture exhibited maximal activation in the presence of 20 μg/ml LPS, as in the absence of 2-mercaptoethanol, the 48 and 96 hour cultures demonstrated maximal activation in the presence of 10 μg/ml LPS compared to 20 μg/ml in the absence of 2-mercaptoethanol.

In view of these results the concentration of LPS chosen for activation of resting B cells in culture, in the presence of 2-mercaptoethanol, was 10 μg/ml.

4.3. SILVER STAINING AS A MARKER FOR MEMBRANE PURIFICATION.

To determine the success of the plasma membrane isolation procedure, a comparison of the protein pattern obtained from B cell homogenate and plasma membrane preparations, separated on SDS-PAGE gels was carried out.

Silver staining is a very sensitive method of protein staining and will detect nanogram quantities (Wray et al., 1981). Therefore, this staining method will detect any remaining non-plasma membrane proteins present in the plasma membrane preparation.

Figure 5 shows a silver stain of cell homogenate and plasma membrane preparations separated by SDS-PAGE. Lane 2 contained 25 μg of cell homogenate while lane 1 contained 25 μg of plasma membrane. Due to the sensitivity of this method it was expected that a decrease in the amount of any homogenate non-plasma membrane protein would be observed upon plasma membrane preparation, rather than a total absence from the membrane lane. A decrease in the quantity of a number proteins was observed upon plasma membrane isolation (see figure 5) and, at least one, and possibly two, proteins appear to have been almost totally removed.

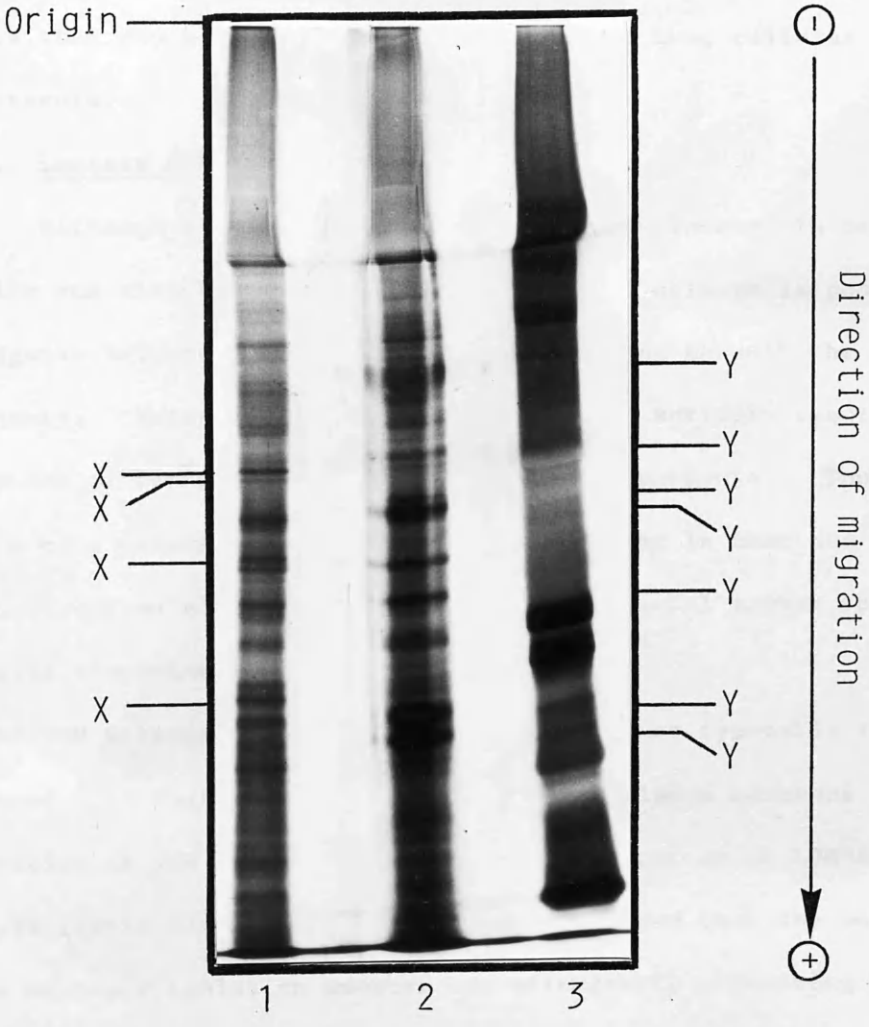
The purification of a given membrane fraction will also result in the enrichment of proteins in this fraction. Therefore, in addition to depleting non-plasma membrane protein, plasma membrane purification should enrich plasma membrane proteins relative to the levels observed in the homogenate sample. A comparison of lanes 1 and 2 indicates that a number of proteins were substantially enriched upon plasma membrane preparation.

In summary, the silver stain indicates that partial purification was achieved upon plasma membrane isolation. However this technique does not identify which compartment the enriched proteins are located in and therefore, is useful only as a supplement to marker enzyme

Figure 5: Silver Stained SDS-PAGE Gel of Resting B Cell Homogenate and Membrane.

Figure 5 depicts a silver stained SDS gel on which 25 µg of resting B cell homogenate (Lane 2) and plasma membrane (Lane 1) were separated. (Lane 3 contained low moleular weight markers.) The proteins depleted upon plasma membrane preparation are noted Y while those enriched in the plasma membrane preparation are noted X.

Silver stained SDS gel of resting B cell homgenate and membrane proteins.



assays which indicate the membrane fraction being enriched.

4.4. EVALUATION OF MARKER ENZYME ACTIVITIES.

In addition to identifying the component required in a cellular fractionation it is necessary to evaluate the purity of that fraction and to determine to what degree other subcellular compartments contaminate the preparation. To distinguish the different compartments it was necessary to identify and evaluate enzymatic markers that can be located in one, or at most two, cellular compartments.

4.4.1. Lactate Dehydrogenase.

Although lactate dehydrogenase (LDH) is located in both the cytosolic and mitochondrial compartments of the cell it is possible to distinguish between the two enzymatic activities through the use of detergents. Measurement of mitochondrial LDH activity requires the disruption of the mitochondrial membrane by detergents. Thus, in the absence of a detergent the LDH activity measured is that due to the cytosolic enzyme alone. Therefore LDH is a useful marker for the cytosolic compartment of cells.

The LDH activity in the B cell homogenate was typically in the region of 0.14 Units/mg (U/mg), while in the plasma membrane preparation it was 0.022 U/mg, a reduction in cytosolic LDH activity of 84.3% (Table 5). Therefore, it was concluded that the method of plasma membrane isolation adopted was effectively separating the cytosolic compartment from the plasma membrane fraction.

4.4.2. Fumarase.

Fumarase is a soluble enzyme in the Krebs cycle responsible for the conversion of malate to fumarate. Due to the location of this enzyme in the mitochondrial matrix it was necessary to carry out the assay in the presence of Triton X-100 to disrupt the mitochondria and allow the substrate, malate, access to the enzyme.

Table 5 indicates that a substantial depletion of mitochondrial

Table 5: Typical Marker Enzyme Activities.

The values presented in table 5 represent single experiments.

MARKER ENZYME ACTIVITIES

Enzyme	Specific Activity (Units/mg)		Percentage Reduction
	Homogenate	Membrane	
Lactate Dehydrogenase	0.14	0.022	84.3
Fumarase	0.017	0.003	82.3
Catalase	4.13	1.23	70.2
Galactosyl Transferase	6.25×10^{-4}	3.3×10^{-4}	47.2
5' Nucleotidase	0.001	0.007	—

Table 5

fumarase activity was achieved upon plasma membrane isolation. The fumarase activity measured in the B cell homogenate was typically 0.017 U/mg compared to an activity of 0.003 U/mg in the plasma membrane preparation, indicating a depletion of mitochondrial fumarase activity of 82.3%.

Thus, as with the cytosolic compartment, the mitochondria are being effectively removed during the isolation of B cell plasma membrane.

4.4.3. Catalase.

Catalase is an enzyme activity present in the peroxisomes of eukaryotic cells responsible for the degradation of hydrogen peroxide, a by-product of fatty acid and amino acid degradation (Darnell et al., 1986). As peroxisomes are membrane bound organelles the catalase activity displays considerable latency (Evans, 1978) and detergent treatment was again necessary to obtain maximal activity. As peroxisomes are difficult to separate from the bulk of the cytosolic compartment catalase activity is sometimes used as a cytosolic enzyme marker.

The high specific activity obtained for this enzyme in cell homogenate, typically 4.13 U/mg (Table 5), is a reflection of the significant amounts of catalase present in peroxisomes (Darnell et al., 1986). However, the activity was typically reduced to 1.23 U/mg in plasma membrane preparations, a decrease of 70.2%.

If catalase is considered a cytosolic enzyme then there is apparently a preferential removal of LDH, the other cytosolic enzyme measured. A more accurate explanation of the discrepancy between the percentage removal of LDH and catalase activities may be that the assay was detecting both cytoslic and mitochondrial LDH activity. Although no detergent was present to disrupt the mitochondrial membrane it was possible that some mitochondria were broken during the homogenisation procedure, thus releasing mitochondrial LDH.

Therefore the value for cytoplasmic LDH in the homogenate may be a slight overestimation, resulting in the apparently more efficient removal of the cytoplasmic fraction than suggested by the catalase assay. If this is so then the percentage depletion for catalase may be a more accurate determination of cytosolic removal. However this value was still high, 70.2%, and therefore the isolation procedure was acceptable.

4.4.4. Cytochrome P-420.

The existence of hepatic microsomal pigments was first reported by Klingenberg (Klingenberg, 1958). However, it was Omura and Sato who demonstrated the haemoprotein nature of these pigments (Omura & Sato, 1962 & 1964a) and also that, when reduced by sodium dithionite, they were capable of combining with carbon monoxide to give a characteristic absorption at 450 nm (Omura & Sato, 1962, 1964a & 1964b).

Cytochrome P-450 has since been identified in tissues other than liver (Nebert & Gelboin, 1969) including lymphocytes where it is linked to aryl hydrocarbon hydroxylase, a microsomal enzyme, located in the endoplasmic reticulum, induced by polycyclic hydrocarbons (Kellerman, et al., 1973).

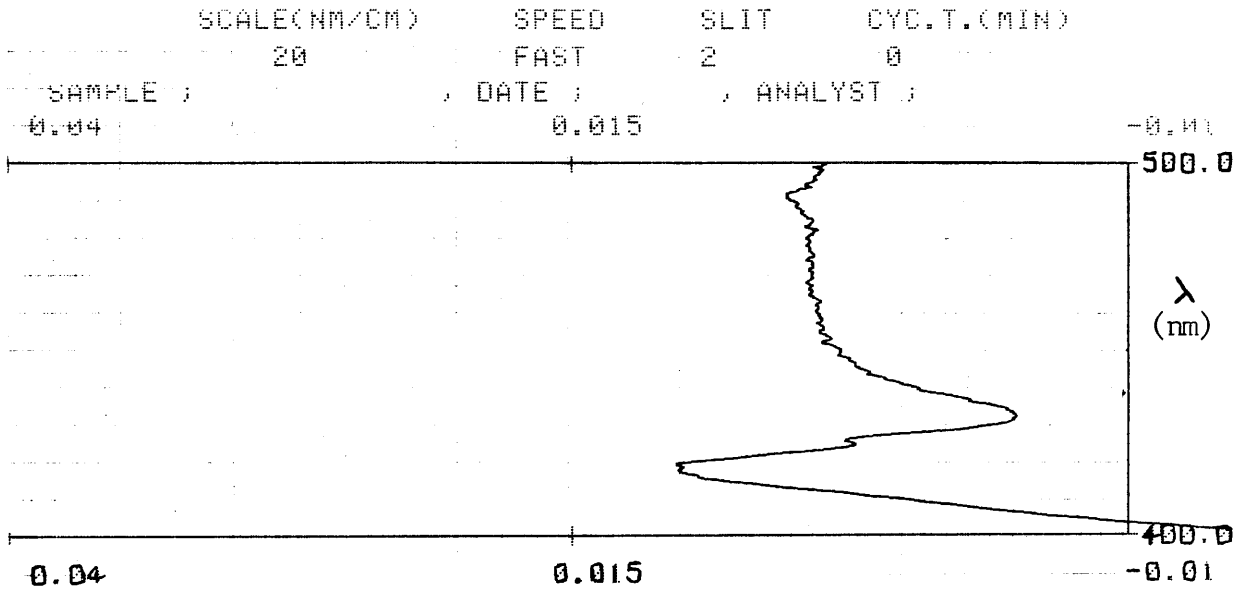
The isolation of cytochrome P-450 in an active form has been demonstrated to be a difficult undertaking (Omura & Sato, 1962) as under conditions of solubilisation the cytochrome P-450 was converted to a spectrally different, inactive form, P-420, so called because the peak of the carbon monoxide difference spectrum of the dithionite reduced pigment was shifted from 450 nm to 420 nm (Omura & Sato, 1964b).

As the plasma membrane isolation procedure utilised was designed to maintain plasma membrane integrity, rather than that of other subcellular membrane fractions, it was not possible to detect active cytochrome P-450 when a carbon monoxide difference spectrum was

Figure 6: Estimation of Cytochrome P-420.

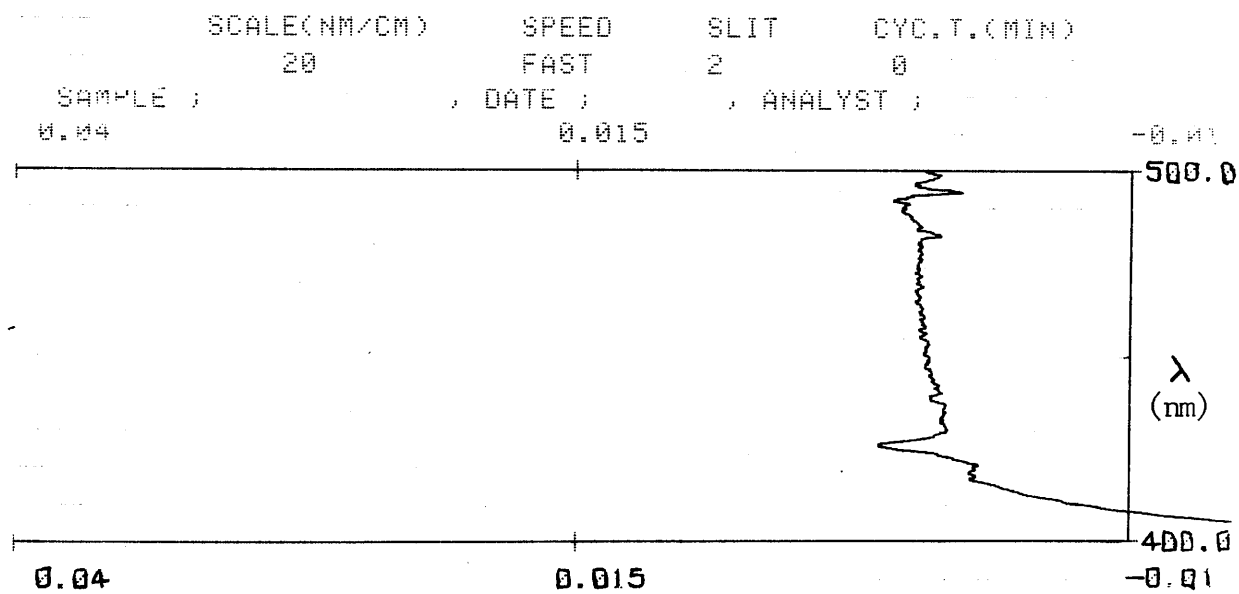
Figure 6 illustrates the carbon monoxide difference spectra of 100 µg of homogenate and plasma membrane preparations. The absorbance range was -0.01 to 0.04 and the wavelength range was 400 - 500 nm.

HOMOGENATE



ABSORBANCE

MEMBRANE



ABSORBANCE

Figure 6 Estimation of Cyt - P420

carried out. However, the inactive cytochrome P-420 form was detected.

Upon comparing the carbon monoxide difference spectrum of cellular homogenate and plasma membrane samples (see figure 6) it was determined that there was a reduction in the 420 nm absorption peak characteristic of cytochrome P-420. Had the plasma membrane isolation procedure purified the endoplasmic reticulum membrane fraction rather than plasma membrane then an increase in absorption at 420 nm, due to an enrichment of cytochrome P-420, would have been observed. Therefore, this assay demonstrated that the isolation procedure adopted was not purifying the endoplasmic reticulum fraction.

4.4.5. Galactosyl Transferase.

Galactosyl transferase was measured as UDP-galactose:N-acetylglucosamine galactosyl transferase which is responsible for the transfer of galactose from UDP-galactose to N-acetyl-glucosamine thus forming N-acetylaminolactose.

The specific activity of galactosyl transferase was typically reduced from 6.25×10^{-4} U/mg, in the cell homogenate preparations, to 3.3×10^{-4} U/mg, in the plasma membrane preparations, a reduction of 48.2% (see table 5). The less efficient removal of golgi membranes from the plasma membrane preparation is a reflection of the observation that golgi and plasma membrane fractions have similar densities (Evans, 1978), thus making the separation of these two compartments more difficult.

4.4.6. 5'-Nucleotidase.

5'-nucleotidase, the enzyme responsible for the conversion of adenosine-5'-monophosphate to adenosine and phosphate, has been shown to be present in both the plasma membrane and golgi fractions of rat liver preparations. However, it was demonstrated that sonication of murine lymphocytes did not cause an increase in 5'-nucleotidase

activity, therefore suggesting that this enzyme activity was essentially confined to the cell surface in lymphocytes (Uusitalo & Karnovsky, 1977a).

Evidence has been presented that there is a heterogeneity of 5'-nucleotidase activity within murine lymphocyte populations (Uusitalo & Karnovsky, 1977b, Allan & Crumpton, 1970). 5'-nucleotidase activity was reported to be similar in B and T lymphocyte populations. Enrichment of these populations gave varying results suggesting that there were sub-populations of both T and B lymphocytes, one which had high plasma membrane 5'-nucleotidase activity and a second that had little or no activity (Uusitalo & Karnovsky, 1977b). It was suggested that some of the 5'-nucleotidase heterogeneity observed within the lymphocyte population, was the result of changes in the proportion of these two sub-populations. However no identification of these sub-populations was presented (Uusitalo & Karnovsky, 1977b).

A typical example of the 5'-nucleotidase activity in resting B cell homogenate and plasma membrane preparations, presented in table 5, indicates an enrichment of this activity. The homogenate 5'-nucleotidase activity was typically 0.001 U/mg, while the activity in the plasma membrane preparations was 0.007 U/mg, a 7-fold enrichment (table 5). The measured plasma membrane 5'-nucleotidase activity of 0.007 U/mg correlates well with that reported by Uusitalo & Karnovsky (1977a).

4.4.7. Conclusion.

A summary of the average activities obtained for the above enzymes, both in cell homogenates and plasma membrane preparations is presented in figure 7. Evaluation of the activity of LDH, catalase and fumarase demonstrated that the procedure adopted for the isolation of plasma membranes was not enriching the cytosolic, peroxisomal or mitochondrial compartments of the cell. Although these activities were present in the plasma membrane preparation the activity remaining was only in the order of 20-30% of that observed in the whole cell homogenate. The reduction in galactosyl transferase activity obtained upon plasma membrane preparation was less than that achieved with the other marker enzymes and this is a reflection of the golgi and plasma membrane fractions exhibiting similar densities (Evans, 1978). As a result of this similarity it is more difficult to separate the golgi from the plasma membrane than it is to remove the other plasma membrane fractions.

By evaluating cytochrome P-420 absorption at 420 nm the degree of cytochrome P-420 present was determined and shown to be reduced during plasma membrane isolation (see figure 6), thus indicating that the membrane fraction isolated was not the endoplasmic reticulum.

Finally, the 7 fold increase in 5'-nucleotidase activity observed was indicative of a substantial purification of plasma membrane. As the results obtained indicated that the membrane fraction being enriched was the plasma membrane the preparation was henceforth referred to as plasma membrane.

Figure 7: Average Specific Activities for Marker Enzymes.

Each enzyme activity was expressed as $\bar{x} \pm SE$, where n = 5.

Clear bars represent the specific activity in resting B cell homogenate preparations.

Hatched bars represent the specific activity in resting B cell plasma membrane preparations.

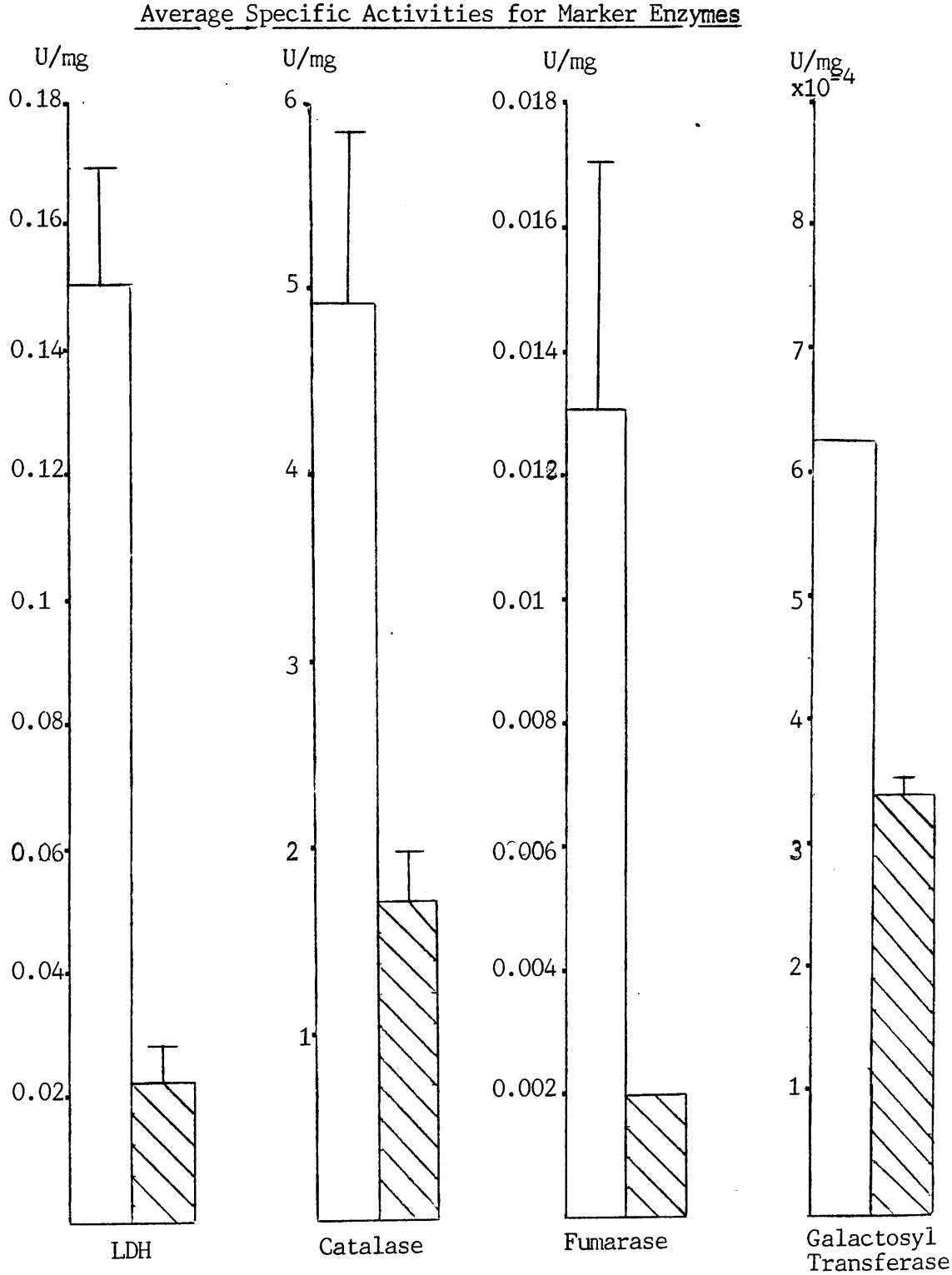

Average Specific Activities for Marker Enzymes
U/mg
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
LDH
U/mg
6
5
4
3
2
1
Catalase
U/mg
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
Fumarase
U/mg x10⁻⁴
8
7
6
5
4
3
2
1
Galactosyl Transferase

Figure 7

4.5. TCA PRECIPITATION OF TOTAL PHOSPHORYLATED MEMBRANE PROTEIN.

After terminating the membrane kinase reaction with loading buffer (see 3.8) 10 µl samples were precipitated with TCA as described (see 3.9). Table 6 illustrates the results of five such experiments.

In three of the five experiments there was a small decrease in total membrane protein phosphorylation with membranes prepared from cells cultured for 24 hour in the presence of LPS. However, in two of these experiments the decrease was very slight. In the remaining two experiments there was an apparent increase in total membrane protein phosphorylation in 24 hour LPS-stimulated B cell membranes. Therefore, it appeared that the increase in total membrane protein phosphorylation, in response to a 24 hour incubation with LPS, was variable.

After an incubation of 48 hours in the presence of LPS all samples exhibited an increase in total membrane protein phosphorylation over resting B cells, while four of the five samples demonstrated an enhancement compared to that observed after a 24 hour incubation.

As B cells are known to be activated asynchronously from the resting state (Andersson *et al.*, 1972, Melchers and Andersson, 1984) it was perhaps not surprising that the results obtained were variable.

The two experiments in which there was a pronounced increase in total membrane protein phosphorylation after 24 hours may be the result of the membrane sample having been prepared from cultures which contained a higher percentage of activated B cells than the others. That all experiments exhibited an increase after a 48 hour incubation with LPS also suggests that the B cells are being activated asynchronously as after 48 hours more cells would have been activated than at 24 hours and therefore one would expect the increased response

Table 6: TCA Precipitation of Membrane Protein.

Kinase reaction was carried out as described in 3.8. 10 µl samples were spotted onto Whatmann filter discs, the dried discs were then washed at 4°C for 20 minutes in 20% TCA followed by two washes in 10% TCA. A final 10 minute ethanol was carried out and the discs dried for counting.

TCA PRECIPITATION OF MEMBRANE PROTEIN

CPM

Resting B Cells	+ LPS 24 Hr	+ LPS 48 Hr
10,532 ± 1,082	15,169 ± 3,488	12,072 ± 910
18,439 ± 1,944	21,799 ± 1,758	22,746 ± 1,399
7,473 ± 81	7,351 ± 761	11,157 ± 942
8,034 ± 330	7,965 ± 195	11,556 ± 1,873
9,696 ± 1,028	7,423 ± 437	12,353 ± 1,432

(Samples expressed as $\bar{X} \pm SE$, n = 3)

Table 6

observed.

From the four experiments that exhibited an increase in total membrane protein phosphorylation between 24 and 48 hours the smallest increase was observed with a sample that had exhibited a pronouced enhancement by 24 hours. This result could have arisen if the initial B cell population had contained a higher percentage of cells that were in a more advanced activation state than those in the other cultures. Therefore, after a 24 hour culture a large proportion of the cells would have been activated, and so a further 24 hour incubation would have less effect on total membrane protein phosphorylation.

In summary, activation of resting B cells, after incubation with LPS for 48 hours results in an increase in total membrane protein phosphorylation. This observation could be visualised in autoradiographs of the SDS-PAGE gels on which the phosphorylated membrane proteins were separated (see figure 9). The 48 hour samples clearly demonstrated an increase in total phosphorylation as suggested by the TCA precipitation experiments.

4.6. TLE OF TOTAL PHOSPHORYLATED MEMBRANE PROTEIN.

In an attempt to identify which phosphoamino acids represented the increase in total membrane protein phosphorylation observed in response to LPS, thin layer elecrophoresis of acid hydrolysed membrane samples labelled with ^{32}P-γ-ATP was carried out as described in 3.12. Table 7 illustrates the results of a series of such experiments. The results for each phosphoamino acid are expressed as a ratio between control values, ie. resting B cells, and stimulated values, ie. 24 or 48 hour LPS-stimulated, or as a ratio between 24 and 48 hour LPS-stimulated cultures.

After incubating B cells in the presence of LPS for 24 hours four of the six experiments illustrated a variable increase in membrane phosphotyrosine levels relative to those observed in resting B cells.

Table 7: TLE Analysis of Phosphorylated Plasma Membrane Phosphoamino-acids.

The individual phosphoamino-acids were scraped from the TLE plate into 3 ml of ecoscint and counted.

Ratios were determined between

i) resting B cell membranes and membrane from 24 hr LPS blasts;

ii) resting B cell membrane and membrane from 48 hr LPS blasts;

iii) membrane from 24 and 48 hr LPS blasts.

Ratios were calculated as X:Y,

where,

$$Y = \frac{\text{cpm in Y}}{\text{cpm in X}}$$

TLE Analysis of Plasma Membrane Phosphoamino-acids

Resting B Cells : 24 Hr LPS Blasts

Ptyr	Pthr	Pser
1:0.69	1:1.33	1:1.65
1:0.48	1:0.78	1:0.32
1:1.5	1:3.4	1:7.15
1:1.48	1:1.3	1:3.67
1:1.17	1:0.71	1:1.35
1:1.4	1:1.29	1:4.9

Resting B Cells : 48 Hr LPS Blasts

Ptyr	Pthr	Pser
1:0.66	1:0.86	1:1.51
1:0.55	1:0.45	1:0.48
1:3.38	1:4.11	1:5.35
1:0.93	1:7.0	1:8.32
1:0.48	1:0.45	1:0.57

24 Hr LPS Blasts : 48 Hr LPS Blasts

Ptyr	Pthr	Pser
1:0.96	1:0.65	1:0.91
1:1.15	1:0.57	1:1.48
1:2.25	1:1.2	1:0.75
1:0.63	1:1.31	1:2.26
1:0.41	1:0.64	1:0.42

Table 7

An increase in phosphothreonine levels was also observed in four of the six samples, while five of the six samples examined demonstrated an increase in phosphoserine levels after a 24 hour incubation in the presence of LPS. The increase in all the phosphoamino acid levels in these 24 hour cultures was, however, variable.

TCA precipitation of two 24 hour LPS-stimulated B cell membrane preparations indicated an increase in total membrane protein phosphorylation levels. However, TLE studies of the membrane samples demonstrated an increase in phosphothreonine and phosphotyrosine in four samples and enhancement in phosphoserine in five samples. Even if the smaller increases observed in the phosphotyrosine and phosphothreonine content were disregarded, the larger increases in phosphoserine occur more frequently than the increases in total protein phosphorylation, as indicated by TCA precipitation. Thus there was no apparent correlation between the increase in total phosphorylation observed and the alterations in the levels of the individual phosphoamino acids.

Upon comparing the phosphoamino acid levels in membranes from cells cultured for 48 hours in the presence of LPS with resting B cells only one of the five samples exhibited an increase in phosphotyrosine content; four samples had reduced levels of phosphotyrosine. This suggests that between 24 and 48 hours there was a reduction in tyrosine phosphorylation rates, or an increase in phosphotyrosine phosphatase activity. This observation was supported by a comparison of 24 and 48 hour culture phosphoamino acid levels, where three of the five samples exhibited a decrease in phosphotyrosine content.

In addition three of the five 48 hour samples exhibited a decrease in phosphothreonine content, while two exhibited a reduction in phosphoserine levels, compared to resting B cell levels. This suggests that there may also be a decrease in threonine and serine

phosphorylation, or an increase in dephosphorylation, between 24 and 48 hours in these samples. These same samples demonstrated relative reductions in phosphothreonine and phosphoserine content between 24 and 48 hours thus supporting this suggestion.

The variable nature of the TLE results make it difficult to derive any firm conclusions about which phosphoamino acids are consistently represented in the changes in total membrane protein phosphorylation observed upon TCA precipitation. Also the reductions in phosphoamino acid levels exhibited after a 48 hour culture in the presence of LPS are in direct contrast to the results of the TCA precipitation experiments, which indicate an increase in membrane phosphorylation.

Using the phorbol ester, TPA (12-o-tetradecanoyl phorbol-13-acetate), a polyclonal activator of human B cells, it has been demonstrated that tyrosine phosphorylation, as a percentage of the overall phosphorylation, was not consistently increased in the triton insoluble material derived from human peripheral B lymphocytes cultured for 24 hours with TPA. This was apparently due to a parallel enhancement of serine and threonine phosphorylation (Nel et al., 1985). If the results obtained from the 24 hour LPS stimulated membranes are expressed in this manner (see table 8a & 8b) only the phosphoserine levels repeatedly exhibited an increase relative to resting levels. The phosphotyrosine level was either unchanged or decreased in response to LPS stimulation. Therefore the increase in total membrane phosphorylation appeared to occur mainly through an enhancement in phosphoserine levels and not via alterations in phosphothreonine or phosphotyrosine content.

The variablity observed with the TLE results may be due to the asynchronous manner in which B cells are activated or, it may result from the method of quantitating the radiolabel incorporated into each phosphoamino acid. As the phosphoamino acids remain close to each

Tables 8a & 8b: Phosphoamino-acid Levels as a Percentage of Total Membrane Phosphorylation.

Percentages were calculated as

$$\frac{\text{cpm of a given phosphoamino-acid}}{\text{cpm of all 3 phosphoamino-acids}} \times 100$$

Phosphoamino Acid Levels as a Percentage of Total Membrane Phosphorylation

Resting B Cells

	Ptyr	Pthr	Pser
Sample 1	24	28	48
2	31	24.5	44.5
3	32	24	44
4	51	20	29
5	18	22	60
6	29	32	39

Table 8a

24 Hour LPS Blasts

	Ptyr	Pthr	Pser
Sample 1	13	28	59
2	31	41	28
3	11	18	71
4	37	13	51
5	18	13	69
6	29	29	42

Table 8b

other upon TLE separation (see figure 8), inaccurate excision of these areas of the TLE plate matrix may result in cross contamination.

There are, however, possible solutions to these technical problems. After culture with LPS any unactivated, ie. resting B cells, remaining could be separated from the blasts by the use of a discontinuous percoll gradient similar to that used in the initial isolation of resting B cells (see 3.3). This would provide a B cell population that was enriched in LPS activated blasts and therefore should reduce variability due to the asynchronous activation of resting B cells. The problem of cross-contamination upon TLE plate scraping could possibly be overcome by increasing the size of TLE plates used thus increasing the distance between the separated phosphoamino acids.

An alternative to TLE for the quantitation of protein kinase activities is the use of exogenous peptides containing a single defined phosphorylation site. The addition of such peptides to kinase reactions allows the quantitation of a given class of kinase activity independent of any other class of kinase present. Nel et al. employing such methods determined that there was an increase in tyrosine kinase activity upon the addition of TPA to human peripheral B cells (Nel et al., 1985). Such a peptide assay system is currently in use in this laboratory for the quantitation of interleukin driven murine lymphocyte kinase activities (G. McGarvie, personal communication). Such a system may help to reduce the inconsistencies observed with TLE studies and allow a more accurate quantitation of alterations in phosphoamino acid levels upon activation of murine B lymphocytes.

Figure 8: Autoradiograph of TLE Separation of Phosphorylated B Cell Membrane.

The autoradiograph presented in figure 8 illustrates that the phosphorylation pattern obtained upon 2-dimensional TLE separation of ^{32}P labelled, acid hydrolysed resting B cell membrane.

Ty = phosphotyrosine

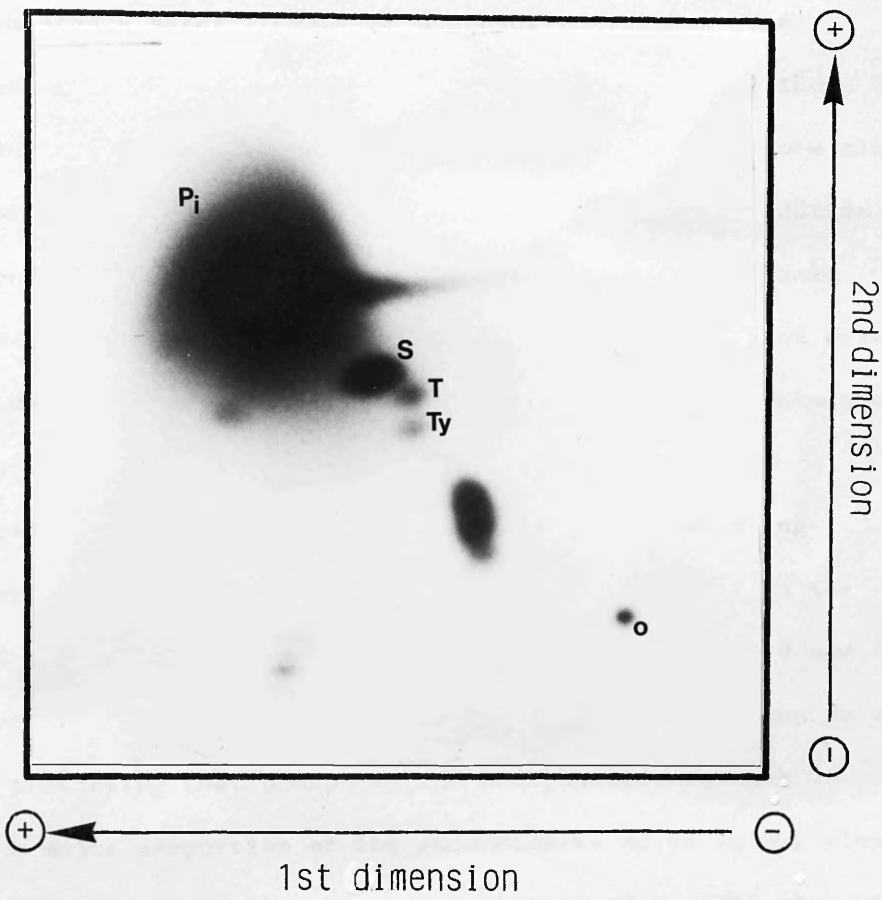
T = phosphothreonine

S = phosphoserine

Pi = free phosphate

O = origin

Autoradiograph of TLE separation of phosphorylated B cell membrane protein.



4.7. COMPARISON OF KOH TREATED AND UNTREATED SDS-PAGE GELS.

Although the TLE studies indicated that the overall level of tyrosine phosphorylation was unaltered upon stimulation of resting B cells by LPS, the possibilty still remained that the distribution of phosphotyrosine within the membrane proteins was altered. Therefore it was necessary to examine the phosphorylation pattern of separated membrane proteins

The phosphate ester linkage of phosphotyrosine has been demonstrated to be more stable under alkali conditions than those of phosphoserine and phosphothreonine (Plimmer, 1941). Therefore alkali treatment of SDS-PAGE gels has been utilised in the identification of proteins containing phosphotyrosine. Autoradiographs of alkali treated SDS-PAGE gels, on which ^{32}P-labelled membrane proteins were separated, will therefore be enriched for phosphoproteins containing phosphotyrosine after such treatment.

A comparison of KOH treated and untreated gels containing membrane samples from resting B cells and B cells cultured in the presence of LPS for 24 and 48 hours is presented in figures 9 and 10. KOH treatment markedly decreased the level of phosphorylation in all gel tracks indicating that phosphoserine and phosphothreonine contribute a major proportion of the phosphoamino acids in the plasma membrane proteins. There was one major protein (M.W. 47K) observed in the KOH treated gel that was present in all samples and appeared to be most heavily labelled in the LPS stimulated 48 hour sample (see lane E of figure 10). A number of other minor bands were also observed. The resolution of these minor bands could possibly be improved by removing any salt present in the gel. These results indicated that although phosphothreonine and phosphoserine are the predominant phosphoamino acids in B cell plasma membranes phosphotyrosine was apparently present in a number of membrane

Figure 9: SDS-PAGE Gel of Phosphorylated Membrane.

Each lane contained 100 μg of phosphorylated membrane protein.

Lane A = resting B cell membrane.

Lane B = resting B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

Lane C = 24 hr LPS stimulated B cell membrane.

Lane D = 24 hr LPS stimulated B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

Lane E = 48 hr LPS stimulated B cell membrane.

Lane F = 48 hr LPS stimulated B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

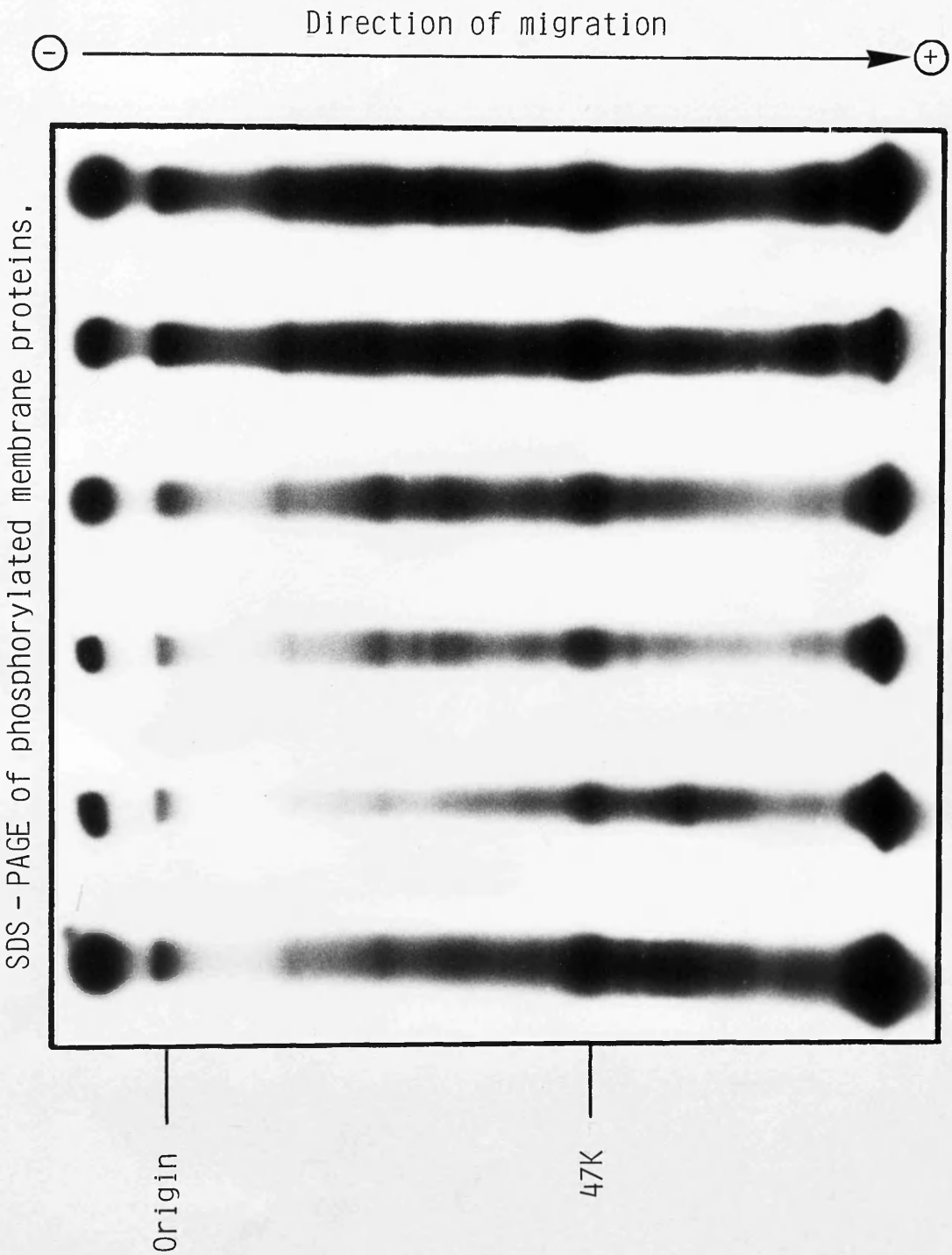

Direction of migration
⊖
⊕
SDS - PAGE of phosphorylated membrane proteins.
Origin
47K
A
B
C
D
E
F

Figure 10: KOH Treated SDS-PAGE Gel of Phosphorylated Membrane.

Each lane contained 100 μg of phosphorylated membrane protein.

Lane A = resting B cell membrane.

Lane B = resting B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

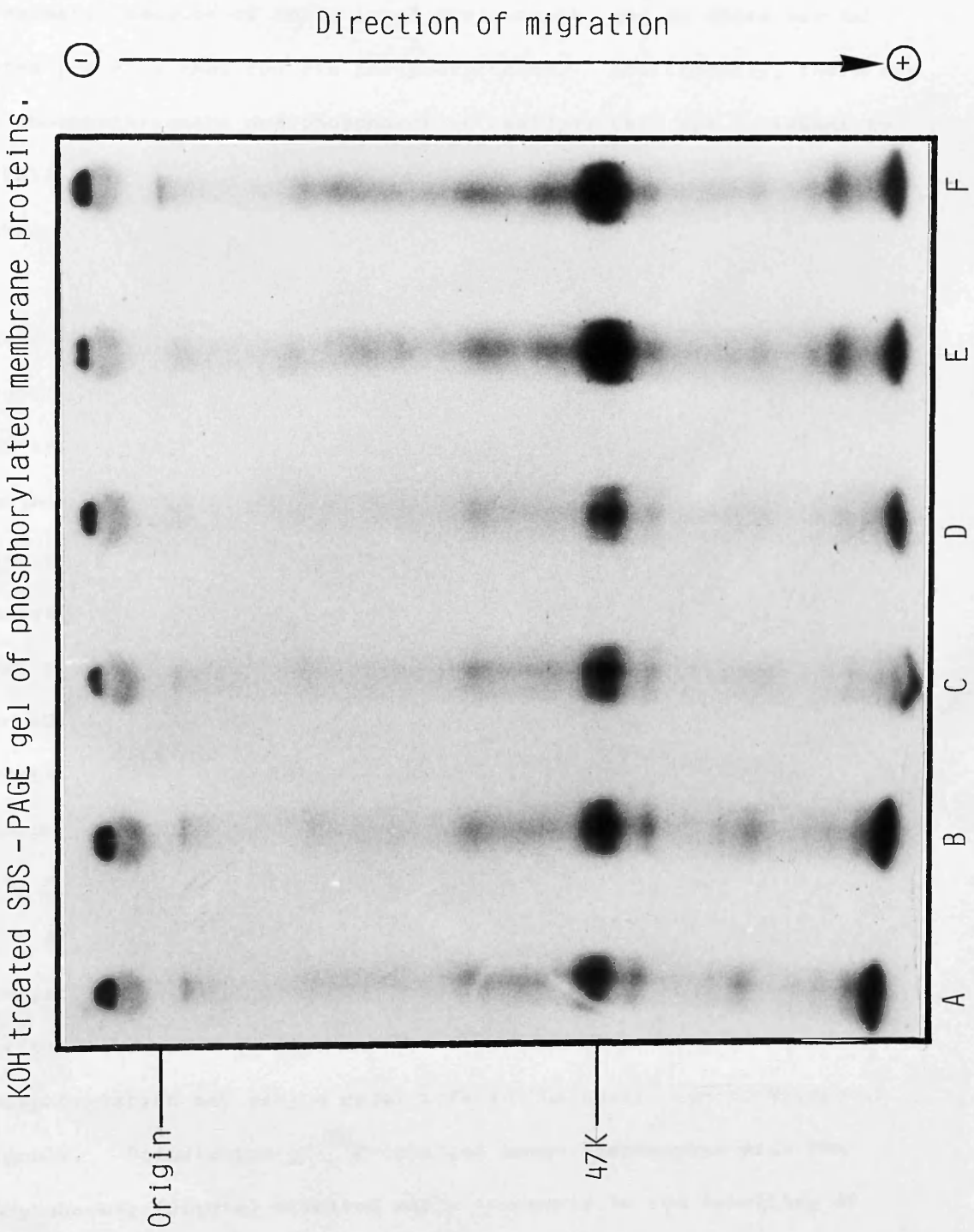
Lane C = 24 hr LPS stimulated B cell membrane.

Lane D = 24 hr LPS stimulated B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

Lane E = 48 hr LPS stimulated B cell membrane.

Lane F = 48 hr LPS stimulated B cell membrane phosphorylated in the presence of 50 U/ml IL-4.

Gel was incubated for 90 minutes in 1 M potassium hydroxide/ 5 mM sodium di-hydrogen phosphate at 55°C.



KOH treated SDS - PAGE gel of phosphorylated membrane proteins.

proteins, and the level of this phosphoamino acid appeared to be increased upon activation by LPS, at least in the 47K protein band.

4.8. TLE OF SDS-PAGE PROTEIN BANDS.

When carrying out alkali treatment of SDS-PAGE gels it should be remembered that not all phosphotyrosine residues are resistant, presumably because of their local environment, and so there may be extra proteins that contain phosphotyrosine. Additionally, there may be phosphothreonine and phosphoserine residues that are resistant to alkali and so one should not assume that the proteins remaining after alkali treatment contain phosphotyrosine (Cooper et al., 1983).

In view of these observations it was necessary to positively identify the phosphoamino acids in the 47K protein. Therefore the protein from the 48 hour LPS-stimulated sample was excised from both KOH treated and untreated gels, as described in 3.13.1 and 3.13.2, and subjected to thin layer electrophoresis, as described in 3.12. Figure 11a and 11b illustrates the pattern of phosphoamino acid analysis obtained from these samples. The autoradiographs indicate that while phosphoserine and phosphotyrosine were present in the non-KOH treated sample only phosphotyrosine was detected in the KOH treated sample. It was not possible to quantitate the level of the phosphoamino acids due to the low level of radioactivity present.

4.9. CONCLUSIONS.

As lymphocytes contain all the machinery required for phosphorylation and dephosphorylation, and exhibit a variety of kinase activities, (Piras et al., 1977) alterations in protein phosphorylation may play a major role in the expression of mitogenic signals. Stimulation of ^{32}P-labelled human lymphocytes with PHA (phytohaemagglutinin) elicited early increases in the labelling of many endogenous proteins. This phosphorylation was dependent on mitogen dose and exhibited a concentration threshold similar to that

Figure 11a: Autoradiograph of the TLE Separation of 47Kd Protein.

The autoradiograph presented in figure 11a illustrates the phosphoamino acid pattern obtained upon 2-dimensional TLE separation of the ^{32}P-labelled 47KD protein from B cell, plus LPS 48 hr, membrane.

Pi = free phosphate

Ty = phosphtyrosine

S = phosphoserine

o = origin

Autoradiograph of the TLE separation of 47Kd protein

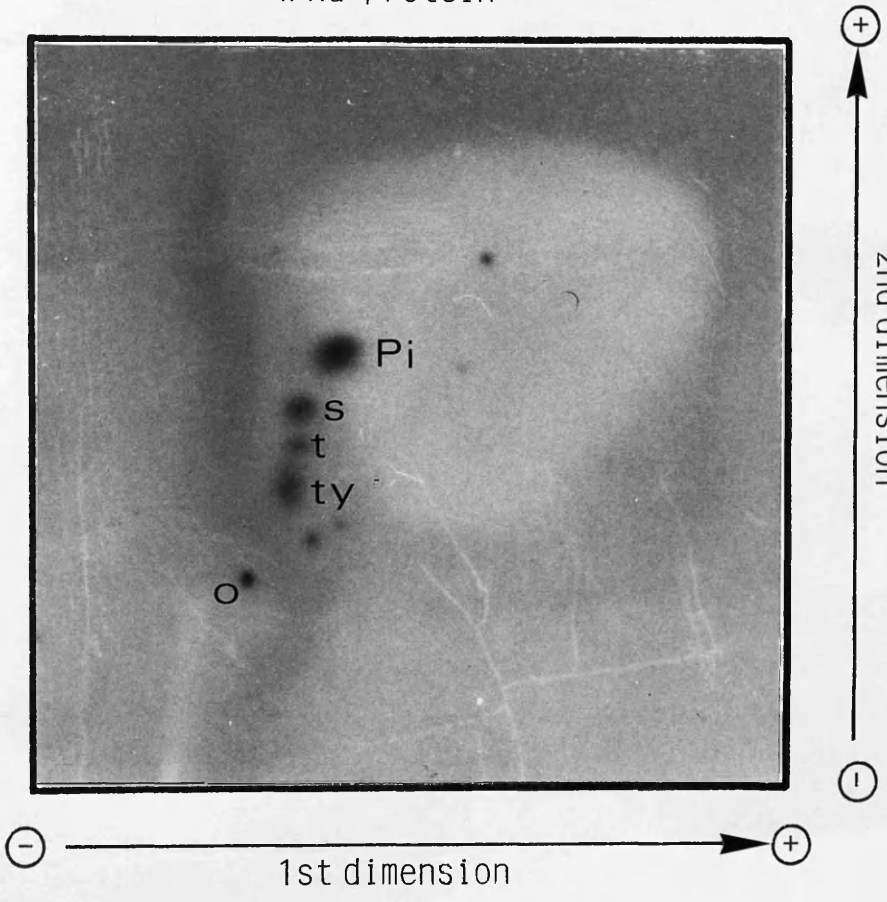


Figure 11b: Autoradiograph of the TLE Separation of KOH Treated 47Kd Protein.

The autoradiograph presented in figure 11b illustrates the phosphoamino acid pattern obtained upon 2-dimensional TLE separation of the ^{32}P-labelled 47KD protein from B cell, plus LPS 48 hr, membrane. The gel from which the protein was eluted was incubated for 90 minutes at 55^{o}C in 1 M KOH.

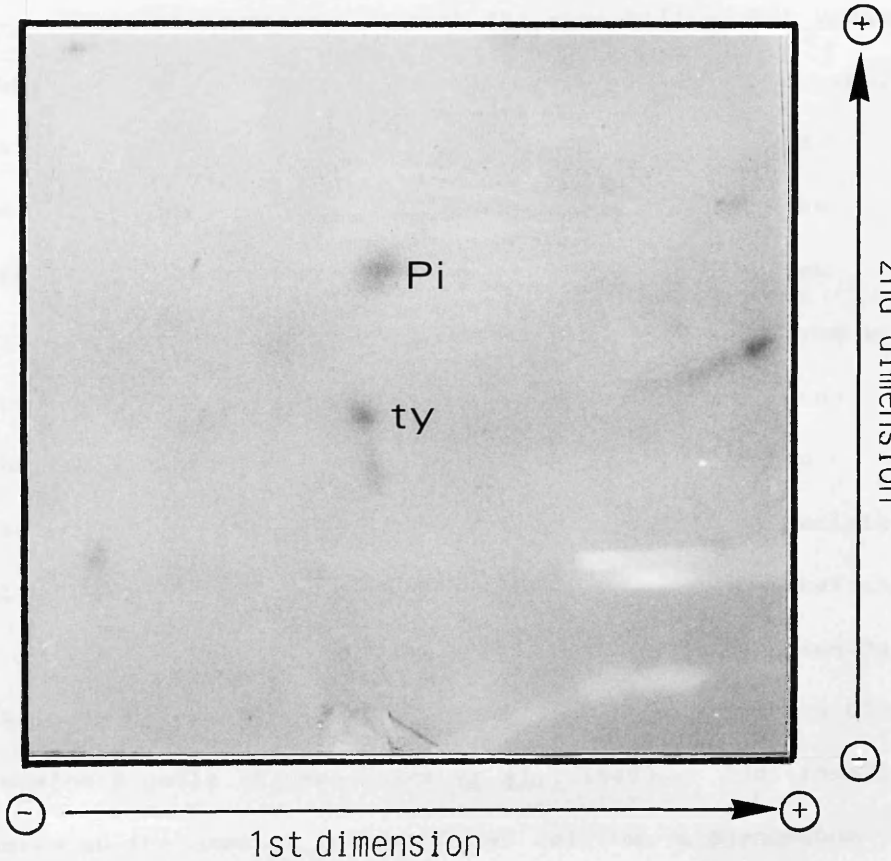
Pi = free phosphate

Ty = phosphtyrosine

S = phosphoserine

o = origin

Autoradiograph of the TLE separation of
KOH treated 47Kd protein



of PHA induced lymphocyte activation, ie. 10 µg/ml (Chaplin et al., 1980). The B cell mitogen anti-Ig has been demonstrated to cause, upon receptor crosslinking, an increase in phosphorylation of cytosolic proteins in rabbit B cells, one of which appears to be membrane associated (Dasch & Stavitsky, 1985). This same mitogen was identified as causing an increase in tyrosine phosphorylation of human B cell membranes upon interaction with surface immunoglobulin (Nel et al., 1984). These observations support the possibility that protein phosphorylation is involved in mitogenic activation of lymphocytes.

The calcium ionophore A23187 mimics mitogen activation of lymphocytes (Ogawa et al., 1981) suggesting that calcium may be important in lymphocyte activation. Protein kinase C has been identified in large quantities in lymphocytes and as this enzyme is calcium dependent there may be a link between mitogen action and protein kinase C activity (Ogawa et al., 1981). PHA has been demonstrated to induce phosphoinositide hydrolysis and to stimulate DAG production in T cells (Fisher & Mueller, 1968), while crosslinking of surface immunoglobulin by anti-Ig has been identified as causing the induction of polyphosphoinositide degradation and elevation of DAG levels in murine B cells (Bjisterbosch et al., 1985). Additionally anti-Ig causes an increase of intracellular calcium, a phenomenon associated with the activation of the PI cycle (see figure 2) (Ransom et al., 1986). This evidence suggests that the mitogenic agents PHA and anti-Ig act through induction of the PI cycle to activate protein kinase C and so cause the enhanced phosphorylation observed upon activation of lymphocytes with these agents.

Protein kinase C has been demonstrated to be specifically activated by TPA (Castagna et al., 1982). As the receptor for this phorbol ester copurifies with protein kinase C it has been proposed that protein kinase C itself is the phorbol ester receptor (Niedel et al., 1983) and that TPA activates protein kinase C directly and not

through DAG production via phosphoinositide hydrolysis. The murine B cell polyclonal activators LPS and PMA (4-phorbol 12-myristate 13-acetate) fail to induce polyphosphoinositide degradation, DAG elevation or calcium mobilisation in B cells (Bjisterbosch et al., 1985), thus it appears that these polyclonal activators by-pass the initial first steps characteristic of lymphocyte activation by PHA and anti-Ig.

Lipid X, a biologically active lipid moiety of LPS which retains B cell mitogenicity, has been demonstrated to activate protein kinase C, as does the free lipid portion of LPS, lipid A (Raetz et al., 1983). The lipid X moiety is able to insert into the plasma membrane of cells and as protein kinase C translocation, from cytoplasm to plasma membrane, has been reported to occur in response to mitogenic stimulation of B cells by anti-Ig, PMA and LPS (Chen et al., 1985) the possibility arises that LPS, like TPA, activates protein kinase C directly.

Anti-Ig crosslinking of surface immunoglobulin has been demonstrated to cause an increase in B cell membrane tyrosine phosphorylation (Nel et al., 1984). Therefore, activation of the PI cycle and protein kinase C are not the only mechanisms involved in the increased membrane phosphorylation observed upon mitogenic activation of lymphocytes.

The phorbol ester TPA has been demonstrated to cause enhancement of tyrosine phosphorylation in both lymphoid (Nel et al., 1985) and non-lymphoid (Gilmore & Martin, 1983) systems. As no receptor for TPA other than protein kinase C has yet been identified, and as protein kinase C has been demonstrated to phosphorylate only serine and threonine residues (Castagna, 1982, Nishizuka, 1984), it is not known how TPA causes this increase in tyrosine kinase activity. It may be that TPA activates a second kinase, one with tyrosine kinase activity, or it may inactivate a tyrosine phosphatase (Gilmore &

Martin, 1983). That the mitogenic agents TPA (Nel et al., 1985) and anti-Ig (Nel et al., 1984) have been reported to cause an increase in tyrosine phosphorylation lent support to the hypothesis that tyrosine phosphorylation was an important mechanism in lymphocyte activation.

The data presented in this report idicates that activation of resting murine B cells by the polyclonal activator LPS, results in an increase in total membrane phosphorylation. TLE analysis of phosphorylated total membrane suggests that this increase is the result of enhanced serine phosphorylation rather than phosphorylation of threonine or tyrosine. However the variability of the results demonstrates that a more sensitive method of quantitation will be required to accurately determine alterations in the phosphoamino acid levels in response to LPS stimulation.

The data from the SDS-Page analysis of LPS stimulated B cell membranes suggests that tyrosine phosphorylation, in addition to serine phosphorylation, may be altered upon such treatment, at least in a 47 Kd protein. Thus the possibilty arises that although no alterations in tyrosine phosphorylation were detected at the overall membrane level there may be changes occurring at the level of individual proteins.

Figure 12 : IL-4 Bioassay.

Figure 12 illustrates the results of a IL-4 bioassay. 10^5 cells were cultured for 56 hours in the presence of

1. medium alone
2. IL-4, (5 U/ml)
3. Goat anti-mouse Ig M ($G_\alpha M$ IgM), (5 μg/ml)
4. IL-4 (5 U/ml) and $G_\alpha M$ IgM (5 μg/ml)

or 5. LPS (10 μg/ml)

before pulsing, for 16 hours, with 1 uCi ^{3}H-methyl thymidine. The cells were then harvested and the level of ^{3}H-methyl thymidine incorporated expressed as counts per minute ($\bar{x} \pm SE$, n = 6).

IL4 BIOASSAY

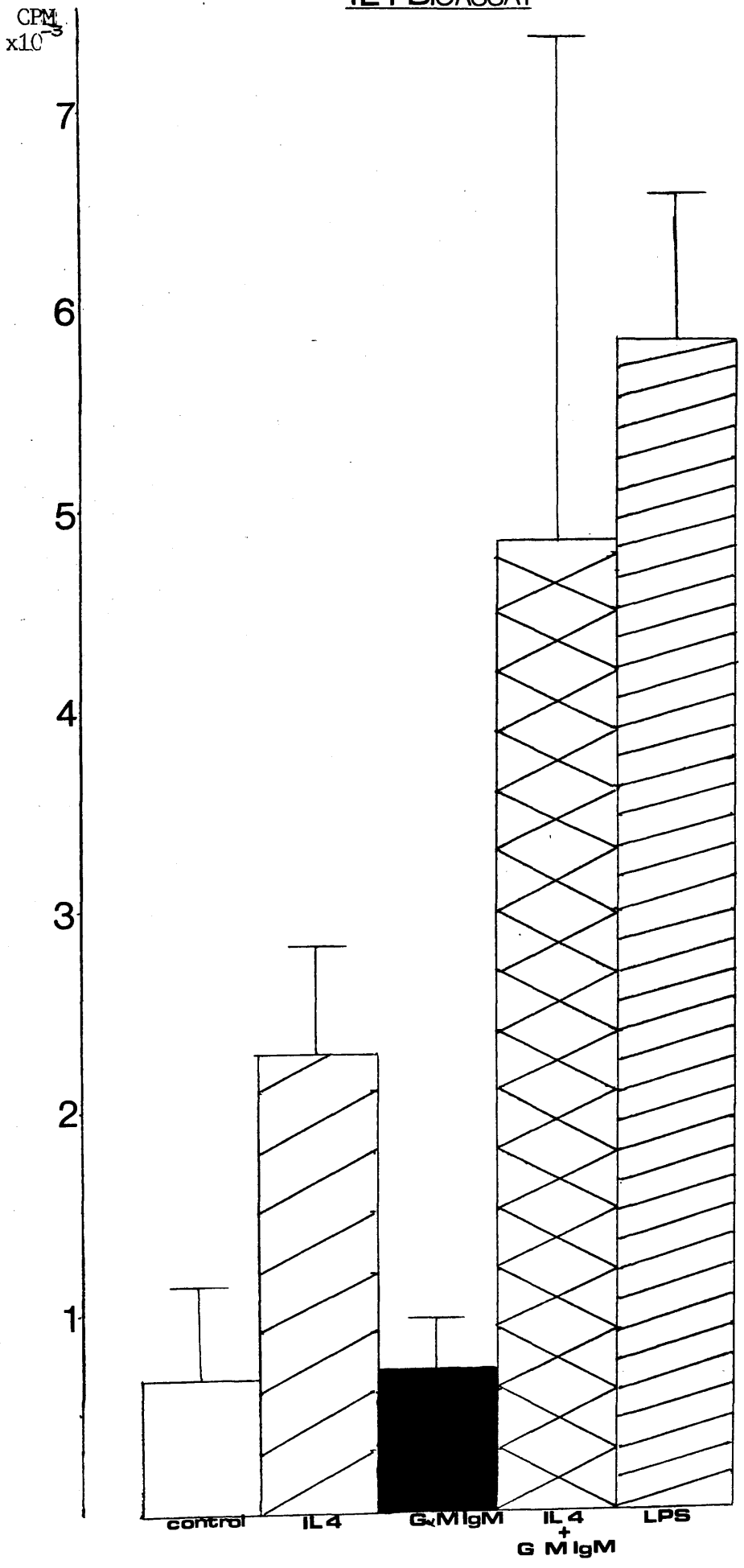


Figure 12

4.10. EFFECT OF INTERLEUKIN-4 ON MEMBRANE PHOSPHORYLATION.

While the investigation into changes in protein phosphorylation upon activation of B cells by LPS was underway, it was reported that murine B cell LPS blasts expressed higher numbers of IL-4 receptors than were expressed on resting B cells. DBA/2 resting splenic B cells were estimated to express 311 +/- 23 receptors per cell compared to 1511 +/- 331 receptors per cell on DBA/2 B cell LPS blasts (Ohara & Paul, 1987). In view of this a parallel study, using some of the techniques developed for the LPS investigations, was undertaken to address the question whether B cells and/or LPS blasts exhibited any alterations in membrane protein phosphorylation patterns in response to IL-4.

4.10.1. Interleukin-4 Bioassay.

Interleukin-4 (IL-4) has been demonstrated to act, with antigen or anti-Ig, as a costimulator in B cell activation (Howard *et al.*, 1982). The bioassay of Ohara *et al.* (1985) was employed to demonstrate that the affinity purified IL-4 was biologically active (figure 12).

It has been demonstrated that antigen alone cannot drive resting B cells into the cell cycle (Noelle *et al.*, 1983, Cambier *et al.*, 1982). Therefore, the minimal activation, above control, observed in the presence of goat anti-mouse IgM was not an unexpected result. As extended contact between B cells and anti-Ig M causes B cells to enter the cell cycle in the absence of lymphokines(Defranco *et al.*, 1982), the small level of activation observed in the presence of antigen alone may have occurred as a result of this mechanism. Alternatively the activation observed was possibly the result of residual contaminating T cells. However, as there was no significant activation, above control levels, the data suggest that the B cell population was successfully depleted of T cells.

Although it was reported that B cells respond to antigenic stimulation after, rather than before, exposure to IL-4 (Oliver et al., 1985), this same study demonstrated a small activation, over control, of B cells cultured in the presence of antigen prior to IL-4 (Oliver et al., 1985). Therefore, although IL-4 may be regarded as a competence factor that prepares cells to respond to antigenic stimulation it may also exhibit growth factor activity and cause proliferation of B cells which have already encountered antigen, either in the presence or absence of IL-4. An explanation for the observation that incubation of B cells with IL-4 alone resulted in an activation of B cells, above that observed for cells incubated with goat-anti-mouse IgM or medium alone, may therefore be that there were cells present which had already encountered antigen and it was these cells that responded to IL-4.

Upon culture in the presence of both IL-4 and goat-anti-mouse IgM there was a large increase, over control, in B cell activation. The level of activation achieved approached that obtained with LPS, a polyclonal activator that drives resting B cells into the cell cycle, thus demonstrating that the IL-4 was acting, with goat-anti-mouse IgM, as a costimulator of B cell activation.

4.10.2. TCA Precipitation of Total Membrane Protein Phosphorylated in the Presence of IL-4.

Kinase reactions were carried out in the presence of IL-4 and samples TCA precipitated as before (see 3.9). The results of five such experiments are illustrated in table 9.

A comparison of the total membrane protein phosphorylation, as indicated by TCA precipitation, of samples phosphorylated in the presence or absence of IL-4 (see tables 6 & 9) indicated that incubation of resting B cell membranes with IL-4, prior to phosphorylation, did not result in an increase in total membrane protein phosphorylation. Only two samples exhibited an increase in

Table 9: TCA Precipitation of Membrane Protein, (Kinase Reaction + IL-4).

Kinase reaction was carried out as described in 3.8.1. (ie. in the presence of 50 U/ml IL-4). 10 µl samples were spotted onto Whatmann filter discs, the dried discs were then washed at 4°C for 20 minutes in 20% TCA followed by two washes in 10% TCA. A final 10 minute ethanol was carried out and the discs dried for counting.

TCA PRECIPITATION OF MEMBRANE PROTEIN

(Kinase Reaction + IL-4)

	CPM	
Resting B Cells	+ LPS 24 Hr	+ LPS 48 Hr
10,210 ± 1,313	22,326 ± 5,207	14,710 ± 1,808
14,478 ± 474	23,275 ± 4,100	19,179 ± 649
7,552 ± 642	9,651 ± 1,748	10,309 ± 583
7,886 ± 263	8,003 ± 601	11,285 ± 891
7,985 ± 1,548	8,500 ± 909	13,918 ± 1,431

(Samples expressed as $\bar{X} \pm SE$, n = 3)

Table 9

response to IL-4 and in three samples a reduction in phosphorylation was observed.

B cells which had been activated with LPS were estimated to express higher levels of IL-4 receptors (1511 +/- 331 per cell) than resting B cells (311 +/- 23 per cell). It was, therefore, hoped that if transmembrane signalling upon IL-4 binding to its receptor was mediated via changes in membrane protein phosphorylation these alterations would be more easily detected in LPS blasts.

All five experiments involving 24 hour B cell LPS blast membranes phosphorylated in the presence of IL-4 exhibited increases in total membrane phosphorylation relative to non IL-4-treated membranes (tables 6 & 9). However the increases observed were variable between samples, and very small in three of the samples. In addition the two samples which exhibited the largest increases also demonstrated variablity within the sample, reflected in the high standard deviation. When 48 hour LPS B cell blast membranes were utilised only two samples exhibited small increases in phosphorylation.

The results obtained however did not convincingly indicate that incubation of B cell membranes with IL-4 resulted in any alterations in total membrane protein phosphorylation. However the possibilty that there were changes at the level of individual proteins was not excluded by this approach.

4.10.4. SDS-PAGE Analysis of ^{32}P-γ-ATP Labelled, IL-4 Treated, B Cell Membranes.

Samples of plasma membrane protein from resting B cell and 24 and 48 hour LPS-stimulated cultures, phosphorylated in the presence of IL-4, were separated on SDS-PAGE gels and a comparison of KOH treated and untreated gels carried out (see figures 9 & 10). There was no apparent enhancement of overall phosphorylation, or alteration in phosphoprotein pattern observed, in either of the gels upon incubation in the presence of IL-4. This suggested that neither phosphoserine,

phosphothreonine or phosphotyrosine levels were significantly altered upon membrane incubation in the presence of IL-4, either at the total membrane protein or individual protein level. This correlated with the results of the TCA precipitation experiments which suggested that IL-4 had no significant effect on total membrane protein phosphorylation, either in resting B cells or in LPS blasts.

There was no apparent alteration in phosphorylation of the 47K protein upon addition of IL-4 thus suggesting, that although phosphorylation of this protein is possibly involved in mitogenic activation of lymphocytes it does not appear to be involved in the action of IL-4. However the possibility that IL-4 phosphorylates membrane proteins in vivo (ie. in the intact cell) has not been excluded by this study.

4.10.4. Conclusions.

The indication that addition of IL-4 to kinase reactions did not result in alterations of membrane protein phosphorylation was initially disappointing as Justement et al. had reported that incubation of a plasma membrane enriched preparation, from BDF_1 B cells, incubated in the presence of of IL-4 resulted in the enhanced phosphorylation of a 44K endogenous protein (Justement et al., 1986). As BALB/c splenic B cells were used in this study, rather than the BDF_1 cells used by Justement et al., there is the possibility that the protein observed in BDF_1 cells is either not present, or is not phosphorylated, in BALB/c cells. Also no phosphoamino acid analysis was presented by Justement et al. and so the identity of the phosphoamino acids in this protein have not been elucidated.

Class I HLA antigens are located at the plasma membrane and are composed of a 44,000 dalton glycoprotein heavy chain and a 12,000 dalton noncovalently associated sub-unit, β2-microglobulin (Ploegh et al., 1981). The 44,000 dalton heavy chain has been identified as a substrate for protein kinase C in vitro and is also phosphorylated in

vivo in response to TPA, a known activator of protein kinase C (Shackelford and Trowbridge, 1986). Although the phosphoamin o acid species identified in the class I HLA antigen was phosphoserine the possibility remains that other undetected phosphoamino acids are present. Therefore the possibility arises that the 44,000 dalton protein identifed by Justement et al. may be a class I HLA antigen.

In addition to the initial report, by Ohara and Paul (Ohara & Paul, 1987), estimating IL-4 receptor number a second report has recently been presented by Park et al. (Park et al., 1987). This latest report indicated that splenic resting B cells from C57BL/6J mice express only 65 +/- 5 receptors per cell, assuming each receptor binds only one IL-4 molecule, compared to the figure of 311 +/- 23 receptors per cell estimated by Ohara and Paul. In addition Park et al. estimated that B cell LPS blasts expressed 320 +/- 65 receptors per cell whereas Ohara and Paul had estimated 1511 +/- 331 receptors per cell, an almost 5-fold difference. One explanation for these discrepencies may be that the initial study of Ohara and Paul utilised affinity purified IL-4 while Park et al. used recombinant IL-4. Therefore the possibility arises that the IL-4 used by Ohara and Paul contained a factor, or factors, other than IL-4 which bound to the B cells resulting in an overestimation of IL-4 receptor number.

Such a difference could also have arisen if, as in the IL-2 system (Robb et al., 1981 & 1984), there are two classes of IL-4 receptor each with different affinities. If this is so then it is possible that Park et al. are detecting only one class of receptor while Ohara and Paul detected two, thus resulting in the higher receptor number estimated.

Alternatively there may actually be differences in IL-4 receptor number between different strains of mice. IL-4 prepares DBA/2 B cells to respond to LPS more efficiently than it does BALB/c B cells (Rabin et al., 1986) and this may be a reflection of differences in

IL-4 receptor number on splenic B cells from different strains of mice.

In view of this latest report on IL-4 receptor number it may be necessary to develop very sensitive methods for the detection of any alterations in tyrosine kinase activity occurring in response to IL-4.

4.11 GENERAL CONCLUSION.

The data presented in this thesis indicate that there is an overall increase in total membrane phosphorylation in response to activation of murine B cells by the polyclonal activator LPS. TLE studies of total phosphorylated membrane suggested that this increase was the result of enhanced phosphoserine content rather than an elevation of phosphothreonine and phosphotyrosine levels. However, the results obtained from these TLE studies were variable and so it will be necessary to utilise more sensitive methods, such as peptide phosphorylation assays, for the quantitation of alterations in kinase activity upon B cell activation.

Although the TLE studies suggested that enhanced phosphoserine levels were responsible for the increase in total membrane phosphorylation the possibility exists that there are alterations in phosphothreonine and phosphotyrosine content at the level of individual proteins. A protein of M.W. 47K was chosen for TLE analysis as this appeared to be a protein that exhibited increased phosphorylation upon activation of resting B cells with LPS. Despite both phosphoserine and phosphotyrosine being identified in this protein it was not possible to estimate the levels of each phosphoamino acid. As the amount of protein involved is very small quantitation of the individual phosphoamino acids will require the development of more efficient methods of protein recovery.

The possib le involvement of phosphorylation in lymphocyte activation in response to LPS will have to be supplemented by *in vivo* studies involving the incubation of intact cells labelled, in the absence and presence of LPS, with ^{32}-P orthophosphate. This is necessary to ensure that any changes in membrane phosphorylation are not artifacts of an *in vitro* assay system.

Although no alterations in phosphorylation levels were detected

in the IL-4 studies the data in this report cannot eliminate the possibility that changes in kinase activity do occur in response to IL-4. Techniques, such as synthetic peptide assays, with increased sensitivity will be required to determine if the action of IL-4 involves phosphorylation either at serine residues, as does IL-2 (Shackelford and Trowbridge, 1986), or at tyrosine residues.

REFERENCES

Aharonov, A., Pruss, R.M. & Herschman, H.R. (1978) Epidermal growth factor. I. Relationship between receptor regulation and mitogenesis in 3T3 cells. Journal of Biological Chemistry, 253, 3970-3977.

Allan, D. & Crumpton, M.J. (1970) Preparation and characterization of the plasma membrane of pig lymphocytes. Biochemical Journal, 120, 133-143.

Andersson, J., Sjoberg, O. & Moller, G. (1972) Induction of immunoglobulin synthesis in vitro by lipopolysaccharides. European Journal of Immunology, 2, 349-353.

Andersson, J., Coutinho, A. & Melchers, F. (1979) Mitogen activated B cell blasts reactive to more than one mitogen. Journal of Experimental Medicine, 149, 553-564.

Andersson, J., Schreier, M. H. & Melchers, F. (1980) T cell dependent B cell stimulation is H-2 restricted and antigen dependent only at the resting B cell level. Proceedings of the National Academy of Science, USA, 77, 1612-1616.

Ashwell, J.D., DeFranco, A.L., Paul, W.E. & Schwartz, R.H. (1984) Antigen presentation by resting B cells. Radiosensitivity of the antigen presenting function and two distinct pathways of T cell activation. Journal of Experimental Medicine, 159, 881-905.

Aqel, N.M., Clark, M., Cobbold, S.P. & Waldmann, H. (1984) Immunohistological screening in the selection of monoclonal antibodies, the use of isotype specific anti-immunoglobulins. Journal of Immunological Methods, 69, 207-214.

Baluyut, A.R. & Subbarao, B. (1986) Synergistic effects of anti-IgM and monoclonal anti-Ia antibodies in induction of B lymphocyte proliferation. Federation Proceedings, 45, 618.

Berridge, M.J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyses polyphosphoinositides instead of phosphatidylinositol. Biochemical Journal, 212, 849-858.

Berridge, M.J. & Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature, 312, 315-321.

Betal, I., Martijnse, J. & van der Berg, K.J. (1974) Absence of an early increase of phospholipid phosphate turnover in mitogen stimulated B lymphocytes. Cellular Immunology, 14, 429-434.

Beung, H. & Hayman, M.J., (1984) Temperature sensitive mutants of avian erythroblastosis virus. Surface expression of the erb-B product correlates with transformation. Cell, 36, 963-972.

Bevan, M.J., Epstein, R. & Cohn, M. (1974) Effect of 2-mercaptoethanol on murine mixed leukocyte cultures. Journal of Experimental Medicine, 139, 1025-1030.

Bjisterbosch, M.K., Meade, C.J., Turner, G.A. & Klaus, G.G.B. (1985) B lymphocyte receptors and phosphoinositide degradation. Cell, 41, 999-1006.

Booth, R.J., Prestidge, R.L. & Watson, J.D. (1983) Constitutive production by the WEHI-3 cell line of a B cell growth and differentiation factor that copurifies with interleukin 1. Journal of Immunology, 131, 1289-1293.

Boss, M.A., Dreyfus, G. & Baltimore, D. (1981) Localisation of the Abelson Murine Leukemia Virus protein in a detergent insoluble subcellular matrix, architecture of the protein. Journal of Virology, 40, 472-481.

Bowen-Pope, D.F. & Ross, R. (1982) Platelet derived growth factor. Specific binding to cultured cells. Journal of Biological Chemistry, 257, 5161-5171.

Braun, J., Sha'afi, R.I. & Unanue, E.R. (1979) Crosslinking by ligands to suface - Ig triggers mobilisation of intracellular $^{45}Ca^{2+}$ in B lymphocytes. Journal of Cellular Biology, 82, 755-766.

Burnet, F.M. (1957) A modification of Jerne's theory of antibody production using the concept of clonal selection. Australian Journal

of Science, 20, 67-82.

Cambier, J.C., Monroe, J.G. & Neale, M.J. (1982) Definition of conditions that enable antigen specific activation of the majority of isolated trinitrophenol binding B cells. Journal of Experimental Medicine, 156, 1635-1649.

Cambier, J.C., Monroe, J.G., Coggeshall, K.M., Ransom, J.T. (1985) The biochemical basis of transmembrane signalling by B lymphocyte surface immunoglobulin. Immunology Today, 6, 218-222.

Cambier, J.C., Chen, Z.Z., Ransom, J.T., Klemsz, M.J., Harris, L.K., Sandoval, V.M. & Newell, M.K. (1986) Molecular evidence for transmembrane signalling by B cell membrane immunoglobulin and Ia antigens. Federation Proceedings, 45, 618.

Cantrell, D.A. & Smith, K.A. (1983) Transient expression of interleukin 2 receptors. Consequences for T cell growth. Journal of Experimental Medicine, 158, 1895-1911.

Carpenter, G., King Jr., L. & Cohen, S. (1979) Rapid enhancement to protein phosphorylation in A431 cell membrane preparations by epidermal growth factor. Journal of Biological Chemistry, 254, 4884-4891.

Casnellie, J.E., Harrison, M.L., Pike, L.J., Hellstrom, K.E. & Krebs, E.G. (1982) Phosphorylation of synthetic peptides by a tyrosine protein kinase from the particulate fraction of a lymphoma cell line. Proceedings of the National Academy of Science, USA. 79, 282-286.

Casnellie, J.E., Gentry, L.E., Rohrschneider, L.R. & Krebs, E.G. (1984) Identifications of the tyrosine protein kinase from LSTRA cells by use of site specific antibodies. Proceedings of the National Academy of Science, USA. 81, 6676-6680.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) Direct activation of calcium activated phospholipid dependent protein kinase by tumour promoting phorbol esters. Journal of Biological Chemistry, 257, 7847-7851.

Chaplin, D.D., Wedner, H.J. & Parker, C.W. (1980) Protein phosphorylation in human peripheral blood lymphocytes, mitogen induced increases in protein phosphorylation in intact lymphocytes. Journal of Immunology, 124, 2390-2398.

Chen, Z.Z., Coggeshall, K.M. & Cambier, J.C. (1985) Translocation of protein kinase C during membrane Ig-mediated transmembrane signalling in B lymphocytes. Journal of Immunology, 136, 2300-2304.

Cobbold, S.P., Jayasuriya, A., Nash, A., Prospero, T.D. & Waldmann, H. (1984) Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. Nature, 312, 548-551.

Coffman, R.L., Ohara, J., Bond, W., Carty, J., Zlotnik, E. & Paul, W.E. (1986) B cell stimulatory factor 1 enhances the IgE response of lipopolysaccharide activated B cells. Journal of Immunology, 136, 4538-4541.

Coggeshall, K.M. & Cambier, J.C. (1984) B cell activation. III. Membrane immunoglobulins transduce signals via activation of phosphatidylinositol hydrolysis. Journal of Immunology, 133, 3382-3386.

Coggeshall, K.M. & Cambier, J.C. (1985) Bcell activation. VI. Effects of exogenous diacylglycerol and modulators of phospholipid metabolism suggests a central role for diacylglycerol generation in transmembrane signalling by membrane immunoglobulin. Journal of Immunology, 134, 101-107.

Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature, 296, 613-617.

Cohen, S., Carpenter, G. & Lembach, K.J. (1975) Interaction of epidermal growth factor (EGF) with cultured fibroblasts. Advances in Metabolic Disorders, 8, 265-284.

Collett, M.S. & Erikson, R.L. (1978) Protein kinase associated with the avian sarcoma virus src gene product. Proceedings of the National Academy of Science, USA. 75, 2021-2024.

Cooper, J.A., Bowen-Pope, D.F., Raines, E., Ross, R. & Hunter, T. (1982) Similar effects of platelet derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. Cell, 31, 263-273.

Cooper, J.A., Sefton, B.M. & Hunter, T. (1983) Detection and quantification of phosphotyrosine in proteins. Methods in Enzymology, 99, 387-402.

Corvera, S., Whitehead, R.E., Mottola, C. & Czech, M.P. (1986) The insulin like growth factor II receptor is phosphorylated by a tyrosine kinase in adipocyte plasma membranes. Journal of Biological Chemistry, 261, 7675-7679.

Coutinho, A. & Moller, G. (1975) Thymus independent B cell induction and paralysis. Advances in Immunology, 21, 113-236.

Czech, M.P. (1977) Molecular basis of insulin action. Annual Review of Biochemistry, 46, 359-384.

Dasch, J.R. & Stavitsky, A.B. (1985) Mitogen induced phosphorylation of cytosolic proteins in rabbit T and B lymphocytes. Molecular Immunology, 22, 379-389.

Darnell, j., Lodish, H. & Baltimore, D. (1986) Molecular Cell Biology, pp166. Scientific American Press.

Defranco, A., Raveche, E., Asofsky, R. & Paul, W.E. (1982) Frequency of B lymphocytes responsive to anti-immunglobulin. Journal of Experimental Medicine, 155, 1523-1536.

Dialynas, D.P., Quan, Z.S., Wall, K.A., Pierres, A., Quintas, J., Loken, M.R., Pierres, M. & Fitch, F.W. (1983) Characterisation of the murine T cell surface antigen designated L3/T4, identified by monoclonal antibody GK1.5. Similarity of L3/T4 to the human Leu 3/T4 molecule. Journal of Immunology, 131, 2445-2451.

DiSabato, G., Chen, D.M. & Erickson, J.W. (1975) Production by murine spleen cells of an activity stimulating the PHA responsiveness of thymus lymphocytes. Cellular Immunology, 17, 495-504.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessing, J. & Waterfield, M. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature, 307, 521-527.

Earp, H.S., Austin, K.S., Buessow, S.C. Dy R. & Gillespie, G.Y. (1984) Membranes from T and B lymphocytes have different patterns of tyrosine phosphorylation. Proceedings of the National Academy of Science, USA. 81, 2347-2351.

Earp, H.S., Austin, K.S., Gillespie, G.Y., Buessow, S.C., Davies, A.A. & Parker, P.J. (1985) Characterisation of distinct tyrosine specific protein kinases in B and T lymphocytes. Journal of Biological Chemistry, 260, 4351-4356.

Eckhart, W., Hutchinson, M.A. & Hunter, T. (1979) An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell, 18, 925-933.

Ek, B. & Heldin, C.H. (1982) Characterization of a tyrosine specific kinase activity in human fibroblast membranes stimulated by platelet derived growth factor. Journal of Biological Chemistry, 257, 10486-10492.

Ek, B., Westermark, B., Wasteson, A. & Heldin, C.H. (1982) Stimulation of tyrosine specific phosphorylation by platelet derived growth factor. Nature, 295, 419-420.

Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. & Rutter, W.J. (1986) Replacement of insulin receptor residues 1162 and 1163 compromises insulin stimulated kinase activity and uptake of 2-deoxyglucose. Cell, 45, 721-732.

Erikson, R.L., Collett, M.S., Purchino, A.F., Brugge, J.S. & Erikson, E. (1979) A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. Proceedings of the National Academy of Science, USA. 76, 3159-3163.

Erikson, R.L., Purchio, A.F., Erikson, E., Collett, M.S. & Brugge, J.S. (1980) Molecular events in cells transformed by Rous Sarcoma Virus. Journal of Cellular Biology, 87, 319-325.

Evans, W.H. (1978) Preparation and Characterisation of Mammalian Plasma Membranes, pp121. Elsevier/North Holland Biomedical Press.

Farrar, W.L., Johnson, H.M. & Farrar, J.J. (1981) Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin 2. Journal of Immunology, 126, 1120-1125.

Farrar, W.L. & Anderson, W.B. (1985) Interleukin 2 stimulates association of protein kinase C with the plasma membrane. Nature, 315, 233-235.

Fisher, D.B. & Mueller, G.C. (1968) An early alteration in the phospholipid metabolism of lymphocytes by phytohaemaggutinin. Proceedings of the National Academy of Science, USA. 60, 1396-1402.

Forsgren, S., Pobor, G., Coutinho, A. & Pierres, M. (1984) The role of I-A/E molecules in B lymphocyte activation. I. Inhibition of lipopolysaccharide induced responses by monoclonal antibodies. Journal of Immunology, 133, 2104-2110.

Foulkes, J.G. & Rosner, M.R. (1985) Molecular Mechanisms of Transmembrane Signalling, pp 217-252. Elsevier Biomedical Press.

Frackelton Jr., A.R., Tremble, P.M. & Williams, L.T. (1984) Evidence for the platelet derived growth factor stimulated tyrosine phosphorylation of the platelet derived growth factor receptor in vivo, Immunopurification using a monoclonal antibody to phosphotyrosine. Journal of Biological Chemistry, 259, 7907-7915.

Galanos, C. & Luderitz, O. (1975) Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. European Journal of Immunology, 54, 603-610.

Gillis, S., Mochizuki, D.Y., Conlon, P.J., Hefeneider, S.H., Ramthun, C.A., Gillis, A.E., Frank, M.B., Henney, C.S. & Watson, J.D. (1982)

Molecular characterization of interleukin 2. Immunological Reviews, 63, 167-209.

Gilmer, T.M. & Erikson, R.L. (1981) Rous sarcoma virus transforming protein, p60src, expressed in E.Coli, functions as a protein kinase. Nature, 294, 771-773.

Gilmore, T. & Martin, G.S. (1983) Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature, 306, 487-489.

Grupp, S.A. & Harmony, J.A.K. (1985) Increased phosphatidylinositol metabolism is an important but not an obligatory early event in B lymphocyte activation. Journal of Immunology, 134, 4087-4091.

Haring, H.U., White, M.F., Kahn, C.R., Kasuga, M., Lauris, V., Fleischmann, R., Murray, M. & Pawelek, J. (1984) Abnormality of insulin binding and receptor phosphorylation in an insulin resistant melanoma cell line. Journal of Cellular Biology, 99, 900-908.

Heldin, C.H., Ek, B. & Ronnsrannd, L. (1983) Characterisation of the receptor for platelet derived growth factor on human fibroblasts. Journal of Biological Chemistry, 258, 10054-10061.

Hill, R.L. & Bradshaw, R.A. (1969) Fumarase. Methods in Enzymology, 13, 91-97.

Hornbeck, P. & Paul, W.E. (1986) Anti-immunoglobulin and phorbol ester induce phosphorylation of proteins associated with the plasma membrane and cytoskeleton in murine B lymphocytes. Journal of Biological Chemistry, 261, 14817-14824.

Howard, M., Farrar, J., Hilfilker, M., Johnson, B., Takatsu, K., Hamaoka, T. & Paul,W.E. (1982) Identification of a T cell derived growth factor distinct from interleukin 2. Journal of Experimental Medicine, 155, 914-923.

Huang, J.S., Huang, S.S., Kennedy, B. & Deuel, T.F. (1982) Platelet derived growth factor, Specific binding to target cells. Journal of Biological Chemistry, 257, 8130-8136.

Hunter, T. & Sefton, B.M. (1980) Transforming gene product of rous

sarcoma virus phosphorylates tyrosine. Proceedings of the National Academy of Science, USA, 77, 1311-1315.

Hunter, T. & Cooper, J.A. (1981) Epidermal growth factor induces rapid tyrosine phosphorylation of protein in A431 Human Tumour Cells. Cell, 24, 741-752.

Hunter, T. & Cooper, J.A. (1985) Protein-tyrosine kinases. Annual Review of Biochemistry, 54, 897-930.

Jacobs, S., Hazum, E., Shechter, Y. & Cuatrecasas, P. (1979) Insulin receptor; covalent labelling and identification of sub-units. Proceedings of the National Academy of Science, USA. 76, 4918-4921.

Jones, B. & Janeway Jnr., C.A. (1981) Cooperative interactions of B lymphocytes in MHC restriction. Nature, 292, 547-549.

Justement, L., Chen, Z., Harris, L., Ransom, J., Sandoval, V., Smith, C., Rennick, D., Roehm, N. & Cambier, J. (1986) BSF-1 induces membrane protein phosphorylation but not phosphoinositol metabolism, Ca2+ mobilisation, protein kinase C translocation, or membrane depolarisation in resting murine B lymphocytes. Journal of Immunology, 137, 3664-3670.

Kahn. C.R. (1976) Membrane receptors for hormones and neurotransmitters. Journal of Cellular Biology, 70, 261-286.

Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Haring, H.U. & Kahn, C.R. (1982a) Insulin stimulation of phosphorylation of the β sub-unit of the insulin receptor. Journal of Biological Chemistry, 257, 9891-9894.

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. & Kahn, C.R. (1982b) Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell free system. Nature, 298, 667-669.

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., White, M.F. & Kahn, C.R. (1983) Characterization of the insulin receptor kinase purified from human placental membranes. Journal of Biological Chemistry, 258, 10973-10980.

Kellerman, G., Shaw, C.R. & Luyten-Kellerman, M. (1973) Aryl hydrocarbon hydroxlase inducibility and bronchogenic carcinoma. New England Journal of Medicine, 289, 934-937.

Kishimoto, T. & Ishizaka, K.J. (1975a) Regulation of antibody response in vitro. IX. Induction of secondary anti-hapten immunoglobulin antibody response by anti-Ig and enhancing soluble factor. Journal of Immunology, 114, 585-591.

Kishimoto, T., Mujake, T., Nishizuka, Y. Watanabe, T. & Yamamura, Y. (1975b) Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. Journal of Immunology, 115, 1179-1184.

Kishimoto, A., Takai, Y., Kakkawa, U. & Nishizuka, Y. (1980) Activation of calcium and phospholipid dependent protein kinase by diacylglycerol, its possible relationship to phosphatidylinositol turnover. Journal of Biological Chemistry, 255, 2273-2276.

Klingenberg, M. (1958) Pigments of rat liver microsomes. Archives of Biochemistry and Biophysics, 76, 376-386.

Kraft, A.S., Anderson, W.B., Cooper, L. & Sando, J.J. (1982) Decrease in cytosolic calcium/phospholipid dependent protein kinase activity following phorbol ester treatment of EL-3 thymoma cells. Journal of Biological Chemistry, 257, 13193-13196.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lanzavecchia, A. (1985) Antigen specific interaction between T and B cells. Nature, 314, 537-539.

Layton, J.E., Vitetta, E.S., Uhr, J.W. & Krammer, P.H. (1984) Clonal analysis of B cells induced to secrete by T cell derived lymphokine(s). Journal of Experimental Medicine, 160, 1850-1863.

Leanderson, T., Lundgren, E., Ruuth, E., Borg, H., Persson, H. & Coutinho, A. (1982) B cell growth factor; distinct from T cell growth factor and B cell maturation factor. Proceedings of the National

Academy of Science, USA, 79, 7455-7459.

Ledbetter, J.A. & Herzenberg, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunological Reviews, 47, 63-90.

Lemke, H. & Opitz, H.G. (1976) Function of 2-mercaptoethanol as a macrophage substitue in the primary immune response in vitro. Journal of Immunology, 117, 388-395.

Lernhardt, W., Corel, C., Wall, R. & Melchers, F. (1982) T cell hybridomas which produce B lymphocyte replication factors. Nature, 300, 355-357.

Maino, V.C., Hayman, M.H. & Crumpton, M.H. (1975) Relationship between enhanced turnover of phosphatidylinositol and lymphocyte activation by mitogens. Biochemical Journal, 146, 247-252.

Marth, J.D., Peet, R., Krebs, E.G. & Perlmutter, R.M. (1985) A lymphocyte-specific protein tyrosine kinase gene is rearranged and over-expressed in the murine T cell lymphoma LSTRA. Cell, 43, 393-404.

McGrath, J.P. & Levinson, A.D. (1982) Bacterial expression of an enzymatically active product encoded by RSV src gene. Nature, 295, 423-425.

Melchers, F., Braun, V. & Galanos, C. (1975) The lipoprotein of the outer membrane of Escherichia coli, a B lymphocyte mitogen. Journal of Experimental Medicine, 142, 473-482.

Melchers, F. & Andersson, J. (1984) B cell activation, three steps and their variations. Cell, 37, 715-720.

Mitchison, N.A. (1971) The carrier effect in the secondary response to hapten-protein conjugates. III. Cellular cooperation. European Journal of Immunology, 1, 18-27.

Moller, G., Lemke, H. & Opitz, H.G. (1976) The role of adherent cells in the immune response. Scandanavian Journal of Immunology, 5, 269-274.

Mond. J.J., Seghal, E., Kung, J. & Finkelman, J.D. (1981) Increased expression of I-region associated (Ia) on B cells after crosslinking of surface immunoglobulin. Journal of Immunology, 127, 881-888.

Mond, J.J., Carman, J., Sharma, C., Ohara, J. & Finkelman, F.D. (1986) Interferonγ suppresses B cell stimulatory factor (BSF 1) induction of class II MHC determinants on B cells. Journal of Immunology, 137, 3534-3537.

Monroe, J.G. & Cambier, J.C. (1983a) B cell activation. III. B cell plasma membrane depolarisation and hyper Ia expression induced by receptor crosslinking are coupled. Journal of Experimental Medicine, 158, 1589-1599.

Monroe, J.G. & Cambier, J.C. (1983b) B cell activation. I. Anti-immunoglobulin induced receptor crosslinking results in a decrease in the plasma membrane potential of murine B lymphocytes. Journal of Experimental Medicine, 157, 2073-2086.

Monroe, J.G., Niedel, J.E. & Cambier, J.C. (1984) B cell activation. IV. Induction of cell membrane depolarisation and hyper Ia expression by phorbol diesters suggests a role for protein kinase C in murine B lymphocyte activation. Journal of Immunology, 132, 1472-1478.

Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. & de Laat, S.W. (1983) Na+/H+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature, 304, 645-648.

Moolenaar, W.H., Tertoden, L.G.J. & deLaat, S.W. (1984) Growth factors immediately raise cytoplasmic free Ca2+ in human fibroblasts. Journal of Biological Chemistry, 259, 8066-8069.

Morgan, D.A., Ruscetti, F.W. & Gallo, R.C. (1976) Selective in vitro growth of T lymphocytes from normal bone marrow. Science, 193, 1007-1008.

Mosmann, T.R., Yokota, T., Kastelein, R., Zurawski, S.M., Arai, N. & Takebe, Y. (1987) Species-specificity of T cell stimulating activities

of IL 2 and BSF-1 (IL 4), comparison of normal and recombinant, mouse and human IL 2 and BSF-1 (IL 4). Journal of Immunology, 138, 1813-1816.

Nebert, D. & Gelboin, H.V. (1969) The in vivo and in vitro induction of aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains and development and hormonal states. Archives of Biochemistry and Biophysics, 134, 76-89.

Neckameyer, W.S. & Wang, L.H. (1985) Nucleotide sequence of Avian Sarcoma Virus UR2 and comparison of its transforming gene with other members of the tyrosine protein kinase oncogene family. Journal of Virology, 53, 879-884.

Nel, A.E., Landreth, G.E., Goldschmidt-Clermont, P.S., Tung, H.E. & Galbraith, R.M. (1984) Enhanced tyrosine phosphorylation of B lymphocytes upon complexing of membrane immunoglobulin. Biochemical and Biophysical Research Communications, 125, 859-866.

Nel, A.E., Navailles, M., Rosberger, D.F., Landreth, G.E., Goldschmidt-Clermont, P.J., Baldwin, G.J. & Galbraith, R.M. (1985) Phorbol ester induces tyrosine phosphorylation in normal and abnormal human B-lymphocytes. Journal of Immunology, 135, 3448-3453.

Niedel, J.E., Kuhn, L.J. & Vanderbank, G.R. (1983) Phorbol diester receptor co-purifies with protein kinase C. Proceedings of the National Academy of Science, USA, 80, 36-40.

Nishimura, J., Huang, J.S. & Deuel, T.F. (1982) Platelet derived growth factor stimulates tyrosine specific protein kinase activity in Swiss mouse 3T3 cell membranes. Proceedings of the National Academy of Science, USA, 79, 4303-4307.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature, 308, 693-698.

Noelle, R.J., Snow, E.C., Uhr. J.W. & Vitetta, E.S. (1983) Activation of antigen specific B cells. Role of T cells, cytokines and antigen in

induction of growth and differentiation. Proceedings National Academy Science, USA, 80, 6628-6631.

Noelle, R.J., Krammer, P.H., Ohara, J., Uhr, J.W. & Vitetta, E.S. (1984) Increased expression of Ia antigens on resting B cells, an additional role for B cell growth factor. Proceedings of the National Academy of Science, USA, 81, 6149-6153.

O'Brien, T.G. & Krzeminiski, K. (1983) Phorbol ester inhibits furosemide sensitve potassium transport in BALB/c 3T3 preadipose cells. Proceedings of the National Academy of Science, USA, 80, 4334-4338.

Ogawa, Y., Taki, Y., Kawahara, A., Kiniura, S. & Nishizuka, Y. (1981) A new possible regulatory system for protein phosphorylation in human peripheral lymphocytes. I. Characterisaion of a calcium activated, phospholipid dependent protein kinase. Journal of Immunology, 127, 1369-1374.

Ohara, J., Lahet, S. & Paul, W.E. (1985) Partial purification of murine B cell stimulatory factor (BSF)-1. Journal of Immunology, 135, 2518-2513.

Ohara, J. & Paul, W.E. (1985) Production of a monoclonal antibody to and molecular characterization of B cell stimulatory factor-1. Nature, 315, 333-335.

Ohara, J. & Paul, W.E. (1987) Receptors for B cell stimulatory factor 1 expressed on cells of haematopoietic lineage. Nature, 325, 537-540.

Oliver, K., Noelle, R.J., Uhr, J.W., Krammer, P.H. & Vitetta, E.S. (1985) B cell growth factor 1 (BCGF 1 or B cell stimulatory factor provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proceedings of the National Academy of Science, USA, 82, 2465-2467.

Omura, T. & Sato, R. (1962) A new cytochrome in liver microsomes. Journal of Biological Chemistry, 237, 1375-1376.

Omura, T. &Sato, R. (1964a) The carbon monoxide-binding pigment of

liver microsomes. I. Evidence for its hemoprotein nature. Journal of Biological Chemistry, 239, 2370-2378.

Omura, T. & Sato. R. (1964b) The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purifaction and properties. Journal of Biological Chemistry, 239, 2379-2385.

Pang, D.T., Sharma, B.R., Shafer, J.A., White, M.F. & Kahn, C.R. (1985) Predominance of tyrosine phosphorylation of insulin receptors during the initial response of intact cells to insulin. Journal of Biological Chemistry, 260, 7131-7136.

Park, L.S., Friend, D., Grabstein, K. & Urdal, D.L. (1987) Characterisation of the high affinity cell surface receptor for murine B cell stimulating factor. Proceedings of the National Academy of Science, USA, 84, 1669-1673.

Parker, D.C. (1975) Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. Nature, 258, 361-363.

Parker, D.C., Fothergill, J.J. & Wadsworth, D.C. (1979) B lymphocyte activation by insoluble anti-immunoglobulin, Induction of immunoglobulin secretion by a T cell derived soluble factor. Journal of Immunology, 123, 931-941.

Perisic, O. & Traugh, J.A. (1983) Protease activated kinase II as the potential mediator of insulin stimulated phosphorylation of ribosomal protein S6. Journal of Biological Chemistry, 258, 9584-9589.

Pike, B.L., Vaux, D.L., Clark-Lewis, I., Schrader, J.W. & Nossal, G.J.V. (1982) Proliferation and differentiation of single hapten specific B lymphocytes is promoted by T cell factor(s) distinct from T cell growth factor. Proceedings of the National Academy of Science, USA, 79, 6350-6354.

Pike, L.J., Kuenzel, E.A., Casnellie, J.E. & Krebs, E.G. (1984) A comparision of the insulin and epidermal growth factor stimulated protein kinases from human placenta. Journal of Biological Chemistry, 259, 9913-9921.

Piras, M.M., Horenstein, A. & Piras, R. (1975) Identification of multiple protein kinases in normal human lymphocytes. Enzyme 22, 219-222.

Plimmer, R.H.A. (1941) Esters of phosphoric acid phosphoryl hydroxy-amino acids. Biochemical Journal, 35, 461-469.

Ploegh, H.L., Orr, H.T. & Strominger, J.L. (1981) Major histocompatibity antigens: the human (HLA-A, -B, -C) and murine (H-2K, H-2D) class I molecules. Cell, 24, 287-299.

Pure, E., Isakson, P.C., Takatsu, K.,Hamaoka, T., Swain, S.L., Dutton, R.W., Dennert, G., Uhr, J.W. & Vitteta,E.S. (1981) Induction of B cell differentiation by T cell factors. I. Stimulation of IgM secretion by products of a T cell hybrid and a T cell line. Journal of Immunology, 127, 1953-1958.

Rabin, E.M., Ohara, J. & Paul, W.E. (1985) B cell stimulatory factor (BSF) 1 activates resting B cells. Proceedings of the National Academy of Science, USA, 82, 2935-2939.

Rabin, E.M., Mond, J.J., Ohara, J. & Paul, W.E. (1986) B cell stimulatory factor 1 (BSF 1) prepares resting B cells to enter S phase in response to anti-IgM and lipopolysaccharide. Journal of Experimental Medicine, 164, 517-531.

Raetz, C.R.H., Purcell, S. & Takayama, K. (1983) Molecular requirements for B lymphocyte activation by Escherichia coli lipopolysaccharide. Proceedings of the National Academy of Science, USA, 80, 4624-4628.

Ransom, J.T., Harris, L.K. & Cambier, J.C. (1986) Anti-immunoglobulin release of inositol-1,4,5-trisphosphate which mediates mobilisation of intracellular Ca2+ stores in B lymphocytes. Journal of Immunology, 137, 708-714.

Ratcliffe, M.J.H. & Julius, M.H. (1982) H-2 restricted T-B cell interactions involved in polyspecific B cell responses mediated by soluble antigen. European Journal of Immunology, 12, 634-641.

Richert, N., Davies, P.J.A., Gilbert, J. & Paston, I. (1979) Inhibition of the transformation specific kinase in ASV-transformed cells by N-α-tosyl-L-lysyl chloromethyl ketone. Cell, 18, 369-374.

Robb, R.J., Munck, A. & Smith, K.A. (1981) T cell growth factor receptors, quantitation, specificty and biological relevance. Journal of Experimental Medicine, 154, 1455-1474.

Robb, R.J. & Greene, W.C. (1983) Direct demonstration of and identity of T cell growth factor binding protein and the Tac antigen. Journal of Experimental Medicine, 158, 1332-1337.

Robb, R.J., Greene, W.C. & Rusk, C.M. (1984) Low and high affinity cellular receptors of IL-2. Journal of Experimental Medicine, 160, 1126-1146.

Rock, K.L., Benacerraf, B. & Abbas, A.K. (1984) Antigen presentation by hapten specific B lymphocytes. I. Role of surface immunoglobulin receptors. Journal of Experimental Medicine, 160, 1102-1113.

Roitt, I., Brostoff, J. & Male, D. (1985) Immunology. Ch.7, pp7.1-7.14. Gower Medical Publishing.

Rosen, O.M., Herrera, R., Olowe, Y., Petruzzelli, L.M. & Cobb, M.H. (1983) Phosphorylation activates the insulin receptor tyrosine kinase. Proceedings of the National Academy of Science, USA, 80, 3237-3240.

Rosenthal, A.S. & Shevach, E.M. (1973) Function of macrophages in antigen recognition by guinea pig T cells. I. Requirement for histocompatible macrophages and lymphocytes. Journal of Experimental Medicine, 138, 1194-1212.

Rosenthal, A.S., Barcinski, M.A. & Blake, J.T. (1977) Determinant selection, a macrophage dependent immune response gene function. Nature, 267, 156-158.

Rubin, J.B., Shia, M.A. & Pilch, P.F. (1983) Stimulation of tyrosine specific phosphorylation in vitro by insulin- like growth factor 1. Nature, 305, 438-441.

Schimpl, A. & Wecker, E. (1975) A third signal in B cell activation

given by the T cell replacing factor. Transplant. Rev. 23, 176-188.

Schimpl, A. & Wecker, E. (1972) Replacment of T cell function by a T cell product. Nature, New Biol. 237, 15-17.

Schreier, M.H., Andersson, J., Lernhardt, W.T. & Melchers, F. (1980) Antigen specific T helper cells stimulate H-2 compatible and H-2 incompatible B cell blasts polyclonally. Journal of Experimental Medicine, 151, 194-203.

Sefton, B.M., Hunter, T. & Rsachke, W.C. (1981) Evidence that the Abelson virus protein functions in vivo as a protein kinase that phosphorylates tyrosine. Proceedings of the National Academy of Science, USA, 78, 1552-1556.

Shackelford, D.A. & Trowbridge, I.S. (1984) Induction of expression and phosphorylation of the human IL-2 receptor by a phorbol ester. Journal of Biological Chemistry, 259, 11706-11712.

Shackelford, D.A. & Trowbridge, I.S. (1986) Identification of lymphocyte integral membrane proteins as substrates for protein kinase C. Journal of Biological Chemistry, 261, 8334-8341.

Shinnick, T.M., Lerner, R.A. & Sutcliff, D.G. (1981) Nucleotide sequence of Moloney murine leukaemia virus. Nature, 293, 543-548.

Sieckman, D.G., Asofsky, R., Mosier, D. E., Zitron, I.M. & Paul, W.E. (1978) Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferation response. Journal of Experimental Medicine, 147, 814-829.

Simpson, I.A. & Hedo, J.A. (1984) Insulin receptor phosphorylation may not be a prerequiste for active insulin action. Science 223, 1301-1304.

Snow, E.C. Noelle, R.J., Uhr, J.W. & Vitetta, E.S. (1983) Activation of antigen-enriched B cells in the B cell population to thymus-dependent antigens. Journal of Immunology, 130, 614-618.

Sprent, J. (1978) Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. I. Failure to collaborate with B cells of the

opposite parental strain not associated with active suppression. Journal of Experimental Medicine, 147, 1142-1158.

Subbarao, B. & Mosier, D.E. (1983) Lyb antigens and their role in B lymphocyte activation. Immunological Reviews, 69, 81-97.

Swain S.L. & Dutton R.W. (1982) Production of a B cell growth promoting activity, (DL) BCGF, from a cloned T cell line and its assay on the BCL1 B cell tumour. Journal of Experimental Medicine, 156, 1821-1834.

Swain, S.L., Howard, M., Kappler, J., Marrack, P., Watson, J., Booth, R., Wetzel, G.D. & Dutton, R.W. (1983) Evidence for 2 distinct classes of murine B cell growth factors with activities in different functional assays. Journal of Experimental Medicine, 158, 822-835.

Swarup, G., Dasgupta, J.D. & Garbers, D.L. (1983) Tyrosine protein kinase activity of rat spleen and other tissues. Journal of Biological Chemistry, 258, 10341-10347.

Takatsu, K. Tanaka, K. Tominaga, A. Kumahara, Y. & Hamaoka, T. (1980) Antigen induced T cell repacing factor (TRF) III. Establishment of a T cell hybrid clone continually producing TRF and functional analysis of released TRF. Journal of Immunology, 125, 2646-2653.

Takayama, S., White, M., Lauris, V. & Kahn, C.R. (1984) Phorbol esters modulate insulin receptor phosphorylation and insulin action in cultured hepatoma cells. Proceedings of the National Academy of Science, USA, 81, 7797-7801.

Uusitalo, R.J. & Karnovsky, M.J. (1977a) Surface localisation of 5'-nucleotidase on the mouse lymphocyte. J. Histochem. Cytochem. 25, 87-96.

Uusitalo, R.J. & Karnovsky, M.J. (1977b) 5'-nucleotidase in different populations of mouse lymphocytes. J. Histochem. Cytochem. 25, 97-103.

Waldman, T.A., Goldman, C.K., Robb, R.J., Depper, J.M., Leonard, W.J., Sharrow, S.O., Bongiovanni, K.F., Korsmeyer, S.J. & Greene, W.C. (1984) Expression of interleukin 2 receptors on activated human B

cells. Journal of Experimental Medicine, 160, 1450-1466.

Waterfield, J.D., Dennert, G., Swain, S.L. & Dutton, R.W. (1979) Continuously proliferating allospecific T cells. 1. Specificty of cooperation with allogenic B cells in the humoral antibody response to sheep erthrocytes. Journal of Experimental Medicine, 149, 808-814.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A.,Westermark, B., Heldin,C.H., Huang, J.S. Deuel, T.F. (1983) Platelet derived growth factor is structurally related to the putative transforming protein, p28sis, of the simi an sarcoma virus. Nature, 304, 35-40.

Wray, W., Boulikas, T., Wray, V.P. & Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. Analytical Biochemistry, 118, 197-203.

Yakura, H., Kawabatta, J., Shen, F.W. & Katagiri, M. (1985) Lyb 2 as a possible B cell growth factor (BCGF) 1 receptor. Fed. Proc. 44, 1532.

Yu, K-T. & Czech, M.P. (1984) Tyrosine phosphorylation of the insulin receptor β sub-unit activates the receptor associated tyrosine activity. Journal of Biological Chemistry, 259, 5277-5286.

Zick, Y., Kasuga, M., Kahn, C.R. & Roth, J.R. (1983) Characterization of insulin mediated phosphorylation of the insulin receptor in a cell free system. Journal of Biological Chemistry, 258, 75-80.

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