B CELL DIFFERENTIATION IN SHEEP.

by

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This thesis is submitted as part of the course requirements for the degree of Doctor of Philosophy at the University of Edinburgh.

June 1988.

DECLARATION.

This thesis was composed by myself and the work which it describes is my own.

Philip A. Jones

ACKNOWLEDGEMENTS.

I would like to thank Professor Ian McConnell for the excellent opportunity which he afforded me. The experience which I have gained under his guidance is irreplacable. Due to Ian's committments, Dr John Hopkins was my day-to day supervisor and I thank him for much assistance, many stimulating discussions and proof-reading this thesis.

I also wish to thank the entire staff of the Department of Veterinary Pathology as they have all had an input into this thesis by way of help, distraction, support and many enjoyable times. I shall miss them all.

Finally, I thank Sheilagh for all that we have shared over the period of these studies. I can only guess how I would have coped without her.

DEDICATION.

I dedicate this thesis to my late father PETER (1935-1983).

ABSTRACT.

The ileal Peyer's patch (IPP) of lambs is a region of intense lymphopoiesis and B cell development. Monoclonal antibodies against ovine lymphocyte antigens have been used to characterise the IPP lymphocyte. Three murine monoclonal antibodies against ovine IgM, IgG1 and Ig light chain were produced and are described fully. IgM and MHC class II antigens are expressed on the vast majority of IPP cells whilst cells bearing other serum Ig isotypes and T cell antigens are rare. A novel Ig molecule appears to be coexpressed with IgM, it is proposed that this is the ovine equivalent of IgD.

IPP cells can be induced to proliferate and differentiate when cultured with lipopolysaccharide (LPS) and interleukin 2 (IL2). Proliferation is inhibited by rabbit anti-sheep Ig antibodies. Using an ELISA for Ig, it has been posible to quantitate Ig synthesis and secretion. Mean cellular Ig increases greater than 25-fold during differentiation. High-rate secretion begins 4 days after initiation of culture and is virtually complete by day 7.

As IPP B cells differentiate to IgM secretion, membrane Ig is rapidly lost so that by day 6, only 15% of cells express Ig on their surfaces. Changes in MHC class II antigens were also studied. Surface expression of MHC class II molecules doubled by 24 hours and slowly declined to resting levels as differentiation proceeded. A large increase in cytoplasmic MHC class II content was noted on day 3. The reasons for this increase are discussed.

Kinetic studies suggest that IL2 responsiveness is acquired approxiately 20 hours after activation by LPS. The concentration required to give half maximal Ig secretion is 125pM indicating that the interaction between IL2 and its receptor is one of high affinity.

During differentiation, the cells enlarge and show an increase in the cytoplasmic:nuclear ratio. The formation of extensive rough endoplasmic reticulum and additional mitochondria is indicative of the functional changes occuring

This is the first description of a sheep B cell differentiation assay. It is proposed that this system is a suitable model on which to base further studies into the molecular biology of sheep Ig genes, Ig isotype switching and lymphokines and their receptors.

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INTRODUCTION.

B lymphocyte development and differentiation is the area in which immunological research, with the possible exception of work relating to HIV infection, has made the most major advances in the last three years. Immunologists now have a much improved understanding of the control of this process and despite the *in vitro* nature of the majority of experiments, some approximation to the *in vivo* situation can be summised. The fascination with B cell differentiation is that it appears to be controlled by a series of non-specific factors; IL1, IL2, IL4, IL5 and gamma-interferon (Howard and Paul 1983b, Leibson *et al* 1984, Swain 1985). These factors mediate several effects on B cells as well as on a wider range of haematopoetic cells. This situation creates a dilemma as it provides no mechanism for ensuring the exquisite specificity of the immune system nor for eliminating potentially harmful 'bystander' effects. It is therefore postulated that cognate interactions between cells play an important role in the homeostasis of the B cell response (Kupfer *et al* 1987).

A major criticism of *in vitro* assays of B cell growth and differentiation in mouse and man is the difficulty in providing purified populations of resting B cells. The conflicting results obtained in assays on B cells from these species have been ascribed to the vastly different sources of B cells used; usually the tonsils in man and the spleen in mice. This thesis is concerned with the establishment of an *in vitro* model for B cell differentiation in the sheep. The distinguishing feature of this assay compared to former

assays is its use of the ileal Peyer's patch (IPP) as a source of resting B lymphocytes. The IPP is widely accepted as being the major area of B cell production and export in lambs. The development and involution of the IPP has many characteristics of the bursa of Fabricius in birds. As such the IPP of lambs is an excellent source of large numbers of newly formed uncommitted B cells. There is little doubt that this assay will play a major role in furthering our understanding of B cell physiology in the sheep.

B CELL PRODUCTION.

In order to appreciate the importance of the IPP in lambs as a primary lymphoid organ and as a source of B cells for <u>in vitro</u> assays, it is important to consider B cell production in other species. Broadly, B cell development can be divided into antigen-independant and -dependant stages. The antigen-independant stages occur in a variety of locations depending on the species under consideration and last until the mature B cell joins the peripheral lymphoid pool. There the B cell acquires the ability to bind antigen, to participate in cell-cell interactions and to respond to soluble factors. These stimuli may cause the cell to undergo clonal expansion and differentiation. The control of B cell production is poorly understood and this is largely due to the difficulties in performing direct studies.

Chapter One.

B CELL ONTOGENY AND DEVELOPMENT IN THE MOUSE.

In the mouse, the early B cell production occurs in the liver and later in the bone marrow. Haemopoietic stem cells (HSC) migrate from the yolk sac and enter the foetal liver on the ninth day of gestation. On entering the liver, these HSC become committed to give rise to cells of the lymphoid lineage. It has been demonstrated that μ+ pre-B cells appear in the liver of mouse embryos at about 12 days (Raff et al 1976). The number of pre-B cells increases over the next few days; and some express light chains to become IgM-bearing B cells by day 17 (Owen et al 1974). Since IgM+ B cells require 3-4 days to become plasma cells, it is suggested that the absolute minimum time from the HSC to the terminal stage of B cell differentiation is 11-12 days. Pre- B cells comprise a minor sub-population in the haemopoietic tissues. In order to study the development of these early B lineage cells it has been necessary to develop methods to rescue clones from the polyclonal admixture occuring in the bone marrow.

The structure of the bone marrow shows remarkable organisation. The blood supply confers a radial symmetry to the marrow. Kinetic studies and continuous [^{3}H]-thymidine labelling experiments have suggested that there is a centripetal maturation of haematopoietic precursors (Osmond et al 1981).

The mechanism that controls the exit of newly mature B cells from their site of production is poorly understood. Osmond (1986) postulated that murine B cells receive a final maturation signal within the

sinusoids of the bone marrow. Once the B cell has entered the circulation, it is able to bind antigen to its membrane antigen receptor. However, there is a considerable turnover of B cells each day as the half life of primary B cells in vivo is only 3 days. This loss requires continuing output of cells from the regions of B cell production.

IMMUNOGLOBULIN GENE REARRANGEMENTS.

Rearrangements of immunoglobulin genes appears to be initiated in a sequential manner. Heavy chain rearrangements commence with the connection of a D_H to a J_H gene segment on one chromosome. This is followed by rearranging a V_H gene to the DJ sequence. The variation in the actual site of joining of the V_H and DJ segments can result in a non-productive gene. If this occurs, rearrangements continue on the paired chromosome. Heavy chain expression terminates further rearrangements and the process is initiated for light chain. If the rearrangements on the second chromosome fail to produce a satisfactory VDJ gene and therefore no heavy chain is expressed, the cell will be non-functional. In the mouse, rearrangements to produce a functional V_L gene occur for the k chain genes first. If both chromosomes fail to give an adequate VJ rearrangement, then rearrangements occur on the l chain genes. As with VDJ genes, production of a functional VJ gene terminates further rearrangements. It has been calculated that each rearrangement has a one in three chance of resulting in a continuous

reading frame (Lewis et al 1985). This suggests that the importance of always obtaining functional B cells has been sacrificed for the advantages of increasing the antibody repertoire beyond that encoded by the germ-line genes. It is proposed that it would be the fate of a considerable number of cells to be non-functional. It is presumed that these cells fail to leave their site of origin and die in situ.

LONG-TERM BONE MARROW CULTURES.

Two types of long-term bone marrow cultures have proved extremely useful for studying B cell ontogeny. The Dexter culture system allows the maintenance of HSC which coninually undergo myeloid and erythroid differentiation (Dexter et al 1977). Whilst B lineage cells are not found in these cultures, stem cells derived from these cultures will give rise to B cells when transferred to irradiated recipients (Nishikawa et al 1985). These stem cells migrate to the bone marrow and produce LPS-responsive B cells after 11 days.

Whitlock and Witte have modified the original Dexter culture conditions to provide a system for the growth of early lineage B cells (Whilock et al 1985). Changing from Dexter to Whitlock-Witte (WW) cultures results in a loss of erythroid and myeloid elements and the development of pre-B and B cells. The growth of adherent cell populations is a crucial feature of both types of long-term bone marrow cultures. Clones of pre-B cells can be derived from WW cultures by A-MuLv transformation (Whitlock et al 1983) and these cells can undergo

Ig gene rearrangements but interestingly they appear not to undergo isotype switching. Alternatively, short-term cultures of fetal liver and bone marrow cells allow the rapid generation of clonally diverse, isotypically diverse and functional B cells (Owen et al 1974, 1977, Melchers 1977, Kincade et al 1981).

Adherent accessory cells have been found to be essential for the growth of HSC precursor and pre-B cells in long and short term culture systems. In addition, a variety of well defined factors influence pre-B cell growth perhaps through accessory cells. These factors include IL3, CSF-1 and GM-CSF (Paige et al 1984).

PHENOTYPIC ANALYSIS OF B CELL ONTOGENY.

Another method for following B cell ontogeny is the use of monoclonal antibodies against cell differentiation antigens. Although Ig is not expressed on pre-B cells, there is an increasing array of monoclonal antibodies and other markers for non-Ig molecules on B lineage cells. The B220 antigen is expressed on B lineage cells, though not exclusively, and can be used to isolate pre-B and B cells from bone marrow (Kincade et al 1981). The surface of pre-B cells in the mouse bone marrow strongly binds the lectin peanut agglutinin (PNA). Binding of PNA is lost soon after expression of IgM. This has led to speculation that lectin-like binding properties may be involved in the retention of pre-B cells within the bone marrow until they are

sufficiently mature to exit (Osmond 1986).

B CELL ONTOGENY IN BIRDS.

In birds, the embryonic liver is never a site of haematopoiesis. B cells are generated from stem cell precursors which migrate into the bursa of Fabricius, an invagination of the cloaca or the terminal gut. These stem cells enter the mesenchymal and epidermal tissue of the bursa at about the eighth day of incubation (Moore and Owen 1965, 1966). Those in the epidermal lining of the bursa form foci of B lineage cells. The immature B cells in the bursa have an average cell cycle time of 8 to 10 hours. This massive rate of proliferation rapidly leads to the formation of approximately 10^4 lymphoid follicles. These cells express MHC class II antigens by day 10 and sIgM by day 11. At hatching virtually all bursal cells (90-95%) express sIgM. As in the bone marrow of mice, B cell development can be investigated by a variety of monoclonal antibodies to non-Ig molecules (Chen and Cooper 1986). In particular, antibodies to the allelic forms of the Bu antigen and IgM have proved useful in defining the clonal development of B cells (Ratcliffe 1985). Very few B cells exit from the bursa before the 16th day of incubation; therefore surgical removal at this stage prevents the peripheralisation of B cells and results in agammaglobulinaemia. (Glick et al 1956, Cooper et al 1969). Later removal can severely deplete the numbers of circulating B cells, although this does not necessarily result in an immunological deficit.

Finally the bursa involutes at the time of the bird's sexual maturation. Therefore it appears that the bursa is solely responsible for the generation of B cells during embryonic and early post-hatching life of the bird.

B CELL ONTOGENY IN LAMBS.

In the lamb, B cell production has been demonstrated to occur in the terminal ileum. The terminal ileum contains a large Peyer's patch from approximately 60 days of gestation onwards. This continuous ileal Peyer's patch (IPP) is composed of tightly packed follicles with little interfollicular tissue and is histogically mature well before birth. Post-natally, the IPP begins to involute and is virtually absent by 18 months of age. By contrast, the jejunal PP (JPP) are single discrete organs and are composed of follicles with large interfollicular spaces. The JPP are present pre-natally but their size vastly increases post-natally due to challenge by exogenous antigen. The JPP continue to be present throughout life (Reynolds and Morris 1983). The removal of the IPP before or shortly after birth results in a severe deficit of Ig-bearing cells in the blood. However these lambs had serum Ig levels within the normal range. In addition, these animals were able to respond to Salmonella muenchen organisms in similar fashion to intact animals (Gerber, Morris and Trevella 1985). The prenatal exit of cells from the IPP accounts for the presence of B cells (and therefore antibody) in ileectomised animals and appears to be analogous to the

situation in the chick embryo when bursectomy is performed after 16 days of incubation. The cells exiting the IPP colonise peripheral secondary lymphoid organs (Maddox, MacKay and Brandon 1987a, 1987b, 1987c).

RATE OF MITOSIS IN THE IPP.

Using the metaphase arrest technique, it has been shown that 2.8% of IPP cells entered mitosis every hour and that the potential doubling time was about 36 hours compared to over 200 hours for the thymus (Reynolds 1986). The enormous growth potential of the IPP must be rigidly controlled to prevent over-production. Since greater than 95 % of cells do not exit from the IPP, enormous cell death occurs in the area. The mechanisms by which cell death is initiated and dead cells removed are unknown. Thymocytes have been shown to undergo a distinct process known as apoptosis (Duvall and Wyllie 1986) and it seems reasonable to suggest that lymphocytes in the IPP undergo a similar well-controlled process, rather than necrosis.

EMIGRATION OF CELLS FROM THE IPP.

When cells formed in the IPP were labelled by an extra-corporeal perfusion system, it was shown that the vast majority of cells do not exit from the IPP. Three days after labelling <u>in vivo</u>, cells that have

emigrated are present in blood, spleen, peripheral and mesenteric lymph nodes and the jejunal PP but not in the thymus or in the non-perfused part of IPP. When a secondary antigen-reactive lymphoid organ, the jejunal PP was labelled by identical techniques, the exiting cells were found 24 hours later in lymph nodes, non-perfused JPP, spleen and blood (Pabst and Reynolds 1986) but not the IPP. These results indicate that

1) the IPP is a region of intense lymphopoiesis,

2) the low percentage of cells which do exit the IPP are quickly distributed among secondary lymphoid organs,

3) cells formed in a secondary lymphoid organ (the JPP) do not recirculate to lodge in the IPP in substantial numbers.

These results confirm the hypothesis that the IPP is an unique part of the lymphoid system, responsible for producing large numbers of cells of which only a few will leave. In accordance with the antigen-independant nature of its role, few mature recirculating cells enter the IPP. Since antigen, cell surface molecules, such as MEL-14 (Gallatin, Weissman and Butcher 1983), and the high endothelial venules of the lymphoid blood vessels all play a role in mediating extravasation of lymphocytes, the reason for the apparent 'splendid isolation' of the IPP is unknown.

PHENOTYPE OF THE IPP LYMPHOCYTE.

The IPP lymphocytes were further characterised by Miyasaka et al. (1984) who prepared a single cell suspension of cells from the terminal

ileum. The lymphocytes were obtained from the interface of a discontinuous Percoll^{R} gradient and stained for lymphocyte membrane molecules. The samples were analysed by flow cytometry and demonstrated that the IPP cell expresses low amounts of IgM and MHC class II molecules compared to peripheral lymphocytes. Cells expressing T cell antigens or other Ig isotypes (IgG and IgA) were rare. Interestingly, more than 95 % of IPP lymphocytes expressed binding for fluorescinated peanut agglutinin (PNA) whereas B cells obtained from peripheral lymph failed to bind PNA unless treated with neuraminidase. It has been postulated that immature B cells may be retained in their site of production by virtue of their lectin-like binding properies (Osmond et al 1986). When IPP cells were cultured overnight with phorbal myristate acetate, the expression of MHC class II and IgM increased substantially (Miyasaki et al 1984). Unfortunately the effect of culturing cells in media alone overnight was not included and this makes the significance of this experiment difficult to judge.

MATURE B CELLS.

After displaying IgM on the cell surface, most mammallian B lymphocytes coexpress IgD. The IgD and IgM heavy chains have the same V_H region (Knapp et al 1982), exhibit allelic exclusion (Luzzati et al 1973) and are encoded on the same chromosome (Hertzenburg et al 1977). It is proposed that membrane IgM and IgD heavy chains may be generated

from a single complex transcription unit (Maki et al 1981, Moore et al 1981, Knapp et al 1982, Tucker 1985). This transcript is approximately 25 kb long although its existence has not been formally demonstrated. Yaoita et al (1982) have proposed that isotype switching is a two-step event. Initially, isotype switching may proceed via differential splicing of long RNA transcipts (Perlmutter and Gilbert 1984). Next, recombinase activity at switch regions 5' to each heavy chain gene deletes the DNA intervening between the V_H gene and the new C_H gene (Davis et al 1980). Hence all clonal progeny of the initial IgM^+ B cell express the same antigen specificity. Interestingly, there is not a switch region 5' to the $C\delta$ gene.

B CELL ACTIVATION BY ANTIGEN.

Antigen binds to membrane IgM and IgD on the surface of specific B cells and can promote B cell activation in two distinct ways. First, antigen-specific B cells can bind the antigen and present it very efficiently to helper T cells (Lanzavecchia 1985). Antigen-specific B cells can present antigen to T cells at concentrations of antigen 1000-fold below that required by non-specific antigen-presenting cells (Rock et al 1984, Tony and Parker 1985, Lanzavecchia 1985). This captured antigen is internalised and selectively degraded by proteases (Metezeau et al 1984, Buus et al 1986) although the pathways by which this occurs is far from clear (Tony and Parker 1985). Certain peptide fragments can

associate with MHC class II molecules (Babbitt et al 1985) which are expressed on the cell surface. The antigenic fragments associate with certain sites on the class II molecule (Germain 1986). The T cell antigen receptor will bind to antigen or antigenic fragments in association with MHC class II. Other interactions between cell surface molecules occur, for example, the T cell antigen CD2/T11 interacts with the LFA-3 molecule on the antigen presenting cell (Springer et al 1987). It is also postulated that the "helper" T cell marker CD4 helps to mediate transient low affinity interactions between cell by binding to monomorphic determinants on MHC class II molecules. It has been demonstrated that once an initial interaction has occurred between two antigen-specific cells, the T cell rapidly accumulates additional TcR, CD4 and LFA-1 molecules so that they are organised opposite to the membrane of the B cell. The ligand for LFA-1 is the ICAM-1 molecule (Springer et al 1987). These events are accompanied with a reorganisation of the cytoskeleton and reorientation of the Golgi apparatus. This sequence of events is similar to the lysis of target cells by cytotoxic lymphocytes (Podack and Koningsberg 1984). Hence it has been postulated that an activating factor may be inserted into the interior of the B cell (Kupfer et al 1987). The mechanisms which allow the initial close contact between the antigen specific cells in the midst of overwhelming numbers of cells specific for other antigens is unknown. The architecture of lymphoid organs is certain to play a role in this "sampling" process.

Secondly, antigen binding to mIgM or mIgD on B cells can generate transmembrane signalling reactions that may participate in the

activation of the B cell. This signalling role for antigen receptors on B cells has been substantiated by recent experiments demonstrating that mIgM binding can activate the phosphoinositide pathway (Berridge and Irvine 1985, Monroe and Kass 1985, Ransom and Cambier 1986). The importance of B cell activation by antigen-binding to mIg has been questioned by Lanzavecchia (1987). Lanzavecchia has calculated that under conditions at which antigen-presentation by B cell occurs, only 0.05% of membrane Ig molecules are occupied by antigen and postulated that B cells operate as selective "vacuum cleaners" to rapidly internalise antigen. Once sufficient antigen has accumulated in the cytoplasm, the re-expression of antigenic peptides with MHC class II molecules (Babbitt et al 1985) is sufficient for antigen-presentation to T cells.

B CELL ACTIVATION BY POLYCLONAL ACTIVATORS.

B cells can also be stimulated to proliferate and differentiate by another class of B cell activators, molecules derived from the surfaces of some bacteria and viruses. The best studied of these compounds is bacterial lipopolysaccharide (LPS). Although a great deal is known of the cellular effects of LPS on B cells and macrophages (Morrison and Ryan 1979), the molecular details by which LPS activates these cells largely remains a mystery. De Franco et al (1987) report that LPS activates one of a class of signalling compounds known as G proteins. As activation of these components is known to be stimulated by a wide

range of receptors for hormones and neurotransmitters, it is likely that LPS also utilises a specific cell surface receptor. Since a wide range of bacterial and viral surface structures function as polyclonal B cell activators, it is attractive to suggest that a mechanism has evolved in the immune system to aid B cells and macrophages to respond to pathogenic microorganisms.

B CELL GROWTH.

The central feature of the humoral immune response is that when quiescent B lymphocytes encounter antigen they become activated, proliferate and differentiate to become antibody secreting cells. Because resting lymphocytes specific for any individual antigen are present at low frequency prior to immunisation, rapid cell expansion is a critical phase of the immune response. Thus, part of the immune system is concerned with a differentiation pathway, the end-point of which is the plasma cell. Each plasma cell is unique in the sense that a clonal specificity relevant for a given antigenic epitope regulates the amplification of a given B cell clone. This is the basis for clonal selection (Jerne 1955, Burnet 1957). Clonal expansion and differentiation requires the participation of helper T cells and accessory cells such as macrophages. Recently, it has become clear that the helper function of T lymphocytes can be explained by their production of growth and differentiation factors (lymphokines) that act on B cells (Melchers and Andersson 1986).

Chapter One.

A MODEL FOR THE REGULATION OF B CELL PROLIFERATION.

A resting B lymphocyte is activated by antigen and a T helper cell to become an activated B cell. This initial step can also be accomplished by using polyclonal B cell activators such as anti-Ig or LPS. Once activated, the B cell has acquired the ability to respond to soluble lymphokines which will support exponential growth and differentiation to immunoglobulin-secreting cells. This model has proved to be over-simplistic. While the early characterisations of B cell acting lymphokines indicated that the same factor could induce both growth and differentiation of B cells (Andersson et al 1980, Melchers et al 1980), later reports indicated that some factors would only support growth or differentiation (Howard and Paul 1982, Lernhardt et al 1982, Leanderson et al 1982). After the isolation of the genes of two of these lymphokines, IL4 and IL5 (Noma et al 1986, Kinashi et al 1986) it is clear that this variation is more related to the assay system used than to the lymphokines themselves. Thus there is no evidence of any lymphokine that will selectively support B cell growth or induce differentiation only. In addition, IL4 has been shown to act at the level of the resting B cell (Roehm et al 1984), as has a separate lymphokine that awaits identification (Leclerq et al 1984).

Three restriction points controlling proliferation of pre-activated B cells have been defined (Melchers and Lernhardt 1985). One of these points was defined by the action of anti-IgM while the other two were defined by the action of soluble factors chronologically referred to as α and β factors. In the absence of any of these signals the cell cycle

progression is arrested at the appropriate point, whilst if present 100% of cells will proliferate for several divisions.

LYMPHOKINES.

As described above, the division of lymphokines into growth and differentiation factors has had to be reconsidered. Interleukin 4 is a good example of a pleiotropic factor. IL4 was first postulated to act as a B cell growth factor because it could costimulate with anti-Ig and cause proliferation of resting B cells (Howard et al 1982). But IL4 can also induce the expression of class II MHC molecules (Roehm et al 1984, Noelle et al 1984) and low affinity receptors for the Fc portion of IgE on resting B cells (Hudak et al 1987, Defrance et al 1987). Moreover the cloning of the cDNA encoding murine IgG1 inducing factor (Noma et al 1986) has indicated that it is IL4. IL4 has also been shown to increase IgE production by activated murine B cells (Coffman et al 1987). Clearly IL4 has both growth and differentiation properties. Human IL4 has been recently identified on the basis of its homology with a murine IL4 cDNA clone (Yokota et al 1986). The properties of human IL4 on human B cells are similar to the murine system although increases in MHC class II expression are not observed.

Murine BCGF II (IL5) was initially described as a growth factor for a murine B cell lymphoma, BCL1 (Swain and Dutton 1982). It has since been shown to cause DNA synthesis and IgM and IgG secretion by preactivated normal murine B cells (O'Garra et al 1986) and also

enhance the production of IgA by activated B cells (Yokota et al 1987, Coffman et al 1987). Its effects are not confined to be B cells as it can induce the differentiation of thymocytes into cytolytic T cells (Takatsu et al 1987) and bone marrow stem cells into eosinophils (Sanderson et al 1986). Human IL5 has also been cloned and mediates similar functions to murine IL5 (Yokota et al 1987). The differences between the species may not be significant as they are likely to be related to

1) the source of B cells, for example, in human assays peripheral blood or the tonsil is used as a source of B cells whilst in murine assays splenic B cells are used.

2) the purity of B cells and

3) the assay system used.

BSF-2 (IL6) was first identified in humans by its ability to induce antibody secretion by preactivated normal and Epstein-Barr virus transformed human B cells (Hirano et al 1987). It is identical to a plasmacytoma/hybridoma growth factor (Van Damme et al 1987) and to a factor with anti-viral activity, interferon $\beta 2$ (Haegeman et al 1986).

Whilst some B cell-acting factors eg IL4 have been shown to have activities on other cell types, other lymphokines originally thought to influence cells other than B cells are now known to modulate B cell function. Interleukin 1 (IL1) causes pre-B cells to express membrane Ig (Giri et al 1984) and enhances the proliferation of activated B cells (Pike and Nossal 1985). However much of the work with IL1 has been performed using crudely purified preparations and requires to be repeated with recombinant material.

Chapter One.

The role of interleukin 2 in B cell physiology has been controversial (Julius et al 1985), but it is now clear that it is involved in B cell proliferation (Zubler et al 1984, Romagnani et al 1985, Nakagawa et al 1985). In some experiments, B cells have been induced to differentiate to Ig secretion in response to IL2 (Jelinek and Lipsky 1987, Bich-Thuy et al 1985).

The importance of gamma-interferon in B cell proliferation and differentiation remains unresolved. Recombinant gamma-IFN enhances antibody secretion in mice and humans. Synergy with IL1 (Leibson et al 1984) and IL2 (Jelinek and Lipsky 1987) has been observed. However, gamma-IFN exerts both stimulatory and inhibitory influences on antibody production. Whilst gamma-IFN enhances the IgG2a response by activated murine B cells (Snapper and Paul 1987), it also suppresses the enhancement of IgG1 and IgE secretion in LPS blasts induced by IL4 (Coffman and Carty 1986). In the mouse, gamma-IFN and IL4 are produced by distinct subsets of helper T cell lines Th1 and Th2 respectively (~~Mossman and Coffman 1987~~ Mosmann et al 1986). The reciprocal interaction of these subsets may determine the outcome of a humoral immune response in vivo.

In addition to factors produced by other cell types, B cell-derived factors may play a role in B cell development. A number of B cell lines produce factors which enhance their growth; soluble Fc Rs (CD23 antigens) have also been reported to stimulate B cell growth (Gordan and Guy 1987). The properties of the major B cell-acting lymphokines are tabled below.

FACTOR	SOURCE	MOL. WT.	CLONED?	EFFECTS ON B CELLS.
IL1	Various	14-17 kD	Yes	Induces proliferation and differentiation with co-stimulants.
IL2	T cells	15-25 kD	Yes	Induces proliferation and differentiation with co-stimulants.
IL3	T cells	28 kD	Yes	Growth of pre-B cells.
IL4	T cells	15 kD	Yes	Activates resting B cells. Increased Ia expression. Increased Fc R expression. Increased secretion of IgG1 and IgE.
IL5	T cells	45-60 kD	Yes	Increased proliferation of activated cells. Increased secretion of IgM, IgG1 and IgA. Increased expression of IL2R on activated cells.
IL6	Various	30 kD	Yes	Induces growth of plasmacytomas

				and Ig secretion in activated B cells.
gamma-IFN	T cells	?	Yes	Induces Ig proliferation and secretion with IL2. Inhibits IL4-mediated effects.

Chapter One.

ASSAYS FOR B CELL-ACTING FACTORS.

There are three basic assay systems for B cell growth and differentiation factors. The first is the tumour model (Hirano et al 1986, Swain 1985), putative lymphokines are added to the target tumour cells (often the murine lymphoma, BCL_1) and improved growth or Ig secretion are measured by conventional means. The advantage with this type of assay is that the target cells are cloned and well characterised. The absence of contaminating cell types is assured but there is difficulty in extrapolating data obtained on such abnormal cells to normal B cells. The second type of assay is the co-stimulatory assay (Howard et al 1983). In this assay, normal resting B lymphocytes are cultured with antigen or mitogen under sub-optimal conditions and the added lymphokine increases or decreases the measured responses. The assays described in this thesis correspond to this type of assay. The third type of assay is the pre-activation assay (Leanderson 1985). Resting B cells are cultured with a mitogen and the blast cells are recovered, usually by density fractionation and cultured in lymphokine-containing supernatants. Since mitogens such as LPS and anti-Ig are extremely difficult to wash off cells, these assays are in reality likely to be co-stimulatory.

INTRODUCTION TO THIS THESIS.

The sheep is an important animal in immunological research. The

size and temperament of sheep make them ideal for studies for which mice and man are equally poorly suited but for differing reasons. The ability to cannulate the efferent lymphatics of sheep allows the events in a single lymph node to be studied. Similarly, the advances in technologies relating to separation, phenotyping and culture of cells has allowed the contribution of various cell types to be determined. The demonstration that soluble factors play an important role in the control of B cell differentiation prompted this investigation on sheep B cells. The events of sheep B cell differentiation have not been described before.

In order to perform this work it was necessary to develop methods for obtaining purified resting B cells. The IPP is an ideal and plentiful source of such cells. This is a major advantage of this assay compared to those previously available. The development of this model required the phenotyping of the IPP cell and methods for quantifying B cell differentiation. This was facilitated by producing and characterising monoclonal antibodies specific for sheep immunoglobulins. These were successfully used to considerably advance our knowledge of sheep B cell physiology. Further studies on this model are proposed.

CHAPTER TWO.

Chapter Two.

INTRODUCTION.

In order to investigate the control of B cell differentiation in sheep, it was necessary to produce reliable specific probes for ovine immunoglobulins. The basic structures of ovine Igs is similar to the mouse and human, being composed of covalently- linked light and heavy chains. As with other species the Ig heavy chains can be divided into isotypes and immunoglobulins of the IgM, IgG and IgA classes have been described. IgD and IgE have not been described in the sheep.

IgM is the earliest Ig to appear during development and is found in serum and on cell surfaces. It is thought that membrane IgM plays an important role in antigen binding and consequently in B cell activation. Serum IgM is a highly efficient agglutinin and fixes complement. The IgG class is split into IgG1 and IgG2. There are such distinct differences between the sub-classes, that it has been proposed that they constitute infra-class rather than intra-class differences (Butler 1986). There is no antigenic cross-reacivity between the constant regions of the heavy chains and the immunoglobulins are easily separated by ion-exchange chromatography due to their distinct isoelectric points. The functions of the classes are also distinct. IgG1 is the predominant immunoglobulin in serum, lymph, colostrum, intestinal secretions, and alveolar secretions. It fixes complement well and is responsible for the passive transfer of systemic immunity to the young ruminant. IgG2 is found in lower concentrations and fixes complement well. However it is 100 times as efficient as IgG1 at inducing antibody-dependant cytotoxicity (ADCC). Macrophages have been

shown to express receptors for the IgG classes.

IgA is found in intestinal, bronchial and other mucosal secretions. As in other species, IgA does not appear to have an array of effector mechanisms but functions as an 'antiseptic paint'. IgA predominates in milk (but not colostrum), and passively protects the neonatal gut by preventing adherence of pathogenic organisms. The IgA found in milk is largely synthesised in the mammary gland reflecting the gut-mammary recirculation of lymphocytes. By contrast, the IgG1 found in colostrum is transferred from serum into the mammary gland lumen at impressive rates during the last stages of gestation. Receptors for IgG1 have been described in the bovine mammary gland epithelium.

One Ig light chain class accounts for the vast majority if not all the Ig light chain in sheep. Based on C- and N-terminal sequence analysis and peptide mapping, Hood et al (1967) reported that 1) the peptide map of ovine light chains is strikingly simple compared to those from species known to have two light chain classes;

2) the N-terminal sequence of ovine light chains is blocked at the amino group. This is characteristic of lambda chains;

3) serine was the C-terminal amino acid in sheep light chains, this is indicative of lambda chains. The authors concluded that the lambda light chain vastly predominates in the sheep. Similarly, Hood et al found only one light chain class in the cow and this is supported by findings from the few bovine myelomas described so far (Beal and Squires 1970)

Initial attempts to obtain polyclonal antisera that defined the

ovine isotypes resulted in the production of an antisera speciic for α chain (Figure 1). This antisera recognised a 59 kD protein in ovine intestinal lymph by Western blotting (data not shown). Further attempts to produce an identical preparation were unsuccessful. Similarly, the contamination of IgA and IgG2 preparations with lgG1 made repeated absorptions necessary. In order to overcome these problems, it was decided to produce and characterise monoclonal antibodies to ovine immunoglobulins.

The advantages of monoclonal antibodies are

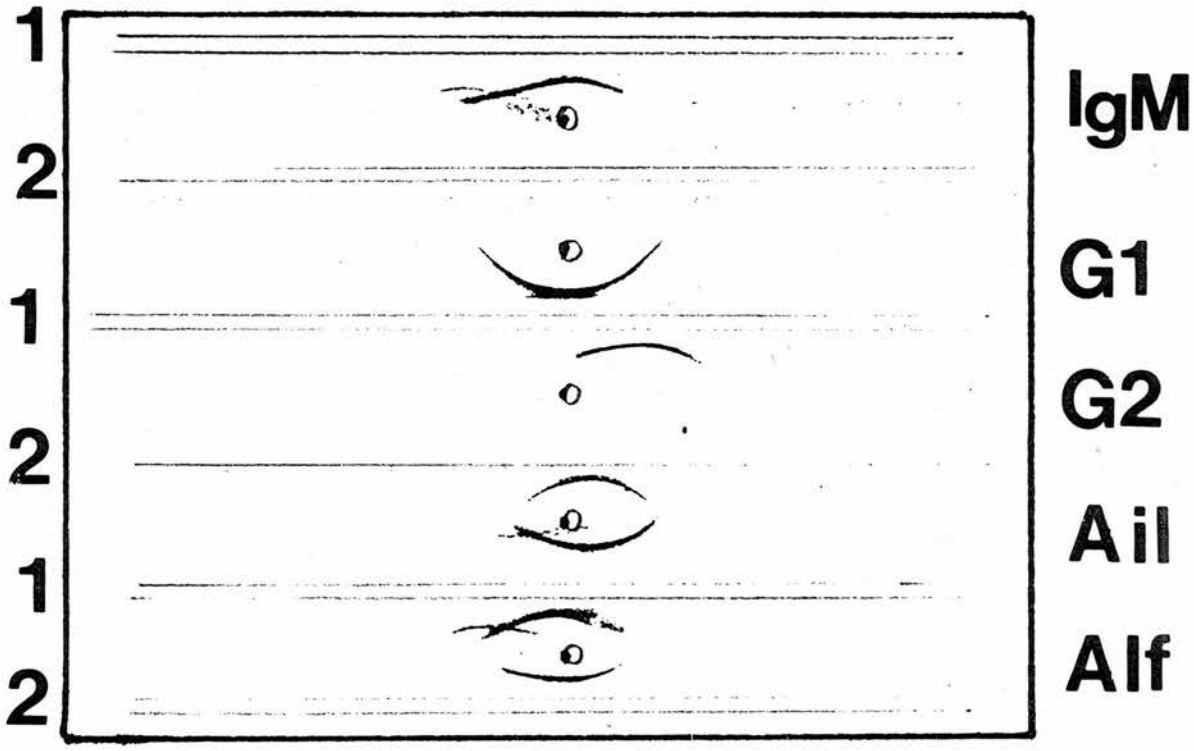
1) the ability to produce highly specific antibodies despite the lack of ovine immunoglobulin standards or highly purified Igs. IgG1 is a common contaminant of preparations of IgA and IgG1 prepared by standard biochemical methods;

2) the ability to produce an antibody probe with unique specificity. Once cloned and characterised, the antibody can be produced with confidence that unwanted specificities will not be present;

3) the ability to produce vast quantities of antibody with this specificity. This allows the reagent to be used in a variety of applications such as affinity chromatography, immunofluorescence, immunohistology and immunoassays. This is in contrast to the situation with monospecific polyclonal antibodies which are usually extremely difficult to produce in large quantities and therefore are used as economically as possible. This almost certainly excludes exchanging reagents with fellow workers and has prevented the establishment of world-wide ovine Ig standards. By contrast, the availability of

Figure 1 . Immunoelectrophoresis of immunoglobulin preparations and precipitation by rabbit antisera.

Preparations of IgM, IgG1 (G1), IgG2 (G2), and IgA were run in an agarose gel as described. IgA was prepared from intestinal lymph (Ail) and from lung fluid (Alf). After electrophoresis, the troughs were removed and filled with 1) rabbit anti-normal sheep serum serum and 2) rabbit anti-sheep IgA heavy chain serum. The gels were washed and stained by Coomassie blue dye.



Immunoelectrophoresis of immunoglobulin preparations and precipitation by rabbit antisera.

monoclonal antibodies to ovine immunoglobulins in the near future will help to correct this situation. It is hoped that the reagents to be described in this chapter will contribute to this advance.

The aim of this work was to produce and characterise monoclonal antibodies to ovine immunoglobulins. In particular, it was wished to produce reagents specific for IgM and Ig light chain. These reagents were successfully used in a variety of applications and were of crucial importance in the work described in following chapters.

PRODUCTION OF MONOCLONAL ANTIBODIES TO OVINE IMMUNOGLOBULINS.

Mice were injected subcutaneously with 50 μg ovine IgG1, prepared by ion-exchange chromatography, in complete Freund's adjuvant. This was followed by twice-repeated intra-peritoneal injections of the same antigen in PBS. The sera from these mice was assayed by ELISA for binding to IgG1. Four days after a final intravenous injection, the mice were killed by cervical dislocation and their spleens were removed by sterile technique. The fusion protocol was as described in the Appendix.

Wells which showed evidence of hybridoma growth were screened for anti-IgG1 antibodies by ELISA, those positive by this assay were transferred to 24 well plates and retested. Hybridomas were cloned by limiting dilution using normal mouse spleen cells as filler cells.

SPECIFICITY OF MONOCLONAL ANTIBODIES.

The supernatants of the cloned hybridomas were tested by protein blotting. Thirty μg of IgG1 was run on a 10% acrylamide gel per track under reducing conditions. The separated proteins were transferred to nitrocellulose paper which was then cut lengthwise and individually probed for two hours. The result of this experiment is demonstrated in Figure 2 for two hybridoma supernatants; VPM 8 and VPM 6.

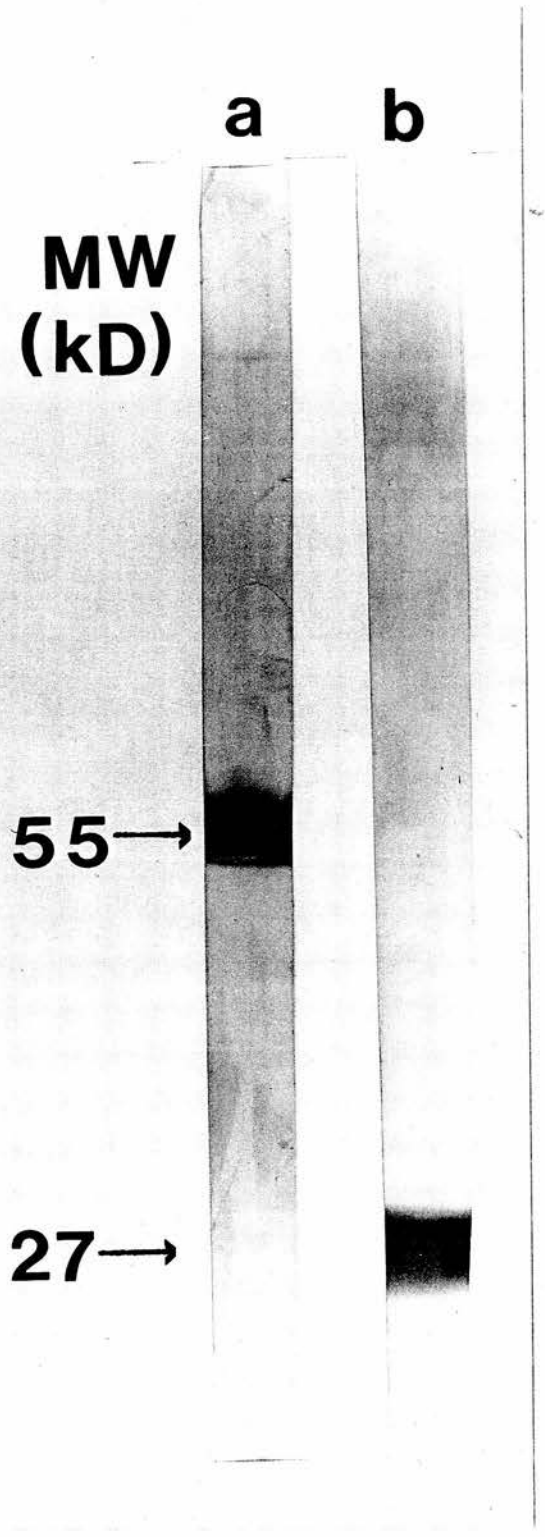
VPM 8 binds to a polypeptide with a molecular weight of approximately 27 kD while VPM 6 binds to a heavier chain at 55 kD. These are the molecular weights of ovine light chain and IgG1-heavy chain respectively (Butler 1984 and personal observations). An identical binding pattern was obtained when a variety of immunoglobulin-containing fluids were used eg serum, lymph, and colostrum (data not shown).

MONOCLONAL ANTIBODY REACTIVE WITH OVINE IgM.

Workers at the Moredun Research Institute isolated a murine hybridoma (VPM13) which produced a putative anti-IgM antibody. Dr. Hugh Millar kindly allowed me to have hybridoma cells in order to do some further characterisation.

Figure 2. VPM 8 and VPM 6 recognise different molecular weight components of IgG1.

Ovine IgG1 was run on a 10 % SDS-PAGE gel under dissociating conditions. After protein blotting, the nitrocellulose paper was probed with a) VPM 6 and b) VPM 8. The molecular weights of the bands are shown.



VPM 8 and VPM 6 recognise different molecular weight components of IgG1.

AFFINITY PURIFICATION OF THE ANTIGEN(S) RECOGNISED BY VPM 6.

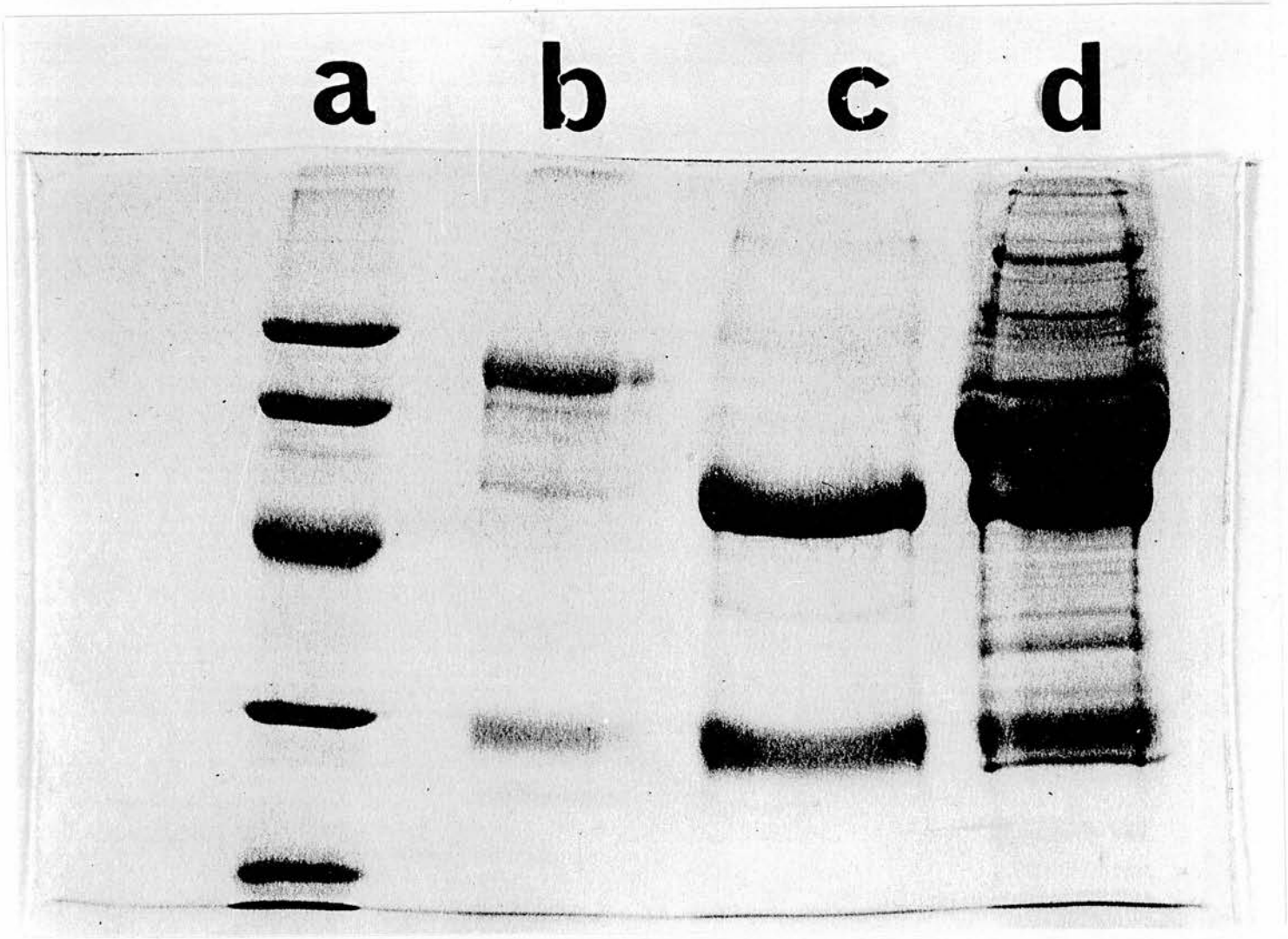
VPM 6 antibody was affinity purified from ascitic fluid produced in mice injected intra-peritoneally with VPM 6 cells. This antibody was coupled to CNBr-activated Sepharose to produce a VPM 6-Sepharose column. Protein was purified from normal sheep serum using this column and this was analysed by SDS-PAGE and immunoelectrophoresis (IEP). By SDS-PAGE, this protein consists of two polypeptides at 28 and 55 kD (Figure 3). When transferred to nitrocellulose paper, VPM 6 binds to the high molecular weight band while VPM 8 binds to the low molecular weight band. By IEP, the protein purified by VPM 6-Sepharose shows mobility characteristic of ovine IgG1 and distinct from that of IgG2 and IgA (Figure 4).

AFFINITY PURIFICATION OF THE ANTIGEN(S) RECOGNISED BY VPM 13.

VPM 13 antibody was purified from ascitic fluid by affinity chromatography on Protein A-Sepharose and insolubilised onto Sepharose beads. This column was used to purify the antigen(s) recognised by VPM 13 from serum and intestinal lymph. When examined by SDS-PAGE (Figure 3), the purified protein was found to consist of two bands at 78 kD and 28 kD. VPM 8 bound to the latter by protein blotting (data not shown). VPM 13 did not bind to any bands in a variety of body fluids or immunoglobulin preparations by Western blotting.

Figure 3. **The antigens recognised by VPM 13 and VPM 6.**

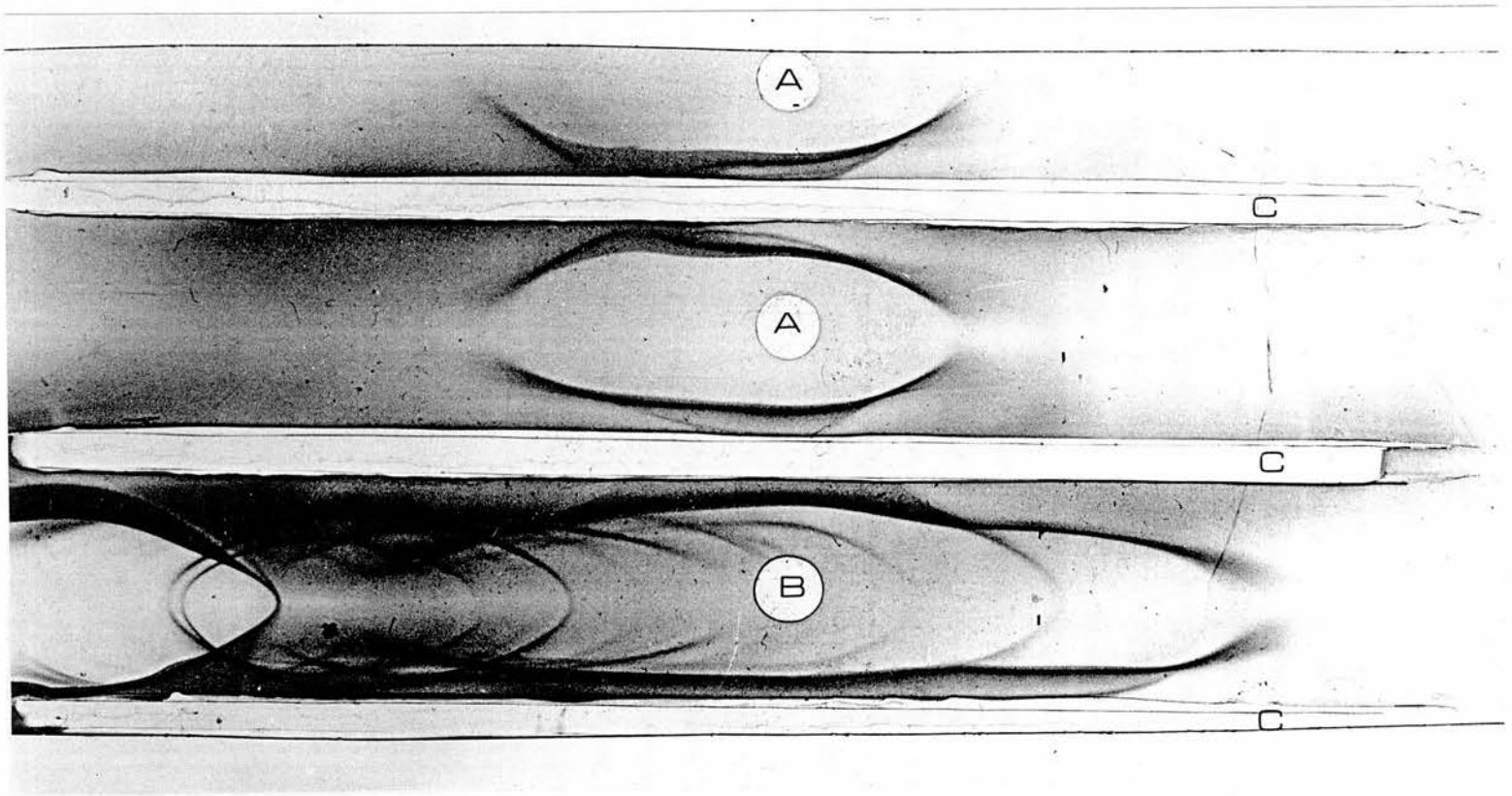
Monoclonal antibodies, VPM 13 and VPM 6 were used to affinity purify their specific antigens b and c respectively, from normal sheep serum (d). The purified material was run on a 10 % SDS-PAGE gel under dissociating conditions. The gel was stained by 0.025% Coomassie blue. Molecular weight markers are shown in track a.



The antigens recognised by VPM 13 and VPM 6.

Figure 4. **Immunoelectrophoresis of the VPM6-antigen.**

The antigen isolated using VPM6-Sepharose was analysed by immunoelectrophoresis. A: affinity-purified VPM6 antigen. B: normal sheep serum. C: trough filled with rabbit anti-sheep serum.


A
C
A
C
B
C

IMMUNOFLUORESCENCE ON OVINE TISSUES.

The supernatants from these positive hybridomas were tested by immunofluorescence for binding to lymphoid cells from various sources. The results by flow cyometric analysis are shown in Table I. Both these reagents recognise cell surface molecules. The anti-light chain reagent and rabbit anti-sheep Ig antibody stain a similar percentage of cells from each source. This was expected as there appears to be only one isotype of light chain in the sheep (Hood et al 1967). An anti-Class II β-chain monoclonal antibody (VPM 16) from Drs.Hopkins and Dutia is included as a positive control.

IMMUNOHISTOLOGY.

The distribution of the antigens recognised by these mAbs was determined by immunohistology on cryostat sections of spleen, thymus, and lymph node. As can be seen in Figures 5 and 6, VPM 8 and VPM 13 recognise follicular accumulations of cells in the B cell areas of spleen and lymph node. In particular, it can be seen that VPM 8 and VPM 13 stain the lymphoid tissue around the arterioles of the spleen. As expected from the immunofluorescence results, VPM-6 resulted in very little staining of organised lymphoid tissues.There was no staining of the thymic follicles with any of the monoclonals (VPMs 8, 6, & 13), though there was variable staining of the interfollicular tissue due to its serum Ig content.

Table I. **Reactivity of monoclonal antibodies specific for sheep immunoglobulin with various lymphoid tissues.**

Lymphocytes from the spleen, thymus and spleen were prepared and stained using murine monoclonal antibodies to sheep lymphocyte antigens. The samples were analysed by flow cytometry. The percentage of positively staining cells is reported.

ND = not determined.

Reactivity of monoclonal antibodies specific for sheep immunoglobulin with various lymphoid tissues.

Specificity.	Percentage of positive cells.				
	Tissue.(Lamb/adult).				
	Spleen (lamb)	Peripheral blood lymphocytes (adult).	(lamb).	(lamb).	Thymus (lamb)
Ig light-chain	27	36	79	59	2
IgM	23	18	72	50	1
IgG1	3	6	13	10	2
MHC class II	ND	42	78	ND	17
Negative	<1	<1	<1	<1	<1

TABLE I.

Figure 5. **Reactivity of monoclonal antibodies to lymphocyte antigens in sheep spleen.**

Cryostat sections of sheep spleen were prepared and stained indirectly using a) VPM-8, b) VPM-13, c) VPM 16 (anti-MHC class II), and d) anti-HCG (negative control). Original magnification x 250. Counterstained with methylene green.

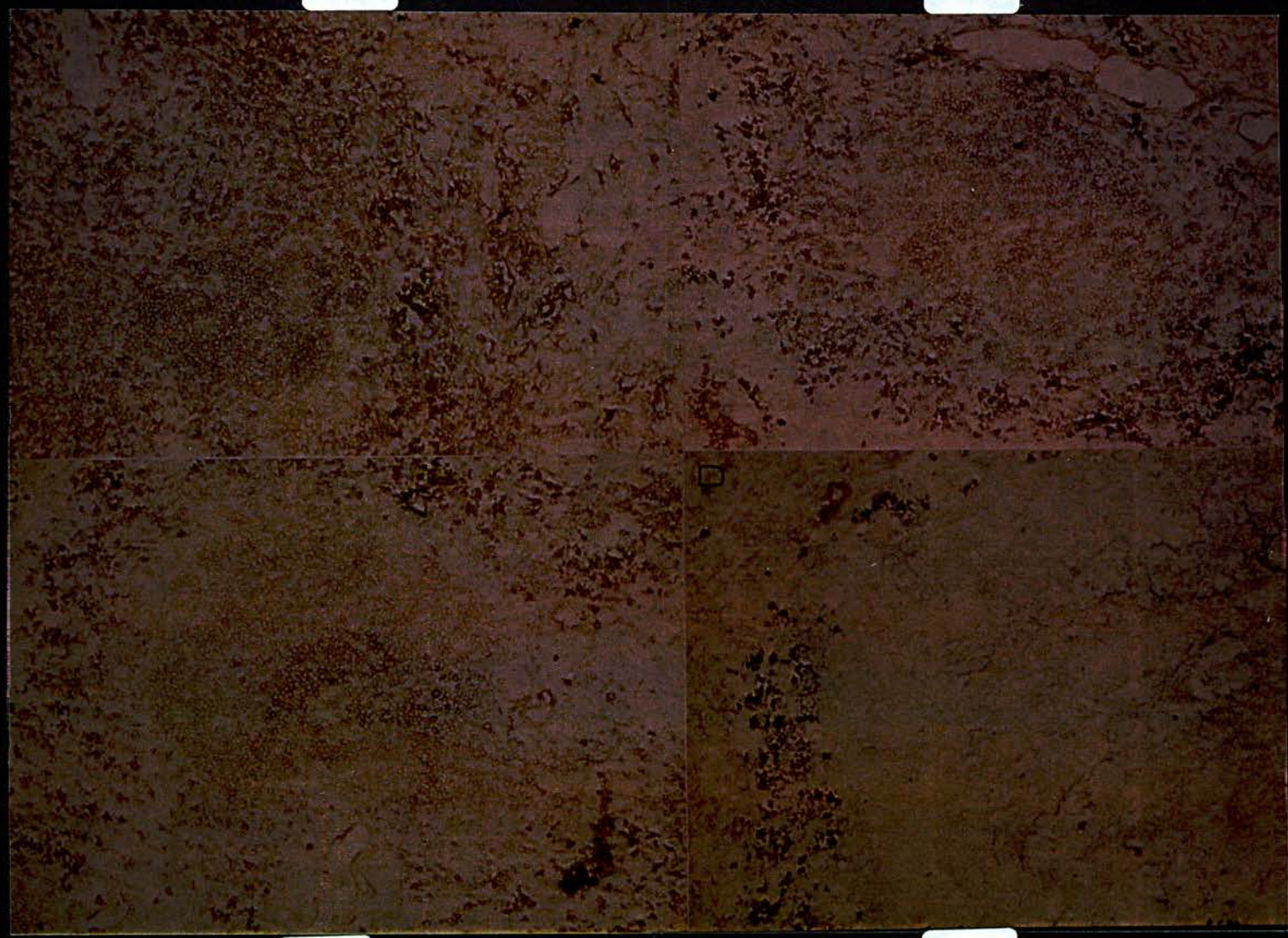

A
B
C
D
D

Figure 6. Reactivity of monoclonal antibodies to lymphocyte antigens in sheep lymph node.

Cryostat sections of sheep mesenteric lymph node were prepared and stained indirectly using a) VPM-8, b) VPM-13, c) VPM 16 (anti-MHC class II), and d) anti-HCG (negative control). Original magnification x 250. Counterstained by methylene green.


a
b
c
d

Chapter Two.

DEVELOPMENT OF AN ELISA TO QUANTITATE IMMUNOGLOBULINS.

A rabbit anti-ovine light chain antisera was prepared by immunising with affinity-purified IgM and sera were absorbed onto IgG1-Sepharose. The specificity of the eluted material was demonstrated by Western blotting. This antibody was diluted in coating buffer (Nakamura, Voller and Bidwell, 1986) to 3µg/ml and 100µl was placed in each well of an ELISA microtitre plate (Dynatech Laboratories Ltd.,England). This was incubated overnight at 4°C in a humid chamber.

The coating solution was removed and two hundred microlitres of 1% bovine serum albumin in PBS (PBS-BSA) were used to reduce non-specific binding in each well. The plates were washed in PBS-BSA with 0.05% Tween-20 (PBS-BSA-Tw20) and then incubated with 100µl of each sample. After 45 minutes the plates were washed three times in PBS-BSA-Tw20 and then incubated with monoclonal anti-sheep light chain (VPM-8) supernatant for a further 30 minutes. Rabbit anti-mouse IgG (H+L) immunoglobulin conjugated to HRPx was either purchased from ICN (Israel) or was prepared in the laboratory by myself. After washing the plates as above, this reagent was diluted in PBS-BSA-Tw20 and 100µl added to each well for 30 minutes. The wells were washed five times and 150µl of freshly prepared substrate added. Forty milligrams of ortho-phenylene diamine (OPD) was dissolved in 100 mls of 0.1 M citrate-phosphate buffer pH 5.0 with 40 µl of hydrogen peroxide. The reaction was allowed to continue in the dark for 20 minutes and was stopped with 50 µl of 2 M sulphuric acid. Absorbance was read at 492 nm on an automatic photometer (Titertek). The optimal concentration of

each reagent was determined using a chequerboard arrangement of ELISA plates with vertical and horizontal titrations. The optimal incubation times and washing proceedures were determined on replica plates.

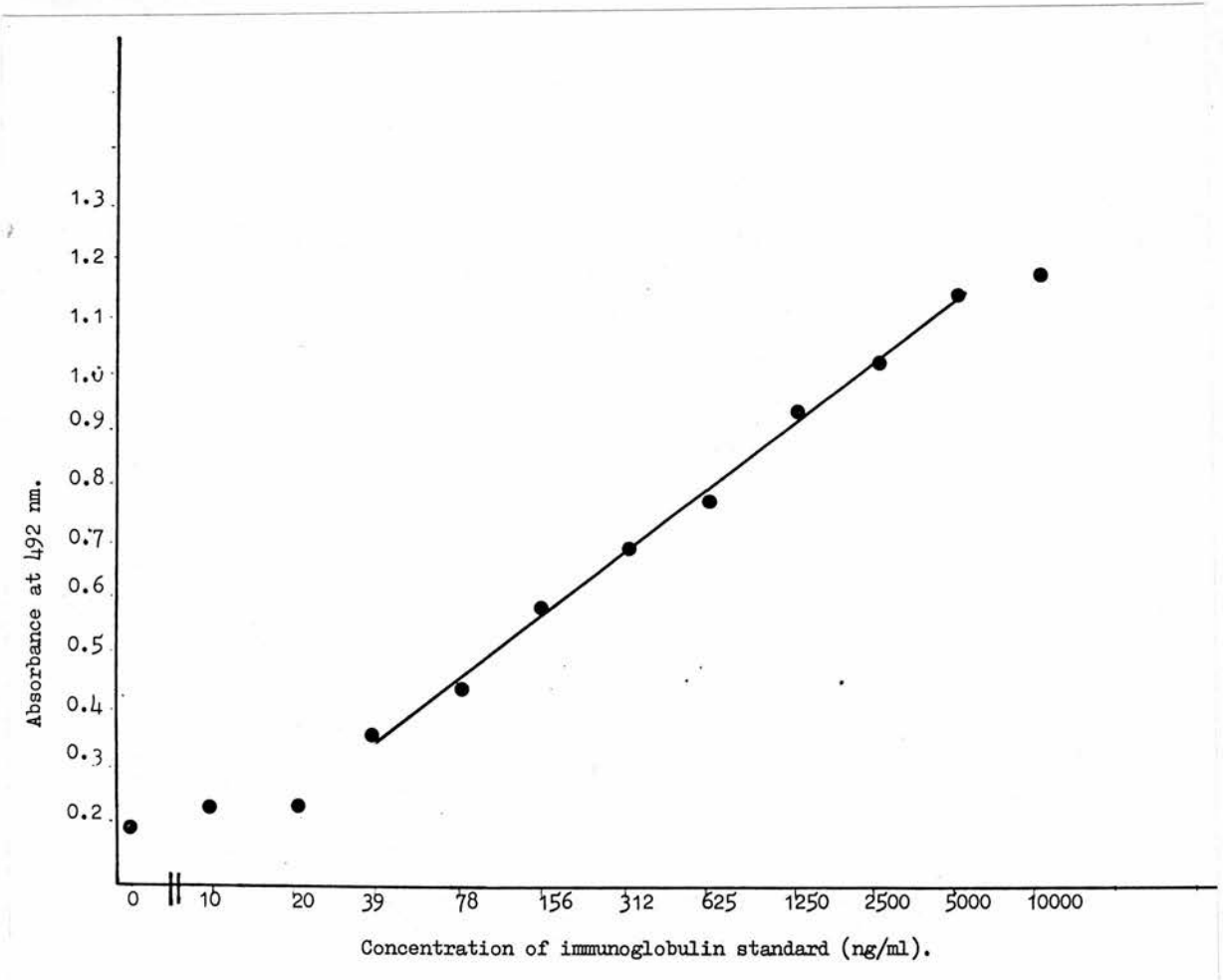
Affinity-purified IgG1 was used as a reference. The immunoglobulin concentration was determined using the Bio-Rad protein assay according to the manufacturer's instructions. The immunoglobulin standard was kept at 4°C in 50 mM tris 0.15 M NaCl pH 8.0 with 1% BSA to aid stability. The standard sample kept under these conditions showed no loss of antigen over six months when compared to a duplicate vial kept at -70°C. Doubling dilutions in the range of 10-10000ng/ml were included in triplicate. The mean and standard deviation of the absorbance were calculated for each reference sample, the standard deviation was always less than 10% of the mean. Immunoglobulin concentrations of samples were derived by plotting a standard curve of $\log_{10}$ immunoglobulin concentration against absorbance at 492 nm. This resulted in a linear correlation over the range 50-5000 ng/ml (Figure 7).

This assay has been principally used to detect total immunoglobulin using the monoclonal anti-immunoglobulin light chain. This is possible as there appears to only one light chain in ruminants, the lambda chain (Hood et al, 1967, Butler 1983).

It was initially intended that this ELISA should be used to determine total Ig concentration in a variety of ovine body fluids. However, in the case of serum for example, it was neccessary to dilute samples to at least 1/100,000 in order to get optical density readings in the desired range. This limits the wider use of this particular

Figure 7 **A representative standard curve derived from the ELISA for immunoglobulin.**

Doubling dilutions of affinity purified IgG1 of known protein concentration, were analysed in triplicate. The mean absorbance at 492 nm of each protein concentration was calculated. Absorbance is plotted against log immunoglobulin concentration.


Absorbance at 492 nm.
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
10
20
39
78
156
312
625
1250
2500
5000
10000
Concentration of immunoglobulin standard (ng/ml).

ELISA for such work. An advantage of using an indirect system to detect the bound murine antibody is that this assay is easy to adapt for other mouse antibodies such as the anti-IgM or anti-IgG1 monoclonal antibodies or for screening large numbers of supernatants of putative anti-immunoglobulin hybridomas.

DISCUSSION.

This chapter describes the use of hybridoma technology to prepare monoclonal antibodies of well defined specificity against sheep immunoglobulins. VPM 8 recognises immunoglobulin light chain under a variety of conditions, including high concentrations of detergent. Similarly, VPM 6 binds to immunoglobulin IgG1 heavy chain. VPM 13 binds to sheep IgM as demonstrated by its ability to affinity purify the whole molecule and its failure to bind to preparations of other immunoglobulin isotypes by ELISA. The molecular weight of ovine μ chain is similar to that recorded in other species. This is not surprising as the μ chain is strongly conserved across species. The polypeptide recognised by VPM 13 could not be determined by Western blotting. This indicates that either the intact epitope requires the IgM heavy and light chains to be covalently linked or that the epitope on the IgM heavy chain is lost under dissociating conditions.

The anti-light chain appears to recognise all IgM bearing cells in the IPP by immunofluorescence (see Chapter Three) and stains a similar number of cells to polyclonal anti-Ig sera. Since serological cross-

reactivity between the lambda and kappa light chains has not been recorded in other species, it appears that one immunoglobulin light chain isotype predominates in the sheep. This confirms the work of Hood et al (1967) who proposed that lambda is the predominant isotype. This is identical to the situation in the bovine. No kappa light chain was present in the only bovine myeloma proteins found so far (Beal and Squires 1970). The reason for this apparent discrimination against kappa light chains is unknown.

In the mouse, lambda light chains are only rearranged when there has been a failure to produce a functional kappa gene on both chromosomes. Hence in mouse, there is a predominance of kappa genes in the approximate ratio of 20:1. The order in which the sheep light chain genes are rearranged is obviously unknown but in other species studied to date have been similar to the mouse. Even if the reverse situation applies in the sheep, it is unlikely that successful rearrangements on the first isotype (lambda) genes could so effectively exclude the second (kappa). Lewis et al (1985) have calcualated that only 33% of light chain rearrangements at a locus will be successful. The implication is that via a mechanism at present unknown, rearrangements of the kappa genes do not occur or are destined for failure. This would mean that for a functional light chain gene to be produced, one of the lambda gene rearrangements must be successful. As discussed above, this is not a frequent occurence and must contribute production of large numbers of non-functional B cells. This may have significance with respect to the low numbers of cells exiting the ileal Peyer's patch compared to the cells produced in that region.

The development of these monoclonal antibodies has allowed the production of useful reagents for the detection of both soluble and membrane immunoglobulin. In the case of soluble immunoglobulin it has been possible to devise an enzyme immuno-assay to detect immunoglobulin present at low concentrations (>10 ng/ml). In addition these reagents have been used to immunopurify sheep immunoglobulins for further studies. It is hoped that continued attempts to produce monoclonal antibodies against sheep immunoglobulin isotypes will be successful and allow further characterisation of sheep immunoglobulins.

Chapter Three.

CHAPTER THREE.

CHAPTER THREE.

INTRODUCTION.

The ileal Peyer's patch is an important source of B cell production in lambs. The phenotype of the IPP lymphocyte has been analysed by Miyasaki et al (1984) using polyclonal sera to sheep Ig and this supports the hypothesis that the IPP is composed almost entirely of B cells, the vast majority of which express IgM and MHC class II antigens. The development of monoclonal antibodies to sheep Igs and in particular to IgM and Ig light chain has allowed more critical analysis of the IPP lymphocyte and this is described in this chapter. In addition, the availability of monoclonal antibodies to other sheep lymphocyte antigens has reinforced the hypothesis that the IPP is almost entirely composed of B cells. As such it can be considered that the IPP is the true equivalent of the bursa of Fabricius in birds, a B cell primary lymphoid organ.

IgD is frequently coexpressed with IgM on B human and murine B cells particularly those which have never encountered antigen or other positive stimuli. Although IgD has not been definately shown to possess a particular function in B cell physiology, it is assumed that it may play an important role in initial binding of antigen to the surface of B cells. This is reinforced by the finding that quantitatively IgD is the major immunoglobulin on the membrane of resting B cells. Since IgD has not been demonstrated previously, the availability of monoclonal

antibodies to sheep Igs allowed an analysis of the total surface Igs of the IPP lymphocyte for the first time. The preliminary findings suggest that there is a major immunoglobulin species on the surface of IPP lymphocytes and this novel Ig is almost certainly coexpressed with IgM.

GROSS ANATOMY OF THE ILEAL PEYER'S PATCH OF LAMBS.

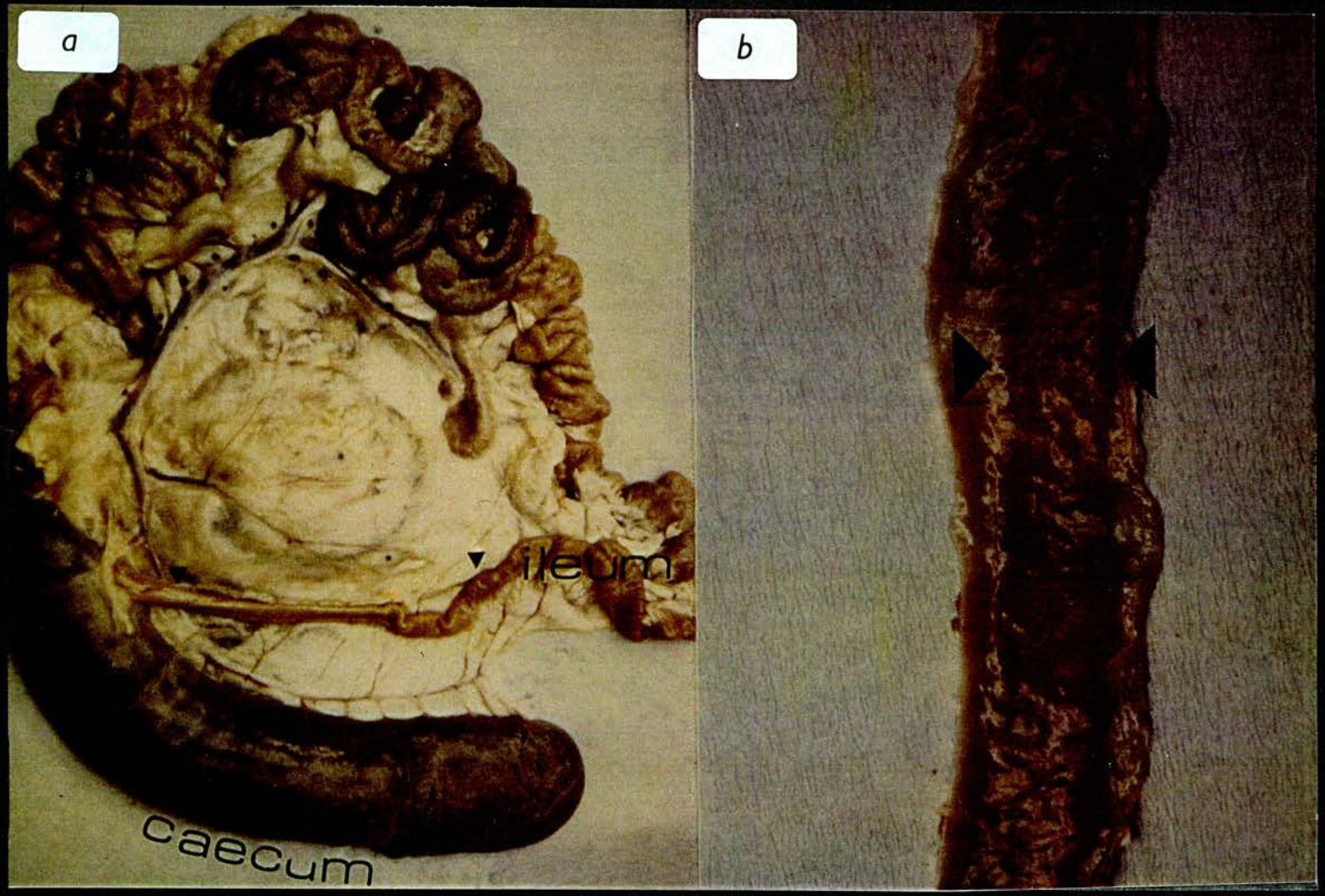
The ileum of lambs is the terminal part of the small intestine and joins the caecum distally at the ileo-caecal valve (Figure 8). At the abbatoir, the abdominal contents are removed within 15 minutes of stunning and bleeding and are placed on moving trays. The ileal Peyer's patch is clearly visible extending along nearly 1 metre of the terminal small intestine. The last 15-20 cms of the ileum has a double mesentery, a major contribution from the common mesentery and a branch from the caecum; the ileo-caecal fold. This "landmark" allowed me to always take specimens from within the same anatomical area.

When the intestine is opened along the attached border, the continuous IPP can be seen on the opposite border occupying approximately 50% of the gut circumference. The IPP is a distinct raised area surrounded on either side by normal intestinal tissue (Figure 8b).

DEVELOPMENT OF THE IPP <u>IN UTERO</u>.

Figure 8. **The gross anatomy of the IPP.**

The abdominal viscera of a lamb are shown in (a). The caecum and ileum are indicated. The area from which the IPP is obtained is marked between the small arrows (approximate distance = 15 cms). In (b), the ileum has been opened along the mesenteric border. The width of the IPP is demonstrated by the large arrows (approximate distance = 2 cms).


a
ileum
caecum
b

The IPP develops early *in utero* (Reynolds and Morris 1983) The samples shown in Figure 9 were taken from a foetus of approximately 90 days gestation as judged by foetal length and hair development (the full length of gestation is 150 days). These show the developing follicles already containing IgM bearing cells. As this IPP was detectable by immunohistology for a considerable proportion of the foetal gut; the cells of the IPP must contribute a high percentage of the total lymphoid population at this stage of *in utero* development. By contrast, the spleen only showed occassional disordered accumulations of cells that stained for immunoglobulin (Maddox, MacKay and Brandon 1987a,b,& c and Figure 10). The liver does not appear to have any Ig bearing cells at this stage of development (data not shown) while attempts to obtain foetal bone marrow were unsuccessful. Al Salami *et al* (1985) reported that the foetal bone marrow and liver are lymphopenic.

HISTOLOGY.

As can be seen from the paraffin section of the IPP of a six month old lamb stained with haematoxylin and eosin (Figure 11), the lymphoid tissue in this area consists of tightly packed follicles in the sub-mucosal area. There is very little inter-follicular tissue characteristic of Peyer's patches in other species and the jejunal Peyer's patch of sheep. Each follicle in the IPP is composed of a dense

Figure 9. **Immunohistology of foetal IPP.**

The IPP was taken from a sheep foetus (approximate gestational age 90 days) and stained for b) Ig light chain, c) IgM, and d) HCG (negative control). Counterstained by methylene green. Foetal IPP stained by H & E is shown in (a). Original magnification in (a) = 250, (b) and (c) = 100, (d) = 40.

C
A
D
B

Figure 10 . **Immunohistology of foetal spleen.**

The spleen was taken from a sheep foetus (approximate gestational age 90 days) and stained for b) Ig light chain, c) IgM, and d) HCG (negative control). Counterstained by methylene green. Foetal spleen stained by H & E is shown in (a). Original magnification x 100.

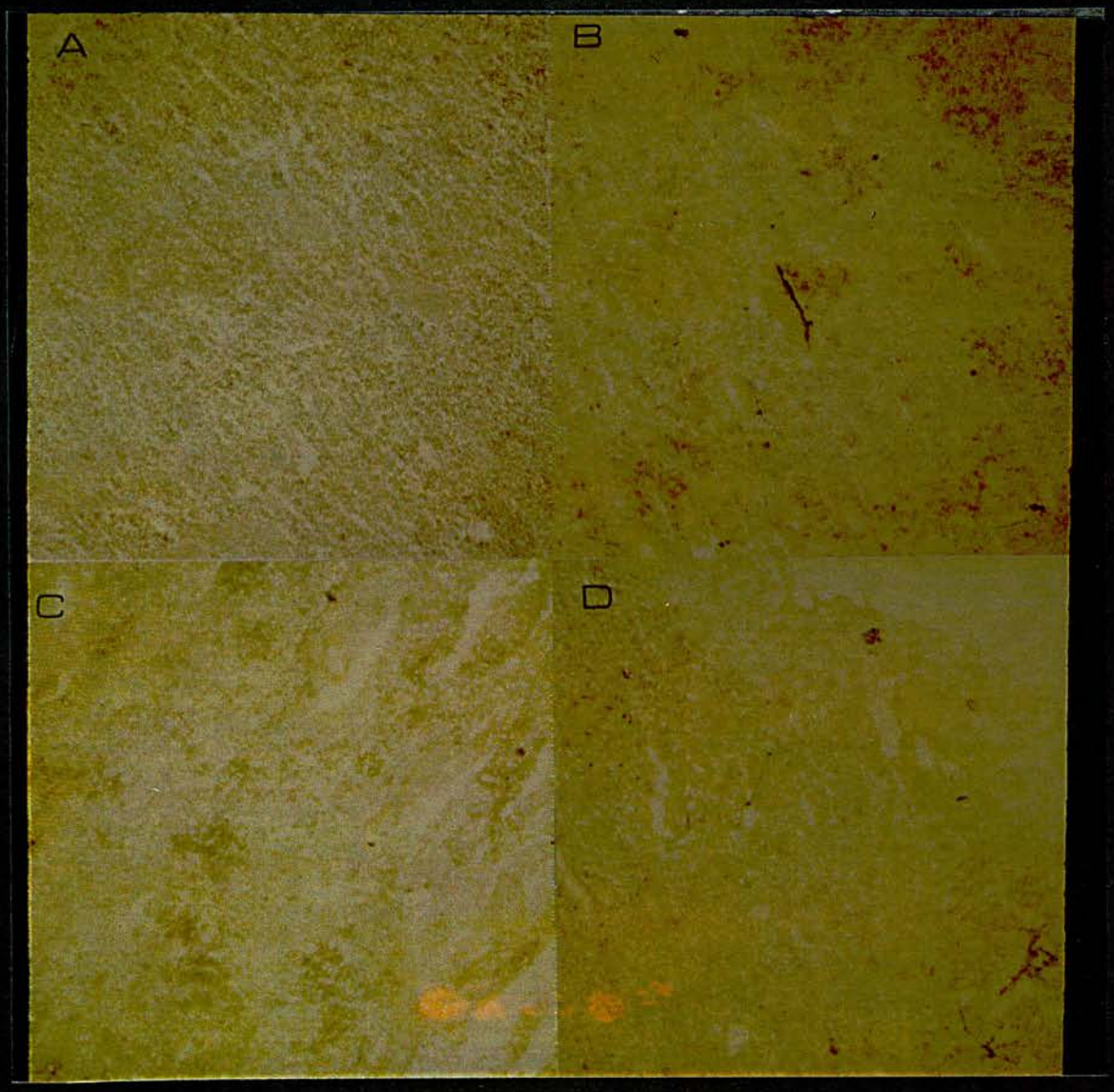
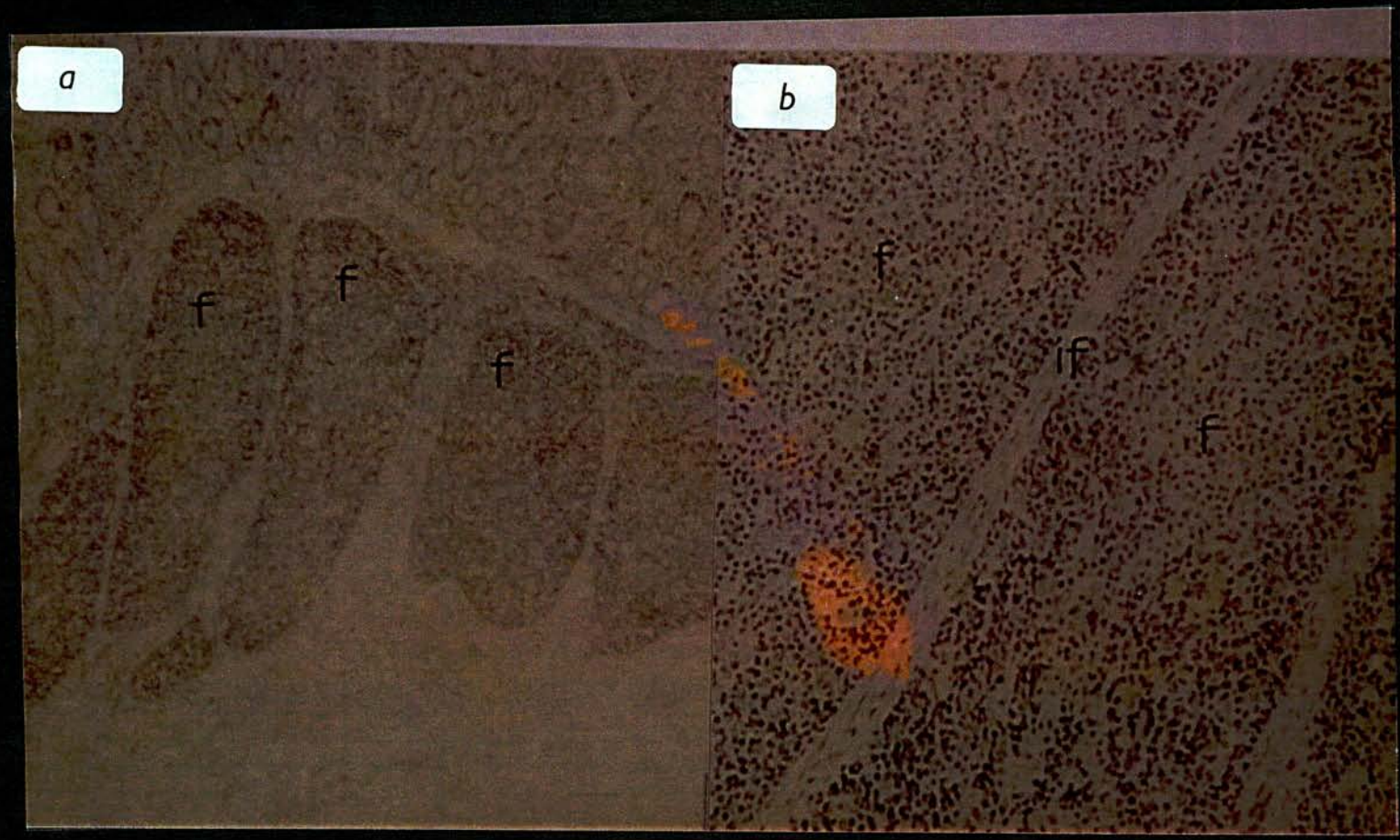

A
B
C
D

Figure 11. Histology of the ileal Peyer's patch.

Paraffin-embedded sections of IPP were stained using haematoxylin and eosin. (f) follicles, (if) interfollicular area. Original magnification (a) = 40, (b) = 250.


a
f
f
f
b
f
if
f

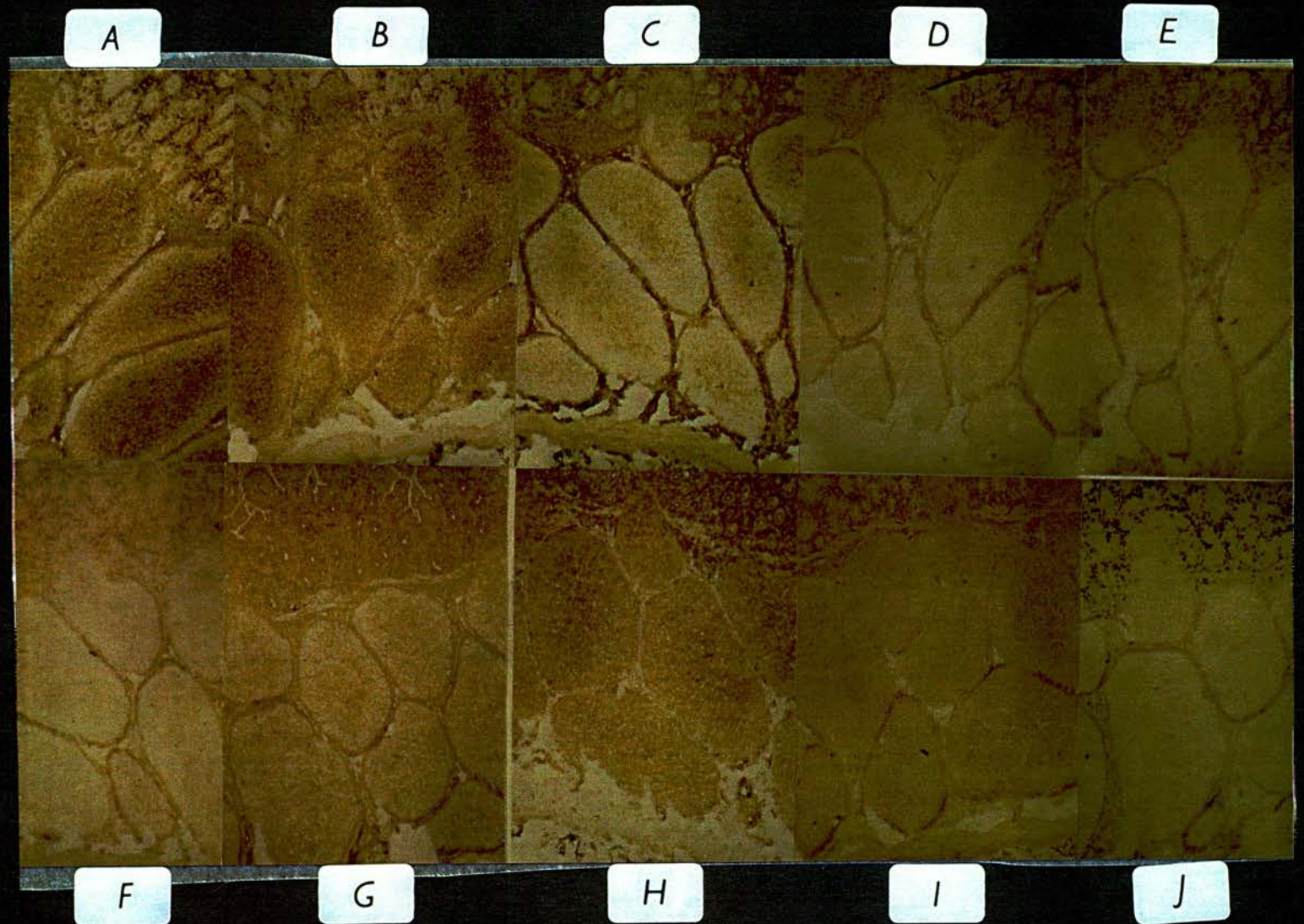
accumulation of cells enclosed by a small amount of connective tissue. The follicles are elongated parallel to the radius of the ileum.

IMMUNOHISTOLOGY OF THE IPP OF A SIX MONTH-OLD LAMB.

The IPP can be stained using the available monoclonal antibodies directed against ovine lymphocyte surface antigens (Figure 12). The monoclonal antibodies used are described in the Appendix. All follicles stain for immunoglobulin light chain with increased density near the centre of the follicle. This may indicate centripetal maturation of the cells prior to exiting the IPP. There is also staining of the mucosa and connective tissue, this is due to the high concentrations of circulating immunoglobulins which penetrate the connective tissue and become fixed there during processing of the section. This complication affects all immunohistological staining for immunoglobulin (Rouse and Warnke 1986) and is a particular problem when staining for IgG1 which because of its size and high serum concentration results in a high degree of staining for the follicle capsule. The anti-IgG1 antibody only stains the very centre of each follicle. On closer examination, it can be seen that this does not resemble the "honeycomb" circum-cellular pattern of staining seen with anti-IgM and anti-light chain. Staining by anti-IgG1 antibody is likely to be due to the increasing amount of connective tissue support at the centre of the follicle. This is substantiated by the immunofluorescence data (see later). On the other hand, due the likely large size of the IgM molecule, it does not

Figure 12 . **Immunohistology of the ileal Peyer's patch.**

Cryostat sections of the IPP were prepared and stained for a) Ig light chain, b) IgM, c) IgG1, d) CD5, e) CD4, f) CD8, g) MHC class I, h) MHC class II, i) LCA, j) HCG (negative control). Counterstained by methylene green. Magnification x 100.


A
B
C
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G
H
I
J

penetrate connective tissue easily. This is demonstrated by the reduced staining of connective tissue with the anti-IgM antibody. All follicles stain well for IgM and demonstrate that the level of light chain staining is due, at least in part, to the expression of IgM.

Staining of IPP sections by anti-MHC class II reagents shows that the follicles are composed of class II positive cells. The staining is more intense centrally. Staining for MHC class I antigens appears to uniform across the follicle but it is evident that staining of the mucosal tissue is more intense.

The mucosal tissues do not stain for leucocyte common antigens while the folliclar tissue is positive.

Using monoclonal antibodies against surface antigens associated with T cells such as T1,T4 and T8 results in no staining of follicles above background. The negative control was a mouse monoclonal antibody directed against human chorionic gonadotrophin hormone supplied by Mr. J.N. Flynn of this department.

PREPARATION OF ILEAL PEYER'S PATCH LYMPHOCYTES;

Short lengths of ileum were taken from healthy lambs being slaughtered at the City of Edinburgh abbatoir. In order to standardise conditions, the region of ileum taken was always between the first attachment of the ileo-caecal mesentery and the ileo-caecal junction, a length of approximately 15 cms (Figure 8). The ileum was opened along its attached border and cut into 3 cm lengths. These sections of

intestine were washed extensively in medium and then placed in Petri dishes. Using two 19-gauge needles with shafts bend at right angles, the mucosa was gently scraped away, disrupting the follicles underneath. The cell suspension was transferred to a 50 ml centrifuge tube, and the clumps were allowed to settle for five minutes and separated. The cells were washed and then laid onto Lymphoprepr and centrifuged for twenty minutes at 300g. The interface cells were removed and washed three times in medium. Viability was determined using trypan blue dye exclusion and was always in excess of 95 %. Total yield of cells was in the range of 2-5 x 108 cells per ileum.

CELLULAR MORPHOLOGY.

The structure of the IPP cell was examined by light and electron microscopy. Photomicrographs of typical cells are shown in Figures 21 and 22. These demonstrate that the unstimulated IPP cells are uniformly small with very little cytoplasm. The nucleus occupies a large percentage of the cell and contains dense nuclear chromatin. The cells usually only contain one or two mitochondria per section and have no visible rough endoplasmic reticulum.

IMMUNOFLUORESCENCE.

A single cell suspension of ileal Peyer's patch lymphocytes was

prepared as described and stained using the available monoclonal antibodies (see Appendix). Early attempts to determine the number of cells staining positively for surface immunoglobulin using u-v microscopy were confounded by the very low amounts of antigen present on the membrane. This low expression had already been referred to by Miyasaki et al. (1984).

A preparation of stained cells were analysed by flow cytometry (FACS IV, Becton Dickinson) at the Department of Zoology at the University of Edinburgh. A typical phenotype of resting IPP cells is demonstrated in Table II and Figure 13.

When using the flow cytometer, a sample of uniform fluorescent particles were run prior to the experiment in order to find optimal adjustment positions and detect problems in the flow system. A logarithmic amplifier was used in order to give good resolution over the wide range of fluorescent signals. Indeed stained cells displayed in log form appear to have a normal distribution and this is helpful when finding break points in order to aid analysis and to discriminate between two or more intermingled populations. The apparent normal distribution of $\log_2$ fluorescence is not surprising since the control of the amount of cell surface proteins is frequently controlled by a series of multiplicative synthesis steps. The logarithmic amplifier was adjusted to give a 30 channel increase for a doubling in fluorescence intensity, in other words a cell at channel number 170 was twice as fluorescent as one at channel 140, assuming the cells were same size.

When analysing a population of cells the first sample was always unstained cells. These cells were treated similarly to the other

Table II. **Phenotype of lymphocytes from the ileal Peyer's patch.**

IPP lymphocytes were prepared and stained indirectly using monoclonal antibodies to sheep lymphocyte antigens. The samples were analysed by flow cytometry. The percentage of positive cells and the channel number corresponding to the positive peak is reported.

nd = not determined.

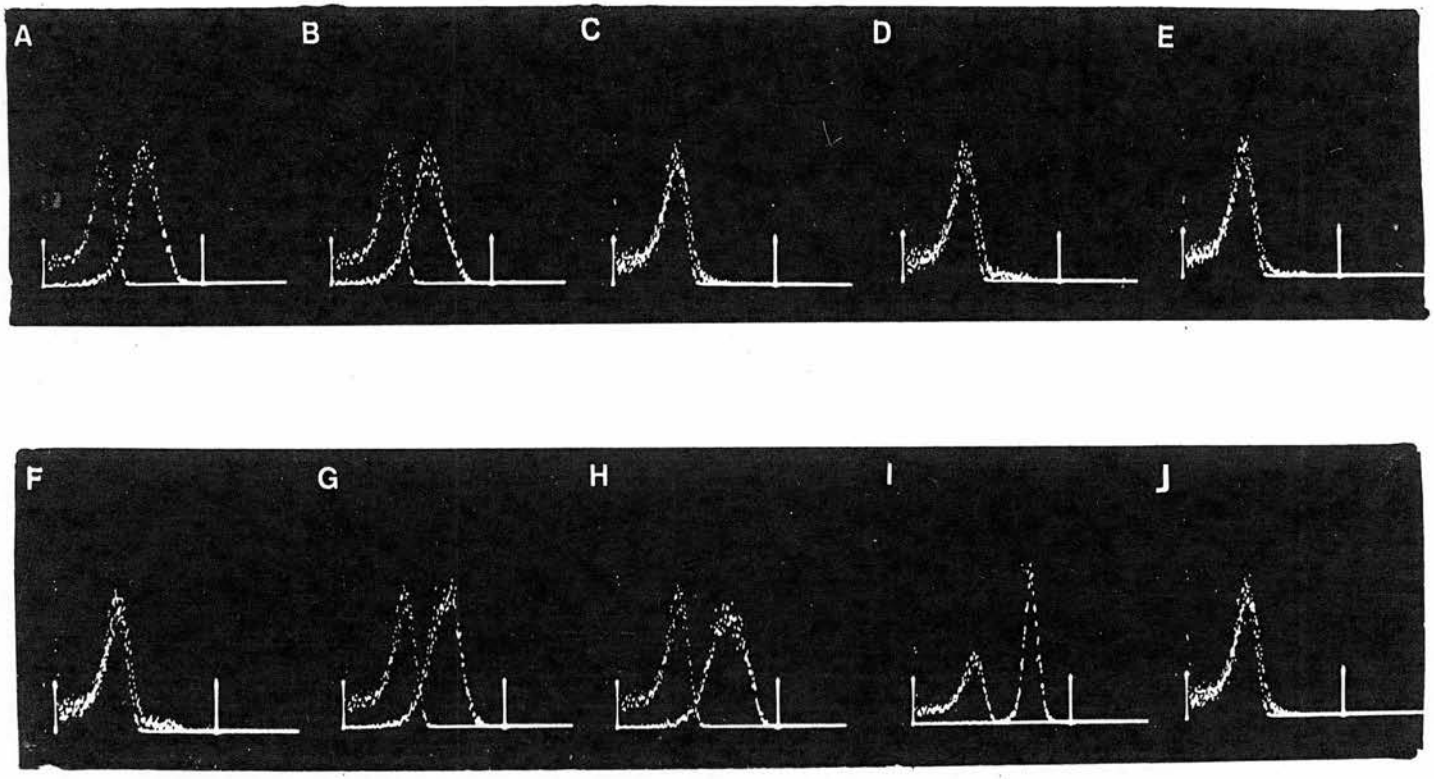
Phenotype of lymphocytes from the ileal Peyer's patch.

Antigen.	Positive cells (%)	Peak fluorescence intensity.
Ig light chain	84.0	163
IgM heavy chain	74.6	155
IgG1 heavy chain	2.6	nd
T1 (CD5)	4.8	150
T4 (CD4)	2.8	nd
T8 (CD8)	5.2	180
MHC Class I	82.8	168
MHC Class II	91.2	178
L.C.A.	99.4	191
Negative	2.6	(103)
2nd Ab Control	2.2	(104)
Unstained	0.08	(101)

TABLE II.

Figure 13. **The phenotype of ileal Peyer's patch lymphocytes.**

A single cell suspension of IPP cells was stained indirectly using mouse monoclonal antibodies to ovine lymphocyte antigens. The samples were examined by FACS. Cell numbers are shown on the vertical axis and log fluorescence intensity on the horizontal. The staining profile for each lymphocyte antigen is superimposed on the unstained histogram for comparison. The vertical scale in sample I has been reduced to accomodate the height of the positive peak. A) Ig light chain. B) IgM. C) IgG1. D) CD5. E) CD4. F) CD8. G) MHC class I. H) MHC class II. I) LCA. J) HCG (negative control).



The phenotype of ileal Peyer's patch lymphocytes.

samples with respect to washing steps and fixation (if performed). This sample was analysed by forward and 90° scatter. Forward light scatter is almost proportional to cell size and provides good live-dead discrimination as well as distinguishing platelets and RBCs (particularly in preparations of blood lymphocytes). Ninety degree scatter is less responsive to cell size but reflects the internal complexity of the cells and therefore is used to detect contamination by granulocytes. Only cells which had scatter profiles expected of lymphocytes were analysed though this excluded less than 2% of the total cells. The final analysis performed on the unstained cells was to examine cell number versus log fluorescence intensity. Conventionally cell number is shown linearly on the vertical axis with log fluorescence on the horizontal. In order to be able to describe the staining pattern of the stained samples, certain criteria are used. In particular the one feature which most people require is the number of positive cells. The channel number above which cells are usually considered positive corresponds to the most highly fluorescent cells in the unstained cell histogram. This channel number usually allows for a small percentage of unstained cells to be considered positive. In this experiment, ten thousand cells were analysed and the percentage of unstained cells considered 'positive' was 0.08 %. This method of defining number of positive cells is satisfactory for bimodal populations as in Figure 23 which demonstrates the loss of cell membrane immunoglobulin from differentiating B cells. In this situation there is a clear division between the negative cells which overlap with the unstained cells and the positive cells which lie to the right of

these cells. However this method of defining the number of positive cells is not appropriate in certain circumstances. An example of this exists in Figure 13 . In this situation the number of positive cells for Ig as determined by the criteria defined above would be 84.0% (Table II) but there is no indication from the histogram or scatter data that two qualitatively distinct populations exist. According to Parks, Lanier and Hertzenberg (1986), the more likely explanation in this situation is that all cells express the antigen but a few express insufficient to be considered "positive" by the criteria adopted above. Hence it is reasonable to deduce that the pattern of anti-light chain staining indicates a population of cells the vast majority of which are expressing low amounts of light chain and therefore presumably low amounts of total Ig on their surface. The staining pattern for several cell surface proteins reveals the homogenous nature of the lymphocytes derived from the IPP. The staining histogram for IgM reveals that 74.6% of cells can be arbitarily defined as positve but the unimodal distribution (with no sub-divisions when examined by forward or wide-angle scatter versus fluorescence) indicate that it is reasonable to define these cells as being virtually 100% positive for IgM. By contrast, the 2.6% positive for IgG1 staining indicates only background levels of fluorescence. In Figure 14c, the stained histogram is almost exactly superimposed onto the unstained histogram. The percentage of positive staining is identical to that obtained with an isotypically matched mouse monoclonal antibody against human chorionic gonadotrophin ("negative" in Table II) and only marginally higher than that obtained with the second antibody alone. This suggests that the fluorescence

derived from the anti-IgG1 staining is probably due to binding between the second fluorescinated antibody and the IPP cells. This second antibody was a sheep anti-mouse IgG preparation and the level of staining may be due to binding of the sheep immunoglubulin and Fc receptors on the IPP cells. Ten percent normal sheep serum was included in the buffer for the second antibody in order to reduce this interaction to these levels.

Binding for cells expressing the CD5 (T1) antigen demonstrated that 4.8% of cells were positive. These positive cells were arranged with a distinct peak at channel number 150. The remaining cells were almost exactly superimposed on the unstained histogram indicating that the majority had not acquired stain and were negative for CD5 expression. For CD4 staining, 2.8 percent of cells were positive; this is only a small increase (0.2%) over the negative control suggesting that contamination with CD4 +ve cells is minimal. Staining for CD8 antigen revealed a distinct positive population of 5.2% with a clear peak (mode) intensity at channel number 180. CD8 positive cells normally also express CD5 but the apparent discrepancy may be attributable to the low peak (mode) fluorescence intensity of the CD5 +ve population. Part of the CD5 positive peak is certain to have been masked by the much larger negative peak and the percentage of CD5 positive cells may have therefore been slightly underestimated.

The IPP cells were stained for MHC antigens. The histogram profile reveals that 83% and 91.2% are positive for Class I and Class II respectively. However the argument applied above for the interpretation of such unimodal distributions can be applied to these antigens making

it extremely likely that virtually all cells express these proteins. The level of staining with these two reagents results in a fluorescence intensity which is approximately 50% of that expected of mature populations eg. peripheral blood lymphocytes and lymphatic efferent lymphocytes (data not shown).

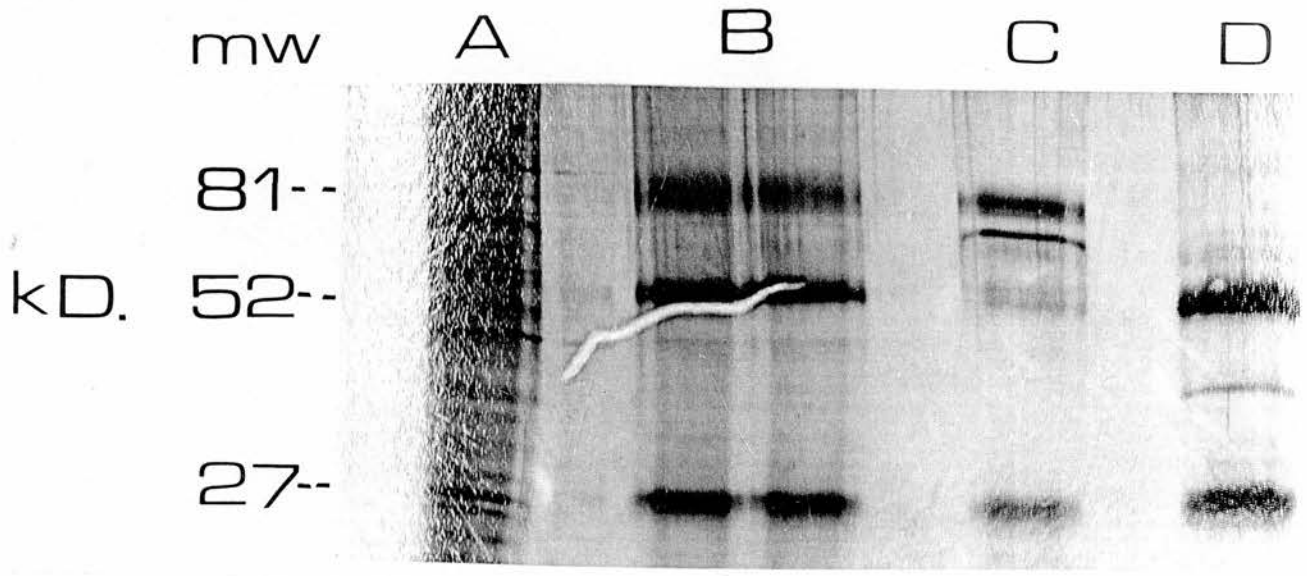
Finally,the histogram for leucocyte common antigen staining leaves little doubt that this antigen is expressed on all cells present in this lymphocyte preparation.

MEMBRANE IMMUNOGLOBULINS OF THE ILEAL PEYER'S PATCH LYMPHOCYTE.

Ileal Peyer's patch lymphocytes were solubilised in PBS containing 0.5% NP-40 (see Appendix). Cellular immunoglobulins were isolated by affinity-chromatography using anti-light chain monoclonal antibody conjugated to cellulose. The eluted fractions were dialysed against 50mM tris 150mM NaCl pH 8.0 containing 0.5% Nonidet P-40 and the protein containing fractions determined by SDS-PAGE and silver staining. Figure 14 shows the pooled protein in track B. The track shows 3 major bands. The least mobile corresponds to a molecular weight of 81 kD. A band is present at this molecular weight when the anti-IgM monoclonal antibody is used to purify IgM from IPP lymphocytes. This band is therefore thought to correspond to membrane IgM heavy chain. The most mobile band has a molecular weight of approximately 27 kD. After transfer to nitrocellulose paper, a band at this molecular weight is bound by monoclonal anti-Ig light chain (data not shown).

Figure 14. **Membrane immunoglobulins of IPP cells.**

10^9 IPP cells were solubilised in 0.5% NP-40 (track A). The membrane immunoglobulins were purified by affinity chromatography using anti-Ig light chain antibodies conjugated to Sepharose. The purified Igs are shown in track B. Track C contains IPP-secreted Ig purified by affinity chromatography (see Chapter Four), and track D contains affinity purified IgG1.



Membrane immunoglobulins of IPP cells.

Finally, the band with a mobility corresponding to a molecular weight of 52 kD is of considerable interest. It is not bound by the polyclonal antisera to IgM by protein blotting, nor is it present when the monoclonal anti-IgM, VPM 13, is used to purify membrane IgM. The protein has migrated further than the heavy chain of IgG1. In addition, the anti-IgG1 monoclonal antibody failed to detect any protein in this preparation either by ELISA or by Western blotting.

DISCUSSION.

This chapter describes the characterisation of the lymphocytes found in the IPP of lambs. My findings support those of Reynolds and Morris (1983) who originally proposed that the IPP was a primary lymphoid organ. The majority of IPP cells express IgM and MHC class II as found by Miyasaki et al (1984). Cells expressing IgG1, the predominant immunoglobulin isotype in sheep and other ruminants (Baker 1983) are rare. Affinity chromatography was used to characterise the membrane immunoglobulins of the IPP lymphocyte. Surface μ chain has a molecular weight of 81 kD and is approximately 3 kD heavier than the chain present in serum immunoglobulins. This result agrees with previous estimates of the difference between membrane and secretory immunoglobulin and is therefore likely to be due to the presence of a hydrophobic transmembrane portion and cytoplasmic tail. Since immunoglobulin light chain does not span the cell membrane, there is no detectable difference between light chains from surface or secreted

immunoglobulins. No IgG1 heavy chain could be detected. The molecular weights of the heavy chains of other serum imunoglobulins (IgG2 and IgA) are also greater than 52 kD (approximately 55-60 kD)(Butler 1983 and data not shown). The membrane forms of these heavy chains would be significantly heavier with the addition of a transmembrane portion and cytoplasmic tail. The cytoplasmic tails of murine gamma chains are 28 amino acids long compared to just 3 residues in IgD and IgM (Tyler et al 1982).It seems unlikely that IgG1, IgG2 or IgA heavy chains could account for the distinct band at 52 kD. It would therefore appear likely that this novel band corresponds to a previously unreported immunoglobulin heavy chain. Since the IPP cells contain sparse amounts of cytoplasmic Ig (see Chapter Five), this Ig is probably expressed on the cell surface. The vast majority of IPP cell have already been demonstrated to bear mIgM. Silver stained gels of the membrane immunoglobulins purified from IPP cells suggest that the heavy chain found at 52 kD quantitatively predominates over the μ chain. Although, the number of cells which contained the 52 kD protein is unknown, the quantity of protein obtained makes it unlikely that it is a small minority. The inference is that the 52 kD protein is co-expressed with mIgM. Hence, it is postulated that this new immunoglobulin is the ovine equivalent of IgD. Havran et al. (1984) found that IgD exceeded IgM on the membrane of murine spleen cells by 10:1.

The molecular weight estimate of putative IgD agrees with the value obtained for native murine δ heavy chain by Goding and Hertzenberg (1980). It has been postulated that the murine Cδ chain consists of only two domains with an unusual intervening hinge structure (Tucker et

al 1980). The discrepancy between these results and previous work (Abney and Parkhouse 1974) is attributable to the presence of a large amount of carbohydrate.

The purified IPP membrane immunoglobulins have been injected into mice. After repeated boosts, the spleens of these mice have been used to perform fusions with the myeloma cell line, NSO. So far no hybridomas secreting antibody reactive with the purified material by ELISA have been found. It is hoped that continuing work will determine the identity of this membrane immunoglobulin and its distribution in various lymphoid organs.

It can be concluded that the IPP is a genuine B cell population without parallel in mammalian immunology. These B cell show the characteristics of newly formed B cells as various stages of development but significantly, before any antigenic stimulus. This conclusion is supported by the finding of putative sheep IgD since IgD is rapidly lost from the surface of activated cells. Therefore the IPP B cell offers an outstanding opportunity to study the differentiation of sheep B cells from the true resting stage through to immunoglobulin secretion. These studies are reported in the following chapters.

Chapter Four.

Chapter Four.

INTRODUCTION.

Most of our present knowledge of B cell physiology in the mouse and human is derived from work performed in vitro and this is reviewed in Chapter One. To date there has not been a suitable assay for critically examining sheep B cell differentiation. However, the demonstration that the ileal Peyer's patch is an area of intense lymphocyte production and that it is composed almost entirely of small IgM+ B cells gives rise to an unique opportunity in mammalian immunology. In particular, it allows us to study the control of B cell growth and proliferation in a population of virgin IgM+ cells. As discussed in the first chapter, the activation of B cells is antigen-dependent and in order to substitute for this in a antigen-non specific population, a polyclonal analogue for antigen is required.

There are two polyclonal B cell activators which have been commonly used; occasionally in combination. The first is lipopolysaccharide (LPS); the mode of action is unknown but it is postulated that there is a specific cell surface receptor for LPS and that this represents an evolutionary adaptation to give prompt immune responses to Gram-negative bacteria (De Franco et al 1987).However the effect of LPS on sheep B cells has not been described. Alternative B cell activators are anti-immunoglobulin antibodies. These anti-Ig reagents mediate their effect through binding to the antigen-receptor of B cells, ie membrane immunoglobulin. The effects of anti-Ig antibodies are extremely variable and are dependant on the following criteria;

1) Source of anti-Ig. Intact rabbit anti-mouse Ig antibodies

inhibit the proliferation of mouse B cells (Klaus et al 1984). This is believed to be due to result from cross-linking of surface Ig and Fc receptors on B cells. $F(ab)_2$ fragments of rabbit anti-mouse Ig antibodies will activate mouse B cells.

2) The nature of the B cells used. Raff (1970) demonstrated that newly formed murine B cells were inhibited by anti-Ig antibodies whilst B cells obtained from the periphery ie mature B cells were stimulated. Raff proposed that crosslinking of the antigen-receptors of newly formed B cells is an early mechanism for deleting possible autoreactive cells since exogenous antigen is not found in the regions in which B cell production takes place. Therefore any crosslinked immature B cells in the bone marrow (or IPP?) are likely to be specific for host antigens and as such are potentially dangerous.

3) The ability of the anti-Ig to cross-link surface Ig molecules. This is controversial. Many investigators consider that the demonstration that cross-linkage of membrane Ig can transduce an activating signal indicates that cross-linkage by antigen is likely to be the major event leading to B cell activation in vivo. However, this suggestion has been criticised on two particular points:

a) Membrane crosslinking is not required for efficient antigen presentation or for responsiveness to MHC-restricted T cell help. Both are equally well induced by monovalent Fab' fragments and haptens (Tony et al 1985).

b) Lanzavecchia (1987) has shown that antigen presentation by B cells occurs at concentrations of free antigen that are 1000-fold below that required for 50% saturation. This is not

compatible with the situation when anti-Ig antibodies are used resulting in a large degree (approaching 100%) of saturation. Lanzavecchia explains the ability of B cells to acts as efficient antigen presenting cells in terms of the rapid internalisation of antigen and the apparent long half-life of processed antigen intracellularly. These two phenomenon result in the "vacuum cleaner" effect, ie the net accumulation of antigen until sufficient antigen has been gathered to trigger T cells.

Hence the importance of membrane Ig is controversial. On one side it is given a pivotal role in B cell activation via its ability to trigger the phosphoinositol pathway (albeit when heavily cross-linked) and the other group see it as no more than a means for concentrating specific antigen within a B cell prior to antigen presentation to T cells. In the latter scenario, B cell activation then proceeds as a result of the cognate and non-cognate interactions as result of antigen presentation. The possible role of lymphokines, particularly IL4, and cell-cell interactions has been discussed in Chapter One. This lesser role for membrane Ig is supported by the finding that rabbit antibodies bound to MHC class I are very efficiently presented to T cell specific for rabbit Ig, although three- to five-fold less efficiently than rabbit antibodies bound to membrane IgM or IgD (Tony et al 1985). However even this small difference can probably be explained in terms of the slow rate of internalisation of MHC class I molecules compared to Ig (Pernis 1985).

Subsequent differentiation of activated B cells is controlled by lymphokines; this is known as the antigen-independent phase of B cell

differentiation. The role and contribution of each lymphokine to the immune response is still the subject of considerable debate and the current situation is reviewed in Chapter One. In particular, interleukin-2 has been shown to play an important role in the control of B cell growth and differentiation of human and murine B cells (Farrar et al 1982, Zubler et al 1984, Kehrl et al 1985, Kishimoto 1985). Since recombinant human IL2 was available and I had already demonstrated that it could significantly enhance the proliferation of sheep Con A blasts, it was decided that this offered a good opportunity to investigate the effect of IL2 on sheep B cells.

This chapter describes the development of an assay for sheep B cell growth and differentiation and its use to characterise the events culminating in immunoglobulin secretion.

B CELL GROWTH ASSAY.

IPP cells were cultured at 37°C in an atmosphere of 5% CO_2 in 96-well flat bottomed plates in RPMI-1640 medium containing 10% FCS with L-glutamine and 2-mercaptoethanol. LPS and human recombinant IL2 were added as indicated in the text. Lipopolysaccharide derived from Salmonella abortus equi was used as this had been shown to stimulate tritiated thymidine uptake by sheep peripheral blood lymphocytes (data not shown). Twenty hours before the end of culture, 1 μCi of [^{3}H]thymidine was added in 20 μl. Cells were harvested onto glass fibre

filter papers, washed, dried and [^{3}H]thymidine incorporation was assessed by scintillation counting.

EFFECT OF LIPOPOLYSACCHARIDE ON IPP CELLS.

IPP cells were prepared as described and set up in culture at a range of cell concentrations from 0.125 x 10^6 to 2.0 x 10^6 per ml. LPS was used at a concentration of 50 μg/ml for two main reasons:

a) this concentration was optimal for inducing proliferation of sheep peripheral blood lymphocytes.

b) work in other species, particularly mouse and human, had indicated that this concentration was optimal for B cells in those species. The chosen concentration is supported by work in the next section.

To determine the ideal conditions for culture of IPP cells with LPS, the experiment was performed in both round- and flat-bottomed 96-well plates. Each well contained 0.2 ml at the initiation of culture. The culture period was 4 days. The results are shown in Table IV and are derived from triplicate samples.

The results indicate that LPS will cause substantial thymidine incorporation in cultured IPP cells particularly in flat-bottomed well at the higher cell concentrations tested ie 1-2 x 10^6 per ml. By comparison, at these cell concentrations there is a definite reduction in proliferation of cells in round-bottomed plates. This is likely to be due to excessive cell-cell contact causing growth inhibition. It was

Table III. **Effect of well geometry and cell density on the proliferation of IPP cells.**

IPP cells were cultured at a range of cell concentrations in flat- or round-bottomed plates. LPS at 50 μg/ml was added as indicated. The culture period was four days. Results in c.p.m. are expressed as the mean of triplicate cultures with the standard deviation in brackets.

Effect of well geometry and cell density on the proliferation of IPP cells.

Cell concentration. ($\times 10^6$/ml).	[^{3}H]-Thymidine incorporation. (cpm).			
	Culture conditions.			
	Round-bottomed		Flat-bottomed	
	Media	LPS	Media	LPS
2.0	16476 (680)	39566 (3618)	29140 (6052)	88756 (4061)
1.0	18148 (2240)	40996 (2134)	10941 (553)	49491 (1271)
0.5	8588 (1578)	30976 (2683)	4521 (642)	24128 (1679)
0.25	2192 (756)	13291 (3168)	2252 (511)	10762 (3391)
0.125	1141 (437)	4972 (1983)	1709 (135)	6345 (135)

therefore decided to culture IPP cells in flat-bottomed wells. The results for the flat-bottomed plates indicate that optimal proliferation is achieved at a cell concentration of 1 x 10^6 per ml. Therefore this cell concentration was adopted for further experiments on the control of the growth of IPP cells.

EFFECT OF RECOMBINANT HUMAN INTERLEUKIN-2 ON IPP CELLS.

Lymphocytes were prepared from the IPP as described and placed in 96-well flat-bottomed plates with LPS in doubling dilutions from a concentration of 200 μg/ml to 0.2 μg/ml in the presence or absence of human recombinant interleukin-2 at 7 ng/ml. Thymidine incorporation was assessed on the fourth day. Cells were at a final concentration of 1 x 10^6 per ml.

All results were derived from triplicate samples (Table IV). Interleukin 2 was included in the culture media at a concentration of 7 ng/ml,this concentration had been found to be optimal for culturing sheep Con A blasts (data not shown). LPS gives modest increases in [^{3}H]-thymidine uptake compared to cells cultured in media alone with a peak response to LPS concentrations in the range of 50-200 μg/ml. IL2 alone causes a greater than five-fold increase compared to the media control. When LPS and IL2 were both included, tritiated thymidine incorporation is synergistically increased.

Table IV. **Proliferation of IPP cells cultured with LPS and IL2.**

IPP cells were cultured at 1 x 10^6 cells per ml in doubling dilutions of LPS. IL2 at 7 ng/ml was added where indicated. Tritiated thymidine was added 20 hours before the end of culture. Results are expressed as the mean counts per minute (cpm) of triplicate cultures with the standard deviation in brackets.

Proliferation of IPP cells cultured with LPS and IL2.

LPS concentration. (µg/m)	[^{3}H]-Thymidine incorporation. (cpm)	
	Culture conditions	
	without IL2	with IL2
200.0	12686 (1395)	48192 (6328)
100.0	10930 (306)	42463 (3495)
50.0	10822 (562)	41767 (6024)
25.0	8546 (588)	35717 (3216)
12.5	8039 (1343)	32963 (2076)
6.3	7568 (461)	31978 (2412)
3.1	6957 (247)	30431 (2708)
1.6	6335 (616)	26489 (1182)
0.8	5981 (604)	25771 (2184)
0.4	5420 (458)	25851 (2810)
0.2	5533 (482)	25188 (1813)
0.0	3798 (440)	20228 (825)

Chapter Four.

EFFECT OF RABBIT ANTI-IMMUNOGLOBULIN LIGHT CHAIN ANTIBODIES ON PROLIFERATION OF IPP CELLS.

Many investigators have used polyclonal and monoclonal antibodies to immunoglobulins as B cell activators. The antibody binds the membrane immunoglobulin molecules and acts as a polyclonal analogue of antigen, leading to the early events of B cell activation discussed in Chapter One. Others have used anti-immunoglobulin and LPS together, either simultaneously or sequentially (Zubler et al 1987). The following experiment was performed to determine the effect of anti-immunoglobulin antibodies in the B cell growth assay

Rabbit anti-sheep Ig light chain antibodies were affinity purified. The concentration of antibody was determined by spectrometry at 280 nm. The immunoglobulin solution was dialysed against culture media. Rabbit anti-mouse IgG antibodies were similarly prepared as a negative control.

The anti-immunoglobulin antibodies were included in the complete media at a range of concentrations from 50 μg/ml to 0.8 μg/ml with LPS (50 μg/ml) and IL2 (7 ng/ml) added as indicated. IPP cells were set up at 1 x 10^6 per ml in a final volume of 0.2 ml. The cells were harvested on day 4.

The results are shown in Table V. Firstly, in Table V(b), it can be seen that the inclusion of rabbit anti-mouse Ig in the culture is without effect. Therefore it can be stated that any effects seen when rabbit anti-sheep light chain are used are due to the binding of the rabbit to the membrane Ig of the IPP cells. IPP cells cultured in

Table V(a). **Effect of rabbit antibodies on the proliferation of IPP cells.** Anti-sheep light chain antibodies.

Affinity-purified antibodies to sheep immunoglobulin light chain were included in doubling dilutions from 50 μg/ml. LPS at 50 μg/ml and IL2 at 7 ng/ml were included as indicated. The culture period was four days. Results in c.p.m. are expressed as the mean of triplicate cultures with the standard deviation in brackets.

Effect of rabbit antibodies on the proliferation of IPP cells.

a) anti-sheep light chain antibodies.

Antibody concentration. (µg/ml).	[^{3}H]-Thymidine incorporation. (cpm).			
	Culture conditions.			
	LPS + IL2	LPS	IL2	Media
50	21240 (2137)	724 (266)	20480 (2179)	191 (38)
25	30566 (3476)	1211 (141)	19412 (959)	338 (64)
12.5	27043 (6570)	1754 (240)	29317 (5447)	667 (51)
6.3	39725 (2211)	3667 (578)	30289 (3448)	1081 (50)
3.1	62841 (8105)	5703 (329)	37120 (4335)	1736 (484)
1.6	71291 (5170)	9014 (606)	45689 (7889)	2923 (223)
0.8	86862 (1149)	11968 (1338)	44590 (3805)	3462 (260)
0.0	94526 (7028)	18098 (2483)	53178 (2114)	5102 (120)

TABLE V(a).

Table V(b). **Effect of rabbit antibodies on the proliferation of IPP cells.** Anti-mouse Ig antibodies.

Affinity-purified antibodies to mouse IgG were present at doubling dilutions from 50 μg/ml. LPS at 50 g/ml and IL2 at 7 ng/ml were included as indicated. The culture period was four days. Results in c.p.m. are expressed as the mean of triplicate cultures with the standard deviation in brackets.

Effect of rabbit antibodies on the proliferation of IPP cells.

b) anti-mouse Ig antibodies.

Antibody concentration. (µg/ml)	[^{3}H]-Thymidine incorporation. (cpm).			
	Culture conditions			
	LPS + IL2	LPS	IL2	Media
50.0	87983 (2031)	17263 (947)	55073 (1610)	4986 (187)
6.3	85561 (3769)	19987 (1359)	56117 (2731)	4849 (427)
0.8	91136 (876)	18916 (327)	56252 (534)	4826 (468)
0.0	92223 (1478)	18905 (1563)	55229 (832)	5129 (515)

TABLE V(b).

medium alone proliferate at at rate equivalent to approximately 5000 cpm. However in the presence of the highest concentration of anti-Ig used there is in excess of 95 % inhibition of this thymidine incorporation. Similarly for IPP cell growth induced by LPS, this is inhibited to a similar degree.

Conversely, the effect of anti-Ig on the proliferation of IPP cells cultured with LPS and IL2 or IL2 alone was less substantial (78 % and 62 % respectively). Interestingly, the uninhibited proliferation in each case was almost identical ie approximately 20 000 cpm. This would suggests that IL2 stimulates proliferation in a population of cells that are not inhibited by anti-Ig. The identity of these cells is unknown. By the final day of culture, it was difficult to locate viable cells by inverted microscopy in those wells cultured with the highest concentrations of anti-sheep immunoglobulin. This cell death was not due to complement-mediated lysis of the bound cells as the foetal calf serum had been heated to 56°C for thirty minutes, a proceedure widely used to inactivate certain complement components (ie. C2 and Factor B). In addition, the same FCS did not result in a drop in the viability of antibody bound IPP cells at 37°C within 2 hours compared to unbound cells (data not shown).

EFFECT OF LPS CONCENTRATION ON IMMUNOGLOBULIN SECRETION.

Preliminary experiments using the supernatants of proliferating IPP cell cultured under optimal conditions had indicated that

immunoglobulin was detectable by ELISA. It was wished to examine the role of LPS and IL2 on the differentiation of IPP cells. A B cell differentiation assay was performed in 24 well plates with a final volume of 2 mls per well. The cells were at a final concentration of 1 x 10^6 per ml, ie two million cells per well. The culture period was seven days. At the end of the incubation, the media was recovered and microfuged at high speed for 3 minutes. The supernatant was stored at 4°C with 0.1 % sodium azide. The effect of LPS was tested at a range of concentrations from 200 μg/ml to 0.2 μg/ml. IL2 at 7 ng/ml was added as indicated. This concentration had been shown to be optimal for inducing proliferation of IPP cells when co-cultured with LPS (data not shown). The results shown in Table VI are representative of the many occasions on which similar experiments were performed.

These results show that Ig production by IPP cells cultured in media alone is very low. The addition of LPS alone to IPP cells promotes differentiation with maximum secretion induced by LPS at 50 μg/ml. Recombinant IL2 alone only gives minor enhancement of the supernatant Ig concentration. However the addition of IL2 to cells co-cultured with LPS at 50 μg/ml results in a substantial increase in secretion to greater than 3700 ng/ml by day 7. These results demonstrate that LPS and IL2 synergistically stimulate the differentiation of IPP cells to immunoglobulin secretion.

EFFECT OF IL2 ON IMMUNOGLOBULIN SECRETION.

Table VI. **Effect of LPS concentration on immunoglobulin secretion.**

IPP cells were cultured at 1 $x10^6$ /ml in the presence of doubling dilutions of LPS. IL2 at 7 ng/ml was included as indicated. Supernatants were harvested after 7 days and concentration of immunoglobulin determined by ELISA. Results are expressed as ng/ml.

TABLE VI.

Effect of LPS concentration on immunoglobulin secretion.

LPS concentration. (µg/ml).	Secreted immunoglobulin. (ng/ml).	
	Culture conditions.	
	without IL2	with IL2
200	1318	3467
100	1258	3630
50	1778	3715
25	1348	3548
12.5	1000	3311
6.3	831	2884
3.1	724	2630
1.5	661	2291
0.8	575	1778
0.4	427	1349
0.2	354	912
0.0	363	741

Chapter Four.

A feature of the effect of lymphokines on target cells is that the response is dose dependant. The human recombinant interleukin 2 has a nominal activity of 7 units per nanogram (product information from Biogen S.A.). One unit is the concentration which stimulates half-maximal tritiated thymidine incorporation in antigen or mitogen-stimulated human T cells. It was useful to determine the activity of IL2 for stimulating immunoglobulin secretion by IPP cells. Recombinant IL2 was used at a range of concentrations from 100 ng/ml to 0.1 ng/ml. LPS at 50 μg/ml was included as indicated. The supernatants were collected after 7 days. The results are shown in Table VII and Figure 15.

As already described, the effect of IL2 alone on Ig secretion by IPP cells is small with only a modest increase in Ig secretion at large doses (25-100 ng/ml). By contrast in the presence of LPS, 6.3 ng/ml IL2 induces substantial Ig secretion from IPP cells. Further addition of IL2 does not substantially increase secretion of immunoglobulin. The IL2 concentration which induces half maximal immunoglobulin secretion is 2.5 ng/ml. This is equivalent to 17.5 units of activity (U) per millilitre in the Biogen assay. Maximal secretion was induced by 6.3 ng/ml (equivalent to 44 U/ml).

EFFECT OF CELL CONCENTRATION ON IMMUNOGLOBULIN SECRETION.

In order to determine the optimal conditions for differentiation to Ig secretion, IPP cells were cultured at a range of cell

Table VII. **Effect of IL2 concentration on immunoglobulin secretion.**

IPP lymphocytes were cultured in the presence of doubling dilutions of IL2. LPS at 50 μg/ml was included as indicated. The supernatants were harvested after 7 days and the concentration of immunoglobulin determined by ELISA. The results are expressed as ng/ml.

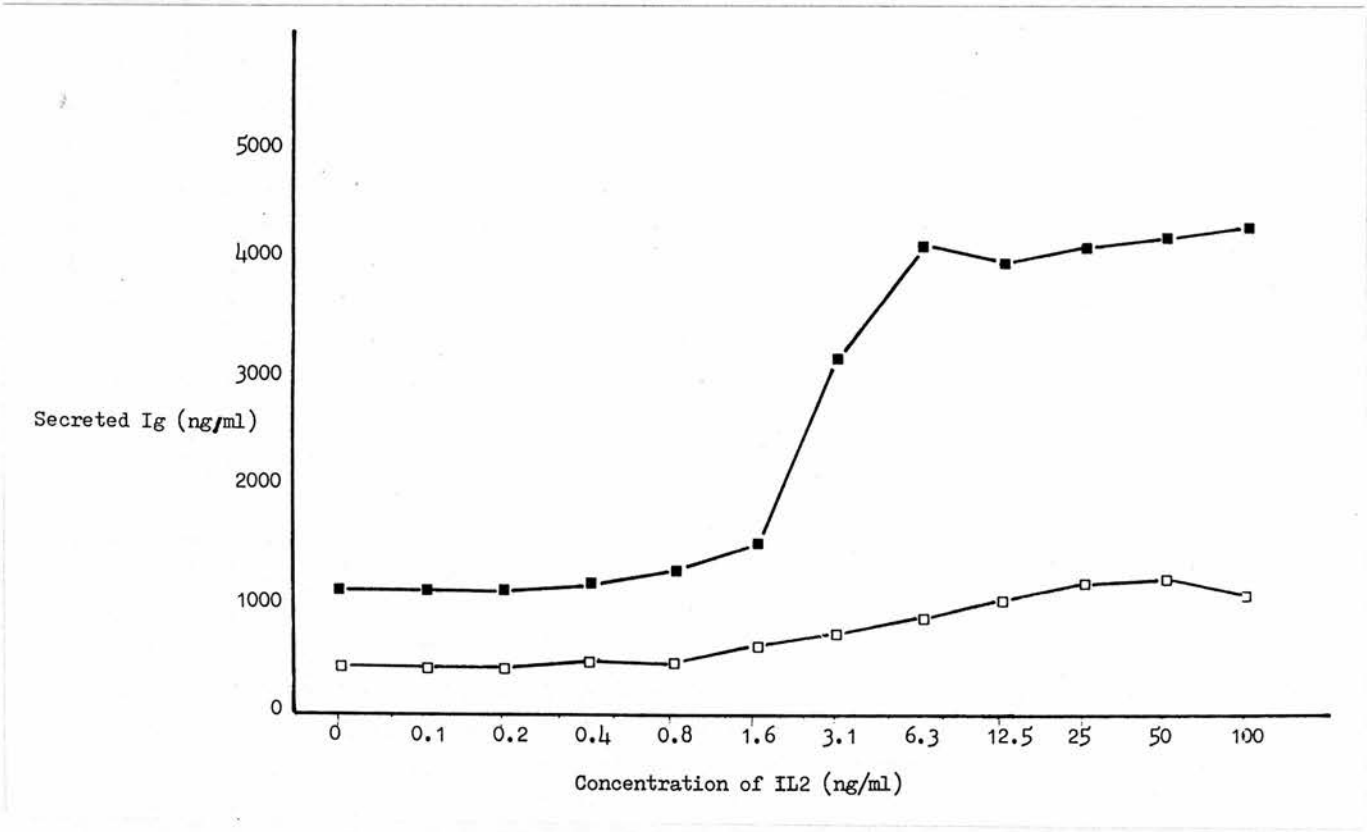
TABLE VII.

Effect of IL2 concentration on immunoglobulin secretion.

IL2 concentration. (ng/ml).	Secreted immunoglobulin. (ng/ml).	
	Culture conditions.	
	without LPS	with LPS (50 µg/ml)
100	1071	4365
50	1202	4265
25	1174	4168
12.5	977	3981
6.3	831	4168
3.1	707	3981
1.6	603	3162
0.8	426	1258
0.4	436	1174
0.2	416	1071
0.1	398	1071
0.0	407	1071

Figure 15. **Effect of IL2 concentration on immunoglobulin secretion.**

IPP cells were cultured in doubling dilutions of IL2 for 7 days in the presence ■ or absence □ of LPS. Supernatants were analysed for Ig concentration after 7 days.


5000
4000
3000
2000
1000
0
Secreted Ig (ng/ml)
0
0.1
0.2
0.4
0.8
1.6
3.1
6.3
12.5
25
50
100
Concentration of IL2 (ng/ml)

concentrations. Immunoglobulin secretion was analysed on day 7. The results are shown in Table VIII. In (a), the results show the total immunoglobulin in ng/ml. In (b), the results are expressed per 10^5 cells/ml.

The results demonstrate that LPS and IL2 synergise to promote B cell differentiation at all cell densities tested except 8.0 x 10^6 per ml. At this concentration, the cells are extremely packed together. Co-culturing of LPS and IL2 with IPP cells resulted in maximum secretion per cell at 1 x 10^6 cells/ml. The effect of LPS was optimal when the cells were at twice this concentration and the effects of IL2 and media alone were maximal when the cells were cultured at a density of 4 x 10^6 per ml.

These results demonstrate the effect of excessive cell density on LPS-IL2 induced differentiation. Similarly, it is clear that the combination of LPS and IL2 promotes the optimal differentiation of IPP cells at a lower cell density than would be necessary for either of the co-stimulants on their own. On the basis of these results, it was decided to culture the IPP cells at a concentration of 1 x 10^6 cells/ml for optimal B cell differentiation.

TIME COURSE OF IMMUNOGLOBULIN SECRETION BY IPP LYMPHOCYTES.

Supernatants were collected daily from cultured IPP lymphocytes to investigate the kinetics of Ig secretion from sheep B cells. As some of the supernatants contained large amounts of sheep Ig, it was

Table VIII(a). **Effect of cell density on immunoglobulin secretion.**

IPP cells were cultured at a range of cell concentrations for 7 days. LPS (50 μg/ml) and IL2 (7 ng/ml) were included as indicated. The supernatant immunoglobulin concentration is expressed as ng/ml.

TABLE VIII(a).

Effect of cell density on immunoglobulin secretion.

a)

Cell concentration. ($\times 10^6$/ml).	Secreted immunoglobulin (ng/ml).			
	Culture conditions.			
	LPS+IL2	LPS	IL2	Media
8.0	5888	5248	4168	2454
4.0	7585	5754	3890	2884
2.0	6607	3020	1905	1230
1.0	3715	1202	851	446
0.5	1750	603	398	151
0.25	479	148	126	49

Table VIII(b). **Effect of cell density on immunoglobulin secretion per cell.**

The results from Table VIII(a) are expressed as ng of secreted immunoglobulin per 10^5 cells/ml.

TABLE VIII(b).

Effect of cell density on immunoglobulin secretion per cell.

Culture conditions.	Secreted immunoglobulin. (ng /10^5 cells /ml).					
	Cell concentration (x 10^6/ml).					
	8.0	4.0	2.0	1.0	0.5	0.25
LPS + IL2.	73	189	330	373	350	191
LPS	65	144	151	120	120	59
IL2	52	97	95	85	79	50
Media	30	72	61	45	30	20

necessary to dilute the samples 1:10 in ELISA washing buffer before analysis. The results are shown in Figure 16 and Table IX. These results are representative of the several occassions on which similar time course experiments were performed.

The majority of Ig is secreted from IPP cells between day 4 and day 6. Secretion is virtually complete by day 7. In similar experiments (data not shown), cultures continued to day 16 only showed a 5 % increase after day 7. When cultures older than 7-8 days are examined by inverted microscopy, live cells (as judged by the ability to refract light) are rare.

THE INCREASE IN TOTAL CELLULAR IMMUNOGLOBULIN PRECEEDS THE ONSET OF SECRETION.

Immunohistological staining of cytospin preparations had shown that the resting IPP cells contained low amounts of immunoglobulin. Since these cells are clearly capable of massive Ig secretion, a large percentage of these immunoglobulin molecules must be synthesized de novo. In order to investigate the appearance of cellular immunoglobulin, IPP cells were set up in 25 cm^2 flasks at 1 x 10^6/ml. Care was taken to ensure that the cell density would be similar to the optimal conditions determined earlier. Cells and supernatants were harvested daily. The cells were washed in medium twice and the number of viable cells recovered was determined by the trypan blue exclusion assay. The cells were solubilised in one ml of 0.5% NP-40. The samples

Figure 16. **Time course of immunoglobulin secretion from IPP cells.**

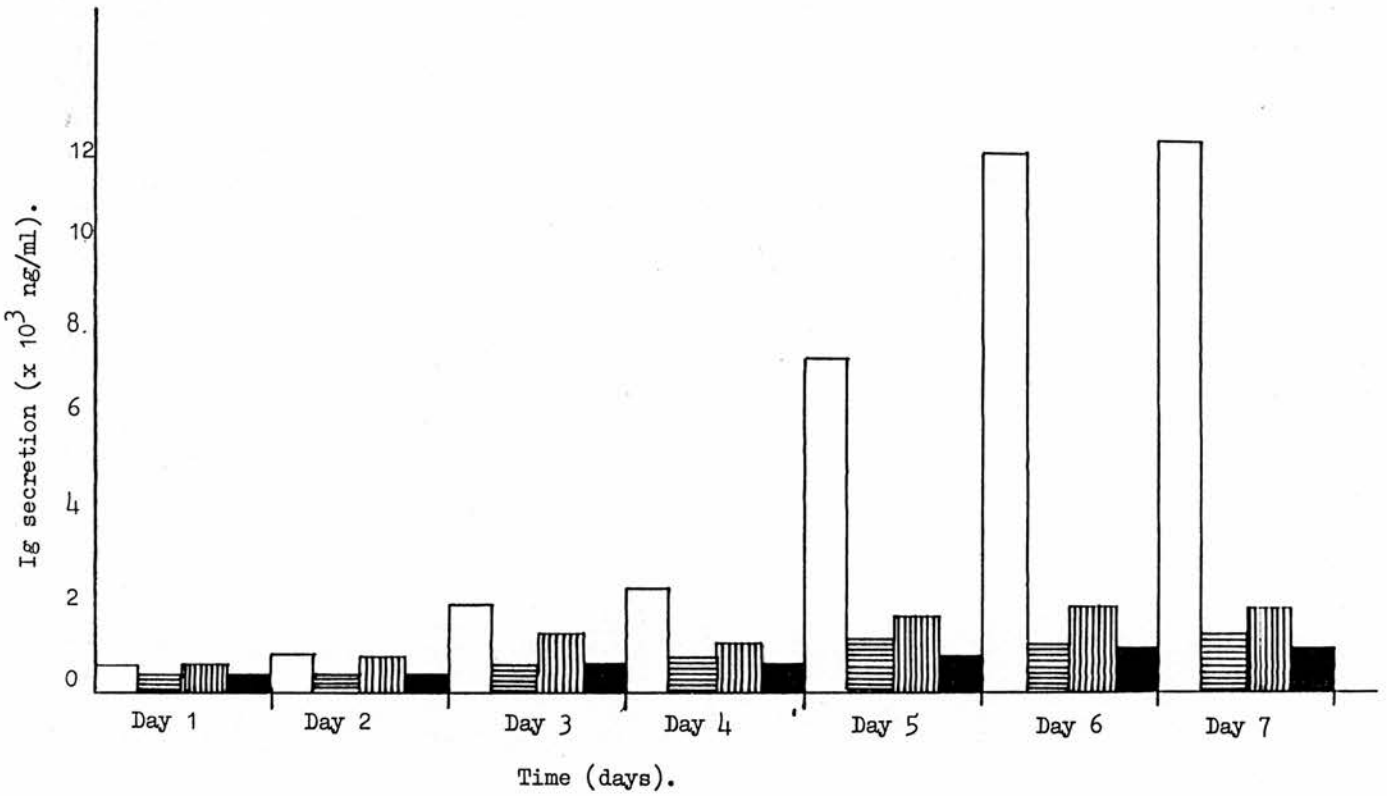
IPP cells were cultured in LPS (50 μg/ml) and/or IL2 (7 ng/ml). Supernatants were collected daily and analysed for Ig. The horizontal axis refers to the time in days since the initiation of culture.

LPS and IL2. 

LPS. 

IL2. 

Media alone 



Time course of Immunoglobulin secretion from IPP lymphocytes.

Table IX. **Time course of immunoglobulin secretion from cultured IPP cells.**

IPP cells were cultured in LPS and/or IL2. Supernatants from replica cultures were collected daily and assayed for immunoglobulin concentration. The results are expressed as ng/ml.

TABLE IX.

Time course of immunoglobulin secretion from cultured IPP cells.

Culture conditions.	Immunoglobulin secretion (ng/ml).						
	Time from initiation of culture (days).						
	1	2	3	4	5	6	7
LPS and IL2	560	831	1949	2344	7413	12022	12302
LPS	389	398	588	741	1230	1122	1258
IL2	575	776	1288	1122	1737	1949	1862
Media	407	407	630	588	758	1000	954

were kept at 4°C with 0.1% sodium azide until analysis. Ovine immunoglobulins stored in this manner showed no loss of antigen over several months (data not shown) even in the presence of detergent. The cell-free supernatants were stored in an identical manner. The samples were analysed for immunoglobulin by ELISA. The results are shown in Figure 17 and Table X. An earlier experiment gave similar results.

The results confirm that resting cells contain little immunoglobulin (mean = approximately 10 fg/cell). The initial drop in total cell-associated immunoglobulin is likely to be due to the greater than 75 % drop in cell recovery after 24 hours. The dead cells probably contribute to the small amount of secreted ("released") immunoglobulin. Compared to day 1, cellular immunoglobulin increases substantially by day 2 and continues to increase until day 4 when the amount of cellular Ig starts to plateau. There is a small fall in cellular Ig on day 7. This fall was reproduced in the replica experiment. Allowing for the low cell recoveries, the peak total cellular immunoglobulin indicated a mean cellular immunoglobulin concentration of 260 fg/cell; a 26-fold increase over resting levels.

By contrast and as already demonstrated, immunoglobulin secretion by IPP cells commences later. The majority of secretion occurs between days four and six. As shown earlier, the rate of secretion is falling by day 7 and this is clearly compatible with a fall in cellular Ig reflecting reduced synthesis of immunoglobulin molecules.

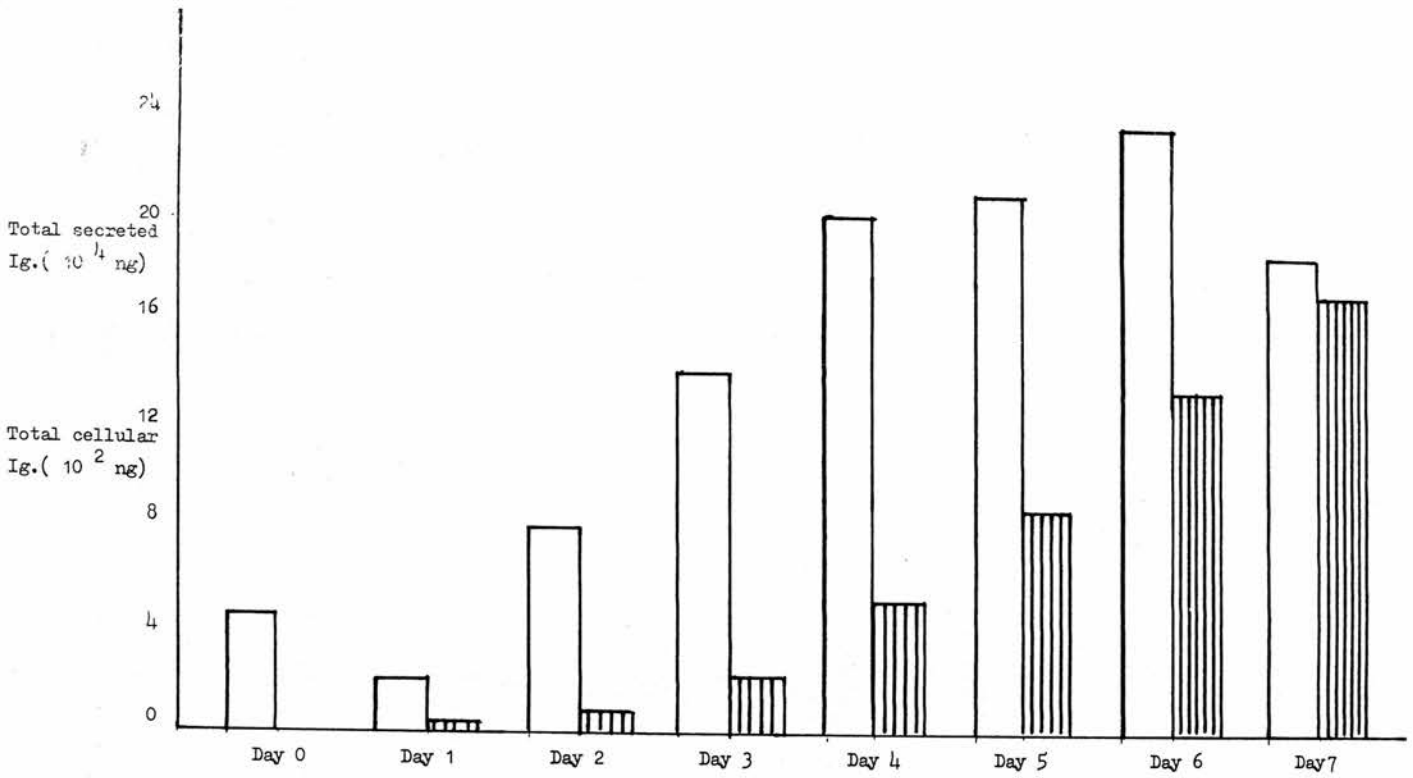
ISOTYPE OF THE SECRETED IMMUNOGLOBULIN.

Figure 17. **Kinetics of the increase in secreted and cellular immunoglobulin in cultures of IPP cells stimulated with LPS and IL2.**

IPP cells were cultured with LPS (50 μg/ml) and IL2 (7 ng/ml). Cells and supernatants were collected daily. The cells were solubilised in 0.5% NP-40. Samples were analysed for Ig concentration by ELISA.

Cellular Ig. 

Secreted Ig. 



Kinetics of secreted and cellular immunoglobulin in cultures of IPP cells stimulated with LPS and IL2.

Table X. **Increase in cellular and secreted immunoglobulin in differentiated B cells.**

IPP cells cultured in LPS and/or IL2 were harvested daily and solubilised in 0.5% NP-40. The lysate and the cell culture supernatant were examined for immunoglobulin. The results are expressed in ng of total cellular Ig and ng/ml of secreted Ig.

TABLE X.

Increase in cellular and secreted immunoglobulin in differentiating B cells.

Culture period (days)	Total cellular Ig (ng)	Secreted Ig. (ng/ml)
0	448	0
1	194	120
2	794	195
3	1412	562
4	2042	1288
5	2188	2187
6	2399	3388
7	1862	4365

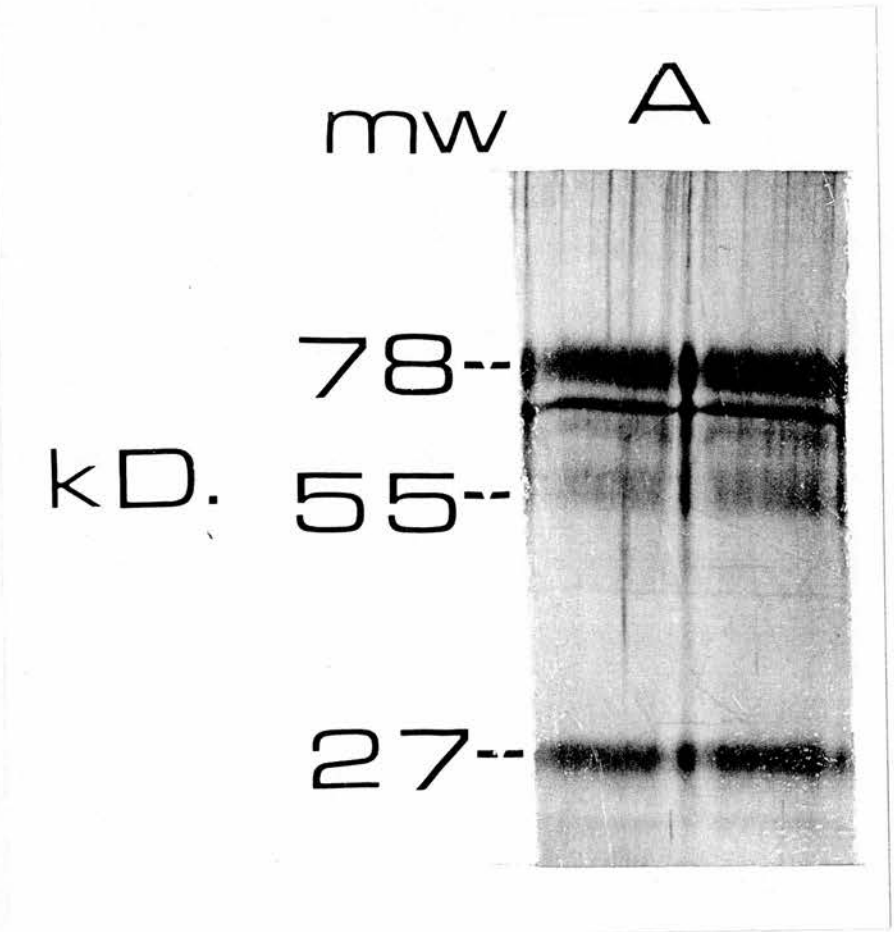
Chapter Four.

By adapting the ELISA for secreted immunoglobulin to determine the heavy chain isotype of secreted immunoglobulins, it had been shown that only IgM was detectable (> 6000ng/ml). IgG1 was not detectable at concentrations above 30 ng/ml. Therefore the concentration of IgM is calculated to be at least 200 times the maximum possible concentration of IgG1. As monclonal reagents for IgA and IgG2 heavy chain were not available, it was wished to confirm this finding by an alternative strategy.

A bulk culture of 2 x 10^8 IPP cells was incubated for 7 days with optimal amounts of LPS and IL2. The supernatant was collected and stored at 4°C in the presence of sodium azide. The supernatant was rotated overnight at 4°C with mouse monoclonal anti-sheep light chain conjugated to Sepharose^R. The beads were packed into a suitable column and washed. The bound protein was eluted with 0.1 M glycine pH 2.5 and dialysed against column running buffer. The protein containing fractions were analysed by SDS-PAGE chromatography and the results are demonstrated in Figure 18. These show that the two major purified bands have molecular weights of 78 and 27 kD corresponding to μ chain and light chain respectively. The band at 67 kD is likely to be albumin and its presence probably reflects the association of IgM with bovine serum albumin in the cell culture media. The presence of other heavy chain isotypes is suggested by the indistinct band at 55 kD. However, repeated experiments have confirmed the initial ELISA results, that IgM is the predominant immunoglobulin isotype produced by IPP cells.

Figure 18. **Isotype of secreted immunoglobulin.**

Monoclonal anti-Ig light chain was used to purify protein from the supernatant of IPP cells cultured with LPS and IL2 for 7 days. The purified protein (A) was analysed by SDS-PAGE under dissociating conditions. The protein bands were visualised by silver staining. The molecular weights are shown.



Isotype of secreted immunoglobulin.

Chapter Four.

EFFECT OF DELAYING THE ADDITION OF LPS OR IL2 ON IMMUNOGLOBULIN SECRETION BY IPP CELLS.

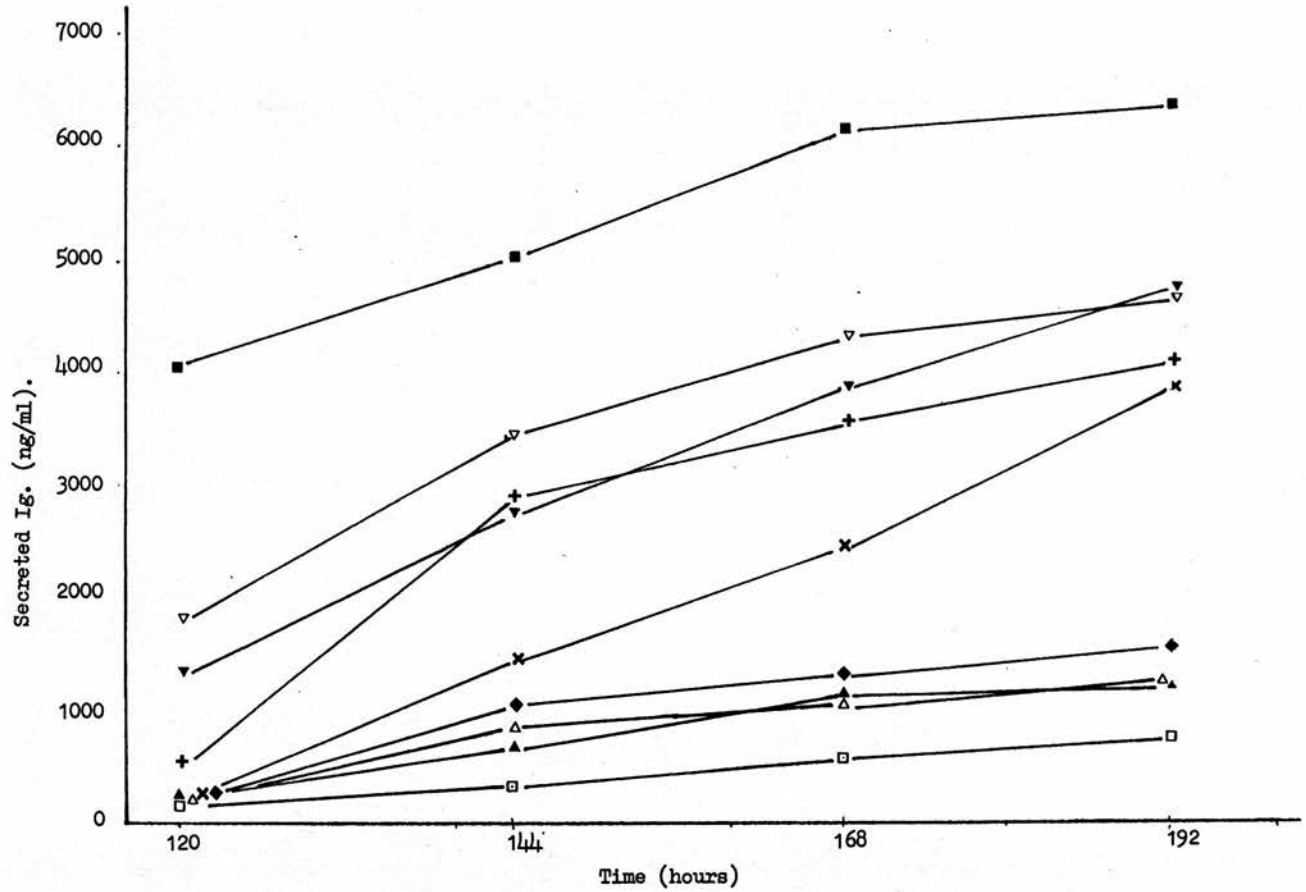
It is clear from the preceeding work that the effects of LPS and IL2 are not merely additive. This indicates that these stimulants are acting on the same cells in a synergistic and possibly sequential manner. In view of other work which has been done using LPS as a polyclonal B cell mitogen, it is predicted that LPS binds to a specific receptor on the IPP lymphocyte and activates the cell. This cell cycle progression in turn leads to the expression of functional receptors for various lymphokines, including IL2. The following experiments were performed to test this hypothesis.

Two million IPP lymphocytes were cultured in 24-well plates in a final volume of 2 mls. In the experiment to investigate the effect of delaying LPS addition, the cells were cultured initially in the presence of IL2 at 7 ng/ml. One hundred micrograms of LPS were added in 20 μl volumes at intervals after the initiation of culture. The supernatants were harvested on days 5, 6, 7, and 8. After microcentrifugation, the cell-free supernatants were stored with 0.1% sodium azide at 4°C until analysis.

Using IPP cells from the same preparation, an identical experiment was performed by delaying the addition of IL2 to cells cultured with LPS at 50 μg/ml. Fourteen nanograms of IL2 were added in 20 μl. The results shown in Tables XI and XII and Figures 19 and 20 are representative of the three times the experiment was performed.

Figure 19. **Effect of delaying the addition of LPS to IPP cells cultured with IL2.**

IPP cells were cultured with IL2 (14 ng/ml) in 2 ml volumes. One hundred g of LPS was added to identical cultures at ten hour intervals. Supernatants were collected at 120, 144, 168, and 192 hours. The horizontal axis refers to the time in hours since the initiation of culture. Key: LPS and IL2 ■ . IL2 alone ▲ . Media alone □ . Time of LPS addition: 0 hours ▮ . 10 hours ▽ . 20 hours ▼ . 30 hours + . 40 hours X . 50 hours ◆ . 60 hours △ .



Effect of delaying the addition of LPS to IPP cells cultured with IL-2.

Table XI. **Effect of delaying the addition of LPS to IPP cells cultured with IL2.**

IPP cells were cultured in 7 ng/ml IL2. Lipopolysaccharide (100 μg) was added at ten hour intervals until 60 hours. Supernatants were harvested at 120, 144, 168, and 192 hours. The immunoglobulin concentration is expressed as ng/ml.

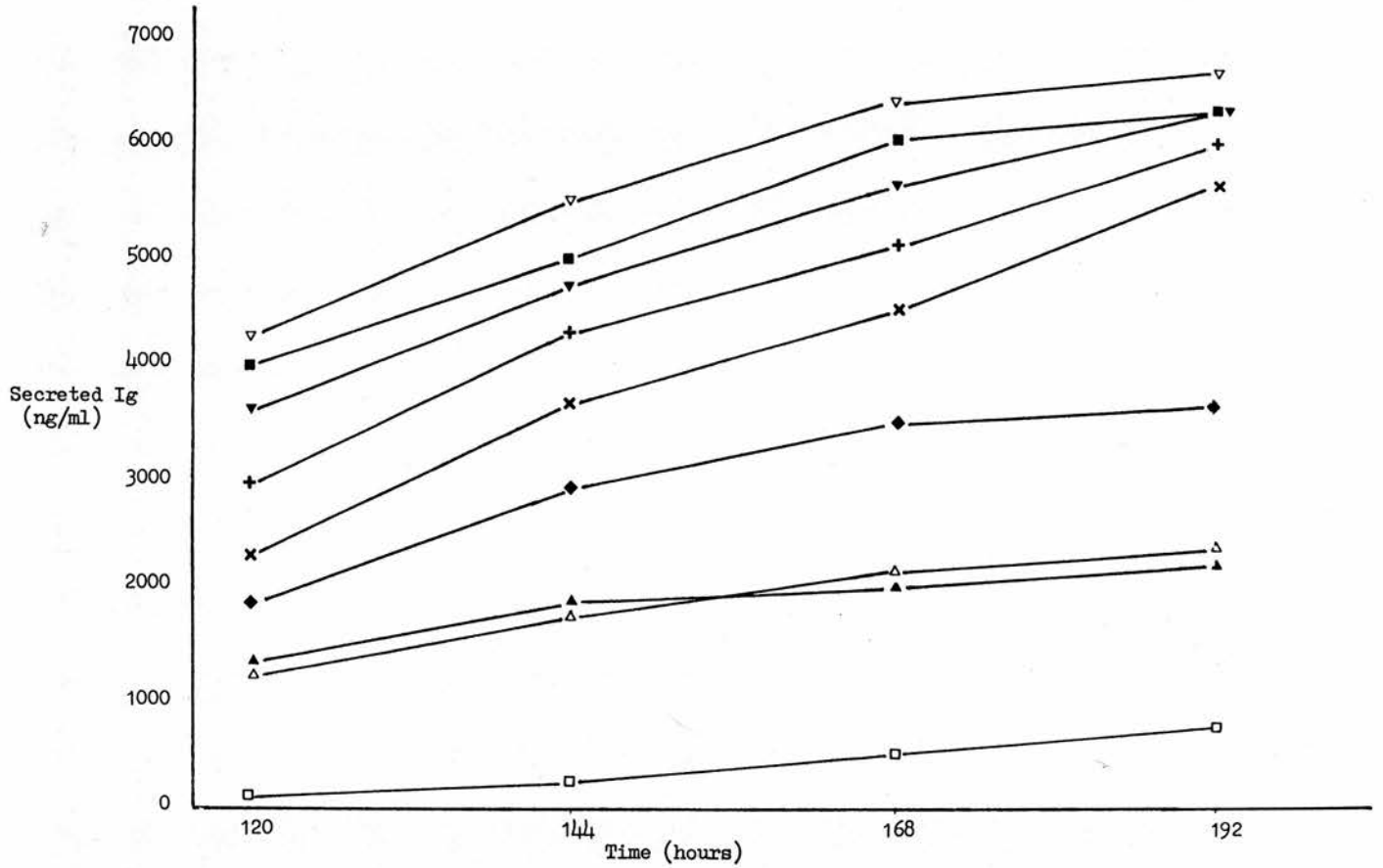
TABLE XI.

Effect of delaying the addition of LPS to IPP cells cultured with IL2.

Culture period. (hours)	Secreted immunoglobulin (ng/ml).								
	Culture conditions.								
	Media	IL2	Time of LPS addition (hours) 0	10	20	30	40	50	60
120	151	204	3981	1778	1348	549	251	288	181
144	281	660	5011	3388	2754	2290	1445	1047	794
168	562	1096	6165	4365	3981	3630	2454	1288	1047
192	776	1202	6456	4677	4786	4168	3890	1549	1230

Figure 20. **Effect of delaying the addition of interleukin 2 to IPP cells cultured with LPS.**

IPP cells were cultured with LPS (50 μg/ml) in 2 ml volumes. Fourteen ng of IL2 was added to identical cultures at ten hour intervals. Supernatants were collected at 120, 144, 168, and 192 hours. The horizontal axis refers to the time in hours since the initiation of culture. Key: LPS and IL2 ■ . LPS alone ▲ . Media alone □ . Time of IL2 addition: 0 hours ■ . 10 hours ▽ . 20 hours ▼ . 30 hours + . 40 hours X . 50 hours ♦ . 60 hours Δ .



Effect of delaying the addition of interleukin-2 to IPP cells cultured with LPS.

Table XII. **Effect of delaying the addition of IL2 to IPP cells cultured with LPS.**

IPP cells were cultured in 50 μg/ml of LPS. Interleukin 2 (14 ng) was added at ten hour intervals until 60 hours. Supernatants were harvested at 120, 144, 168, and 192 hours. The immunoglobulin concentration is expressed as ng/ml.

TABLE XII.

Effect of delaying the addition of IL2 to IPP cells cultured with LPS.

Culture period. (hours).	Secreted immunoglobulin (ng/ml).								
	Culture conditions.								
	Media alone	LPS alone	Time of addition of IL2 (hours).						
			0	10	20	30	40	50	60
120	151	1380	3981	4266	4169	3630	2951	2290	1862
144	281	1819	5011	5495	6370	4786	4365	3715	2951
168	562	2041	6165	6456	6309	5754	5128	4677	3548
192	776	2238	6456	6760	6607	6456	6165	5754	3715

These results confirm previous results regarding the kinetics of immunoglobulin secretion by IPP cells cultured with LPS and IL2 from the start of culture. However, when LPS was withheld for 10 hours, the potential for secretion was considerably diminished. This large reduction was not repeated when LPS addition was delayed for a further 10 hours. Rather, it would appear that the slightly reduced Ig concentration found at 120, 144 and 168 hours was a kinetic result of withholding LPS for an extra ten hours. By interpolating the amount of secreted Ig onto the horizontal (time) axis it can be deduced that the delay in secretion was indeed approximately ten hours. This process appeared to continue when LPS was withheld for up to forty hours. After delays of fifty and sixty hours, the addition of LPS fails to stimulate Ig secretion substantially above that found with IL2 alone.

The effects of delaying the addition of IL2 are apparently less complex. A ten hour delay results in a small (<10%) increase in secretion. However, the withholding of IL2 until 20 hours after initiation causes a slight delay in the secretion profile equivalent to 6-8 hours. This delay was appropriately increased when the IL2 was added at 30 and 40 hours. After 40 hours, the ability of IL2 to synergistically promote Ig secretion is reduced or lost.

DISCUSSION.

Cells derived from the ileal Peyer's patch can be induced to proliferate and differentiate under the influence of LPS and IL2. The

optimal conditions for these processes have been determined. The use of polyclonal anti-Ig as a putative B cell activator suggests that the IPP population has a characteristic in common with newly formed mouse B cells (Raff et al 1976). The massive 96% inhibition of tritiated thymidine incorporation by IPP cells cultured in media with 50 g/ml anti-Ig may be an important physiological event in the avoidance of autoreactivity. Since exogenous antigen is not introduced into the ileal Peyer's patch, binding of membrane immunoglobulin on newly formed B cells within the IPP is likely to be due to interaction with endogenous or self-antigens. This mechanism to delete B cell clones is probably one of the earliest that operate to avoid autoreactivity. It might therefore be predicted that B cells exiting the IPP quickly lose this property in order to respond to exogenous antigen. Alternatively, the effect of anti-Ig antibodies may be due to the cross-linking of mIg and Fc receptors on the same cell. This mechanism may have important consequences in reducing a B cell response in the presence of antigen-antibody complexes. This suppression is removed by using $F(ab)_2$ fragments instead of the entire molecule (Klaus et al 1984).

IL2 acts on IPP cells cultured with LPS in a saturable dose-dependant fashion. Maximal secretion was induced by 44 U/ml IL2. It is interesting to note that Romagnani et al used recombinant IL2 at 25 U/ml from the same source to stimulate proliferation of anti-IgM stimulated human B cells.

Assuming that the molecular weight of IL2 is 20 kD, the concentration of IL2 causing half-maximal secretion by IPP cells is approximately 125 pM. This is the approximate concentration at which

only high-affinity receptor-ligand interactions takes place (Yamada et al 1986, Wang and Smith 1987). Conversely, intermediate and low-affinity interactions generally require concentrations 10-1000 fold greater. Therefore it seems likely that the human IL2 binds to functional receptors of high affinity on the activated IPP lymphocyte. These are likely to be receptors specific for ovine IL2. The nature and the distribution of the ovine IL2R remains to be established. It is interesting to note that the majority of the effect of IL2 alone on the proliferation of IPP cells is attributable to cells that are inhibited by anti-Ig treatment (Table V). This would appear to indicate that IL2 exerts a direct effect on IPP cells to cause proliferation. However, this result does not eliminate the possibility that IL2 induces the release of one or more lymphokines from contaminating T cells.

The onset of secretion by IPP cells occurs on day 4 and the maximum rate of secretion was greater than 5 g/ml/day. The molecular weight of sheep IgG1, used as the reference immunoglobulin in the ELISA, is approximately 150 000 daltons. Therefore, 235 x 10^6 Ig molecules per ml are released every second during the period of highest secretion. Although there were 10^6 cells per ml at the initiation of culture, substantial cell death results in a 75 % drop in cell recovery by day 4 of culture. Therefore, the rate of secretion per cell is likely to be greater than one thousand molecules per second. This is comparable with other estimates of high-rate secretion from terminally differentiated B cells. Not surprisingly, the cells synthesized large amounts of Ig prior to the onset of secretion. This was noticable by day 2 of incubation. Mean cellular Ig increased approximately 26-fold. The

increased production of secretory Ig is likely to have resulted from an increase in cellular mRNA specific for heavy and light chain proteins and an increase in the ratio of RNA for secretory Ig compared to membrane Ig (Nakanishi et al 1984).

Mu (μ) is the predominant heavy chain isotype of the secreted immunoglobulins. By SDS-PAGE, the molecular weight of the secreted μ heavy chain is approximately 3 kD smaller than the membrane μ heavy chain. The difference is probably due to the production of a truncated μ chain lacking the transmembrane portion and cytoplasmic tail. The precursors of IgG and IgA secreting cells in vivo are derived from immature B cells originating in the IPP (Reynolds 1986). Hence the absence of cells secreting significant amounts of isotypes other than IgM requires explanation. Firstly, it may be that the protocol used to induce B cell differentiation only activates those cells committed to IgM secretion and which have lost the ability to switch heavy chain isotypes. Secondly, the cells may retain the ability to secrete other Ig isotypes but have not received the combination of factors and interactions necessary to induce this switch. In this context, IL2 has been described as a maturation factor for IgM secretion in vitro by human B cells although the fate of the genes encoding other heavy chain isotypes was not determined (Nakanishi et al 1984). In other species, our knowledge of the control of isotype expression is still very limited. Certainly, microenvironmental influences are likely to be important and these cannot be reconstructed in vitro. The degree of committment of the target cells for putative isotype switch factors is uncertain. For example, murine IL4 appears to act on cells that lack

surface IgG but are committed to secrete IgG1 by LPS activation (Vitetta et al 1984). The effects of IL4 in IgG1 regulation are therefore only to expand and differentiate the already determined cells. A genuine switch factor must operate on a cell previously showing no committment to secretion of that isotype. This committment is widely believed to only occur after antigen stimulation (Nossal et al 1964, Kearney and Lawton 1975, Kearney et al. 1976, Andersson et al 1978, Coutinho and Forni 1982). In view of the primary lymphoid source of the sheep B cells used in these experiments, the IPP cell would appear to be an ideal target cell for putative ovine switch factors.

The experiments examining the effects of delaying the addition of one of the co-stimulants gave complex but interesting results. The results obtained by delaying the addition of either LPS or IL2 require further explanation. The diminished secretion when LPS was withheld for ten or more hours could be due to at least two reasons. The secretion curve parallels that of the positive control suggesting that fewer cells are responding. Firstly, the drop in immunoglobulin secretion may be due to the loss of cells during the first ten hours of culture. This loss is clearly avoided or at least diminished if LPS is present from the initiation of culture. It has already been discussed that the IPP lymphocyte is an immature B cell and whilst some of the cells recovered from the IPP would have soon left via the efferent lymphatics, other cells would probably require further maturation before exiting. It is possible that LPS replaces a maturation signal or factor which causes the cells to attain a level of maturation which allows survival in culture. These cells respond to the presence of LPS

and IL2 by differentiating to Ig secretion. The absence of LPS from the start of the culture period results in the death of this cellular subset and therefore a reduced potential for secretion. Alternatively, IL2 acting alone on unstimulated B cells may inhibit cell differentiation in all or a subset of IPP lymphocytes. This inhibitory effect of IL2 may be normally reversed by the simultaneous presence of LPS. Evidence supporting the maturation-inducing role of LPS early in the culture period will be advanced in the next chapter.

The addition of LPS after 20-40 hours resulted in a secretion profile that lagged behind the 10 hour curve by a similar period. Taken together, these results suggest that LPS responsive populations are present at the start of culture. Murine primary B cells have a short half-life both *in vivo* and *in vitro* of approximately 2 days (Osmond 1986). Similarly for sheep B cells, when LPS addition did not occur until 50 and 60 hours, LPS failed to promote Ig secretion above the levels from cells cultured with IL2 alone. This mechanism appears to operate remarkably swiftly between forty and fifty hours. This sudden cut-off may reflect the functional homogeneity of this B cell preparation.

By contrast, IL2 is not required early in B cell activation. Reductions in the Ig-secretion profile are not noted until 10-20 hours. These observations are consistent with the finding in other species that IL2 is a factor which acts in the late G_1 stage of the cell cycle (Melchers and Andersson 1986). It is therefore presumed that IL2 exerts its effects following activation of sheep B cells by LPS. As previously discussed, it has not been demonstrated that IL2 acts directly on sheep

B cells. The possibility of IL2 inducing the release of one or more B cell growth and/or differentiation factors from a contaminating T lymphocyte population or B cell sub-set cannot be eliminated.

Chapter Five

Chapter Five.

Chapter Five

INTRODUCTION.

The previous two chapters have dealt with the phenotypic characteristics of the IPP lymphocyte and the effect of cell culture on the immunoglobulin secretion by these cells. It has been demonstrated that sheep B cells can mature to secrete immunoglobulin in the presence of optimal amounts of LPS and recombinant IL2. At the initiation of culture, the resting IPP cell is a small IgM+ cell which contains little cytoplasmic Ig. However, the function of these cells changes quite dramatically once the cells are stimulated. By 48 hours, cytoplasmic Ig is accumulating and high-rate secretion commences after a further two days. This change from a comparatively inactive cell to a cell synthesizing and secreting large amounts of immunoglobulin requires the development of cellular apparatus not present in the resting cell. In particular, plasma cells are larger, have extensive rough endoplasmic reticulum and Golgi apparatus. Since the IPP cells can be induced to secrete immunoglobulin, it was an opportunity to identify some of the morphological changes in these cell as they differentiate.

As well as the more obvious morphological changes, there are also important alterations in the expression of cell surface markers as differentiation proceeds. Of particular importance are the changes in expression of membrane Ig and MHC class II. Membrane Ig is the antigen receptor for B cells and its expression on the cell membrane is therefore only required for the period of B cell differentiation which is dependent on antigen. As discussed earlier (see Chapter One and the

introduction to Chapter Four), activation of B cells is directly or indirectly due to binding of antigen (or a polyclonal analogue) to the B cell antigen receptor. Subsequent events are controlled by interactions with antigen-specific T cells and their soluble products. Therefore once B cells have accumulated sufficient antigen for presentation to T cells (Lanzavecchia 1987), there is no role for membrane Ig and expression diminishes. Murine plasma cells do not express surface Ig. Similarly for murine MHC class II, maximum expression on B cells coincides with the period of T cell-B cell interaction ie antigen presentation. This is logical since antigen is presented to T cells bound to MHC class II molecules. As with Ig, expression of MHC class II diminishes subsequently until murine plasma cells express no MHC class II. The loss of important cell markers on plasma cells is a reflection of the terminally differentiated position of the plasma cell. Plasma cells have a life span of approximately 2 days.

Since the IPP is such an excellent source of large numbers of B cells, there is the opportunity to culture B cells under a variety of conditions and then to recover sufficient cells to perform morphological and phenotypic analysis. This chapter describes some experiments designed to investigate these changes. The results indicate that data obtained in the mouse cannot always be applied to another species.

MORPHOLOGY OF DIFFERENTIATING SHEEP B CELLS.

Chapter Five

These experiments were performed to determine the morphological changes occuring in IPP cells prior to the onset of high-rate secretion. IPP cells were induced to differentiate under optimal conditions as defined earlier. After culture, the cells were harvested and the viable cells were recovered after centrifugation over Lymphoprep^{R}. The cells were counted and cytospins were made. Alternatively, the cells were fixed by glutaraldehyde in cacodylate buffer and sent for processing in the Electron Microscopy Unit in the Faculty of Veterinary Medicine.

The results are shown in Figures 21 and 22. The Giemsa-stained cytospins of cells after 2 days in culture with LPS and IL2, revealed a large increase in cell size and a decrease in the nuclear:cytoplasmic ratio. These observations were confirmed by the FACS scatter profiles of resting and differentiating cells (data not shown). An increase in forward-angle scatter demonstrated an increase in cell size while the 90° scatter increase indicated that the cellular complexity had increased.

These conclusions were reinforced by the transmission electron microscopy morphology of differentiating cells after 3 days of culture. At low powers of magnification, the difference in cell size, nuclear:cytoplasmic ratio, and the electron density of the cytoplasm is demonstrated. It was also noted that in a large percentage of cells the nucleus occupied an eccentric position. At higher power (magnification x 35500), the cytoplasmic changes were evident, these included the formation of extensive rough endoplsmic reticulum (RER) and an increase in the number of mitochondria per section.

Figure 21. **Cellular morphology of resting and differentiating IPP cells.**

Cytospins of resting (a) and differentiating (b) cells were stained by the Giemsa method. Original magnification x 400.

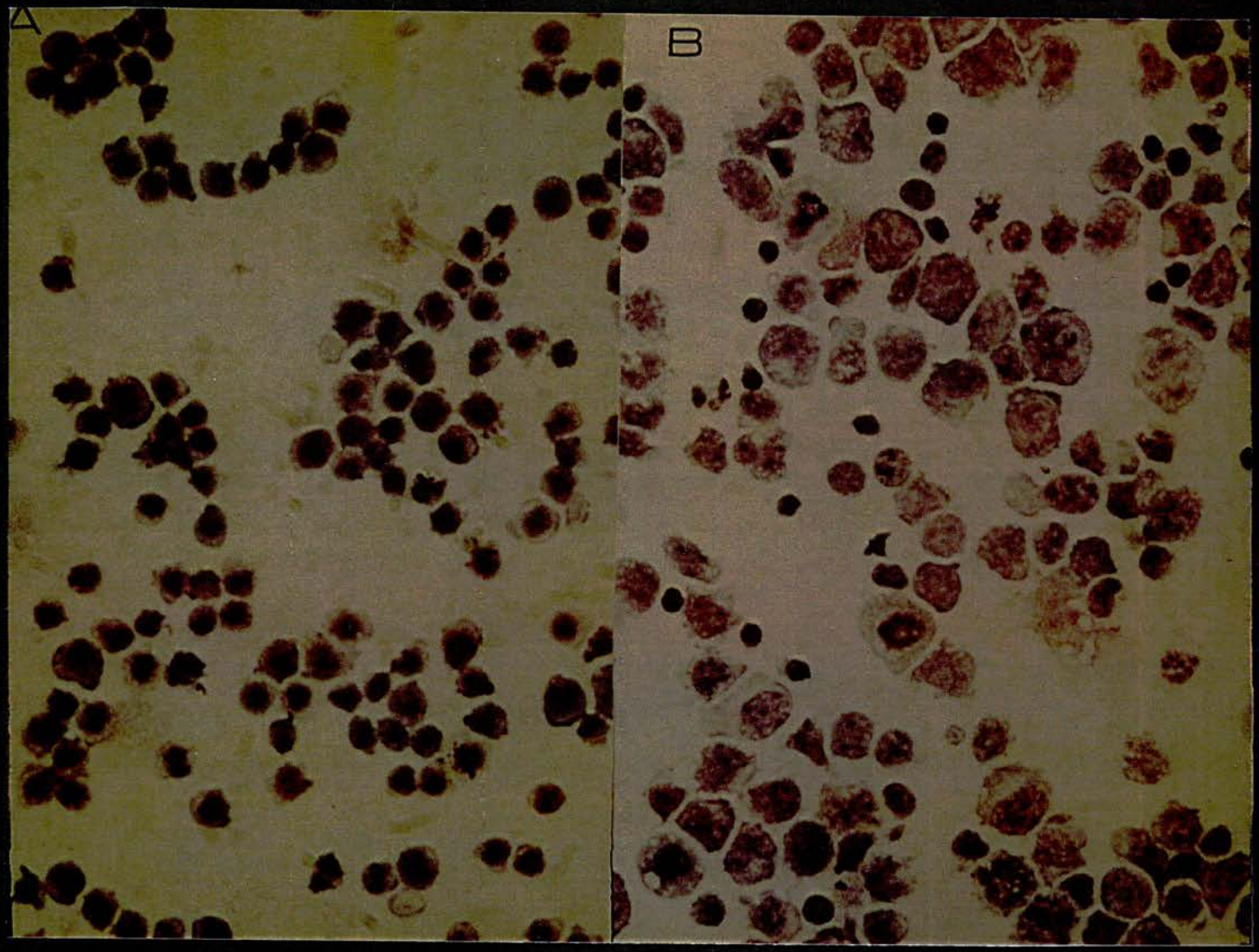
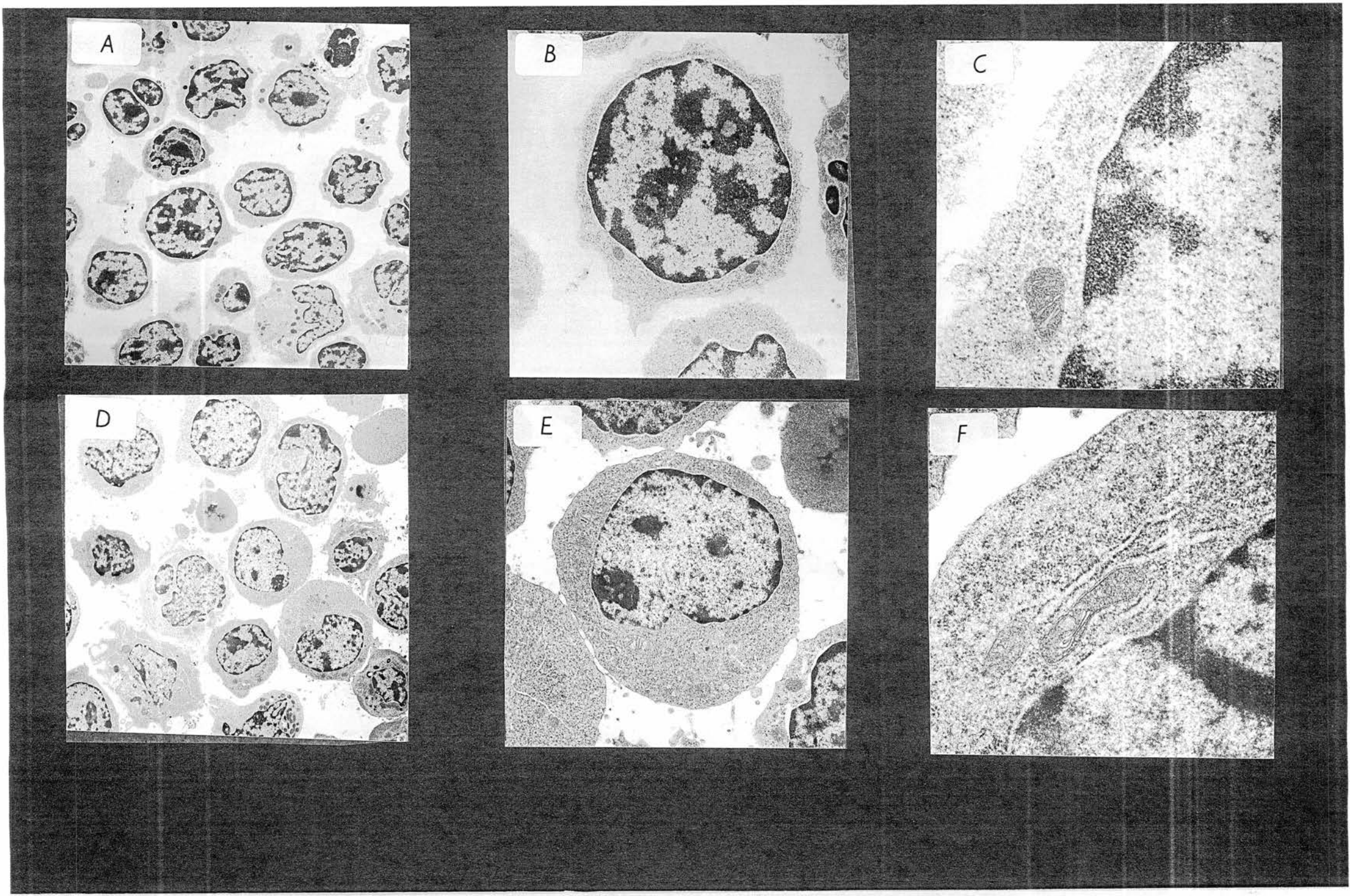

A
B

Figure 22. **Electron micrographs of resting and differentiating cells.**

Resting (a, b, and c) and differentiating (d, e, and f) were examined by transmission electron microscopy. Magnifications: x2750 (a and d); x7700 (b and e); and x35500 (c and f). Stained by uranyl acetate.


A
B
C
D
E
F

Chapter Five

CHANGES IN EXPRESSION OF MEMBRANE IMMUNOGLOBULIN AND MHC CLASS II DURING CULTURE WITH LPS AND IL2.

The following experiment was performed to examine kinetic changes in the expression of surface molecules as sheep B cells differentiate to immunoglobulin secretion. IPP cells were cultured at 1 x 10^6 with 50 μg/ml LPS and 7 ng/ml recombinant IL2 in four identical flasks. On days 1, 2, 4 and 6, the cells were harvested by centrifugation. Viable cells were recovered by centrifugation over Lymphoprep^R and washed three times in Hank's balanced salt solution (HBSS). The recovered cells were stained and fixed. The results of the analysis by flow cytometer are shown in Table XIII and Figure 23.

The phenotype of the resting cells is shown as day 0 and is similar to the preparation described previously. The most immediate noticable effect in this experiment is the substantial increase in surface MHC Class II expression after only 24 hours. The change in the log mode (peak) fluorescence intensity was 36 channels. This indicates a more than doubling in mode fluorescence intensity. This increased expression of MHC class II antigens persists until day 2. A drop in F.I. by day 4 is followed by a larger decrease by day 6 to levels below those of resting cells.

The changes in surface immunoglobulin occur more slowly. The rise in surface Ig expression of the positive population takes 48 hours to become maximal: one channel below a doubling in fluorescence intensity.

Table XIII. **Changes in the phenotype of cells cultured with LPS and IL2.**

a) Phenotypic changes.

IPP cells were cultured in 4 x 25 cm^2 flasks in LPS (50 g/ml) and IL2 (7 ng/ml). The viable cells were recovered daily and stained for sheep lymphocyte antigens. The samples were examined by flow cytometry. The results are expressed as the percentage of positive cells and the median (peak) fluorescent intensity (MFI). The percentage of positive cells in unimodal distributions overlapping the negative histogram is not given (*).

b) Immunoglobulin secretion by cells cultured with LPS and IL2.

The immunoglobulin concentration in the supernatant on days 0, 1, 2, 4, and 6 was determined and is expressed as ng/ml.

ND = not determined.

TABLE XIII.

Changes in the phenotype of cells cultured with LPS and IL2.

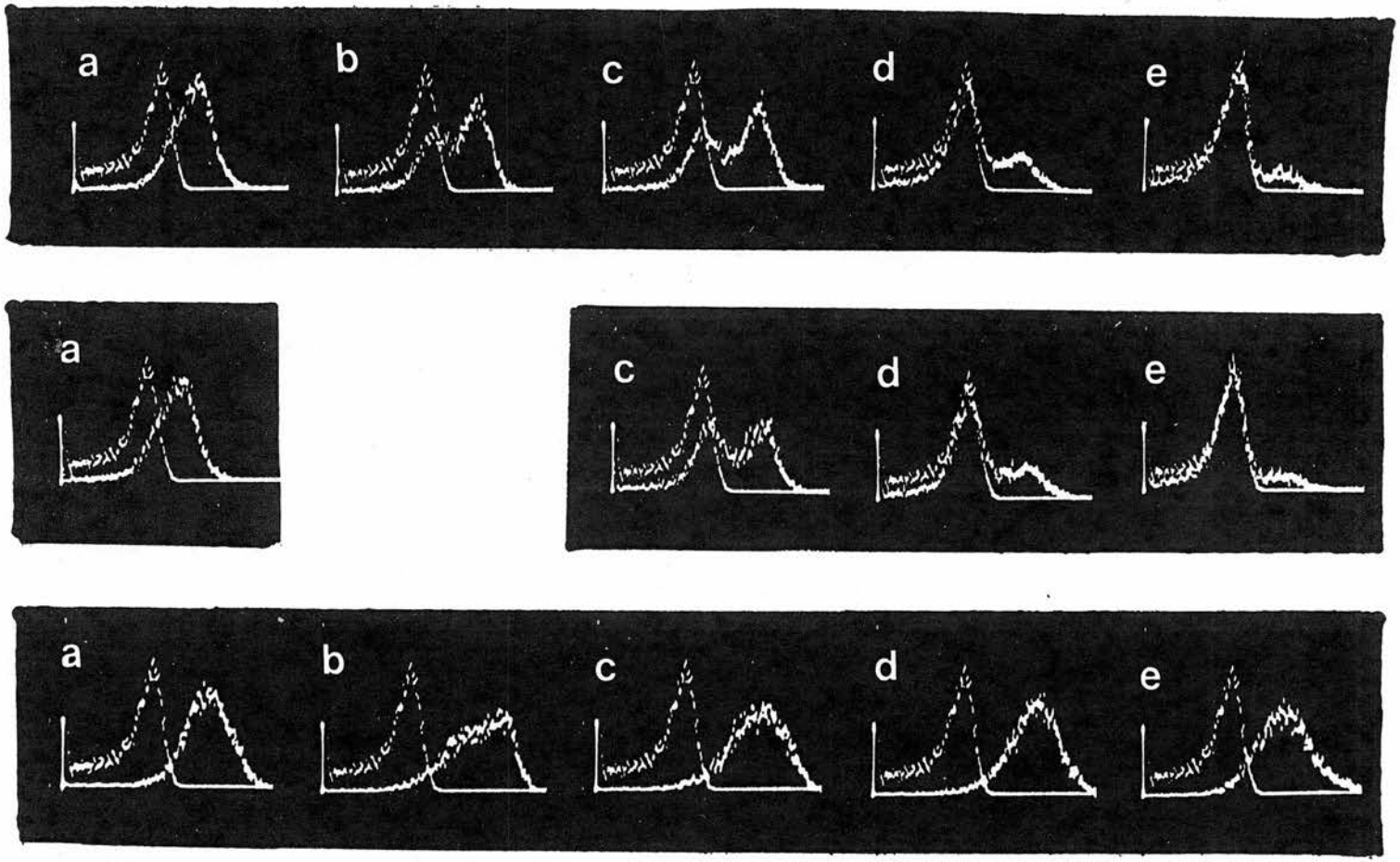
Surface antigen	Phenotype of cells recovered on									
	Day 0		Day 1		Day 2		Day 4		Day 6	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
Light chain	*	143	55	157	58	172	27	163	11	149
IgM	*	141	ND	ND	49	171	26	162	11	163
CD5 (T1)	10	147	20	147	26	142	30	141	27	143
Class II	*	162	*	198	*	186	*	181	*	157
Negative	1	104	2	103	3	102	4	96	2	97
Unstained.	0	100	1	94	0	92	0	90	0	93

b) Immunoglobulin secretion by cells cultured with LPS and IL2.

Time (days)	Immunoglobulin secreted. (ng/ml).
0	0
1	71
2	113
4	1154
6	1361

Figure 23. **Changes in the phenotype of cells cultured with LPS and IL2.**

Resting IPP cells (a) were cultured with LPS and IL2 under optimal conditions. Cells from identical cultures were recovered on days 1 (b), 2 (c), 4 (d), and 6 (e) and stained for i) (top row) Ig light chain. ii) (middle row) IgM and iii) (bottom row) MHC class II. The samples were examined by flow cytometry. The staining profile for each antigen is superimposed on the unstained histogram for comparison.



Changes in the phenotype of cells cultured with LPS and IL2.

Again the F.I. gradually drops but at day 6 it is still higher than resting levels although the profile had become considerably flattened making an exact peak difficult to discern. Of considerable importance, is the early appearance of a sIg^- population. By 24 hours, 45% of cells had lost surface immunoglobulin from their surface; this process continued until by day 6, only 11% of cells bore sIg. Although the profile for IgM staining was not determined on day 1, the response of surface IgM during culture mirrors that seen with total sIg with only 11% of cells positive for sIgM on day 6. By contrast, after 4 days 60-70 % of cells were positive for cytoplasmic Ig by immunohistology of cytospin preparations (Figure 25)

The number of $CD5^+$ cells also increases during the culture period. Although there were 10% present in the resting population, this quickly increased by day 1 to 20% and by day 4 reached 30% of all cells present. The peak fluorescence intensity of these cells varied very little over the culture period. The increase in $CD5^+$ cells is clearly insufficient to explain the very marked decrease in the number of sIg^+ cells.

The controls for the immunofluorescence data are shown in Table and reveal only small changes in fluorescence intensity of the negatively stained and unstained preparations. Contrary to expectations, the cultured cells showed a slight drop in autofluorescence.

ANALYSIS OF THE EARLY INCREASE IN SURFACE MHC CLASS II EXPRESSION BY DIFFERENTIATING B CELLS.

The above experiment revealed that differentiating cells showed a remarkable increase in cell surface expression of MHC class II antigens early in the differentiative process. This heightened expression persisted until the onset of high-rate secretion. As previously discussed, increased expression is an early event in B cell activation and can be induced by IL4. Finkleman et al. (1986) used this property of IL4 to assay its activity in the supernatants of antigen-specific T cells activated in vivo.

This apparent increase in the level of class II expression took place simultaneously with a large drop in cell recovery. Approximately 30 % of cells were recovered after 24 hours. It is possible therefore that the observed increase in expression was due to the death of cells expressing low levels of MHC class II. This would leave the high-expressing cells and account for the apparent increase by 24 hours. Alternatively, cell death may have occurred randomly among the IPP cells and the increase in MHC class II may be related to the effects of LPS and/or IL2. These mechanisms are not mutually exclusive and both may be responsible for the observed increase.

The following experiment was performed to test these hypotheses. IPP cells were set up at 3 x 10^6 per ml in bulk culture flasks with LPS and/or IL2. A higher cell concentration was adopted as preliminary experiments had demonstrated that cell recoveries were higher, particularly for cells cultured in media alone. Viable cells were recovered by standard means and stained for membrane immunoglobulins and MHC class II. Table XIV(a) shows the cell recoveries after 24 hours. It is clear that LPS alone enhances cell recovery sustantially

Table XIV. **a) Cells recovered after 24 hours in culture under various conditions.**

Sixty million IPP lymphocytes were set up in culture at 3 x 10^6 /ml for 24 hours. The number of live cells was determined by trypan blue exclusion. The results are expressed as the mean of three counts (x 10^6). The standard deviation is in brackets. The number of live cells recovered is expressed as a percentage of initial cell number.

b) Expression of immunoglobulin and MHC class II on the membrane of cells recovered after 24 hours of culture under various conditions.

The viable cells were recovered by centrifugation over Lymphoprep^R and stained for lymphocyte antigens. The samples were examined by flow cytometry. The results are expressed as the median fluorescence intensity.

TABLE XIV.

a) Cells recovered after 24 hours in culture under various conditions.

Culture conditions.	Cells recovered.	
	($\times 10^6$)	(%)
LPS+IL2	30.5 (1.6)	51
LPS	23.8 (1.2)	40
IL2	15.8 (0.0)	26
Media	14.3 (0.4)	24.

b) Expression of immunoglobulin and MHC Class II on the membrane of cells recovered after 24 hours of culture under various conditions.

Surface antigen	Median fluorescence intensity.				
	Culture conditions.				
	Resting	LPS+IL2	LPS	IL2	MEDIA
Ig light chain	150	160	161	159	163
MHC Class II	179	206	212	208	207
Negative	88	105	108	103	105
2nd antibody	88	102	106	103	102
Unstained	90	80	84	81	81

whilst IL2 alone gives only a marginal increase in recovery compared to media alone. IL2 and LPS in combination synergistically promote cell recovery.

Table XIV(b) shows the results of the flow cytometric analysis. The phenotype of the resting cells was analysed in full and was similar to results reported in Table II. The data in Table XIV is presented as the median fluorescence intensity. This is given as the channel number above and below which lie equal numbers of cells. This is suitable for the description of unimodal distributions of cells and eliminates the difficulty in accurately determining the peak (mode) fluorescence intensity. However, changes in the median fluorescence intensity were precisely mirrored by changes in the peak fluorescence intensity (data not shown).

The results demonstrate that there is little difference in the M.F.I. of MHC class II or immunoglobulin expression on IPP cells cultured with LPS and IL2, LPS alone, IL2 alone or media alone. There is however a substantial increase in the median fluorescence intensity of these parameters compared to the resting cells. As noted previously, the increase in the intensity of staining for class II approximately doubles by 24 hours. The intensity of staining for membrane Ig only shows a small increase by 24 hours confirming earlier data that changes in Ig expression occur less rapidly than changes in MHC class II expression.

Although these results appear to demonstrate no role for LPS and IL2 in the early increase in MHC class II expression, LPS and IL2 clearly promote cell recovery. In order to analyse the recovered cells

further, cells with a fluorescence intensity for anti-class II staining greater than 206 were arbitarily considered to be MHC class II-high expressers. The results of this calculation are demonstrated in Table XV. These results show that the numbers of class II-high cells recovered from media alone and IL2 -stimulated cultures can be explained solely by the survival of these cells from the initial population. However, the numbers of class II-high cells recovered from LPS alone or LPS with IL2 cannot be attributed in this way. There are two alternatives as to the origin of the additional "high-expressing" cells. Firstly, division by 60% of the surviving MHC class II "high" cells could account for the phenomenon. Secondly, LPS or LPS and IL2 may promote the expression of MHC class II on a subset of IPP cells which were previously "low-expressers". The latter suggestion is supported by data in the previous chapter and is further discussed at the end of this chapter.

PHENOTYPE OF DIFFERENTIATING CELLS.

The previous experiment showed that there was little distinction between IPP cells that had been cultured for 24 hours with LPS and IL2 and those that were cultured for an identical time with medium alone. Since it is known that the LPSand IL2 are potent inducers of B cell differentiation which will not occur in medium alone, it is interesting to discover at which stage a difference between stimulated and unstimulated cells appeared. It was decided to investigate the

Table XV. **The number of cells expressing "high" amounts of membrane Class II antigens after 24 hours in culture.**

The percentage of cells with a fluorescent intensity for MHC class II staining greater than 206 was obtained from the flow cytometric analysis. The number of MHC class II-"high" cells was determined using the information in Table XIV(a).

TABLE XV.

The number of cells expressing 'high' amounts of membrane Class II antigens after 24 hours in culture.

Culture conditions	Number of cells (x 10^6)
LPS + IL2	15.2
LPS	16.3
IL2	8.0
Media	7.2
Resting	9.5

phenotype of IPP cells after approximately 3 days in culture. This interval was chosen as it is after the onset of Ig synthesis and just prior to the start of Ig secretion.

IPP cells were set up at 1 x 10^6/ml under the usual variety of conditions ie LPS and IL2, LPS alone, IL2 alone, and medium control. After 66 hours, the cells were harvested and the viable cells recovered. Table XVI reveals the increased cell recovery from cultures stimulated with LPS and IL2 together compared to the other culture conditions. The cells were stained for surface molecules and the results are shown in Table XVII and Figure 24. The phenotype is reported as the percentage of positive staining cells with the peak (mode) fluorescence intensity in brackets. The peak fluorescence intensity refers to the positive peak with the exception of the unstained control. The percentage of positive cells in an unimodal distribution overlapping the negative histogram is not given (***) for reasons previously discussed.

The phenotype of the resting population was typical of other IPP preparations reported here. The changes in MHC class II expression are clear. Cells cultured with LPS and IL2 lost membrane class II molecules as differentiation proceeded. However, cells cultured with IL2 or in media alone still bore large amounts of class II as revealed by the high peak fluorescence intensity.

The expression of MHC class I antigens on the differentiating cells increased as shown by a doubling in the fluorescence intensity. Unfortunately the profile for cells cultured with IL2 alone or media could not be obtained due to lack of cells. Staining for the leucocyte

Table XVI. **Cell recoveries after 66 hours in culture under various culture conditions.**

One hundred and fifty million IPP cells were cultured in LPS and /or IL2 for 66 hours. The number of viable cells was determined and is the mean of triplicate counts. The standard deviation is shown in brackets. The percentage recovery is also calculated.

TABLE XVI.

Cell recoveries after 66 hours in culture under various culture conditions.

Culture conditions	Cells recovered after 66 hours. (x 10^6)	(%)
LPS + IL2	41.2 (2.0)	27
LPS	31.1 (1.6)	20
IL2	25.8 (0.8)	17
MEDIA	17.2 (1.1)	11

Table XVII. Changes in the phenotype of cultured cells after 66 hours.

The cells recovered after 66 hours in culture were stained for ovine lymphocyte antigens and examined by flow cytometry. The results are expressed as the number of positive cells and the mode (peak) fluorescence intensity in brackets ( ). The percentage of positive cells in a unimodal distribution is not given (***).

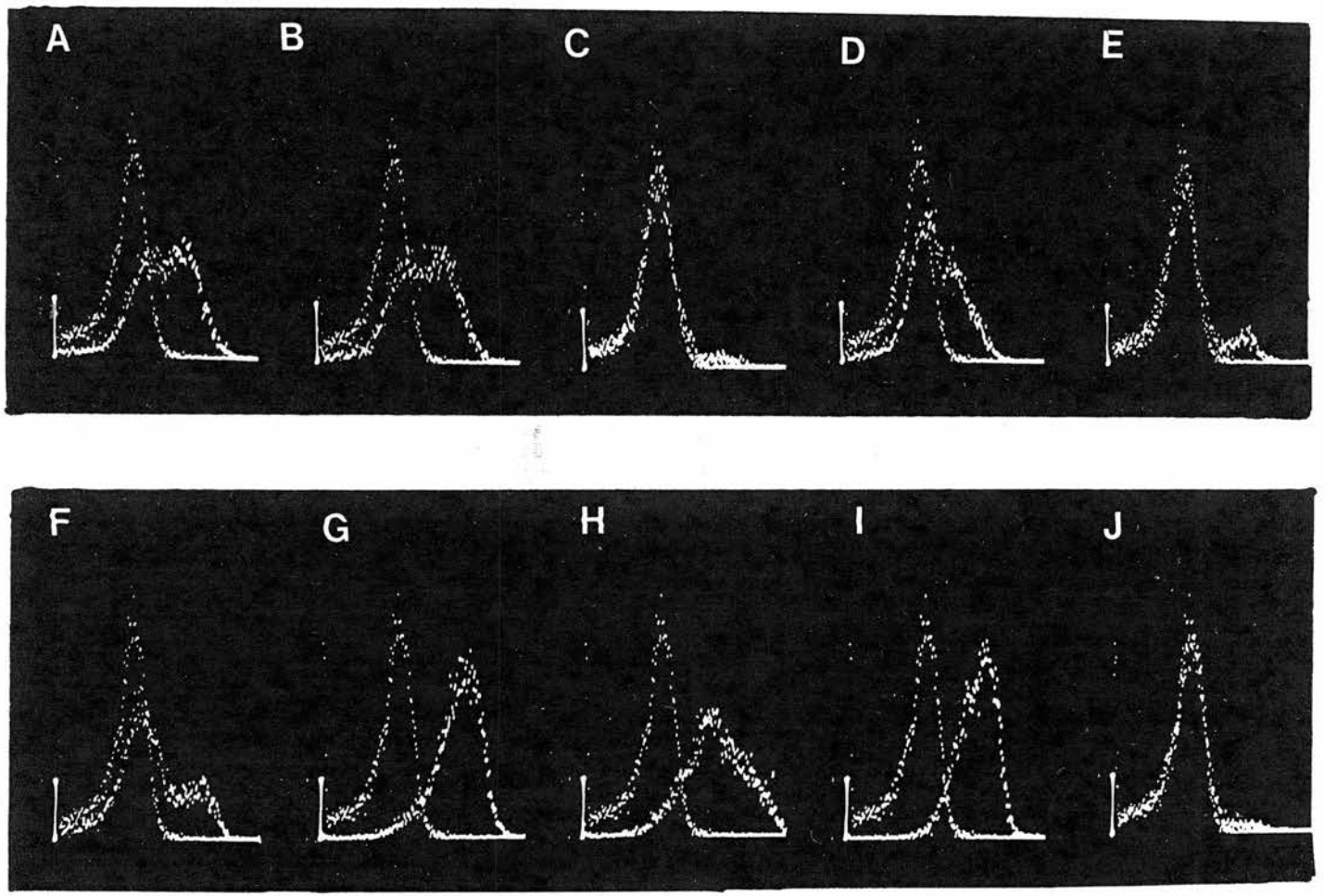
TABLE XVII.

Changes in the phenotype of cultured cells after 66 hours.

Surface antigen.	Phenotype.				
	Culture conditions.				
	Resting	LPS +IL2	LPS	IL2	Media
Light chain	*** (146)	47 (160)	66 (160)	36 (155)	70 (158)
IgM	*** (140)	42 (158)	63 (159)	35 (160)	68 (157)
IgG1	5 ND	5 ND	4 ND	5 ND	5 ND
Class II	*** (176)	*** (159)	*** (169)	*** (211)	*** (212)
Class I	*** (155)	*** (185)	*** (188)	ND	ND
L.C.A.	*** (168)	*** (173)	*** (177)	ND	ND
T1 (CD5)	12 (153)	35 (141)	18 (153)	37 (144)	29 (143)
T4 (CD4)	4 (177)	11 (180)	11 (167)	ND	ND
T8 (CD8)	5 (156)	27 (183)	10 (174)	ND	ND
Negative	1 ND	4 ND	5 ND	3 ND	3 ND
2nd antibody	1 ND	4 ND	6 ND	3 ND	4 ND
Unstained	0 (94)	0 (95)	1 (89)	1 (88)	1 (90)

Figure 24. **Phenotype of differentiating IPP cells.**

IPP cells were cultured for 66 hours with LPS and IL2. The viable cells were recovered and stained indirectly using monoclonal antibodies to sheep lymphocyte antigens. The samples were examined by flow cytometry. Cell number is shown on the vertical axis and log fluorescence intensity on the horizontal axis. The staining profile for each antigen is superimposed on the unstained histogram to aid comparison. A) Ig light chain. B) IgM. C) IgG1. D) CD5. E) CD4. F) CD8. G) MHC class I. H) MHC class II. I). LCA. J). negative (anti-HCG).



Phenotype of differentiating IPP cells.

common antigen (LCA) showed little change between the differentiating and resting populations.

The percentage of cells expressing Ig differed between the culture conditions. After almost three days, with LPS and IL2, less than half the cells recovered expressed surface Ig. Previous work described this chapter shows that by day 6 the percentage of Ig^+ cells can fall to approximately 10 %. This is attributable to a switch in the synthesis of membrane Ig to secretory Ig. When cells were cultured with LPS or media alone, the drop in Ig^+ cells was less substantial and indicates the lack of induced differentiation under these conditions. IL2 alone caused the loss of Ig from the cell surface in a large number of cells. However it has already been demonstrated that IL2 alone will not cause differentiation to the high-rate of Ig secretion found when cells are cultured with LPS and IL2. The results for expression of IgM were similar to those for total Ig and will not be discussed further. By contrast, the percentage of cells expressing IgG1 remained unchanged at approximately 5 %.

The percentage of cells expressing T cell antigens increased markedly during the differentiative process particularly under culture conditions involving IL2. When IPP cells were cultured with LPS and IL2 the percentage of CD5-bearing cells increased to 35 %. This CD5 positive population appeared to be composed mainly of $CD8^+$ cells with much fewer bearing CD4. Similarly IL2 alone increased the proportion of $CD5^+$ cells to 37 %.

Chapter Five

INCREASES IN CYTOPLASMIC MHC CLASS II AND IMMUNOGLOBULIN DURING DIFFERENTIATION.

Cells remaining after the immunofluorescence experiment described above were used to prepare cytospin preparations. These were stained for Ig light chain and MHC class II (Figure 25). A substantial proportion of cells showed a marked increase in cellular Ig. By ELISA, this increase has been determined to be approximately twenty six-fold. In addition, there was a large increase in the level of staining for cytoplasmic MHC class II by immunohistology (Figure 25). Assuming the number of epitopes per molecule has not changed during differentiation, this is indicative of an increase in the number of MHC class II molecules per cell. However, the same preparation of cells stained for surface MHC class II molecules using the same monoclonal antibody showed a slight fall in the mode fluorescence intensity (Table XVII) indicating a fall in the density of MHC class II molecules on the cell surface. Possible reasons for the large increase in total cellular class II in the apparent absence of increased surface expression will be discussed.

This observation was confirmed by Western blotting analysis of resting and differentiating cells. One hundred million cells from both samples were solubilised in 0.3 mls of lysis buffer. These were diluted similarly in SDS-PAGE sample buffer and run on a 12 % polyacrylamide gel. The separated proteins were transferred to nitrocellulose paper and after blocking were probed by VPM 16, a mouse monoclonal anti-sheep MHC class II β-chain antibody. The result is shown in Figure 26.

Figure 25. **Changes in the cellular content of Ig light chain and MHC class II during differentiation.**

Cytospins were prepared of resting (a, b, and c) and differentiating cells (d, e, and f). The preparations were stained indirectly for Ig light chain (a and d), MHC class II (b and e) and HCG (negative control) (c and f). Original magnification x 250.

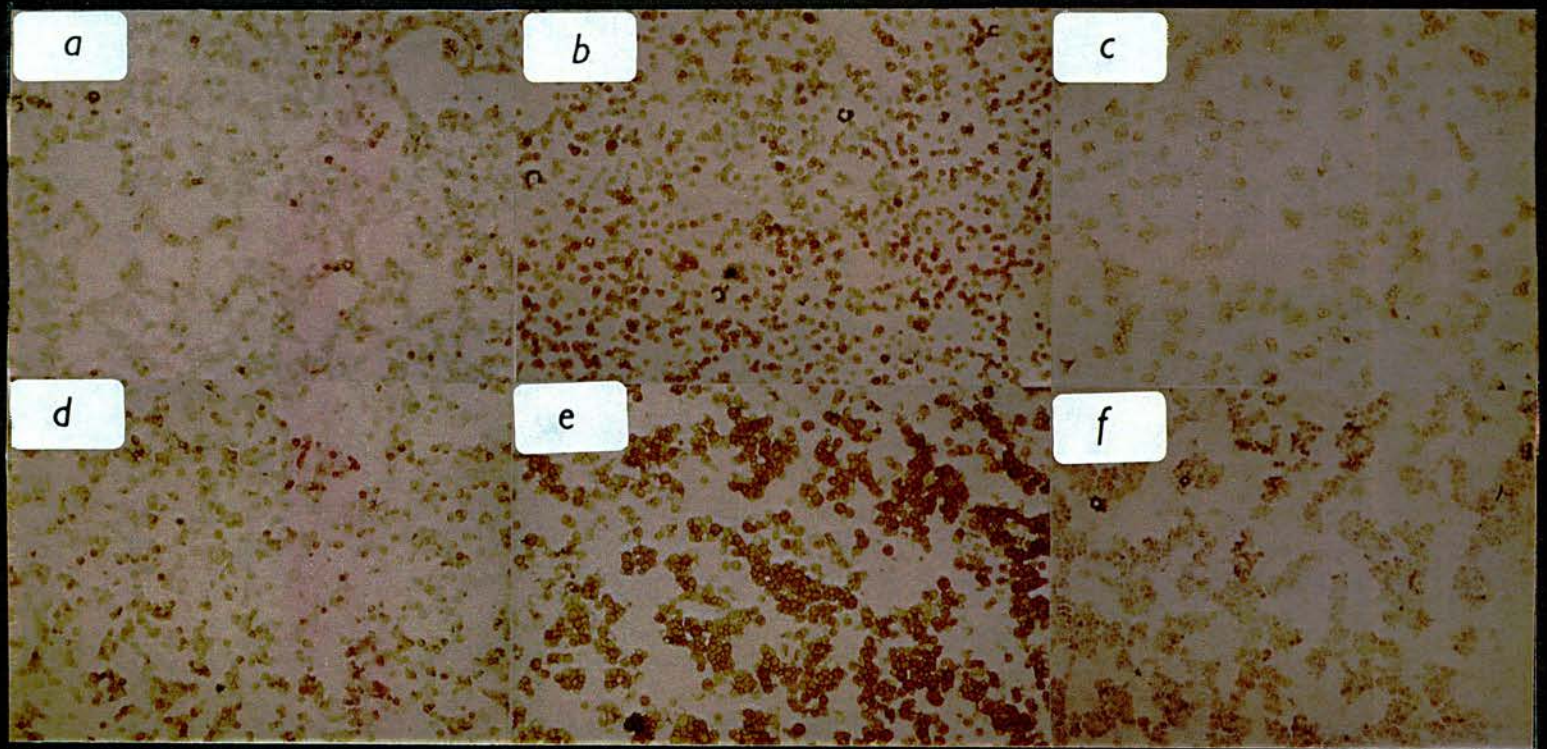

a
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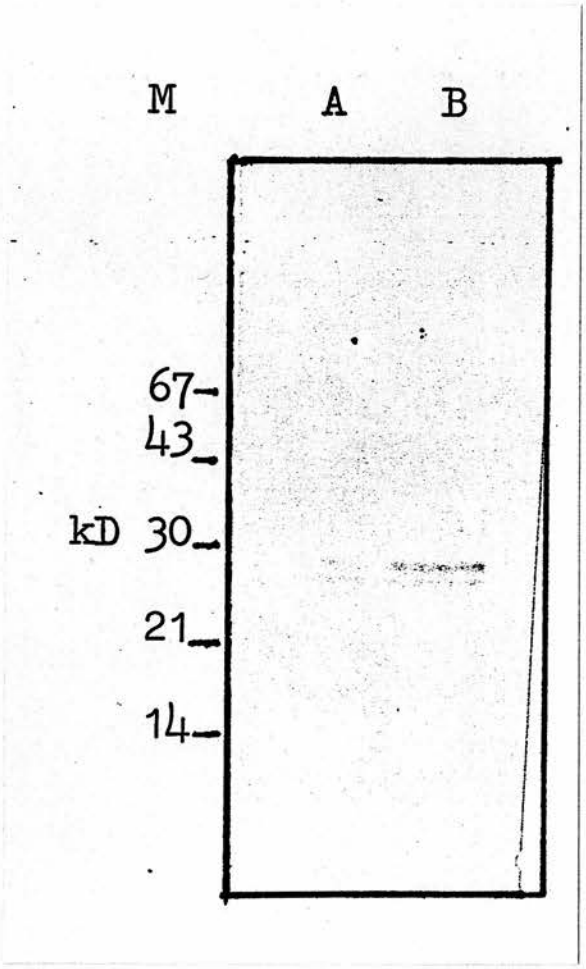
Figure 26. **Increase in MHC class II content of differentiating IPP cells compared to resting IPP cells.**

One hundred million resting (A) and differentiating cells (B) were solubilised in 0.3 ml of 0.5% NP-40. The differentiating cells were obtained after 3 days in culture with LPS and IL2 under optimal conditions. The samples were run on a 12.5% acrylamide gel under dissociating conditions. The distance migrated by certain marker proteins and their molecular weights is shown in track M.



Increase in MHC class II content of differentiating IPP cells compared to resting IPP cells.

The blot confirms that there is a marked increase in MHC class II ß-chains in differentiating cells.

DISCUSSION.

The ileal Peyer's patch lymphocyte can be induced to differentiate to IgM secretion. Cells recovered after 24 hours of culture with LPS and IL2 showed a doubling in fluorescent intensity when stained with a monoclonal anti-MHC class II antibody. Dr J. Hopkins (personal communication) has shown by Scatchard analysis that this approximately corresponds to a doubling in the number of MHC class II molecules on a cell assuming that the number of epitopes per molecule does not change. However, it is uncertain as to whether this corresponds to the early increase in MHC class II antigens seen in other species as a consequence of B cell activation (LeClerq et al 1986) as this increase is at least partly attributable to culturing IPP cells in medium alone and results from the death of an immature MHC class II-low population. It therefore appears that cell recovery after 24 hours is a more accurate estimate of sheep B cell activation than an early increase in membrane class II as used by other investigators. However, the number of MHC class II-high cells recovered after 24 hours of culture with LPS or LPS and IL2 cannot be explained merely by selective survival. This LPS-mediated effect may be due to the induction of cell division or to the promotion of MHC class II expression and survival in a population of cells previously bearing low amounts of class II. The latter

suggestion is clearly compatable with the finding in a previous experiment (Table XI) that LPS may promote the survival of cells which would otherwise die in culture. This mechanism operates within the first ten hours of culture. Hammerling et al (1976) has already shown that LPS increased the expression of Ia antigens on immature murine B cells. Similarly, Miyasaki et al (1984) demonstrated that PMA increased MHC class II expression on IPP cells after overnight culture, apparently in the absence of substantial cell death. Therefore, it is proposed that LPS acts on at least a subset of IPP cells early in the culture period to promote MHC class II expression and presumably cell-cycle progression until the cells reach an appropriate arrest point (Melchers and Andersson 1986). IL2 appears to be required later than LPS (Table XII) and allows the cells to proceed to cell division (Table III). As these later stages of the cell cycle are associated with a fall in class II expression, the addition of IL2 to LPS-stimulated cells results in fewer MHC class II-high cells at 24 hours.

B cell-helper T cell interactions occur early in the formation of an antibody response. Increases in MHC class II are therefore synchronised with the period of maximal cell-cell contact. Subsequent to this period, the expresion of MHC class II molecules on murine B lymphocytes falls until the fully differentiated plasma cell bears little or no MHC class II. In the sheep, there does not appear to be a large MHC class II negative population even at day 6 of culture when about 85 % of the B cells have completely lost surface Ig. This suggests that there is a difference between the plasma cells of sheep and mice, or that the IPP cells are not fully differentiated. In view

of the high-rate of secretion capable by these cells, the latter would be very unlikely. It is important to note that activated sheep T cells express large amounts of MHC class II antigens whilst murine T cells do not. The reasons for these differences may not be unrelated, and are possibly due to the large size of lymphoid organs in sheep compared to mouse. The continued expression of MHC class II molecules by a differentiating B cell in vivo (a sheep spleen is approximately 7-10 times the mass of an adult mouse) may play an important role in processes controlling differentiation.

While it is clear that the expression of membrane MHC class II falls as differentiation proceeds, it was surprising to find that there is a large increase in cellular MHC class II content. This was demonstrated by the increased immunohistological staining of differentiating cells compared to resting cells and confirmed by protein blotting analysis of solubilised cells. The reason for the discrepancy is unknown. One suggestion is that following antigenic or mitogenic stimulation, increased synthesis of MHC class II molecules occurs. Increased expression of class II molecules on murine B cells can occur independantly of antigen, for example as a result of IL4 treatment. The model of sheep B cell differentiation described in this thesis does not require antigen-specific stimulation of the B cells and it may be that activation under these unusual conditions causes a perturbation of the normal mechanisms controlling the surface expression of membrane class II. Since activated cells cannot be distinguished on the basis of their surface expression of MHC class II molecules, the control of the increase in cellular class II should be

investigated. It is not known whether this increase in cellular MHC class II molecules occurs in cells cultured under conditions which do not give rise to immunoglobulin secretion. If this is not the case, this would suggest that LPS and IL2 stimulate synthesis of class II molecules as part of the differentiative process but for unknown reasons fail to promote increased surface expression.

The loss of Ig from the cell surface has been referred to earlier. This can occur within 24 hours of the initiation of culture and by the latest time point examined there are only 15 % of B cells expressing membrane Ig. Hence it can be concluded that sheep plasma cells do not express Ig. This change in the production of heavy chains is likely to result initially from a change in the differential splicing pattern of the primary mRNA transcript of the heavy chain genes. This alteration results in the accumulation of cytoplasmic IgM. The substantial change in function of the B cell is marked by an increase in the number of mitochondria per cell and the development of rough endoplasmic reticulum. The morphology of the sheep plasma cell is demonstrated in Figure 22.

The increased numbers of cells bearing CD5 antigens confounds this description of B cell differentiation. The influence of these contaminating T cells on B cell differentiation is unknown and requires further work. It is interesting to note that the majority of these cells are $CD8^+$, this molecule is normally associated with the suppressor/cytotoxic subset of T cells. The percentage of $CD8^+$ cells in the starting populations usually exceeded $CD4^+$. The role or origin of these cells is unknown. It has not been possible to demonstrate cells

bearing T cell antigens within a follicle and therefore it is concluded that these cells originate in the lamina propria of the overlying mucosa. Since the gut is a region of immunological surveillance where inappropriate immune responses are extremely undesirable, it is not surprising to find a CD8:CD4 ratio higher than in other parts of the immune system. In addition, it has been demonstrated that $CD8^+$ cells retain responsiveness to IL2 while $CD4^+$ cells quickly lose responsiveness (Smith and Cantrell 1985), this would explain the greater expansion of the $CD8^+$ subset compared to the $CD4^+$ cells.

If it is assumed that the $CD5^+$ and B cell populations are mutually exclusive and that the $CD5^+$ cells include all the non-B lineage cells, then the number of surface Ig^- B cells after 3 days can be calculated. The results are shown below.

Culture conditions.	Number of sIg^- B cells. ($\times 10^6$).
LPS and IL2	7.4
LPS	4.0
IL2	6.8
Media	0.2

Although these results only apply to the situation after 3 days in

culture, it is surprising that so many IPP B cells respond to stimulation by IL2 alone by losing membrane Ig. It is clear from work in the previous chapter that this event does not lead to substantial secretion. However IL2 alone does cause proliferation of IPP cells (Table III) and since this proliferation is inhibited by anti-Ig (Table V) it seems very likely that the responding cells are B cells. Hence it may be that at least in vitro, cells of the B cell lineage can be induced by IL2 alone to differentiate down an Ig+ to Ig- pathway which does not terminate in the formation of an immunoglobulin-secreting cell. The role of the T cell population or its soluble factors in this event is unclear; as is the function of the B lineage cell produced.

This chapter describes the application of many techniques (eg FACS, immunohistology, electron microscopy, polyacrylamide gel electrophoresis and protein blotting) to determining the morphological and phenotypic changes occuring in IPP cells as they differentiate to immunoglobulin secretion. The results provide valuable information but leave several questions unanswered. For example, what is the role of MHC class II molecules in the cytoplasm of differentiating cells and on the surface of sheep plasma cells? With the current interest in sheep immunology, it is hoped that these questions can soon be answered.

Chapter Six.

Chapter Six.

Chapter Six.

INTRODUCTION.

This thesis has described the establishment and the characterisation of an in vitro model for B cell differentiation in sheep. The discussions at the end of Chapters Two to Five have dealt with the results obtained and they will not be repeated here. However, the main findings are incorporated in the table below. This work has considerably advanced our knowledge of sheep B cell differentiation. In particular, it is possible to propose a pathway from IPP to terminally differentiated plasma cell.

The IPP is undoubtedly the major source of newly formed B cells in the lamb but only a small proportion of cells formed within the IPP ever leave. It must be remembered that the IPP cells obtained by the method described probably reflect virtually all stages of B cell development, however the finding that the vast majority of cells express membrane IgM indicates that the cell preparation is biased towards cells in the later stages of intra-IPP development to the apparent exclusion of the earlier B lineage cells ie membrane IgM-ve pre-B cells. Of the cells obtained by the method described, I propose that some would be about to exit the IPP. Logic dictates that these more mature B cells are most likely to be those that survive in cell culture system used in this thesis; therefore these cells are those that I describe as MHC class II-high in Chapter Five. Other less mature B cells will die off if cultured in standard medium alone. However, it appears that the presence of LPS can permit the survival of some of these less mature cells if the LPS is present in the first 10 hours of

TIME (hrs)	SIZE and CONTENTS	IMMUNOGLOBULIN	MHC CLASS II	OTHER REMARKS
0	Small IgM+ cells. Low cytoplasmic nuclear ratio. Few organelles.	Surface: <100% + for IgM. also possibly IgD. other isotypes rare. Cellular: very low. (<10 fg/cell) Secreted: virtually zero.	Surface: 100% +ve. Cellular: Very little by immunohist. or blotting.	Proliferation of unstimulated cells is inhibited by anti-Ig.
<10				Requirement for LPS to achieve max. secretion. (? MHC class II low to high expression + survival)
15-20				IL2 responsiveness is acquired.
24		Surface: 30 % Ig-ve.	Surface: Increased density.	
48	Increased size and complexity.	Cellular: Increased x 20.		
72	High cytoplasmic:nuclear ratio. Nucleus eccentric. Increase in number of organelles eg mitochondria. Formation of RER.		Surface: Fall to below resting levels. Cellular: Large increase.	
96		Surface: 60% Ig -ve. Secreted: Onset of high-rate secretion.		
144		Surface: 85% Ig -ve. Secreted: Majority of secretion complete.		
168		Secreted: End of secretory period.	Surface: 100% + ve. ie sheep plasma cells express MHC class II.	

SUMMARY OF MAJOR EVENTS IN SHEEP B CELL DIFFERENTIATION.

culture. These cells increase their expression of membrane MHC class II by 24 hours after the initiation of culture.

Up to this stage in development in the IPP, the developing B cells do not encounter exogenous antigen. Therefore the crosslinking of membrane Ig of B cells within the IPP must indicate that the B cell has a specificity for endogenous or host antigen(s). The finding that rabbit anti-sheep Ig causes massive inhibition of thymidine incorporation by resting B cells suggests that a mechanism operates to delete such cross-linked cells (Raff 1970). This mechanism would reduce the risk of autoimmune disease. It would be important to determine whether this inhibition requires cross-linking of membrane Ig or merely binding of Ig; this could be tested by using monovalent Fab fragments rather than divalent intact molecules. Alternatively, the inhibition may be a result of crosslinking of Fc receptors and Ig. Klaus (1984) proposes that this occurs to limit further immune stimulation in the presence of excess bound antigen; this could be tested by using $F(ab)_2$ fragments instead of whole immunoglobulin molecules. It therefore appears that this marked inhibition by anti-sheep Ig reflects a physiological event in B cell differentiation.

There is certainly a requirement for LPS early in the culture period for a proportion of the cells and it appears that Ig secretion is not diminished or delayed if IL2 is not added until 15-20 hours after the addition of LPS to the cells. It is therefore suggested that LPS acts on the B cells to induce IL2 responsiveness. Whilst it has not been demonstrated that IL2 acts directly on the B cells, it has been demonstrated that LPS and IL2 act synergistically to promote Ig

secretion and that this synergism is abolished by the presence of anti-sheep Ig. Further work is required to determine the role of IL2 in sheep B cell differentiation and this is discussed below.

By 48 hours after the initiation of culture, there are major changes in cell size and cytoplasmic complexity. In addition, the cells have started to synthesize new immunoglobulin molecules as shown by the large increase in intracellular immunoglobulin by ELISA and immunohistology. When differentiating IPP cells were examined by transmission electron microscope after 3 days, formation of endoplasmic reticulum and additional mitochondria was evident. The latter developments reflect the requirements for a synthesis and secretion function. That this new immunoglobulin is destined for secretion is indicated by the rapid loss of membrane immunoglobulin from the surface of differentiating cells. It would seem likely that this occurs, at least initially, via a differential splicing mechanism for the RNA encoding the entire IgM molecule ie including the transmembrane region.

As well as the loss of surface Ig, IPP cells express less MHC class II as differentiation proceeds. This is not surprising as it is proposed that the role of MHC class II is principally in cell-cell interactions and in antigen presentation in particular. These events occur early in the pathway. In fact, it is not known what factors, if any, influence the latter stages of differentiation. Therefore it was surprising to find the large increase in intracellular MHC class II at a comparatively late stage of differentiation. This is further discussed below.

Finally, high-rate secretion of Ig starts on approximately day 4

and continues until day 6-7. This is consistent of other estimates of plasma cells lifespan of 3-4 days. The limited life span of the plasma cell clearly plays an important role in the homeostasis of the immune response as a method for avoiding prolonged and dangerous levels of circulating immunoglobulins. Obviously in the continued presence of free antigen, there will be continuing B cell activation to initiate further secretion. As antigen levels fall, only the cells with the highest affinity for antigen will bind antigen, this probably plays a role in the phenomenon known as affinity maturation.

The above description refers to work performed in this thesis and proposes how it accords about what is known in other species. However, an additional value of this model of sheep B cell differentiation is in the work which it now enables to be performed. The following discussion is not intended to be complete but indicates some work which was planned or even started during my AFRC fellowship but was not included in the main chapters.

LYMPHOKINES.

The nature of the target cell for IL2 in this assay is unclear. However this is not a unique conclusion. A recent workshop at the Basel Institute of Immunology was similarly undecided although the concensus opinion was that IL2 does have direct effects on B cells (Julius et al

1986). If it assumed that recombinant human IL2 has a direct effect on IPP cells, then this suggests that ovine IL2 will also have B cell differentiation factor activity. This property can be used to screen preparations (cell supernatants or body fluids) for BCDF activity. As stated in Chapter One, a principal advantage of the ovine immune system is the ability to study events occuring in single lymph nodes. A preliminary study has been performed. Dr John Hopkins cannulated the efferent lymphatic of the popliteal lymph node of sheep which had been previously immunised with ovalbumen. The drainage area of the lymph node was injected with 10 μg of ovalbumen in PBS and the cells exiting via the efferent lymphatic were collected and cultured for 24 hours in medium. This was done daily and the harvested supernatants were tested for BCDF activity. A marked increase in BCDF activity was observed on days 4-6 with a large peak on day 5. However, studies of this kind are confounded by the large amount of Ig present in the supernatant of the in vivo activated cells. The calculation of BCDF activity therefore has to be performed by subtraction which would obviously increase errors. Secondly, the presence or absence of a lymphokine at a particular stage in a physiological process can be misleading. Finkleman et al (1986) found that considerable absorption of IL4 could occur by the in vivo activated cells. Therefore the absence of BCDF activity does not indicate that none was released. It seems likely that this complication will plaque attempts to dissect the immune system by this approach. The purification of ovine lymphokines is important to the understanding of B cell differentiation. This assay is easily adapted to screening large numbers of fractions from chromatographic separations. I

performed all secretion assays in 24-well plates but they can equally easily be performed in 96-well plates.

The characterisation of lymphokines will inevitably raise questions regarding their cellular receptors. The availability of large numbers of IPP cells will allow their characterisation by radio-labelling and cross-linking studies.

IMMUNOGLOBULINS.

In Chapter Four, it was demonstrated that the predominant Ig isotype secreted was IgM. Unfortunately the absence of reliable reagents for several isotypes of sheep Ig made it difficult to determine the contribution from isotypes other than IgM. As discussed above the screening of supernatants from a variety of sources may generate Ig isotype secretion markedly different to that obtained by recombinant IL2 alone. The generation of monoclonal antibodies to the other ovine Ig isotypes is clearly a priority. In addition the development of alternative assays such as plaque forming assays would allow analysis despite the presence of Igs in the test supernatant.

The apparent appearance of a novel Ig molecule which is coexpressed with IgM suggests that the IPP cells express the ovine equivalent of IgD. Since the IPP is a region of B cell development, this finding is not entirely unexpected. The IPP cells are likely to be suitable for further studies on this immunoglobulin.

Chapter Six.

MAJOR HISTOCOMPATABILITY COMPLEX.

The finding that IPP cells cultured with LPS and IL2 accumulate large amounts of MHC class II molecules in their cytoplasm was unexpected as these cells do not express additional surface MHC class II compared to cells cultured in medium alone. Since MHC class II is believed to play its major role while on the cell surface the importance of vast amounts of cytoplasmic MHC class II is unknown. Studies into the control of expression of MHC antigens on sheep B cells should be pursued.

In addition, the finding that sheep plasma cells express MHC class II provides an important distinction with murine plasma cells. The continued expression of MHC class II is likely to be relevant to the control of the terminal differentiation of sheep B cells. This is dissimilar to the murine situation whereby the terminal differentiation of B cells occurs in a predetermined manner and is apparently without extracellular influence. It remains to be determined at which stage does differentiation to Ig secretion become inevitable for sheep B cells. To this end, it is interesting that IL2 alone will act on sheep B cells to give rise to an Ig- population. It is clear that the IL2 is indeed acting on B cells since the proliferation induced by IL2 alone is substantially inhibited by rabbit anti-sheep Ig. However there is little enhancement of Ig secretion by IL2 alone despite the Ig+ to Ig- progression. The role of the Ig -ve B cell so formed is unknown and this should be investigated.

Chapter Six.

LYMPHOCYTE POPULATIONS.

The role of the contaminating T cell population has been referred to. Improved cell purification techniques may help to answer some questions raised. In addition, it has been proposed that the occurence of T cell contamination is related to the age of the animal and that new-born animals would therefore be an ideal source of purified B cells. Unfortunately, this would obviously limit experiments to within the lambing season and would be considerably more expensive.

MOLECULAR STUDIES.

These studies were initiated before the conclusion of my experimental work but could not be pursued due to lack of time. Initial studies were aimed at studying mRNA levels in resting and differentiating cells. A probe for the C_μ gene had been obtained from Dr Terry Rabbits at the MRC in Cambridge. It is hoped that due to the length of this probe and the considerable amount of homology which occurs between the μ chains of even distantly related species that cross hybridisation will occur. This approach may lead to important advances in our understanding of immunoglobulin gene regulation in sheep B cells.

Appendix.

Appendix.

Appendix.

MEDIA AND CELL CULTURE REAGENTS.

RPMI 1640 was obtained from Gibco or Flow Laboratories.It was supplemented with 10% foetal calf serum (FCS),5 x 10-5M 2-mercaptoethanol and 2mM L-glutamine. Recombinant interleukin 2 (IL2) was received from Biogen SA of Geneva (batch number P50/51). Lipopolysaccharide (LPS) purified by phenol extraction and Concanavalin A (Con A) were purchased from Sigma Chemical Company Ltd. (Dorset, England).

MONOCLONAL ANTIBODIES.

Monoclonal antibodies against sheep T cell antigens CD5, CD4, and CD8 were obtained from Dr. M. Brandon of the University of Melbourne (Maddox, MacKay and Brandon 1985, MacKay, Maddox, Gogolin-Ewens and Brandon 1985). Drs Hopkins and Dutia of this department supplied monoclonal antibodies VPM 19, VPM 16 and VPM 18. These are reactive with ovine MHC class I, MHC class II and leucocyte common antigens respectively (unpublished data). Other monoclonal antibodies used, (VPM-8, VPM-6, VPM-13) are described in Chapter Two.

ANIMALS AND THE SOURCE OF OVINE TISSUES.

All sheep were out-bred animals and were obtained from various

sources. During experiments, they were housed in the animal house of the Department of Veterinary Pathology. Rabbits and Balb/c mice were bred and reared in the animal house.

Wherever possible the Edinburgh District Council slaughterhouse at Gorgie was used as a source of normal ovine tissues. All animals were being killed for human consumption and were examined both pre- and post-mortem for signs of disease. Unless otherwise stated, these tissues were obtained from animals less than 12 months old. In particular, it was possible to obtain samples from the ileal Peyer's patch, thymus, spleen, and mesenteric lymph node.

PREPARATION OF OVINE IMMUNOGLOBULINS.

Purification of IgM: Ovine IgM was purified from serum obtained at the City of Edinburgh slaughterhouse by affinity chromatography using VPM 13 antibody conjugated to Sepharose.

Preparation of IgG1: Ovine IgG1 was obtained from ovine colostrum by ion exchange chromatography (Watson, Brandon and Lascelles 1974). Briefly, the colostrum was centrifuged at 20000 g for 60 minutes to remove the chylomicrons and then the immunoglobulins were precipitated in 18% sodium sulphate. The precipitate obtained after centrifugation was washed in 18% sodium sulphate and then resuspended in distilled water. This solution was dialysed against 20 mM phosphate pH 7.4 and applied to a DEAE-cellulose column equilibrated against the same buffer. After the initial unretarded protein run-through was monitored

by spectrophotometry, a linear gradient was started from 20mM phosphate to 200 mM phosphate (all at pH 7.4). Elution of IgG1 at an ionic strength of approximately 0.125 M was determined by immunoelectrophoresis as it has a characteristic migration in agarose gels. The IgG1 was precipitated using a rabbit anti-sheep serum antiserum prepared by David Veitch. Lines were stained by Coomassie blue.

Pure IgG1 was obtained by affinity chromatography using VPM-6 antibody conjugated to Sepharose.

Preparation of IgG2: IgG2 was enriched from normal sheep serum by a similar method to that employed to obtain IgG1. The sodium sulphate precipitable fraction was dialysed against 20 mM phosphate pH 8.3 and applied to a DEAE-cellulose column equilibrated against the same buffer. After washing the column a linear gradient was run from 20 mM to 100 mM phosphate (all at pH 8.3), the IgG2 was eluted at approximately 50 mM phosphate.IgG2 containing fractions were analysed by immunoelectrophoresis versus rabbit anti-sheep serum antiserum. Ovine IgG2 has a distinct cathodal migration by IEP.

Preparation of IgA: IgA was obtained from lung fluid from a clinical case of pulmonary adenomatosis. This condition is characterised by the production of large amounts of bronchial secretions. The lung fluid was centrifuged to remove chylomicrons and then a standard sodium sulphate precipitation was performed. The solution was dialysed against 20 mM phosphate pH 7.4 and applied to a DEAE column. After washing, a linear gradient was run to 200 mM phosphate at the same pH. The IgA was eluted slightly later than the

IgG1. IgA content was monitored by IEP with antisera against whole sheep serum and IgA-heavy chain specific antiserum prepared as described below. The IgA-containing fractions with the least ammounts of IgG1 were pooled and dialysed against 50 mM tris, 0.15 M NaCl pH 8.0.

PRODUCTION OF ANTISERA.

Antisera were produced in rabbits by two intra-muscular injections of antigen in complete Freund's adjuvant (CFA) 14 days apart. After a rest of 1-3 months, 50µg of antigen was injected intravenously. Bleeds of 40 mls were taken every 7 days thereafter while titres remained good. The blood was allowed to clot at room temperature for one hour and then kept at 4°C overnight. The serum was collected and stored at -20°C.

Production of an antisera specific to IgA-heavy chain. A rabbit was immunised with IgA as described above. In order to remove reactivity to light chains and any contaminating IgG1 in the original preparation of IgA, the sera was exhaustive absorbed against IgG1-Sepharose until the sera showed no reactivity to IgG1 by IEP and ELISA. By Western blotting, the sera only bound to a single band in the region of 59 kD. Ovine IgG1 has a molecular weight by SDS-PAGE of 55 kD.

PRODUCTION OF MONOCLONAL ANTIBODIES (Kohler and Milstein 1975).

Appendix.

Spleen cells from appropriately immunised mice were fused with cells of the aminopterin-sensitive myeloma, NSO, with 50% polyethylene glycol 1500 (various sources). Mice were given an intravenous injection of antigen four days prior to being killed by cervical dislocation. The spleen was removed by sterile techniques and placed in a Petri dish with warm RPMI-1640 medium. A single cell suspension of spleen cells was prepared using two 19-gauge needles with shafts bent at right angles. These were used to disrupt the capsule and to gently scrape the cells into the medium. Cells were transferred to a plastic universal tube and clumps allowed to settle for five minutes. The cell suspension was removed, washed and counted. The spleen cells were mixed with NS0 cells in the ratio of 3:1 and centrifuged. The disrupted cell pellet was warmed to 37°C. Polyethylene glycol 1500 was added slowly over one minute with shaking to prevent agglutination of cells and after a further at 37°C,warm RPMI-1640 medium was added slowly, 5 mls over five minutes followed by 8mls over three minutes. After further washing the cells were resuspended in 100 mls of RPMI-1640 with 20% FCS, 2-ME, L-glutamine and HAT. The cells were plated out in 96 well flat-bottomed plates and cultured at 37°C with 5% carbon dioxide.

After an appropriate period, supernatants were taken and tested. Hybridomas secreting antibodies with the required specificity were grown up into larger volumes and cloned by limiting dilution using normal mouse spleen cells as filler cells. All hybridoma cell lines were regularly recloned and fresh vials laid down to replace those which were more than 12 months old.

Appendix.

CRYOPRESERVATION OF HYBRIDOMA CELL LINES.

Cell growing under optimal conditions were collected by centrifugation at 300g. The pellet was disrupted and slowly cooled. Freezing mixture (7% DMSO in RPMI-1640 with 30% FCS) was added dropwise to give a final cell concentration of approximately 10^7 per ml. The cell suspension was aliquoted into Linde vials and gradually cooled at -30°C overnight inside a polystyrene box.The vials were then transferred to a -70°C or a liquid nitrogen freezer.

IMMUNOHISTOLOGY.

Tissue samples were obtained from the abbatoir as described below. These were brought back to the laboratory on ice in order to minimise post-mortem artifacts. The sections were prepared by Brian Kelly of this department. Sections were allowed to warm to room temperature and then fixed in acetone at -20°C for 2 minutes. Sections were then incubated for 30 minutes with an appropriate dilution of the antiserum or monoclonal supernatant in a final volume of 50 μl. The slides were washed three times in PBS for 5 minutes each. The area surrounding each section was carefully dried, taking care to avoid touching the section. The appropriate peroxidase conjugated anti-immunoglobulin was diluted and applied to the section for 15 minutes. After washing three times as above the slides, were washed in 50 ml of Tris buffer for 10 minutes. The slides were developed in 0.05% 3,3'-diaminobenzidine

tetrahydrochloride in tris buffer containing 0.03% hydrogen peroxide. After 15 minutes at room temperature the slides were rinsed twice in tris buffer. On occasions, the slides were counterstained using methylene green in order to aid orientation of the section. Slides were mounted and viewed using transmitted light optics.

AFFINITY CHROMATOGRAPHY.

Cyanogen bromide-activated Sepharose 4B was purchased from Sigma Chemical Company Ltd. The powder was swollen by washing with 1 mM HCl over a sintered glass filter using 200 ml per gram of dry gel. The protein to be coupled was dialysed against conjugation buffer (0.1 M sodium bicarbonate pH 8.3 with 0.5 M sodium chloride) to give 5 mgs of protein per ml of gel. The swollen gel was washed quickly with conjugation buffer and then added to the protein solution. The mixture was rotated overnight at 4°C to allow coupling. The remaining active sites were blocked using 0.2 M glycine pH 8.0. Adsorbed protein was removed by alternately washing the gel with conjugation buffer and 0.1 M glycine pH 2.5. The Sepharose conjugate was stored in the presence of 0.1% sodium azide at 4°C.

Before use the gel was transferred to a small plastic column or syringe barrel and washed with running buffer (50 mM tris with 0.15 M NaCl pH 8.0). Any adsorbed protein was then removed using 3 column volumes of 0.1 M glycine pH 2.5; the gel was then washed with running buffer again. The solution containing the protein to be purified was

allowed to run through the gel at the approximate rate of five column volumes per hour. Where there was a large discrepancy between the volume of the solution and the gel, the gel was removed from the column and rotated overnight at cold room temperatures. The column was then washed extensively with greater than fifteen column volumes of running buffer, ensuring that the reservoir of buffer at the head of the column was minimal. The gel was eluted using eluting buffer and 2 ml samples collected. Protein elution was monitored using spectrophotometry and protein-containing samples were dialysed against running buffer in the first instance.

When purifying proteins from solubilized cells, 0.5% Nonidet P40 was included in all buffers. As NP-40 interferes with spectrophotometry it was neccessary to determine protein content by other means; usually SDS-PAGE.

SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS.

The mini-gel apparatus was purchased from Bio-Rad Laboratories and was used according to the method of Laemmli (1970). The gel moulds were assembled and the separating and stacking gels prepared as described below. Ten per cent gels were most commonly used.

Appendix.

Separating gel.	volume(ml) to give final polyacrylamide concentration.		
	7.5%	10%	12.5%
acrylamide stock.	6.0	8.0	10.0
1M tris-HCl pH 8.8	15.0	15.0	15.0
water	17.7	15.7	13.7
10% w/v SDS	0.4	0.4	0.4
1.5% ammonium persulphate	0.9	0.9	0.9
TEMED	0.025	0.025	0.025

Stacking gel.	volume(ml)
acrylamide stock	1.0
1M tris-HCl pH 6.8	1.25
water	7.3
10% SDS	0.1
1.5% ammonium persulphate	0.35
TEMED	0.01

After the gel has polymerized, the gel moulds were removed and fitted to the tank assembly. The electrode compartments were filled with electrode buffer and the samples were prepared by diluting in sample buffer and heating at 100°C for 3 minutes. After loading 10μl of each sample, the electrodes were connected to a power pack. Electrophoresis

was carried out at 20-30 mA per gel (two gels per tank) and was stopped when the bromophenol blue reaches the bottom of the gel; this was usually within one hour. The gels were removed from the moulds and processed as required.

COOMASSIE BLUE STAINING OF SDS-POLYACRYLAMIDE GELS.

The gel was incubated with 0.025% Coomassie brilliant blue R in 50% v/v methanol,5% v/v acetic acid for 60 minutes on an orbital shaker. The gel was then briefly washed in distilled water and then destained in 5% v/v methanol 7.5% v/v acetic acid until a clear background was obtained. The gel was stored after being dried onto filter paper using heat and suction.

SILVER STAINIING OF SDS-POLYACRYLAMIDE GELS.

Gels were incubated with 50% v/v methanol,10% v/v acetic acid for 15 minutes followed by a further incubations in 5% v/v methanol,7.5% v/v acetic acid and then 10% v/v glutaraldehyde. After extensive washing in distilled water the gels were placed in a 0.1% w/v solution of silver nitrate for 15 minutes. The gels were washed again and then incubated in developer (50μl of formaldehyde in 100 mls of 3% w/v sodium carbonate). The developer was changed 2-3 times. When the colour change appeared to be complete, the reaction was stopped using five mls

of 2.3M citric acid per 100 mls of developer. After washing, the gels were placed in 10% v/v Ilford Fixer for two minutes. The gels were washed and dried and stored away from sunlight.

PROTEIN BLOTTING OF SDS-POLYACRYLAMIDE GELS.

At the end of electrophoresis, the gels were removed from their moulds and washed in transfer buffer (80% v/v 192mM glycine 25mM tris pH8.3, 20% v/v methanol). The proteins were transferred to nitrocellulose paper using a Semi-Dry Electroblotter (Ancos, Denmark). All materials were soaked in transfer buffer before assembling the blotter. The bottom electrode (anode) was covered by six 14 cm^2 pieces of 3MM paper; on top of this was laid a nitrocellulose sheet 0.45 m pore size. The gels were placed onto the nitrocellulose and covered by a further six sheets of 3 MM paper. The top electrode (cathode) was fitted and the apparatus was electrophoresed for 60 minutes at 150 mA. An appropriate part of the nitrocellulose paper was removed and stained using 0.5% w/v naphthol blue black in 50% v/v methanol 5% v/v acetic acid; washed and then destained in 50% v/v methanol 5% v/v acetic acid. This allowed the quality of transfer to be assessed and also assign molecular weights to bands seen after immunochemical staining.

The remainder of the sheet was incubated in 5% w/v dried non-fat milk powder in PBS for 60 minutes to block unbound sites. If the sheet was to be probed with more than one antibody or antiserum, it was then cut. Incubations were performed in sealed plastic bags as these

conserved valuable reagents. With indirect staining, the paper was washed in 1% skimmed milk powder in PBS and then incubated with the appropriate anti-immunoglobulin conjugated to horseradish peroxidase (HRPx). After washing again, the nitrocellulose was incubated in tris buffer (50 mM tris-HCl pH 7.3) for 5 minutes. The blot was developed in tris buffer containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide. This developer was prepared immediately prior to use. After the bands had appeared, the paper was washed and allowed to dry. The molecular weights of any bands could be derived from the markers visualised by naphthol blue black staining. Stained blots were stored in the dark.

IMMUNOELECTROPHORESIS.

A 1% solution of agar in barbitone buffer pH 8.3 was melted in a pressure cooker and a layer of this agar was poured onto Gelbond^{r} and allowed to cool on a level surface. Using a gel cutter, wells and troughs were marked out on the gel, the plugs of agar were removed using a vacuum line attached to a Pasteur pipette. Five 1 of antigen were placed in the wells and was electrophoresed at 6 V/cm of gel. The progress of the electrophoresis was monitored by adding 2μl of 0.001% bromophenol blue to one of the samples eg. normal serum. The troughs were carefully removed by suction and then filled with an appropriate dilution of antiserum and incubated overnight in a humid chamber. Lines were stained by Coomassie blue after washing and drying.

Appendix.

IMMUNOFLUORESCENCE STAINING AND FLOW CYTOMETRY.

Cells were washed in Hank's buffered salt solution with 1% bovine serum albumin and 0.1% sodium azide, and 2 x 106 were placed in a Rhesus tube. Fifty microlitres of appropriately diluted antibody solution were added and incubated for 15 minutes at 4°C. Cells were washed in medium twice and incubated in fluorescein isothiocyanate-conjugated second antibody for 15 minutes at 4°C. After further washes, the cells were examined. For ultra-violet microscopy, the cells were transferred to a slide after being resuspended in 10% glycerol in phosphate bufferred saline (PBS) plus azide. A coverslip was added and sealed using nail varnish.

For flow cytometry, where possible the cells were studied as soon as possible after staining. When this was not the case, the cells were fixed in 1% freshly prepared paraformaldehyde in medium and stored in the dark at 4°C until analysis. Ten thousand cells were analysed using the FACS IV in the Department of Zoology, University of Edinburgh. Polaroid photographs were taken of the monitor in order to demonstrate staining profiles.

SOLUBILISATION OF LYMPHOCYTES.

The cells to be solubilised were suspended in 0.5 ml PBS and cooled

on ice for ten minutes. One millilitre of the detergent solution, 1% w/v Nonidet P40 in PBS containing 1mM EGTA was cooled separately. A 0.1 M solution of phenylmethylsulphonyl fluoride (PMSF) in acetone was made. One microlitre of this protease inhibitor and 0.5 ml of the cooled detergent solution was added to the lymphocytes. The tube was inverted gently to mix the contents and incubated on ice for 30 minutes. The mixture was centrifuged at 200g for 10 minutes at 4°C and the supernatant re-centrifuged at 100 000g for 30 minutes at 4°C. The supernatant was stored at -70°C if neccessary.

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ABBREVIATIONS.

BCDF	B cell differentiation factor
BCGF	B cell growth factor
BSA	Bovine serum albumen
C	Constant
Ci	Currie
Con A	Concanavalin A
CPM	Counts per minute
CD	Cluster designation
CSF	Colony stimulating factor
D	Diversity
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
g	Gravity
GM-CSF	Granulocyte-macrophage CSF.
H & E	Haematoxylin and eosin
HIV	Human immunodeficiency virus.
HSC	Haematopoietic stem cell
ICAM	Intercellular adhesion molecule
IEP	Immunoelectrophoresis
IFN	Interferon (eg IFN-gamma)
Ig	Immunoglobulin (eg IgM)
Ig+	Immunoglobulin-positive
Ig-	Immunoglobulin-negative
IL	Interleukin (eg IL2)
IPP	Ileal Peyer's Patch
J	Joining
JPP	Jejunal Peyer's Patch
LCA	Leucocyte common antigen
LFA	Lymphocyte function antigen
LPS	Lipopolysaccharide
kD	Kilodaltons
MFI	Mode fluorescence intensity
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NP-40	Nonidet P40
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PMA	Phorbal myristate acetate
PNA	Peanut agglutinin.
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
sIg	Surface immunoglobulin.

uv	Ultra-violet
V	Variable
v/v	Volume in volume
w/v	Weight in volume

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