ACTIVATION OF B CELL LOCOMOTION

IN VITRO

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LIST OF ABBREVIATION

^0C	Degrees centigrade.
a	Anti
AET	2-AminoEthylisoThiouronium.
Ag	Antigen.
BC	Buffy Coat.
BCGF	B-Cell Growth Factor.
BCRs	B-Cell Receptors.
BM	Bone Marrow.
BSA	Bovine Serum Albumin.
BSF-1	B cell Stimulatory Factor-1
CD	Cluster Determinant.
CFU	Colony Forming Units.
CTLA-4	Cytolytic T-Lymphocyte Association antigen-4.
EDTA	Ethylenediaminetetraacetic acid.
$F(ab`)_2$	Antigen Binding Fragment.
FACS	Fluorescence Activated Cell Sorter.
FCS	Foetal Calf Serum.
FDC	Follicular Dendritic Cell.
FITC	Fluorescein Isothiocynate.
FT	Fetal Liver.
g	Gram.

GET	Gelatine, EDTA, tris buffer.
GM-CSF	Granulocyte-Macrophage- Colony Stimulating Factor.
HBSS	Hanks` Balanced Salt Solution.
HEV	High Endothelial Venule.
Hrs	Hours.
HSA	Human Serum Albumin.
HSC	Haemopoietic Stem Cell.
ICAM	Intercellular Cell Adhesion Molecule.
IFN-	Interferons.
Ig	Immunoglobulin.
IL-	Interleukin.
l	Litre.
LFA	Lymphocyte Function-Associated Molecule.
LPS	Lipopolysaccharide.
M	Molar.
M-CSF	Macrophage- Colony Stimulating Factor.
mab	Monoclonal Antibody.
mg	Milligram.
mIg	Membrane Immunoglobulin.
min	Minute.
MIP	Macrophage Inflammatory Protein.
ml	Millilitre.

mm	Millimetre.
Mm	MilliMolar.
MNC	Mononuclear Cell.
MOPS	3-[N-Morpholino]Propane - Sulfonic acid.
NK	Natural Killer cell.
PAGE	Polyacrylamide Gel Electrophoresis.
PBS	Phosphate Buffered Saline.
PE	Phycoerythrin.
pH	Negative logarithm of hydrogen ion concentration.
PLSC	Pluripotent Lymphoid Stem Cell.
PMN	Polymorphonuclear cells.
PNA	Peanut Agglutinin.
RANTES	Regulated upon Activation, Normal T Expressed and presumably Secreted.
RPMI	Roswell Park Memorial Institute, medium number 1640.
SDS	Sodium Dodecyl Sulphate.
sIg	Surface Immunoglobulin.
SRBC	Sheep Red Blood Cell.
TBS	Tris Buffered Saline.
TCRs	T Cell Receptors
TdT	Terminal deoxynucleotidyl Transferase.
TEMED	N,N,N`,N`-Tetramethylenediamine.

TGF-	Transforming Growth Factor.
T_H	T Helper
TNF	Tumour Necrosis Factor.
Tris	Tris (hydroxymethyl) methylamine.
U	Unit.
V/V	Volume per Volume.
VCAM	Vascular Cell Adhesion Molecule.
VLA	Very Late antigens.
W/V	Weight per Volume.

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DECLARATION

The original work reported in this thesis was completed through my own efforts and has not been submitted to any other University. Where use has been made of material provided by others, this has been acknowledged. Part of this work has been published in the Journal of Immunology (1995), 155; 1110-1116.

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SUMMARY

This project looks at the signals that induce locomotion in resting B cell populations and in germinal centre B cells, both from human tonsil. Signals that induce locomotion in blood B cells compared with high-density tonsil B cells were also studied. Polarization studies of the response of cells from immunized mice to antigen are also included.

B cells were purified from tonsil by established procedures to yield a high-density fraction (resting cells out of cycle) and low-density (activated) fraction. Germinal centre cells were present in, and were purified from, the latter fraction. Cells from these fractions were assayed for locomotor activity.

Two methods were used to study the locomotor activity of B cells;

(1) Polarization assay. Measurement of shape-change from a spherical to a polarized shape on stimulation with an attractant. (2) Invasion of collagen gels. Lymphocytes overlaid on a collagen gel containing an attractant will migrate into the gel in larger numbers than into control gels.

Previous studies of T cells showed that the full development of the capacity for locomotion and chemotaxis in lymphocytes requires two stages. (a) Resting cells require to be cultured with a growth activator and move from G_0 into the G_1 phase of cell cycle. After overnight culture, a locomotor population of cells is obtained. (b) These cells are now capable of responses to chemoattractants and show immediate (<30 min) polarization and locomotion when incubated in their presence.

(1) High density B cells. These are small surface IgM+ and surface IgD+ cells which are not in cycle. When freshly purified from the tonsil, very few of these cells show locomotor capacities. The results presented here demonstrate that culture overnight in IL-4, aCD40 or IL-13 induces locomotor shape change in a high proportion of high-density B cells. The proportion of polarized cells increases slowly over a period of 24-48 hours suggesting that locomotor capacity is activated as the cells pass from G_0 to the G_1 phase of growth. Anti-IL-4 and anti-IL-13 inhibit the locomotion induced by their respective cytokines. IFN-γ inhibits the locomotion response induced by IL-4. Culture in combination of IL-4 and aCD40 stimulates polarization of more cells than culture in either alone. A combination of IL-4, aCD40, and aIgM stimulates polarization of still more cells (up to 60-70% of the population). Adding aIgM to cultures with aCD40, or with IL-4 does not increase the polarization significantly compared with either aCD40 or IL-4 alone.

In addition to study of the effect of locomotor activators on locomotion in overnight culture, the immediate (<30min.) effects of attractants on locomotion of the resting B cell fraction were studied, using either cells direct from the tonsil or cultured B cells. The polyclonal activators, aIgD and aIgM, were tested in short term assays (30 min. incubation) on freshly isolated cells and on cells cultured in IL-4. Both populations showed immediate polarization to anti-Ig, but cultured cells responded more strongly than cells direct from the tonsil. The optimum attractant concentration of anti-Ig was 100ng-1μg/ml. There was no response to

the appropriate isotype controls, mouse IgG2a and sheep Ig. Cells cultured in IL-4 also polarized in a short-term assay to aCD40 (100ng-1μg/ml) within 30 minutes but not to isotype control mouse IgG1. The results suggest that in contrast to IL-4 and IL-13, anti-CD40 acts not only as a locomotor activator but also as a chemoattractant. Cells direct from the tonsil showed no chemoattractant response to anti-CD40.

To measure locomotion itself, cells cultured in IL-4 were layered on top of collagen gels incorporating aIgM, aIgD, aCD40, and HBSS alone, and were allowed to invade for 18 hours. The number of cells invading gels incorporating any of the three stimuli was greater than that invading gels containing medium alone. FACS analysis on invaded cells show that 75 ± 12% of cells were IgM+ and 82% IgD+. The gel invasion assay selects the locomotor population and demonstrates clearly that small resting IgM+ and IgD+ B cells not only change-shape in response to anti-IgM and anti-IgD, but also show invasive locomotion in response to these antibodies.

To study the relation between locomotion and cell cycle, freshly isolated B cells and cells cultured in IL-4 were pulsed with uridine and thymidine. Autoradiography showed that there were very few cells heavily labelled (>10 grains per cell) with uridine before culture and this increased to about 40% after overnight culture in IL-4. Spherical (non-locomotor) cells were usually unlabelled or very lightly labelled, whereas the polarized cells were heavily labelled. Many such cells were seen after IL-4 culture, but even the few polarized cells before culture showed

heavy uridine labelling. There was no labelling with thymidine in these experiments. These findings suggest that the cell population activated for locomotion also contains the cells most active in RNA synthesis but not DNA synthesis.

(2) Germinal centre B cells. Studies on germinal centre B cells showed that like resting B cells, they could be activated to locomotion by 6-12 hours culture in IL-4 and aCD40. Cells in G_0 phase of growth responded poorly to attractants until they became activated and entered into the G_1 phase. Once again, autoradiography analysis shows that the cells which incorporated uridine were cells capable of polarization. Cells which incorporated thymidine were not polarized. Thus, proliferating cells (centroblasts) are not able to recognize attractants. Following culture of the germinal centre B cells in IL-4 and aCD40, the activated centrocytes were able to recognize aIg as an attractant. They responded to aIgA (1ng/ml) and aIgG (100ng/ml) at very low concentrations, whereas cells responding to aIgM required higher concentrations of antibody (1μg/ml). This suggests that cells that have switched isotype in germinal centres are much more sensitive to locomotor activation by anti-Ig, and therefore probably by antigen, than non-switched cells. In overnight polarization, aCD40 induced polarization better than IL-4 and the former also increased the viability slightly, but combination of IL-4 and aCD40 not only rescued the germinal centre B cells but also induced a significant polarization response.

(3) Blood B cells. Polarization studies on blood B cells showed similar responses to IL-4, IL-13, and aCD40 in overnight culture, but fewer cells responded, compared with the high-density B cells. Cells cultured overnight in IL-4 responded to anti-Ig in 30 minute assays, but freshly isolated cells did not.

(4) Mouse B cells. These studies were carried out to investigate the locomotor response of mouse spleen or lymph node B cells to antigen. In one out of three experiments, the immunized mice responded to the administered antigen (ovalbumin) significantly compared with non-immunized mice, but the preliminary experiments were not impressive and further work is required.

CHAPTER ONE

INTRODUCTION

and

LITERATURE REVIEW

INTRODUCTION

Immunity is a term that applies to all mechanisms which protect the body against environmental agents that are foreign to the body, and leukocytes play the vital role in this protection. The functions of leukocytes in response against micro-organisms which have invaded differ, depending on the cell type. Leukocytes as a whole can be divided into two major groups- myeloid cells, and lymphoid cells. Cells of the myeloid system, chiefly, neutrophils and macrophages (the tissue-based form of monocytes) eliminate foreign materials that have passed through the physical and chemical barriers, by phagocytosis. These cells exist in all individuals from birth and are maintained throughout life. The second major group of leukocytes are the lymphocytes; T-lymphocytes, natural killer cells, and B-lymphocytes. These mediate acquired immunity, and their interactions by cell-cell contact and lymphokine production cause the humoral and cell-mediated immune responses.

Lymphocytes are the key cells of the immune system and millions of them are produced daily from the maturation and multiplication of stem cell and lineage-restricted progenitor cells of the bone marrow. B lymphocytes represent one arm of the acquired immune response in vertebrates. In the presence of antigen, the appropriate clones of cells are activated, and induced to proliferate and to mature into antibody secreting plasma cells. This process of differentiation controls the production of antibody of a particular class and isotype; the generation of memory cells and self/non-self discrimination. The production of long lived memory cells

depends on the nature of the antigens. In addition to production of Ig, these cells can also act as antigen presenting cells (APCs), and can secrete cytokines such as IL-6, etc.

The humoral immune response can be either T-cell independent, such as those directed against bacterial surface polysaccharides, in which the differentiation process to plasma cells is independent of T-cell help, or T-cell dependent , for example the activation of B-cells by antigens such as proteins. This induces a much more complex sequence of reactions which require the help of T-cells for differentiation of the B-cell to a plasma cell or memory B-cell to occur (Parker, 1993). Furthermore, it seems that memory cells are not established in the B-cell independent response.

The aim of this thesis is to study the locomotor properties of human B cells;

(1) To define locomotor properties of different populations of B cells purified from human tonsils, namely-

(a) High-density B cells direct from tonsil. This is the resting cell population.

(b) High-density B cells after activation by a period of culture in vitro, e.g. in the presence of IL-4, anti-CD40 or other activating agents.

(c) Cells activated in vivo, both germinal centre B cells and those with non-germinal centre phenotype, e.g. IgD+ cells isolated from the low-density fraction.

(2) To define agents which control cell locomotion;

(a) Activating agents that change the B cell population from a non-locomotor to a locomotor phenotype. This requires a period of culture in vitro.

(b) Chemoattractants for B cells, acting either on the resting population or on cells following a period of culture with an activating agent.

(3) It is hoped that these in vitro experiments on B cell locomotion will help to understand B cell migration in vivo and to define a possible role for antigen in stimulating B cell locomotion. They may help to understand homing of B cells to sites of antigen or to accessory cells or FDCs. It will be shown that activation of locomotion in B cells is related to activation of their function judged by other parameters. To provide a background to this study, an outline of B cell development and of the factors that influence it is given below.

SECTION ONE

1-1 ANTIGEN-INDEPENDENT PROLIFERATION OF B-CELLS

Proliferation of B-cells in humans occurs in two steps: The first step, the development of stem cells into mature IgM+ IgD+ B-cells is independent of antigens and occurs primarily in the bone marrow and/ or fetal liver. It is characterised by an orderly cascade of rearrangements of immunoglobulin variable region genes that encode antibody specificity and polyclonal proliferation. B cell development is the ordered progression of a stem cell through a number of stages, such as single productive immunoglobulin (Ig) gene rearrangement at the heavy and one of the two light chain loci, expression of a number of cell surface markers which can be used to define discrete stages along the pathway, finally resulting in a mature B cell. The second step, from the

mature resting cell to plasma cells and memory B-cells, is antigen dependent and occurs mostly in secondary lymphoid organs such as lymph nodes, tonsils, spleen, and Peyer`s patches.

1-1-1 HAEMATOPOIESIS

Haematopoiesis, the production of blood cells, begins in the yolk sac of the human embryo from the 14^{th} -19^{th} day of gestation. Lymphopoiesis has not been observed in the yolk sac, but is present in lymph plexuses at 9 weeks, and in the lymph glands at 11 weeks, of gestation. Circulating lymphocytes have been seen in 9 week old embryos (Gilmor, 1942). The fetal liver becomes the main site of haemopoiesis in the second trimester of pregnancy. Lymphocyte subpopulations may be detected after 13 weeks of gestation. Finally the bone marrow plays the main role in haemopoiesis in the healthy adult individual. Haematopoietic stem cells (HSC) in fetal liver (FL) seem to differ from bone marrow (BM) HSCs. For example, molecular markers such as terminal deoxynucleotidyl transferase (TdT) are expressed on BM HSCs, but not on FL HSCs. Also in mouse B-cell development, FL cells are better able to give rise to B-1a cells (formerly called CD5+ B-cells) than adult BM cells (Kantor et al, 1992). Haematopoietic stem cells (HSC) of the mouse as well as the human (Till and McCulloch in 1961) express very low levels or no surface markers for committed blood cell lineage such as: TER119 (an erythroid cell marker); B220 (a B-cell marker); Mac-1 (a monocyte marker); Gr-1(a granulocyte marker); and CD3, CD4 or CD8 (T-cell markers).

These cells may be functionally heterogeneous in vivo as a result of exposure to different stimuli, cytokines and the microenvironment in which they reside. It is not clear precisely what is responsible for heterogeneity, but intrinsic changes in cells as a result of their passage through changing microenvironments may help to explain this. However, responses by cell surface receptors to cytokines or other ligands may also be important. HSCs are characterised by their capacity for self renewal and their ability to generate differentiated daughter cells of all haematopoietic linage.

Uchida, et al (1993) in their haemopoiesis model, suggest four stages of development of all blood cells: (1) quiescent HSC; (2) expanding HSC; (3) multipotent progeny; and (4) maturing blood cells. In this model, HSCs are subdivided into functional subsets by the microenvironments in which they reside. The quiescent HSCs are sessile and located in the "self-renewing stroma" (S) niche which occupies a limited space. When quiescent HSCs are activated into cell division, daughter cells may find themselves either within the same microenvironment, or in an adjacent environment-the expansion stroma (E). An expansion of cell numbers by division occurs there. Stem cells may divide either symmetrically to form two new stem cells, or asymmetrically to form one differentiating cell and one further stem cell. With each cell division, progenies are located at a greater and greater distance from the S niche and enter other microenviroments which promote lineage-committed progenitors to proliferate and differentiate. Thus proliferation in the expansion stroma (E) continues, daughter cells enter the next niche, the maturation stroma, (M) niche, to

differentiate further. In adult life, primary stem cells are probably situated in the bone marrow, which is the main site of blood formation. The mechanisms controlling these processes in the bone marrow are still obscure. The morphological appearance of HSC is not defined but the existence of such a cell was demonstrated functionally in mice by Till and McCulloch in 1961.

T-cells and B-cells originate from a common lymphoid precursor cell which has differentiated along different developmental lines. One line matures in the thymus (T-cells), the other matures in the bone marrow in mammals (B-cells). The sites which are responsible for B-lymphocyte production are strikingly different among species. For example, in the chicken, B-lymphocytes differentiate from pluripotent stem cells only during embryonic life, and, thereafter a specific organ called the Bursa of Fabricius becomes the site of B-lymphocyte production, while in the mouse as well as human the bone marrow generates the haematopoietic stem cells continuously. In human being, like the mouse, colony forming units or CFU, have been identified and characterised which produce small colonies of one or more cell types in semi-solid media containing appropriate haematopoietic stem cells. The pluripotent lymphoid stem cells (PLSC) go through various stages of differentiation and give rise to lymphocyte progenitor cells that may eventually mature into all types of T, B, and non-T, non-B lymphocytes. Unlike the stem cells, the lymphoid progenitor cells have only a limited capacity for self-renewal. B-lymphopoiesis in the mouse and in human can be divided into two phases. In the first phase, the B-cell lineage

arises from very few (0.05%) of the pluripotent HSCs, during embryonic and neonatal life. Fetal omentum, liver, and bone marrow are the primary site of B-cell development and are the first sites which the B-cell lineage can be found. The second (neonatal) phase of B-cell generation mainly occurs in the bone marrow, while the neonatal spleen is also important in B lymphopoiesis (Solvason & Kearney, 1992; Kantor et al, 1992; MacLennen and Chan, 1993). The differentiation of stem cells to pre-B cells, B lymphocytes and, finally, plasma cells and memory cells proceeds through multiple stages which can be identified by sequential immunoglobulin gene rearrangement, and the acquisition or loss of various B-lineage differentiation antigens. The sequence of B-cell differentiation in the bone marrow may be summarised as follows:

HSC ---->PLSC ----> pro B-cells ---->Early pre B-cells ---> late pre B-cells---> Immature B-cells ----> Mature B-cells

1-1-2 Phenotype of bone marrow cells during differentiation

In humans, analysis of B-cells differentiation has benefited from the phenotypic characteristics of leukaemia cells which represent the various stages of the developmental pathway as well as B-cell precursors which were obtained from normal adult and fetal bone marrow. According to studies of Pontvert-Delucq (1993), the B-cell pathway in adult bone marrow can be divided into two subpopulations ; CD34+ and CD34- cells. CD34 is the earliest marker in the human for precursors of colony forming cells in the bone marrow and it is a marker

for HSCs. CD34+ cells can be subdivided into three different groups: (1) CD34+ CD10- CD19- cells which are a heterogeneous population, and contain myeloid colonies; (2) CD34+ CD10+ CD19- cells that give rise to macrophage colonies. This population mainly expresses CD33. This finding suggests that CD10 might be present on myeloid progenitor cells, and suggests the existence of a common progenitor to the macrophagic and lymphoid lineage in human bone marrow; and (3) CD34+ CD10+ CD19+ cells which represent B-lymphoid committed progenitors. These cells were shown to have a blast morphology as do other CD34+ cells but were smaller in size and were more homogeneous. It was shown that CD10 was expressed before CD19 since no CD34+ CD10- CD19+ population was evident. CD10 is expressed on early B cells, B blasts, macrophages, some granulocytes, bone marrow stromal cells and various epithelial cells. It is a type II integral membrane protein. It can be supposed that some of CD34+ are committed to the myeloid lineage and others represent a common progenitor to the macrophages and lymphoid lineage. The latter cells still have the ability to generate CD34+ CD10+ macrophage and might coexpress either CD33 or T and B associated markers including CD7, CD19, and TdT. However 2% of CD34+ CD10- CD19- cells were also TdT+. These results suggest that TdT is either expressed before the acquisition of CD19 and CD10 antigens, or on CD34+ cells that probably include progenitor cells for both T and B lineages. Finally CD34+ CD10+ CD19+ cells which have an exclusively specific characteristic differentiation into CD34- CD10+ CD19+ cμ+ pre B cells. Then cells by losing the CD10 marker differentiate to

immature B cells (sIgM+, sIgD-) and finally become mature (sIgM+, sIgD+) and flow into the circulating system.

1-1-3 MATURATION OF THE B-CELL ANTIGEN RECEPTORS

B-cell antigen receptors (BCRs), are integral membrane proteins. In developing B-cells, gene rearrangements follow a stringent order. The heavy and light immunoglobulin chains are encoded at least by three (V,D,J) and two (V,J) separate germline DNA elements, respectively. The assembly of a functional IgM heavy chain gene starts with the rearrangement of a single DH (diversity) region gene segment with a single JH (joining) region gene segment. The cells carrying only D-J rearrangements are referred to as early pre-B cells. The next stage of the B-cell differentiation pathway involves the rearrangement of the several hundred V (variable) region genes. These become juxtaposed to the D-J elements to give rise to pre-B cells which express IgM H-chain on the first allele and in the case of non-productive (VDJ-) rearrangements, on the second allele. Even more the B-cells or cell line which had generated two non-productive rearrangements are not able to express K light chain (probably 2/3 of VDJ H are non-productive; Yancopoulos & Alt, 1986). Since pre-B cells express μ protein but not Ig light-chain, it became clear that the expression of the two Ig chains is not synchronous. It was also demonstrated that the expression of μ protein is necessary for induction of light-chain (Reth, et al, 1985). According to several investigations (Reth, et al, 1985, Jongstra and Misener, 1993), it appears that the

expression of μ protein and its associated pseudo light chains (Lambda 5) and V-pre B are necessary for pre-B cells to develop into the mature mIg+ B-cells.

1-1-4 REGULATION OF B LYMPHOPOIESIS

The models of haemopoiesis in vitro derive from the development of long term bone marrow culture techniques (Dexter, et al, 1977) which established conditions for maintenance of multipotential stem cells and granulopoiesis. Subsequently, these techniques were adapted for selective growth of B lymphocytes (Whitlock and Witte, 1982). In such culture systems, pre-B cells grow on a complex layer of adherent cells composed of macrophages, endothelial cells, fat cells, fibroblasts, and various cell types collectively designated as `stromal cells'. These cells have been shown to produce many different cytokines including M-CSF, GM-CSF, TGF ß, IL-4, IL-6, and IL-7 (Kincade et al, 1989). Control of B lymphopoiesis by cytokines is not completely understood. Some may have direct and some others indirect effects. For example, using various soluble growth factors to induce proliferation of B cell precursors, some studies demonstrated that IL-3 was a growth factor for the earliest B cell progenitors (Palacios and Garland, 1984), but this study was inconsistent with others indicating that the IL-3 receptor was restricted to non-lymphoid cells (Ihle et al, 1983) and only a small proportion of cells could be differentiated to mature B cells. In humans, IL-3 could induce short-term proliferation of some CD10+ progenitor B cells from foetal liver (Uckun and Ledbetter, 1988). However, the precise role of IL-3 in B

lymphopoiesis is presently unclear and its effect may be indirect. IL-7 plays a major role in B lymphopoiesis. The early studies showed that this cytokine served as a growth factor for early lymphoid cells of both B- and T-cell lineage and because of its lack of activity towards myeloid cells, suggested that there may be a lymphoid stem cell under the regulatory control of IL-7. IL-7 caused proliferation of both pre-B and pro-B cells, but not mature B cells (Henney, 1989). The functionally active IL-7 produced by bone marrow adherent cell layers was inhibited using neutralising anti IL-7 antibody in vitro and vivo (Ryan et al, 1994; Grabstein et al, 1993). IL-4 inhibited proliferation of fetal CD10+ B cell precursors cultured without a supportive adherent layer (Pandrau et al, 1992), but stimulated pro-B cell proliferation in mouse culture systems containing a supportive adherent layer (Peschel et al, 1989). King et al (1988) reported that stromal cell supernatants induced maturation of mouse pre-B cells into sIgM+ B cells, and this activity was blocked by an anti-IL-4 antibody. The blocking effect of anti IL-4 suggested that IL-4 could be involved in the maturation process. Taken together, these results suggest that IL-4 induces the differentiation of late pro-B cells into early pre-B cells or late pre-B cells into mature IgM+ B cells. This effect may be indirect by inducing a proliferation factor by stromal cells which in turn induces proliferation of progenitor B cells (Peschel et al, 1989). From these studies, it may concluded that (1) a single cytokine is not enough for full development, but a combination of cytokines is essential, (2) individual cytokines

may have different functional effects on lymphopoiesis, (3) some cytokines may have an indirect effect by causing stromal cells to release soluble factors.

SECTION TWO

1-2 ANTIGEN-DEPENDENT PROLIFERATION

Millions of newly formed lymphocytes leave the bone marrow daily. Many of these cells (B-cells) immigrate in a short time days after production, passing by way of the blood to the spleen and after a further 1-2 days to the lymph nodes (Osmond, 1986). The microenvironment of secondary lymphoid organs plays an essential role in the regulation of B lymphocytes by antigen. The traffic and regulation of lymphocytes are controlled in part by the selective interaction of circulating lymphocytes with specialised high endothelial venule (HEV) cells at sites of lymphocyte exit from the blood (Jalkanen et al, 1986). Migration and homing receptors are important not only allowing the full repertoire of clonal lymphocyte specificity to be available to respond to antigen but also probably to facilitate the interactions between lymphocyte subsets and accessory cells that are required for effective expansion and regulation of antigen-specific immune responses. Most B cells found in secondary lymphoid organs live longer than a week. It has been shown that the bone marrow of adult mice produces about $5x10^7$ B cells per day (Opstelten & Osmond, 1983). It follows that a high proportion of these newly formed B cells have a very short life-span, those which are not selected within a finite period die and formation of new cells balances the

loss of old ones (MacLennan and Gray, 1986). Specific recognition of foreign antigen by cell-surface immunoglobulin (Ig) induces B cells to proliferate and differentiate either into plasma cells, which produce soluble immunoglobulin to fight infection, or into memory B cells which can respond rapidly to subsequent encounters with the same antigen. Maturation of B-cells into the antibody-secreting cells is a complex process that requires antigen and the collaboration between B-cells, T-cells and antigen presenting cells which can themselves be B cells. Once T cells are activated, they in turn promote B cell activation by releasing T cell-derived cytokines such as IL-2, -4, -5 or by direct intercellular contact (Clark andLedbetter, 1994; Linsley, 1991; Banchereau, 1994).

1-2-1 Collaboration of Cells in Immune Response

Cellular collaboration is an important feature of the immune system and it involves the recognition of cell surface-associated molecules on one cell by specific receptors on another. Two well-characterised cellular interactions are those occurring between T_H cells,/antigen presenting cells (APCs) and T_H cells/B cells. These cellular interactions regulate cell activation, differentiation, and suppression.

Cognate interactions between antigen-presenting cells (APCs) and T cells play crucial roles in immunological responses. It is well known that T cells become activated only after recognising degraded fragments of native protein antigen (Allen, 1987). Professional antigen-presenting cells such as dendritic cells and

macrophages are potent presenters of antigen to both virgin and memory T cells (Inaba and Steinman, 1984; Inaba and Steinman, 1985; Larsen et al, 1992). B cells also play an important role in antigen presentation. However, previous studies indicated that small resting B cells are poor APCs for primary responses, but may be effective after the responding T cell population has been activated (Inaba and Steinman, 1984; Inaba and Steinman, 1985). Thus, the presentation capacity of B cells has been controversial. Whereas some studies indicate that B cells (Abbas et al, 1985; Chesnut & Grey, 1981), especially activated B cells can present antigen to activated T cells and some fresh T cells (Larsen et al, 1992; Liu and Janeway, 1991; Kakiuchi et al, 1983), others have reported situations in which B cells were unable to activate T cells (Inaba and Steinman, 1984; Lassila et al, 1988). Recent studies have shown that activation and differentiation of both T_H and B lymphocytes are dependent upon direct intracellular interactions between these cell types. The signals required for a T_H cell antigenic response are usually provided by APCs. The first signal is initiated by interaction of the T cell antigen receptor (TCR) complex with antigen presented as a processed peptide in the context of class II MHC molecules on the APCs (Allen, 1987). Recognition of antigen by T cell antigen receptor (TCR) is not only insufficient for activation but also may lead to clonal inactivation or anergy (Schwartz, 1990). The molecular nature of these second signals is not fully understood, although both soluble molecules, such as IL-1 (Mizel and Ben-zvi, 1980) and membrane-bound adhesion receptors can provide costimulatory signals in some systems.

Costimulatory receptors on the T cells and their counter-receptors on the APCs involved in B-T_H cell interactions include; intracellular adhesion molecule 1 (ICAM-1) (CD54) and leukocyte function associated antigen-1 (LFA-1) (CD11a/CD18), LFA-3 (CD58) and CD2, very late antigen 4 (VLA-4) (CD_W49d) and vascular cell adhesion molecule 1 (VCAM-1), the B7/BB1 and CD28 family, and CD40-CD40L (Damle et al, 1992; Noelle et al, 1992a). As yet, it is not clear whether these molecules perform the equivalent functions or are expressed simultaneously on the surface of APCs.

1-2-1-1 CD40 and CD40 Ligand

CD40 is a type I integral membrane glycoprotein which belongs to a cysteine-rich receptor family which includes nerve growth factor (NGF)-R, TNF-R, and Fas antigen. This mitogenic surface molecule was originally identified in B cells, some malignant cells and carcinoma cell lines (Clark and Ledbetter, 1986). CD40 has also been detected in other cell types including FDCs, dendritic cells, interdigitating cells in T cell zones of secondary lymphoid organs , and thymic epithelial cells (Callard et al, 1993; Noelle et al, 1992b). The ligand for CD40, gp39, was found on CD4+ T cells, with an approximately equal distribution between CD45RA+ and CD45RO+ T cells. There was low expression of CD40L on CD8+ cells. CD40 ligand is a type II membrane protein which has significant sequence homology with tumour necrosis factor α and β (TNF $\alpha.\beta$) (Callard et al, 1993; Noelle et al, 1992b). Signals via the TCR play a primary role in the

upregulation of CD40L on T cells, since antibodies that block the contribution of CD4, LFA-1, and MHC class II completely block CD40L expression (Durie et al, 1994). The expression of CD40L on activated T cells seems to be tightly regulated. B cells during T/B interactions also down regulate the expression of CD40L mRNA expression by releasing soluble CD40 which binds to surface CD40L. However, it is not clear that other cells expressing this marker have such an activity (Van Kooten et al, 1994). Studies with mAb to CD40 or CD40L have shown that CD40 plays a critical role in activation and proliferation of B cells, including IL-6 secretion (Clark and Shu, 1990) and rescue of GC B cells from apoptosis after somatic mutation in germinal centres (Liu et al, 1989). Anti CD40 or activated T cells caused an increase in expression of B7/BB1 on B cells which was three times greater than the maximum expression of this surface antigen induced by crosslinking sIgM or HLA-DR (Ranheim and Kipps, 1993). Anti-CD40 also enhanced the expression of CD54, and induced the activity of adhesion molecules such as LFA-1 (Barrett et al, 1991). B cells cultured with mAb to CD40 alone produce marginal levels of Ig, and B cell proliferation, suggesting that anti CD40 could not provide an activation signal for the B cells strongly by itself but it could costimulate with other activating stimuli (Gordon et al, 1988; Valle et al, 1989). CD40 on B cells is of critical importance for Ig heavy chain switching in culture with IL-4, *Staphylococcus aureus* (SA), IL-10 or transforming growth factor β(TGF-β) (Splawski et al, 1993; Defrance et al, 1992). Thus the combination of anti CD40 and cytokine and SA or aIg provides both

cognate and non-cognate stimulatory signals, which can substitute for T cell help to B cells. The recent study by Foy et al (1993) demonstrated that administration of anti gp39 reduced the primary as well as secondary response to exogenous TD antigens, but not T-independent (TI)-Type II antigen, in vivo. Therefore, it appears that the interaction of gp39 and CD40 are critical for the thymus-dependent response of B cells. The importance of CD40-CD40 ligand interactions in the generation of Ig secretion is highlighted by studies of patients with X-Linked hyper-IgM syndrome. This is a rare immunodeficiency disorder characterized by normal or elevated serum concentrations of polyclonal IgM and markedly decreased concentrations of IgA, IgE, and IgG with an absence of germinal centres (Conley, 1992). These patients show extremely low or no expression of CD40 ligand. Thus these is a failure of activation of B cells by CD40 ligand-bearing T helper cells which results in an inability to switch from IgM/IgD+ cells to other Ig isotypes and/or failure to rescue switched B cells from apoptosis after affinity maturation in the germinal centre (Callard et al, 1993). Gray et al (1994) in extensive experiments demonstrated that blocking of CD40-CD40 ligand interaction by daily injection of soluble CD40 fusion protein (sCD40-γ1) had a profound effect not only on quantity but also on quality of the primary antibody response. Finally, they proposed two pathways of immune response, one CD40-dependent and one CD40-independent.

1-2-1-2 Interactions Between the CD28 and the CD80 Receptor Families

Two members of the CD28 gene family, CD28 and CTLA-4 (cytolytic T-lymphocyte associated antigen-4), have been described to date. The majority of human and mouse resting T-cells express CD28, whereas expression of CTLA-4 depends on costimulation of CD28 and anti-CD3 or mitogens (June et al, 1994). CD80 family receptors, previously termed B7, are generally limited to professional APCs such as macrophages, dendritic cells, and activated B cells (Larsen et al, 1992). Resting B cells do not express B7, but the expression of this marker is enhanced by crosslinking of HLA-DR antigens within 6 hours (Koulova et al, 1991). Cytokines such as IL-2 and IL-4 also enhance the induction of B7 expression on mitogen stimulated B cells (Vallé et al 1991). Lack of expression of some counter-receptors such as CD54 and B7 on resting B cells may explain why these cells are not able to present antigen to T cells. Activated human B lymphocytes express at least three distinct B7 antigens termed B7-1, B7-2, and B7-3 whose expression depends on activation time (Freeman et al, 1993; June et al, 1994). Many studies show that B7 and related molecules are counter-receptors for activation of T cells through CD28 and CTLA-4. Interaction between the CD80 family and the CD28 family allows T cells to respond to activated B cells by producing cytokines required for T cell differentiation. The hallmark of B7 mediated signal tranduction is the production of IL-2. This function was abolished using mAbs to both CD28 and B7 (June et al, 1994; Linsley et al, 1991).

A reciprocal interaction can ensue as follows. Activated T cells expressing CD40L induce resting B cells to express CD80 and activated B cells expressing CD80 induce T cells to express CD40L. Thus, such stimulated B cells may engage non activated T cells specific for an antigen (s) presented by such cells. This, in turn, may induce CD40 ligand expression on these previously non-activated antigen-specific T-cells, allowing for an amplification of both T and B cells in the immune response. Collectively, these surface phenotype studies show that activated T cells not only help B cells to differentiate into Ig secreting cells, but also may induce B cells to become stimulatory APCs of other resting T cells. Finally, this cellular interaction leads T cell to proliferate, to produce cytokine for isotype switching and in turn to cause B cells to proliferate and switch isotype (Clark & Ledbetter, 1994; Ranheim and Kipps, 1993; Linsley, 1991; Banchereau, 1994).

1-2-2 B CELLS IN LYMPHOID TISSUE

1-2-2-1 Primary Follicles

B cell follicles are found in secondary lymphoid organs throughout the body, including the spleen, lymph node, Peyer`s patches, the appendix, tonsils, and other mucosa-associated lymphoid tissues. Follicles are always in contact with T-dependent areas which are meeting places at which antigens, T cells, and dendritic cells encounter and interact. B cells are probably activated in the T cell areas and then migrate to the centres (van den Eertwegh, 1993). The structure of primary

follicles, those in which no antigen-driven processes are taking place, are relatively simple. Their chief cellular components are a network of follicular dendritic cells (FDCs), which are dispersed towards the periphery of the follicle, and the recirculating surface immunoglobulin + (sIgM+, and sIgD+) small cells (Gray et al, 1982). The recirculating B cells spend a transit time in the primary follicles unless exposed to antigens (Howard et al, 1972). Besides these cells a small number of CD4+ cells and some macrophages are also found in the primary follicles (Namikawa et al, 1986; Johnson et al, 1986). Primary follicles are present in lymph nodes as early as the second trimester of human foetal life (Namikawa et al, 1986).

1-2-2-2 The Resident Cells of The Germinal Centres

The structural and cellular composition of the GC reflects its function, which is Ag-driven B-cell proliferation and differentiation. Secondary follicles, unlike primary follicles, are mainly populated with activated B-cells expressing different phenotypes and morphological characteristics such as clefted or non-clefted nuclei, division and maturation, etc. The GC B-cells bind peanut agglutinin strongly (PNA) (Coico et al, 1983). These cells bear CD45, CD20, CD21, CD38, CD77 (de-los-Toyos et al, 1989; Liu, et al, 1992; MacLennen, 1994; Holder et al, 1991 & 1993a). They express low or absent levels of CD44 (de-los-Toyos et al, 1989) and CD39 (Holder et al, 1993a). The FDCs represent the main non-lymphoid resident cell in germinal centres and can be distinguished from other

accessory cells by a characteristic set of cell surface markers such as complement receptors and adhesion molecules. Antigen-antibody complexes seems to be important for development of secondary immune responses (Kunkl and Klaus, 1981). The trapping of immune complexes is mediated through the Fc receptor; accordingly several types of Fc receptors are present on FDC, such as the low affinity IgE receptor (CD23), type III IgG receptor (CD16), type II IgG receptor (CD32). Complement factors such as C1q, C3(C3b, C3bi, C3d) are also present and may contribute to surface retention of immune complexes (Petrasch et al, 1990; Maeda et al, 1988). FDC also express several adhesion molecules including CD54 (ICAM-1), and members of the β_1 integrin family; VLA-3, VLA-4, VLA-5, and VLA-6, as well as a cell surface molecule termed INCAM-110 (Schriever et al, 1989; Petrasch et al, 1990) and these molecules have been shown to be involved in the interaction between FDC and activated B lymphocytes (Freedman et al, 1990). DRC-1 (R4/23) and KiM4 are human FDC-specific molecules. However these markers exist in B-cells and macrophages (Heinen and Bosseloir, 1994). Expression of Ia antigen on the surface of FDC is still controversial (Humphrey et al, 1984; Petrasch et al, 1990).

The origin of FDC is not fully understood and is still controversial. As yet, there is no totally convincing answer to this question. Some believe that FDC originate from a heamatopoietic cell lineage because of the surface phenotype of these cells. It was shown that FDC express antigens which are common to macrophages as well as B-cells, but lacked T and NK cell antigen (Schriever et al,

1989; Petrasch et al, 1990). In contrast to the haematopoietic theory for FDC formation, there is some strong evidence that these cells originate from mesenchymal cells. As yet, there is no evidence that FDC cells are able to divide and it is believed that a subpopulation of myofibroblasts forms a stromal pool for generation of FDC during the formation of germinal centre cells, keeping in mind that fibroblasts are able to retain immune complexes (Heinen and Bosseloir, 1994; Humphrey et al, 1984, Dijikstra et al, 1984).

There are not many T cells in the germinal centre (≅ 1-5%); these cells are mostly located in the surrounding mantle zone of the follicle at the edge of the germinal centre. Most of them are helper cells, specially CD4+ CD45RO+ (memory phenotype). The necessity for T cells is illustrated by observation that GC are virtually absent and can not be induced in athymic "nude" mice and rats (Klaus and Kunkl, 1982). Furthermore, T cell-independent antigens lead only to minimal GC development. T cells could possibly interact with a subpopulation of GC B-cells, and then provide the impetus for proliferation of B-cells by physical contact and secretion of cytokines (Poppema et al, 1981). Two mechanisms might explain this localisation; (1) recruitment of cells which have already migrated through this area; for instance, memory T cells, may be increased in frequency in the afferent lymph (Mackay et al 1990). (2) Cytokines such as RANTES which has been reported to attract memory cells selectively, might explain the migration of T cells through the paracortex. (Schall et al, 1990). Macrophages are among the cells having access to the GC; tingible body macrophages are mainly cited (Kotani

et al, 1977). CD5+ B cells, IgD+ B cells, interdigitating or dendritic cells, mast cells, polymorphonuclears are rare or absent in germinal centre. Thus the germinal centre, like the thymus, appears to be a closed environment. The entry into the centres is thus restricted to cell types expressing obligate phenotypes. Molecules reach the germinal centres in two ways. Either they are carried by cells, or by the intercellular fluids from the subcapsular sinus. However, the capillaries are developed inside the centres. Soluble substances, except for those filtered through the vascular endothelium, do not have easy access to the germinal centres. For example, primary injected non-antigenic (colloidal carbon or gold) or antigenic material (horse-radish peroxidase, bovine albumin) are not found in the centres, whereas they are located in neighbouring macrophages (sinus, reticular tissue)which have taken up this material (Heinen et al, 1983; Kamperdijk et al, 1987). It was reported that lymphoid cells (mainly B cells) fix immune complexes via Fc receptors, migrate towards the centres and establish contact with follicular dendritic cells and transfer the immune complexes to the latter (Heinen et al 1986; Braun et al 1987).

1-2-2-3 Germinal Centre Formation

Germinal centres form in lymphoid tissue after injection of an antigen, starting at day 4 and reaching peak development by day 10. Antigen re-injection on day 10 reactivates their development, but in the absence of a second antigen injection, or of adjuvant, the proliferation of germinal centres wanes by day 14. The

maximal development of GC is later than the peak of primary antibody production (Coico et al, 1983). It is proposed that the PNA+ GC cells in response to antigen quickly develop into PNA- B cells (memory B cells) which are able to respond to the priming antigen move strongly than virgin B cells (Coico et al, 1983; Baine and Thorbecke, 1982). Antigens appear to have multiple functions in the induction of GC and this was confirmed in germfree animals (Thorbecke et al, 1957). Once antigen is localised on the surface of FDC, it can stimulate B cells directly, particularly in the form of immune complexes together with C3 (Klaus and Humphrey, 1977; Kunkl and Klaus, 1981). C3 is required for retention of antigen-antibody-C3 complexes with no specificity by FDC. They bind immune complexes independently of the nature of the complexed antigen (Klaus and Humphrey, 1986). After a first antigen administration, a primary humoral immune response is initiated and immune complexes are formed as soon as antibodies appear in the circulation. Most of these antigen-antibody complexes are rapidly ingested and digested by cells of the mononuclear phagocyte system, but a small number are trapped on the surfaces and cell processes of follicular dendritic cells located in the follicles of lymph nodes and spleen (Humphrey and Frank, 1967; Van Rooijen, 1990). It is only in this form that antigen may be preserved in the body for long periods of time up to one year after the onset of antibody production (Donaldson et al, 1986; MacLennan, 1994). The antibody-complexed antigens in the follicles are not internalised and degraded, but remain immobilised on the cell surfaces of follicular dendritic cells (Chen et al, 1978). The

unprocessed form of antigen may be taken up from FDC by B cells, which can process this and re-present it to T cells (MacLennan, 1994). In the spleen, complexes move from sites of their initial localisation, e.g. in the marginal zone surrounding the follicles, toward the follicle centre (van Rooijen, 1973).

Resting B cells circulate in the blood, migrating across high endothelial venules to sites of trapped antigen in secondary lymphoid organs such as lymphoid nodes, spleen, tonsils, and Peyer`s patches. In the primary response, B cells bearing immunoglobulin specific for trapped antigen enter the T cell-rich paracortical regions below the outer layer, or cortex, of lymphoid tissues (Liu et al, 1992). Analysis of the site of B cell activation in TD antibody responses shows that B-cells proliferate both in the T cell-rich zones in association with interdigitating cells and in the follicles (Liu et al, 1991b). Interdigitating cells (extra-follicular dendritic cells) show high constitutive expression of class II MHC and are known to be highly efficient at processing and presenting antigen to T-helper cells. These cells are found in T zones of all secondary lymphoid tissue (Knight et al, 1982). MacLennan et al (1992) proposed a sequence of maturation of B cells in GC. They divided the sequence into three stages based on immunohisto-chemical studies of the follicular response to two hapten-protein conjugates in carrier-primed rats; (1) follicles are colonised by fewer than five primary B cell blasts which proliferate rapidly to fill the spaces in the FDC network, to total about 10^4 cells within three days (Liu et al 1991b). This can only be activated with a cell cycle time of about six hours. (2) By around day four after

antigenic challenge, the B cell blasts metamorphorse into centroblasts which express little or no surface immunoglobulin and express CD77. These cells migrate to one pole of the FDC network, forming the dark zone where the rapid B cell proliferation takes place. Centroblasts are in rapid cell cycle and give rise to centrocytes (maturing B cells) which do not increase in numbers and pass into the dense central part of the FDC network, forming the light zone of the germinal centre that contains abundant FDCs and some T cells. At this stage of differentiation, they re-express surface Ig, and interact with the FDC. This is the last judgement of B cells. Cells that interact with antigen expressed on the surface of the FDC are selected into the third stage of development. (3) At this stage, secondary follicular blasts are formed within the follicular network, leading to both memory cells and plasma cells. The light zone is divided into two compartments; the basal light zone near to proliferating centroblasts in the dark zone which lack CD23 and the apical light zone closer to the follicular mantle containing FDC which expresses large amounts of CD23. The FDC in both areas retain antigen as immune complexes. Centrocytes encounter antigen held on FDC and pass across the basal light zone to the apical light zone. Here, the subsequent signals differentiate them either toward the plasmacytoid pathway, depending on soluble CD23 and IL-1α signals, or with IL-2 (Holder at al, 1991), or to the memory B cell pathway, depending on ligation to surface CD40. Surviving memory cells emerging from the light zone reside in the follicular mantle. Cells that fail to interact with FDC and T cells die by apoptosis (Fig 1-2) and are

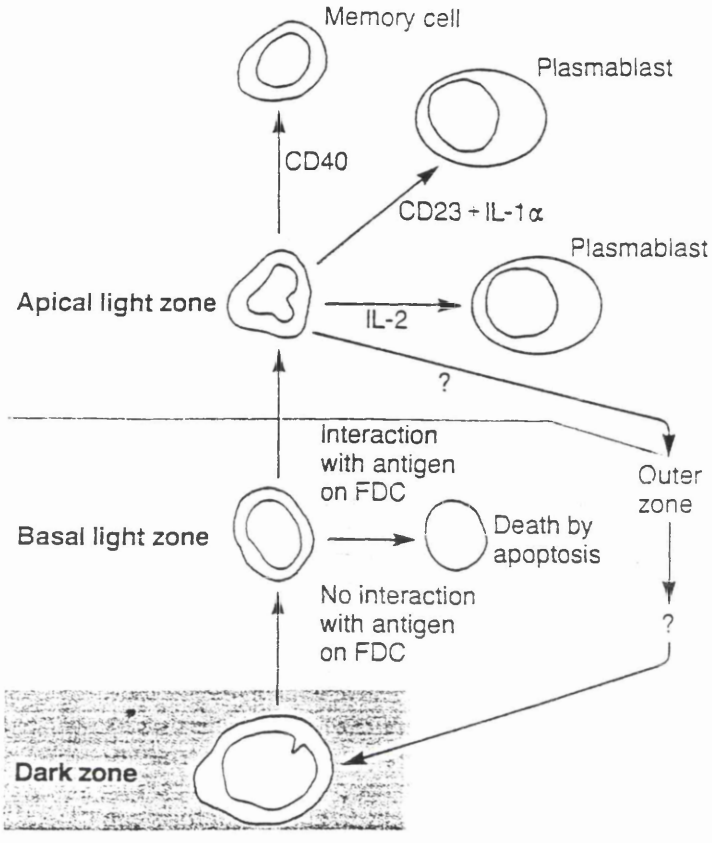
eliminated by macrophages (Liu at al, 1991b; Armitage et al, 1993; Lane et al, 1993). Another explanation, offered by Van Rooijen (1990), suggested that free antigen fixed in the form of immune complexes on the FDC acts synergistically to stimulate differentiation into plasma cells, whereas immune complexes alone would predominantly produce B memory clones. Several results show the vital role of the combination of anti CD40 and IL-4 for the expansion of the sIgD- B cells in the apical light zone of GC (Holder et al, 1991; Butch et al, 1992). It is interesting that GC T cells both express IL-4 and a ligand for CD40 (Butch et al, 1993; Butch at al, 1991; Holder et al, 1991; Liu et al, 1992). To address the phenotype of the precursors of memory cells, there is support for the idea that antigen-activated cells which turn off their IgD synthesis after activation are the precursors of memory cell generation and GC formation (Coico et al, 1988; Jacobson et al, 1981). GC formation as well as memory B-cell generation are T-dependent processes. Athymic mice lack GC, even after immunisation with T-independent antigens such as *Brucella abortus*, but are enabled to produce both GC and B2-cell memory to this antigen after reconstitution with T cells (Jacobson et al, 1974). The main function of the G.C may be to generate plasma cells destined to secrete Ig, as well as being the site of Ig heavy chain class switching, maturation of antibody affinity associated with V region somatic mutation, and the generation of memory B-cells, a series of events which make them able to response rapidly to re-challenge with the same antigen together with appearance of higher affinity antibodies of for example, IgG class (Butcher et al, 1982).

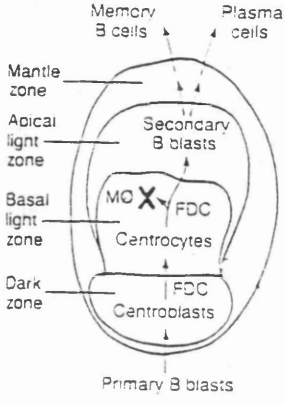
1-2-2-4 Apoptosis and Clonal Selection

All cells die. In general, cell death can be accidental or programmed in a multicellular organism. Two major forms of cell death can be distinguished. (1) Necrosis which is associated with major perturbations in the cellular environment as a result of complement attack, hyperthermia, etc. The plasma membrane may be the major site of damage, losing its ability to regulate osmotic pressure. (2) Apoptosis (programmed cell death), a morphologically distinct process of cell death that seems to be important in controlling immune responses. Dependence on specific survival provides a simple way to eliminate unwanted cells and select the fittest ones. The mechanism of these phenomena differs from one cell to another and still is not fully understood. Cell death is triggered by the appearance or loss of an external signal, leading to the activation of an internal cell death program. Moreover, the stimulus that initiates the apoptotic signal at one developmental stage may activate an entirely different response in the same cells at a different time. It follows that the same signal transduction pathway can be linked to a different effector system. Apoptosis is different from necrosis since it requires new gene expression. This has been demonstrated using inhibitors of RNA or protein synthesis (Raff, 1992; Schwartz & Osborne, 1993).

GC B cells like other mammalian cells undergo apoptosis (Maclennan & Gray, 1986). These cells may commit suicide because of antiself receptor expression, faulty gene rearrangement or lack of stimulation. In T-dependent immune responses, B cells seem to need signals from other cells in order to proliferate.

Figure 1-2 shows schematic and functional compartments in germinal centres.





Thus they need signals from other cells in order to survive, and in the absence of such a signal, the cells kill themselves or commit suicide. These signals may be provided by FDCs and T cells (Liu et al, 1991). In contrast to necrosis, the hallmark of apoptosis is the collapse of the nucleus by activation of an endonuclease which cuts DNA at different intervals. The morphological features of these cells can be identified using conventional and electron microscopy that demonstrate chromatin condensation with nuclear fragmentation (Liu at al 1989). A relation between loss of expression of Bcl-2, a protooncogene intimately linked to haematopoietic cell survival (Korsmeyer, 1992), and apoptosis has been reported (Raff, 1992; Liu et al, 1991a). In many tissues apoptotic cells are phagocytosed before they lyse. This might be related to changes in the expression of sugar residues upon the cell surface that are recognised by endogenous macrophage `lectins` (Duvall & Wyllie, 1986). There is a high death rate among centrocytes and centroblasts in vitro after culturing for few hours at 37^0C. This process can be delayed using immobilised polyvalent antibody to surface antigen receptors, or soluble mAb to the CD40 molecules, or a combination of both (Liu et al, 1989). Induction of Bcl-2 seems to be a secondary product to the survival signal, since the Bcl-2 induction appears after the second day of culture (Holder et al, 1993b), using a combination of recombinant soluble CD23 (sCD23) and interleukin-1α (Liu et al, 1991a). These observations are believed to reflect events that, in vivo, would allow for the selection of centrocytes which have undergone somatic mutation of Ig V-region genes to generate antigen receptor of high

affinity. Transforming growth factor-β (TGF-β) was found to inhibit sIg-mediated rescue of GC cells but had no effect on survival promoted through CD40. Both routes of rescue were blocked by the glucocorticoid, prednisolone (Holder at al, 1992). Recently it was reported that a subset of CD21 antibody (BU-33) promotes the survival of GC cells by up-regulating Bcl-2 expression (Bonnefoy et al, 1993). The latter report is consistent with the effect of sCD23 and IL-1α, since the CD23 can specifically interact with membrane-bound CD21 (Aubry et al, 1992). Finally, studies by Zupo et al (1994) demonstrate that a subset of anti-CD38 (IB4) can rescue GC cells from apoptosis by up-regulating Bcl-2 expression.

SECTION THREE

1-3 ACTIVATORS OF B-CELLS

In general, helper T cells provide stimulatory signals that induce B cell proliferation and differentiation into Ig producing cells. These stimulatory signals are transmitted through cognate interactions with direct physical contact between B and T cells by means of accessory molecules which are known to play a pivotal role in initiating B cell activation during antibody responses to T-dependent antigens. The bioactivity expressed on activated T helper cells is now believed to be largely due to the ligand for CD40, interacting with CD40 on the surface of B cells (Noelle et al, 1992a). The mAbs to CD40 have been shown to mediate direct activation of resting human B cells (Gordon et al, 1988). These signals act

in synergy with those delivered via cytokines such as IL-4 and IL-5 (noncognate mechanism) produced by T helper cells. Both cognate and noncognate stimuli most probably are necessary to activate and differentiate resting B cells into terminal Ig secreting cells (Noelle et al, 1990; Van den Eertwegh et al, 1993; Parker, 1993). In vivo, the activation of native B cells is initiated by antigens which are important for heavy chain switching in the germinal centres. However, the accessory molecules then play their role in the ongoing process. Following activation of IgD+ and IgD- cells in vitro by anti CD40 and IL-4, the latter, but not the former cells, proliferate but do not exhibit GC features and keep their sIgD profile (Galibert et al, 1994). Wheeler et al (1993) proposed two models of activation; (1) A processed antigen on B cells is recognized by a T helper cell which in turn activates B cells through ligation of CD40 and CD40 ligand, (2) Interaction of interdigitating cells presents antigens to the sIg of native B cells and possessed antigens to the T helper cells. Antigen-specific B cells and T helper cells are then brought into physical interaction through CD40 receptor on B cells and its ligands on T helper cells. Anatomical sites of B cell activation revealed that B cells are triggered to respond to activated T helper cells in the outer periarteriolar lymphocyte sheath (outer PALS) and around the terminal arterioles (TA) of the spleen. Following antigen recognition, naive surface IgM+/IgD+ B cells or memory B cells (IgD- cells) proliferate in the T cell rich areas of the secondary lymphoid organs and mature in the medulla to become plasma cells. This process, called extrafollicular reaction, yields a few B cells blasts which enter

primary follicles to form the germinal centres. The CD40 ligand and cytokine producing cells are also found in close proximately to antigenic B cells (Van den Eertwegh et al, 1993). It has been shown that, in the secondary immune response as well as the primary response, differentiation of antibody forming cells in the spleen is localized to the outer parts of the PALS. In the case of normal protein antigens, memory cells migrate from the marginal zone into the PALS, where they further develop into antibody-forming cells (Van Rooijin, 1990; Liu at al, 1988; Blanchard et al, 1994). Thus the process of B cell activation can be divided into two different forms. The first forms by cognate interaction and the second by T cell derived signals such as cytokines.

1-3-1 SURFACE ANTIGENS RECEPTORS ON B CELLS

In addition to the B cell antigen receptor, it is clear that a number of B cell associated surface molecules such as CD19, CD20, CD21, CD22, CD23, CD40, CD45, CD80 and other differentiation CD antigens have been identified which may regulate activation and adhesion of B cells (Clark and Lane, 1991). Many of these CD antigens are receptors for known ligands and others do not have defined ligands.

1-3-1-1 B CELL ANTIGEN RECEPTOR (mIg)

The specificity of a humoral immune response depends on each B lymphocyte binding and responding to a particular antigen determinant. For this purpose B

cells express an antigen receptor composed of a membrane immunoglobulin molecule complexed with several other molecules, the two best-defined of which are products of the mb-1 and B29 genes. The mb-1 gene product is a 32-KDa phosphoprotein found in association with mIgM and mIgD and is termed Ig-α (CD79a). The B29 gene is expressed throughout B cell development as at least two differentially processed proteins, one called Ig-β (CD79b) and a small fraction of the Ig/β molecules are present as slightly low apparent molecular weight forms (37 kDa) and termed Ig-γ that associate with mIg, forming a disulphide-linked dimer. Because of the short cytoplasmic tails of mIgM and mIgD, the Ig-α and Ig-β/γ have been shown to be important in B cell activation and entry into the cell cycle (Reth, 1995; Reth, 1992). Most mature B cells coexpress the IgM-BCR and IgD-BCR on their cell surface. Whether the two BCR classes have different functions on B cells is still controversial. Several early studies suggest that the engagement of mIgM and mIgD induces similar responses (Sieckmann, 1980; Mond et al, 1981). Other studies, however, show that mIgM, but not mIgD, can induce negative responses in B cells, such as anergy and apoptosis (Bell & Goodnow, 1994; Carsetti et al, 1993). The two BCR classes are heterogeneously expressed on normal mature B cells with IgD-BCR generally being more abundant than IgM-BCR (Havran, 1984).

Crosslinking of surface Igs by antigens plays a major role in the antigen specific activation and differentiation into antibody secreting plasma cells as well as survival, since activation of immature B cells (mIgM+ cells) results in either

clonal unresponsiveness or in deletion of the clone which seems to be important to eliminate self-reactive clones (Hasbold and Klaus, 1990; Ales-Martinaze et al, 1991). But mature B cells (IgM+/IgD+ cells) respond positively to ligation of the antigen receptor and in the case of a thymus-dependent antigen, the nature of the response is shaped by T cell-derived cytokines. However, it was recently reported that hyper-cross-linking of surface IgM and IgD receptors on mature B cells induces apoptosis (Parry, 1994). The induction of apoptosis is apparently dependent on the absence of T helper cell signals, because it can be partially reversed by either IL-4 or ligation of CD40 and almost completely abrogated by these two stimuli together (Parry et al, 1994). It has also been reported that the inhibition effect of anti-CD40 depends on expression of LFA-1/ICAM-1 adhesion molecules (Barrette et al, 1991). The same result is obtained from activation of the DND-39 human B lymphoma cell line which resembles mature B cells in respect of their sIgs (IgM+/IgD+ cells) (Sumomoto et al, 1994). The B cell receptor for antigen, membrane-associated immunoglobulin (mIg), serves two functions: To deliver an antigen-induced signal through mIgM and mIgD and to internalize bound antigen via its endocytic receptor function. The antigen and membrane Ig are then degraded (processed) by protease in the late endosome or lysosome into peptide fragments. Some of these processed peptides are bound to MHC class II molecules and such peptide/MHC complexes are then transported to the cell surface and subsequently presented to helper T cells, which in turn activate T cells. T cell activation leads to expression of CD40 ligand, which

provides a cell contact-dependent activation signal to the B cell for synthesis and secretion of cytokines that promote B cell activation and differentiation into antibody secreting cells (Clark & Lebetter, 1994; Campbell and Sefton, 1990). Membrane IgM but not mIgD appears to be important for internalization of antigen particles. This function is absolutely dependent on the presence of IL-4 or IL-2 (Luxembourg and Cooper, 1994).

1-3-1-2 Other Surface Differentiation Antigens

Antigen receptors allow lymphocytes to bind to foreign antigens, but additional molecules are still required to ensure efficient signal transduction and appropriate cellular responses subsequent to the binding of antigens by antigen receptors. An array of accessory molecules has been identified on the surface of T and B cells (Clark & Ledbetter, 1994; Parker, 1993; Clark & Lane, 1991). Most accessory molecules also participate in cell adhesion by interacting with specific ligands present on other cells. These non-specific antigen receptors are involved in activation, proliferation, or tolerance. Table 1-2 summarizes the expression of many of these antigens during B lymphocyte development. The key role of CD40 and CD80 family receptors in interaction with T cells was discussed earlier, other surface molecules include the following:

CD19 is a B cell specific member of the immunoglobulin gene superfamily. CD19 is expressed from about the time of Ig H chain rearrangement until plasma cell differentiation. It is an accessory molecule that has been shown to associate

with mIg and act as a co-stimulatory molecule. Anti-CD19 antibodies also reduce the dose of anti-Ig necessary for optimal stimulation of human B cell proliferation (Carter & Fearon, 1992).

CD20: The expression of CD20 in pre-B cells occurs later than that of CD19 CD20 antibodies have both stimulatory and inhibitory effects on B cell activation, depending on the monoclonal antibody used. The CD20 mAb (IF5), induces B cells to transit from G_0 to the G_1 phase of the cell cycle (Golay et al, 1985), whereas another CD20 mAb (B1) inhibits B lymphocyte proliferation. However, both of these antibodies inhibit B cell differentiation into Ig-secreting cells (Tedder et al, 1985). Anti-CD20 together with anti-CD40 has been shown to initiate both B cell growth and differentiation (Valle et al, 1989).

CD21 is a receptor for complement fragments C3d and iC3b (complement receptor 2) and the Epstein Barr Virus, as well as ligand for CD23. Triggering of CD21 either with an anti-CD21 antibody or with recombinant soluble CD23 increases the interleukin-4 induced IgE production in human peripheral blood B cells (Aubry et al, 1992). In addition, CD21 mediates Ca2+ mobilization after cross-linking with surface Ig (Carter et al, 1988).

CD22, a B cell-specific receptor is expressed in the cytoplasm of pre-B cells and on the surface of mature B lymphocytes. CD22 is an adhesion molecule and is important in communication with other cells in regulation of the immune response. At least one of its ligands on T cells seems to be CD45 including CD45RO, and it was reported that CD22 stimulates T cells in association with the

T cell receptor interaction (Aruffo et al, 1992). Recent studies show that CD22 on B cells induces an increase in proliferation and increases intracellular free calcium upon cross-linking the sIgM/BCR (Pezzutto et al, 1988).

CD23, the low affinity receptor for IgE (FcεRII), is mainly expressed on activated B lymphocytes and FDCs and involved in isotype switching of IgE (Gordon et al, 1989a). Following appropriate activation and subsequent proteolysis, B cells release the soluble form of CD23 (sCD23) which possesses multiple biological activities (Gordon et al, 1989a).

Human **CD38**, is a 45-Kda glycoprotein expressed by a variety of cell types (Malavasi et al, 1994), in T and B lymphocytes. CD38 represents a differentiation marker, being found on cells at different stages of maturation (Table 1-2). In human, immature B cells, GC B cells and plasma cells express abundant CD38, whereas mature B cells are either negative or weakly positive for this marker. Anti CD38 mAb is unable to induce GC B cell proliferation although it prevents cell apoptosis (Zupo, et al, 1994).

1-3-2 CYTOKINES

Cytokines are a diverse group of small or medium size proteins or glycoproteins which show potent biological activity. This activity is mediated by interaction with specific receptors on cell surfaces which trigger intracellular events. Cytokines are rarely released singly. An individual cytokine is able to stimulate the product of many others, generating a network that interacts with

Table 1-2

B cells antigen receptors

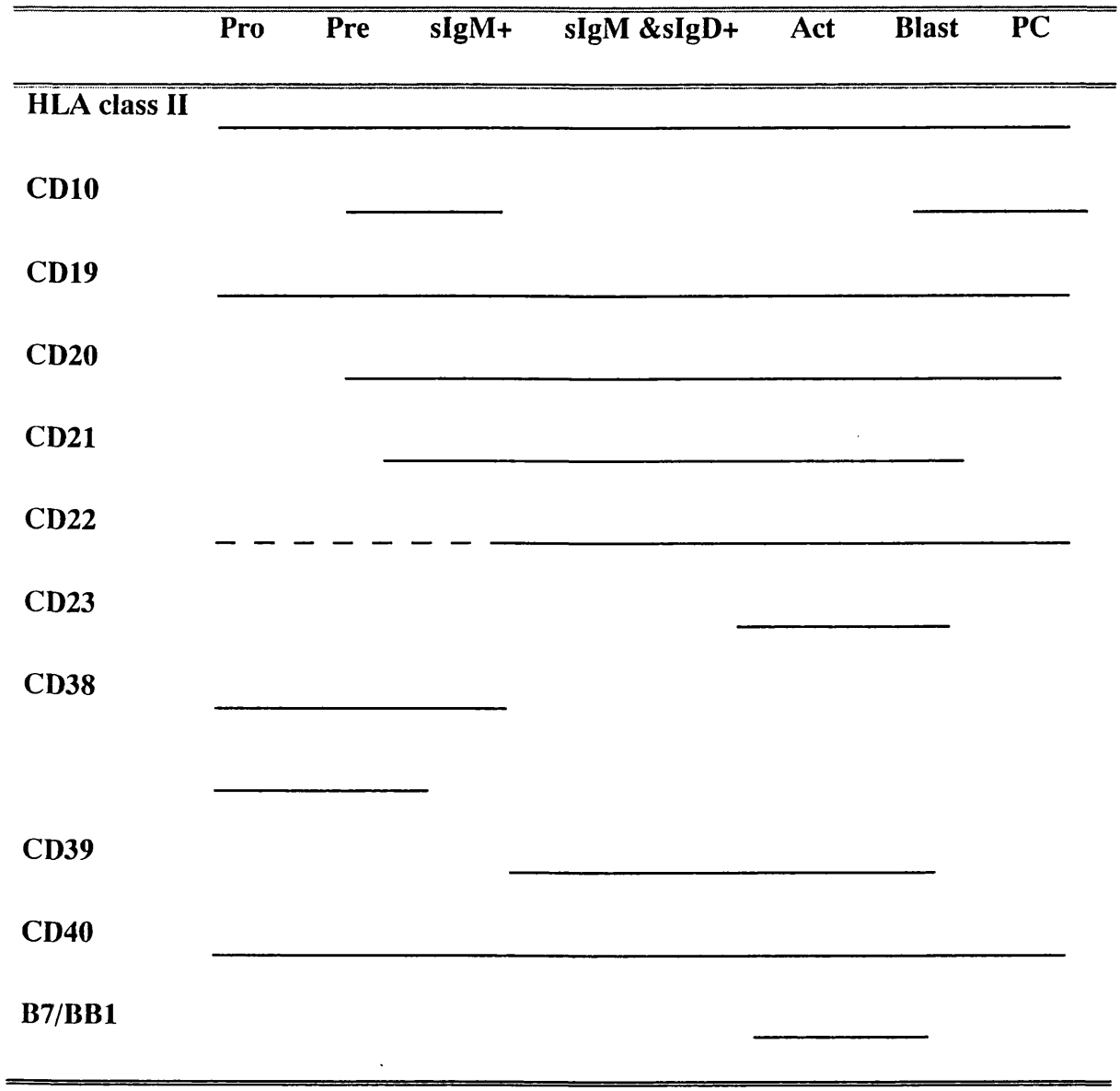


Fig 1-2 Expression of major B cell surface antigens on B lymphocytes at various stages of development (positive is shown by continuous line). The broken line designates expression only in cytoplasm. Act = activated PC = plasma cells

other cell regulators such as hormones, and neuropeptides. In addition, a specific receptor is often found on more than one cell type which makes it difficult to analyse the function of cytokines in the network. Cytokines are secreted by many different cell types into the extracellular fluid where they exert their effects on the same cells (autocrine) or on the neighbouring cells (paracrine). A classification scheme introduced by Mosmann et al (Mosmann et al, 1986; Mosmann and Coffman, 1989) divides mouse CD4+ T cells into two distinct subpopulations, T_{H1} and T_{H2} based on the arrays of secreted lymphokines. Recent studies indicate that this scheme applies in the human as well (Parronchi et al, 1991; Haanen et al, 1991). T_{H1} cells produce mainly IL-2, IFN-γ, TNF α and β. These cytokines are involved in activation of macrophages and induce delayed hypersensitive responses (Kuchroo et al, 1995). T_{H2} cells secrete IL-4, IL-5, IL-6, IL-10, strongly promote and regulate B cell responses, and stimulate the production of mast cells and eosinophils (Powrie and Coffman, 1993). The mechanism which determines the differentiation of T_H precursor cells into T_{H1} and T_{H2} cells is not fully understood, but IFN-γ and IL-4 were reported to have an autoregulatory effect on differentiation of T_H precursor cells into either T_{H1} or T_{H2} effector cells. IFN-γ enhances the T_{H1} type of response and inhibits the T_{H2} response, while IL-4 has the opposite effect (Kuchroo et al, 1995; Rabin et al, 1986; Powrie and Coffman, 1993; Finkelman et al; 1986). I will give a brief description of the cytokines which were used in this project.

1-3-2-1 Interleukin 4 (IL-4)

IL-4, was initially known as B-cell growth factor-1 (BCGF-1) because of its ability to act with anti-IgM antibody to induce proliferation of resting B-cells cultured at high density (Howard, 1982). It was known later as B-cell stimulatory factor 1 (BSF-1) when it was shown to act on resting B-cells to induce expression of class II MHC molecules and to enhance their subsequent responsiveness to anti-IgM antibodies (Oliver et al, 1985; Rabin et al, 1985). Finally these names were replaced by the name IL-4 (Lee et al, 1986). IL-4 is made in response to immunological recognition by T-lymphocytes (Ben-Sasson et al, 1990) and is involved in T-cell-B-cell collaboration. IL-4 production is a property of T_{H2} cells and T-cells isolated from the tonsil are also capable of producing IL-4 (Secrist et al, 1994). Another major set of cells which are capable of producing IL-4 in response to cross linkage of immune recognition receptors are mast cells (Plaut et al, 1989; Brown, 1993), and basophils (Brunner, 1993). The high affinity receptor for IL-4 is present on many cells including T- and B-cells (Ohara & Paul 1987; Zola et al, 1993). IL-4 is a very pleiotropic molecule that induces responses in a wide variety of cell types, including both haematopoietic and non-haematopoietic cells (Paul, 1991). In addition to many activities in other cell types IL-4 has multiple effects on resting and activated B-cells. The expression of IL-4 receptors on resting B-cells and T-cells in contrast to very limited expression of IL-2 receptors on such cells, is consistent with the demonstration that resting B lymphocytes are a major target of IL-4 activity (Roehm et al, 1984; and Oliver, et

al, 1985). In the context of the work in this thesis, reports that IL-4 acts on resting (G_0) B cells, and causes them to move into the G_1 stage of the cell cycle are of special interest (Defrance at al, 1987). IL-4 has a regulatory role in the generation of the T-cell dependent humoral response (Fernandez-Botran et al, 1986) and acts on B-cells at several phases during their activation and differentiation (Isakson et al, 1982; Noelle et al, 1984; Roehm et al, 1984, Sideras et al, 1985; Oliver et al, 1985 and Rabin et al, 1985). On resting cells IL-4 induces an increased expression of class II MHC antigens (Noelle et al, 1984 and Roehm et al, 1984), prepares the cells to enter the S phase of the cell cycle following stimulation with Ig antibodies (Oliver et al, 1985 and Rabin et al, 1985). IL-4 induces class switching to IgG1 (Yuan et al, 1985) and IgE (Coffman and Carty, 1986) and upregulates adhesion molecules such as ICAM-1 and LFA-1 (Branden & Lundgren, 1993; Björck, 1992; Björck, 1993a).

1-3-2-2 INTERLEUKIN 13 (IL-13)

Interleukin 13 is a cytokine secreted by activated T cells including CD8+ and CD4+ cells and is a potent modulator of human monocyte and B cell function (Mckenzie at al, 1993). Human IL-13 has been cloned and has 66% nucleotide sequence identity to mouse IL-13 cDNA and about 30% homology to human IL-4 (Zurawski & de Vries, 1994). Several studies on mouse T_H cells lines indicated that mIL-13 mRNA was produced by T_{H2} cells, but not by most T_{H1} cells (Cherwiniski et al, 1987). A kinetic study of production of these cytokines shows

that IL-4 mRNA expression levels in most T cell clones peaks after 24 hours of activation and are transient (Yssel et al, 1992), whereas expression of mRNA for IL-13 is much earlier and peaks after 2 hours of activation and lasts to up 72 hours (Zurawski & de Vries, 1994). IL-13 has anti-inflammatory activities and shares many biological activities with IL-4, such as morphological and cell surface phenotype changes on human monocytes. It induces proliferation, differentiation, and also facilitates immunoglobulin production by human B cells (Zurawski & de Vries, 1994; de Waal Malefyt et al, 1993; Defrance et al, 1994). Despite the similarities between IL-4 and IL-13, IL-13 fails to activates human T cells. In contrast to IL-4, IL-13 induces IFN-γ production by large granular lymphocytes cells, whereas IL-4 inhibits this function (Minty, 1993; Zurawski & de Vries, 1994).

1-3-2-3 INTERFERONS (IFNs)

The interferons belong to a heterogeneous family of proteins that were discovered and measured on the basis of their anti-viral activity. Three types of interferon have been identified in human and their genes have been characterized. IFN-α and IFN-β are classified as type I and IFN-γ is classified as a type II IFN. IFN-γ has little antiviral activity, in contrast to IFN-α and IFN-β which have strong antiviral activity and are produced by different cell types as a result of viral or LPS activation. Like other cytokines IFN-γ must first bind to specific, high affinity cellular receptors in order to exert its regulatory effects. IFN-γ interacts

with unique type II receptors expressed on a wide range of cells including T cells, B cells, macrophages, polymorphonuclear leukocytes, Platelets, and many other somatic and tumour cells (Valente et al, 1992). IFN-γ is a pleiotropic cytokine that is important in regulation of immune responses, cell mediated immunity, non-specific responses in host defence against bacterial and parasitic infection, inflammatory responses and autoimmune diseases. The multiple functions of IFN-γ were reviewed by Trinchieri & Perussia (1985) and by Farrar & Schreiber (1993). Besides activation of macrophages (Bancroft et al, 1992), it also blocks the upregulation of surface class II MHC expression and cell size induced by IL-4 (Rabin et al, 1986). IFN-γ and IL-2 have been found to stimulate polyclonal IgM secretion in vitro by activated human B cells (Nakagawa et al, 1985). IFN-γ inhibits both IgG1 and IgE secretion induced by IL-4 and LPS, has no effect on IgM production but enhances IgG2a production (Snapper and Paul, 1987; Finkelman et al, 1988).

IFN-γ is produced by all CD8+ cells and the T_{H1} subset of CD4+ cells following activation by antigen (Mosmann et al, 1986; Mosmann and Coffman, 1989). NK cells are another source of IFN-γ producing cells following activation by IL-12 and TNF-α (Bancroft et al, 1992).

SECTION FOUR: Cell Motility and Locomotion

In this project, it was mostly tried to define the locomotor properties of the tonsillar B cells. Tonsil cells were chosen because the proportion of B cells in

tonsil was higher than that in blood and it was easier to purify them. We were able to compare the locomotor behaviour of tonsillar B cells with that of B cells in blood.

1-4-1 Locomotion of lymphocytes

In general the success of the immune system is dependent on the intrinsic ability for locomotion present in immune cells. All leukocytes are capable of locomotion. However, the locomotor capacity of leukocytes differs from one cell type to another. More than 95% of blood neutrophils respond to stimuli immediately (McKay et al, 1991; Keller et al, 1983), whereas the proportion of responding lymphocyte is low until the cells are activated (Wilkinson and Newman, 1992; Wilkinson and Islam, 1989). The locomotor capacity of individual cells is also related to different stages of cell development and differentiation. Many studies both in vivo and in vitro suggest that the locomotor capacity of small lymphocytes from blood or unprimed lymph nodes is not fully expressed. Most cells isolated from blood or lymph nodes are non-motile unless activated following challenge with antigen in vivo or with mitogen in vitro (Wilkinson, 1988; Wilkinson and Newman, 1992; Wilkinson and Islam, 1989).

Cellular locomotion may be stimulated by chemical substances. These substances determine both the direction of locomotion, a reaction known as chemotaxis, and the speed of locomotion, a reaction known as chemokinesis. In addition physical properties of the surfaces that cells move on can determine the

direction of locomotion (contact guidance). In the presence of a chemotactic gradient, cells migrate towards the gradient source. In the presence of the same substance at uniform concentration, the cells show random locomotion (Devereotes and Zigmond, 1988; Wilkinson, 1990a; Wilkinson 1990b). All types of leukocytes show chemotaxis. Chemotactic factors are usually soluble and diffusible and can thus form concentration gradients up which the cells migrate. The same substance could be both chemokinetic and chemotactic, depending on whether a good gradient is established or not (Haston and Wilkinson, 1988b). In order to show these responses, a cell must be capable of locomotion and must be capable of recognizing the attractant. Since resting lymphocytes may not have this capacity, and may require to be activated before they acquire it, a second group of agents with action on leukocyte locomotion consists of locomotor activators, which are not chemotactic factors, but whose effect is to confer locomotor capacity on previously non-motile lymphocytes. Studies of lymphocyte locomotion in vitro are important; (a) for recirculation and the traversing of high endothelial venules in lymphoid tissue; (b) for recruitment of lymphocytes into inflammatory sites (In the latter situation, activated lymphocytes are more active as locomotor cells than resting lymphocytes); (c) in cell-cell interactions in initiation of immune response (Springer, 1994; Wilkinson and Newman, 1992; El-Naggar et al, 1980). All these studies suggest that following activation in vitro or vivo, the lymphocyte populations become more motile than the preactivated population. This is consistent with other studies showing that

lymphocyte locomotor capacity is growth-dependent, since resting cells that are in the G_0 phase of growth require to begin protein and RNA synthesis but not DNA synthesis before they show full locomotor activity (Wilkinson, 1986; Masuyama et al, 1992; Komai-koma et al, 1995; Islam and Wilkinson, 1992). These studies suggest that the proportion of cells in G_1 responding to attractants is higher than in G_0. However, this is in contrast to some reports that found a similar proportion of responding cells in G_1 and G_0 and also that small number of cells in G_2 and M phase of cell cycle were also capable of responses (Ratner, 1992).

1-4-2 Previous Studies of B Cell Locomotion in Vitro

To date, very few reports exist on B cell chemotaxis. The first successful report describing stimulated locomotion of B cells was made in the early 1970s when Russell et al (1975) showed that both human lymphoblasts from B cell lines and mouse lymphoblasts migrated into filters toward chemoattractants, such as endotoxin-activated plasma and casein. In contrast to human B cell lines, T lymphocytes taken from immunized mice did not show directional locomotion. Mouse lymphocytes showed polarization or migration in response to anti-Ig, but not to anti-histocompatibility antibodies or anti-lymphocyte serum (Schreiner & Unanue, 1975), who reported that anti-Ig stimulates random but not directional movement. These authors also reported that locomotor shape-change in rat spleen B cells was induced by anti-Ig. A later paper suggested that a chemotactic response is found at relatively low doses (around 100ng) of anti-Ig, whereas high

concentrations of anti-Ig gave random migration (Ward et al, 1977). Wilkinson et al (1977) reported that lymphocytes derived from immunized mice gave a positive chemotactic response to the immunizing antigen, but that non-immunized mice showed no response. The cells responding to antigen at three days of challenge were large lymphocytes. The first definitive description of the locomotor capacity of human B cells was a study made by Wilkinson and Islam (1989). They demonstrated that B cells responded overnight by polarization in culture in IL-4 and IFN-γ. Clinchy et al (1991 & 1994) showed a similar response of mouse spleen cells to IL-4. The response of these cells to IL-4 was fibronectin and β_1 integrin dependent (Elenström-Magnusson, et al, 1994). This group also reported that the effect of IL-4 on motility of human and murine B-cells could be inhibited using IL-10 (Clinchy, et al, 1994). They also showed that a lot of other cytokines such as IL-1, IL-2, IL-3, IL-5, IL-6 did not induce migration either in small or in large B cells (Clinchy et al, 1993). A recent report (Burton et al, 1995) has shown that physical contact between FDCs and high-density B cells mediates maximal signals for both chemokinetic and chemotactic responses and that these responses were independent of T-cells. It is now evident that B cell activation is optimal in response to multiple signals, and it was therefore of interest to see if the locomotor response like other activation responses could also be optimized using multiple signals (e.g. IL-4 + CD40 etc).

The following cytokines and other stimuli have been reported to stimulate locomotion of B cells. IL-4 (Bacon et al, 1990; Wilkinson and Islam, 1989;

Clinchy et al, 1991), IL-6 (Bacon et al, 1990), IL-10 (Jinquan et al, 1993; Clinchy et al, 1993), IFN-γ (Issekutz and Stoltz, 1989; Wilkinson and Islam, 1989), MIP-1α (Taub et al, 1993, Schall et al, 1993), anti-Ig (Unanue et al, 1974), LPS (Issekutz and Stoltz, 1989), Protein A (Wilkinson et al, 1976), Fibronectin (Davies et al, 1990), C5a (Kupp et al, 1991; El-Naggar et al, 1980) and FMLP (El-Naggar et al, 1980). About many of these, there is disagreement and a consensus has not been reached about which are the important B cell attractants.

1-4-3 Methods For Study of Lymphocyte Locomotion in vitro

Study of locomotion in vitro goes back more than a century. Most of the investigators before 1950 used some kind of visual assay to observe the movement of cells (Lewis & Webster, 1921; McCutcheon, 1923). Boyden (1962) introduced the filter assay, which did not require direct observation of cells, using cellulose ester filters about 100-150μm thick and consisting of pores of different diameters depending on cell type (3-12μm). This technique was used to separate the cells above from the attractant below the filter. The use of this assay for defining chemotactic responses was controversial, since it did not give direct evidence for a chemotactic response (Keller et al, 1972). Zigmond and Hirsch (1973) introduced the checkerboard assay, based on calculation of the velocity of cells in different gradients and uniform concentrations of attractants, to demonstrate directional migration in filters. A number of assays are available to study leukocyte locomotion and chemotaxis, each of which is based on different

principles (Haston and Wilkinson, 1988a). The choice of methods depends on the nature of the cells and the kind of questions being asked. Early studies usually used time-lapse cinematography, but other assay systems which have also been used, are the polarization assay, filter assay, and the collagen gel invasion assay. All these methods except time-lapse cinematography, are indirect assays in which the paths taken by cells and their speed and direction are not examined. In the filter and collagen assays, the end-point distribution of the cell population after exposure to the attractant is determined.

1-4-3-1 Time Lapse Cinematography

Time-lapse cinematography allows a visual demonstration of leukocyte motility as well as orientation towards the source of chemotactic gradients and can provide unequivocal evidence of the leukocyte response to chemoattractants. This assay allows direct measurement of the direction of chemotaxis or chemokinesis. The number of cells which can be sampled in these assays is smaller than in the indirect assays. This technique has been successfully used for many years to study the chemotactic responsiveness of both human blood neutrophils and lymphocytes (Islam at al, 1985; Wilkinson, 1985). This is most frequently done on flat surfaces (e.g. protein coated glass) or the chemical substance can be placed in or on collagen gels and the chemotactic response of leukocytes towards the attractants incorporated in the gel can be studied visually.

1-4-3-2 Polarization Assay

The polarization assay (Haston & Shields, 1985) is the simplest method available for measuring lymphocyte locomotion. Addition of the attractant results in a change in shape from a spherical to a polarized morphology of cells in suspension. Unstimulated cells have a spherical morphology. When spherical non-motile leukocytes are stimulated by a attractant, the cells form a pseudopod towards the gradient source and then if they are on a surface, they move in the direction of pseudopod formation within a few minutes due to the localization of polymerized actin at the head of the cell (Zigmond, 1974; Allen and Wilkinson, 1978). It was reported that some of the membrane proteins and receptors for chemotactic factors become concentrated toward the head of the cell (McKay et al, 1991). It was reported that cells in suspension respond to uniform concentrations of attractants by taking up a similar polarized morphology (Smith et al, 1979; Keller et al, 1983; Haston & Shields, 1985). This is a useful and accurate assay to score the proportion of locomotor cells, and the degree to which they become polarized. It also provides a rapid assessment of the immediate locomotor response to attractants. This is easy, independent of adhesion, and gives excellent dose-response data. The disadvantage of this method is that it does not give direct information about the locomotor events which follow polarization, nevertheless, it correlates well with other more direct methods like the collagen assay (Haston & Wilkinson, 1988b). The polarization assays was used in two different ways; (a) in a short term assay (<30 min. incubation) which

was used to study the immediate response of cells on addition of attractants either directly after separation from biological sources or after overnight culture. This provides valuable information about the locomotion of cells towards chemoattractants before and after culture. (b) in a long term assay (24-72h) which shows the effect of growth-activators on locomotion. These studies show that long term culture enables a high proportion of cells to respond to chemoattractants, and also that chemoattractants are secreted into the culture medium during long-term incubation of peripheral blood mononuclear cells (Wilkinson & Higgins, 1987, Wilkinson and Newman, 1992).

1-4-3-3 Collagen Gel Assay

The polarization assay measures shape-change, but not locomotion directly. The locomotor capacity of polarized cells was determined by using 3-D gels of fibrillar proteins such as collagen (Haston and Wilkinson, 1982; Schor et al, 1983) or fibrin (Wilkinson & Lackie, 1983). This technique allows the relationship between polarization and locomotion to be investigated. Type I collagen from rat tail tendons was used in this thesis as has been described earlier (Haston, Shields, Wilkinson, 1982). In contrast to protein-coated glass or plastic which is ideal for studies of locomotion of more adherent cells such as monocytes and neutrophils, locomotion in collagen gels does not require strong adherence. Thus lymphocytes which do not attach to most 2-dimensional tissue culture substrates, may locomote readily through the matrices of type I collagen gels (Haston et al, 1982; Schor et

al, 1983). The collagen assay is similar to the micropore filter assay, but more physiological and provides a 3-dimensional collagen matrix in which cell migration in response to diffusing gradients of attractant can be observed (Haston et al, 1988; Schor, 1983; Wilkinson, 1986). In contrast to the filter assay, the collagen gel allows more accurate study of the locomotion of leukocytes and also the invasive cells can be recovered for phenotyping using collagenase. Thus this assay is ideal to study locomotion behaviour in mixed populations.

1-4-4 Cell Motility and its Importance in Regulation of Immune Response

Most mature lymphocytes spend the majority of their time in transit, and the circulation and accumulation of lymphocytes are not random. Both T and B lymphocytes recirculate continuously from blood into the lymph nodes and back again. Some studies have indicated that the recirculation speed of B cells is slower than that of T cells (Sprent, 1973) and B cells present in germinal centres do not recirculate (Reichert et al, 1983). Furthermore, activated or memory cells show different migration pathways from inactivated or virgin cells. The inactivated cells are the recirculating population. After activation by antigen, lymphocytes no longer recirculate, but are able to migrate into inflammatory sites (Springer, 1990; Bevilague, 1993). In vitro assays show that activated T cells respond more vigorously to chemoattractants than naive T cells (Wilkinson et al, 1976; Wilkinson and Newman, 1992; Schall et al, 1990). In vivo, labelled

lymphocytes injected into the circulation go back to the same tissue from which they were originally derived (Shimizu etal, 1992). Mucosal lymphocytes preferentially return to mucosa associated lymphoid organs and lymphocytes from peripheral lymph nodes home back to lymph nodes (Kraal et al, 1983). Recirculation between the blood and lymphatics is predominantly a property of small lymphocytes (Gowans and Knight, 1964). Activated lymphocytes appear capable of relocation in the tissue in which they were activated (Gowans and Knight, 1964; Shimizu et al, 1992). Tissue restricted homing migration may be one way to enhance the efficiency of the immune response keeping in mind that each type of leukocyte responds to a particular set of traffic signals and the nature of the stimulus determines which cells predominate (Springer, 1994). The continuous traffic of recirculating B cells increases the chances for encounter with the antigen for which any B cell is specific and ensures many cell-cell interactions which are important in regulation of immune responses.

The molecular explanation for these distinct migration patterns lies in the interaction of lymphocytes with endothelial cells living the blood vessels. Gowans and Knight (1964) identified specialised endothelium, high endothelial venules (HEV), as the specialized site of entry of small lymphocytes into the lymph nodes (tonsils, Peyer`s patch and appendix) from blood. The small virgin lymphocytes which circulate from blood across HEV into lymph nodes then back via lymphatics to the blood consists mainly of small nonactivated cells (Gallatin et al, 1987). These recirculating lymphocytes do not cross nonspecialized endothelia

or migrate into inflammatory sites, whereas activated lymphocytes have been reported to lose their markers for HEV (Dailey et al, 1983) but acquire a capacity to cross inflammatory endothelia (McGregor & Logie, 1974). The lymphocyte-HEV interaction was studied by Stamper and Woodruff (1976) in vitro using frozen sections of endothelial cells. It was suspected that unique adhesion receptors on lymphocytes and endothelial cells provided the sorting mechanism by which only selected lymphocytes were allowed to adhere and subsequently pass (Butcher et al, 1980). Since the lymphocyte endothelial interaction has become the focus of intensive research, an increasing number of monoclonal antibodies (mab) specific for adhesion receptors has become available. It is worth noticing that passage through HEV in lymphoid organs is only permitted for lymphocytes and not for macrophages or granulocytes. However, binding to HEV may not be an absolute requirement for extravasation since lymphocytes pass through spleen lacking HEV even faster than through lymph nodes (Pabst and Binns, 1989).

Extravasation of lymphocytes and endothelial cells is governed by adhesion receptors on lymphocytes and its ligands on the endothelial cells and the mechanisms controlling such interactions are currently being investigated intensively. (Harris, 1995; Shimizu et al, 1992; Springer, 1994; Bevilacaua, 1993). All of the identified adhesion receptors can be classified into three major groups of molecules; selectins, integrin and members of the Ig supergene family (springer, 1990; Shimizu et al, 1992). Besides these groups, another broadly distributed adhesion molecule of importance in lymphocyte migration is CD44

(de-los-Toyos et al, 1989) which does not fit to any of the above groups but is related to cartilage protoglygan core proteins and binds to hyaluronic acid, collagen and fibronectin.

The current hypothesis of molecular mechanisms underlying leukocytes extravasation proposes a multiple step, sequential chain of events based on adhesion receptors (Shimizu et al, 1992; Springer, 1994). According to this scenario four steps were recognized; (1) Tethering which is the initial interaction mediated by selectins binding to their carbohydrate ligands; (2) Rolling along the vessel until the cells finally stop and adhere firmly. This adhesion step is still mediated by selectins. It allows the leukocytes to slow down sufficiently to sense activation signals from chemoattractants. When the chemoattractants bind to their specific receptors the lymphocytes became activated (Bargatze and Butcher, 1993). This triggers up-regulation and conformational changes in integrins, leading to increased adhesiveness. Integrins, here exemplified by LFA-1 and VLA-4, bind to their ligands ICAM-1/ICAM-2 and VCAM-1, respectively. These steps may result within several minutes or hours; (3) Flattening, Integrin receptor-ligand interactions are strong and arrest the rolling of lymphocytes. Since the activation of integrins is transient, the adhesion will reverse and allow lymphocytes to transmigrate; (4) Diapedesis, migration out of the vessel through the pores between endothelial cells. The lymphocytes pass through the space between endothelial cells and the basement membrane, then enter into the extracellular matrix.

CHAPTER TWO:

Materials and Methods

1- Preparation of reagents.

1-1 Cytokines

Lyophilized recombinant human IL-1α(1mg) was obtained from R & D (British Biotechnology Products Ltd, Oxford, UK). This was dissolved in HBSS/HSA and stored at -20^{0}C. Human recombinant IL-2 was a gift from Dr. D.I. Stott (Dept. of Immunology, Western Infirmary, Glasgow, UK), and was originally obtained from Glaxo (Geneva, Switzerland) and also from R&D. IL-4 was a generous gift from Dr W. Niedbala (Dept. of Immunology Western Infirmary, Glasgow, UK) and was originally obtained from Immunex (Seattle, Washington, USA). It was stored at 1000U/ml at -20^{0}C. To avoid a repeated freezed/thaw cycle, IL-4 was diluted at 8,000 u/ml in HBSS/HSA and stored at -20^{0}C in several aliquots. A fresh working solution at an appropriate concentration was made up in Hanks/HSA. Mouse IL-4 was a kind gift from Prof. F.Y. Liew (Dept. of Immunology, Western Infirmary, Glasgow). It was stored at 100mg/ml with a specific activity in excess of 1X10^7 U/mg. Polyclonal rabbit anti-human IL-4 was purchased from Genzyme Corporation (Cambridge, UK) in 0.5mg. This was dissolved in 0.5ml of HBSS and stored in aliquots at -20^{0}C before use. Anti mouse IL-4 (11B11) was also used to block the effect of IL-4 on B-cell activation. Lyophilized human recombinant IL-5, IL-6, and mouse aIL-4 (11B11) were donated by Prof. F.Y. Liew and the former two were originally obtained from

NIBSC (South Mimms, UK) at approximately 500ng per ampoule (5,000 units) and 1.25mg per ampoule (6250 units), respectively. The total content of the IL-5 ampoule was dissolved in 0.5ml of distilled water, then the ampoule was rinsed with PBS to make up the total volume to 1ml. Aliquots were made and stored at -70^{0}C. IL-6 was reconstituted in 0.5ml of HBSS and stored at -70^{0}C. Lyophilized recombinant human IL-7 was purchased from NBS (Hatfield, UK) (5mg) with a specific activity of 2-4 x10^8 units/mg. This was reconstituted in HBSS at 10mg/ml and stored at -20^{0}C in several aliquots. Lyophilized human recombinant IL-8 was purchased from R+D Systems (Abingdon, UK). This was dissolved in 1ml of PBS and stored at -20^{0}C in aliquots. IL-13 & aIL-13 were a kind gift from DNAX Research Institute (Palo, Alto, CA, USA) at 25mg/ml and 0.37ng/ml, respectively. IL-15 was a gift from Prof. F.Y Liew. Recombinant (*E.coli*-derived) human interferon gamma (IFN-γ), with more than 99% purity and with a specific activity equal to 2x10^7 IU/ml, was obtained from Dr G.R. Adolf (Bender Wien). This was reconstituted with 1ml sterile normal saline and stored at -20^{0}C in several aliquots. Recombinant (*E.coli*-derived) human TNF-α was from Bender Wien, with more than 99% purity and with specific activity equal to 5x10^7. This was aliquoted and stored at -20^{0}C. It was diluted immediately prior to use. Purified recombinant sCD23 was a kind gift from Dr. J.Y. Bonnefoy (Glaxo). This was aliquoted and stored at -20^{0}C. Human MIP-1a (LD78) & MIP-1ß were a gift from Dr. G. Graham (Beaston Institute, Glasgow). RANTES was purchased from Peprotech Inc (Rocky Hill, N.Y./USA) and prepared at 10mg/ml, stored at -20^{0}C.

1-2 Anti-Immunoglobulins

The antibodies shown in Table 2-1 were used for functional studies on human B-cells and were prepared as monoclonal anti-human antibodies except anti-human IgD & anti human IgG,A and M, both from the Binding Site, which were polyclonal. All the antibodies containing sodium azide were dialysed against several changes of HBSS for three days to remove the preservatives. For further dilution, all reagents were carried out in a protein containing medium (HBSS/ HSA) to avoid loss of bioactivity of reagents. Mouse IgG_1 purified immunoglobulin, mouse IgG_{2a} purified immunoglobulin, and sheep IgG purified immunoglobulin were purchased from Sigma and were used to show any non-specific response as a negative control. These were applied at the same concentration as the corresponding test antibodies. A variety of antibodies were used to differentiate subpopulations of B-cells, and other mononuclear cells. Different antibodies were used to identify different B-cell populations in terms of their activation and maturation. Some of these primary antibodies were conjugated and those which were not conjugated were used in conjugation with appropriate secondary antibodies (Fluorecein-conjugated $F(ab`)_2$ fragments of rabbit immunoglobulins against mouse immunoglobulins, Dako). The primary antibodies were obtained from different sources and species shown in Table 2-2. CD14 and CD56 were used as markers for monocytes and natural killer cells, respectively. All antibodies derived from mice were detected using FITC-conjugated rabbit anti-mouse immunoglobin[$(Fab)_2$ fragment, Dako] at 1:50

Table 2.1

Antibody	Source	Isotype	species
ahIgG,A and M	Binding Site ✪	IgG Fraction	Sheep
ahIgD	Binding Site	IgG Fraction	Sheep
ahIgD	Dako ☆	IgG_1, Kappa	Mouse
ahIgG	Dako	IgM, Kappa	Mouse
ahIgA	Dako	IgG_1, Kappa	Mouse
ahIgM	Binding Site	IgG_{2a},	Mouse
ahCD20	Coulter ❋	_____	Mouse
ahCD21	SAPU	IgG_1, Kappa	Mouse
ahCD32	Immunotec ⊕	IgG_{2a}	Mouse
ahCD40	Serotec ❖	IgG_1, Kappa	Mouse
ahCD40	Prof J.Gordon ✶	IgG_1	Mouse

Addresses of sources of antibodies

✪Binding site (Birmingham, UK) ❋ Coulter (Hialeah, FL, USA)

❖ Serotec (Kidlington, Oxford, UK) ⊕ Immunotec (Binding Site, Birmingham, UK)

✶ Department of Immunology, University of Birmingham

☆ Dako (Bucks, UK)

dilution in PBS/azide. FITC-conjugated aCD3 and PE-conjugated aCD19 were used as pan T- and B-cell markers, respectively.

The following antibodies were used to study mouse B cells: goat anti mouse Ig (polyvalent) biotin-labelled (Crawley Down, Sussex, UK) was diluted to 200μg/ml and dialysed against PBS for functional studies and phenotyping of mouse spleen B cells. Biotin conjugated hamster anti mouse CD3 monoclonal antibody (Pharmingen, UK), FITC conjugated anti mouse CD8a (Ly-2, Pharmingen), and PE conjugated anti mouse CD4 (L3T4, Pharmingen) were used to phenotype T-cells, T helper cells, and CTL T-cells, respectively. Non-conjugated antibodies were then labelled with fluorescein streptavidin - FITC (Vector Laboratories, Peterborough, UK). This was diluted 1 in 100 of staining buffer. FITC conjugated F 4/80 antigen (Serotec) was used to identify macrophages in mouse spleen cell populations. Mouse monoclonal antibodies to mouse Thy-1.2 (Serotec) was used to lyse T-cells. It was diluted 1:20 in RPMI containing 15% FCS, then aliquoted at 0.2ml and stored at -70^{0}C.

1-3 Antigens and other reagents

LPS was obtained from Sigma and was made up to a 10mg/ml dilution in Hanks. Pneumovax II which contains 23 different Polysaccharides was donated by L.G. Smart (Ruchill Hospital, Pneumococcal reference, Glasgow). Lyophilized protein A from *Staph aureus* (Cowan strain) cell walls, (1mg, Sigma) was diluted with Hanks at 1mg/ml. Ovalbumin, grade V was obtained from

Table 2-2

Antibody	Conjugated	Source	Antibody	Conjugated	Source
ahIgD	None	Dako	ahCD20	None	Coulter
ahIgM	None	Binding Site	ahCD21	None	SAPU
ahIgA	None	Dako	ahCD22	None	Dako
ahIgG	None	Dako	ahCD23	None	Dako
ahCD3-CD19	FITC-PE	Dako	ahCD32	None	Immunotec
ahCD4	PE	Dako	ahCD38	None	Immunotec
ahCD8	PE	Dako	ahCD39	None	Birmingham
ahCD10	None	SAPU	ahCD45	FITC	Becton Dickinson
ahCD14	None	SAPU	ahCD56	None	Dako
ahCD19	PE	Dako	ahCD69	None	Becton Dickinson

Table 2-2 Primary antibodies for phenotyping of human B cells.

Sigma. ISCOMS ovalbumin was donated by Dr A., Mowat. (Dept. Immunology, Western Infirmary, Glasgow). Low-tox rabbit complement was obtained from Cedarlane (Hornby, Ontario, Canada). Each lyophilized vial was reconstituted in one ml of ice cold distilled water on the day of experiment. Type I collagenase derived from *Clostridium histolyticum* (clostridiopeptidase A) with a specific activity of 280 units/mg was obtained from Sigma . This was dissolved in HBSS at 5000U/ml and stored at -20^{0}C. To find the optimal time and dose for digestion, the collagenase was incubated with collagen at different concentrations with various intervals. Collagenase dissolved collagen gels completely at 20u/ml in 20-30 minutes. [5,6 ^{3}H]uridine was purchased from Amersham International PLC (Buckinghamshire, UK). Its activity per dose was 9.25MBq (equal to 250μCi) and it was stored at 4^{0}C. [^{3}H]-thymidine TRA 120, was purchased from the Dept. of Clinical physics and Bio-engineering, Western Infirmary, Glasgow, with an activity per dose of 37 MBq equal to 1000μCi and was stored at 4^{0}C.

2 : Preparation of buffers

2-1 Hanks balanced salt solution :

HBSS (ICN Flow Ltd, High Wycombe, UK) was obtained as a 10 x strength solution without sodium bicarbonate, and mixed 1:9 with distilled water. MOPS (Morpholinopropane Sulfonic Acid, Sigma, Poole, Dorset, UK) was used as a buffer for HBSS. 1M MOPS was prepared by dissolving 209.3g MOPS in one

litre of distilled water. The solution was sterilized by filtration through 0.22μm filters (ICN Flow) and stored at 4^0C before use. It was then diluted 1 in 100 in Hanks to a final dilution of 10mM. The pH was adjusted to 7.4 by either 2M HCl or NaOH. This solution, is designated as Hanks throughout this project. Hanks/HSA was prepared by adding 10mg/ml of HSA (Behringwerke AG, Marburg, Germany) to the Hanks. RPMI 1640 (ICN Flow) was the most common medium used to study mouse B cell locomotion. The high-glucose formulation of this media was supplemented shortly before use with 10mg/ml HSA. It was supplied as a 1 X strength solution containing sodium bicarbonate, but not L-glutamine.

2-2 Normal Saline :

was prepared as a 10 X strength solution by dissolving 85g NaCl (BDH) in one litre of distilled water. The working solution was prepared from this by a 1:10 dilution in distilled water.

2-2 Phosphate buffered saline (PBS):

PBS was prepared by mixing 140mM NaCl, 9.2mM Na_2HPO_4, and 1.3mM NaH_2PO_4 (BDH), and the pH was adjusted to 7.4.

2-4 PBS-Azide:

Sodium azide (BDH) was prepared at 10% (w/v) in distilled water and stored at

4^0C. This solution was diluted in PBS at 1 in 100.

2-5 Lysing Buffer :

Ammonium chloride in the presence of potassium bicarbonate (155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA)at pH 7.4 causes specific lysis of erythrocytes. Lysis does not affect the leukocytes, if the temperature is maintained at 4^0C thus minimising the diffusion. This was mixed and sterilised by filtering through 0.22μm filters.

3- Siliconization of Glassware

Glass universal bottles and test tubes were cleaned before dipping into Repelcote (Hopkin and Williams, Chadwell Health, UK) for 1-2 minutes and allowed to dry inside a fume cupboard for one hour at room temperature. After complete evaporation of fluid, the glassware was washed twice in tap water and finally with distilled water to remove the small quantity of hydrochloric acid formed during hydrolysis of the Repelcote to silicone. The glassware was dried overnight at room temperature and sterilised by autoclaving (Islam, 1986).

4- Discontinuous Density Gradient Centrifugation

Percoll was obtained from Pharmacia LKB, Biotechnology AB (Uppsala, Sweden). A stock solution was made by mixing the following substances; 90ml Percoll, 8.965ml 10 X HBSS, 1ml 1M MOPS, and 455 ml 1N HCL. The physico-

chemical properties of this stock solution are as follows; density = 1.1230 g/ml, osmolarity = 301 mosm/kg, pH=7.3. By the equation given below, any desired density can be obtained from the stock solution. 70%, 66%, 60%, and 50% Percoll were made to separate high and low density of mouse B cells. All gradients were kept on ice in order to have ideal separation. 70% Percoll was prepared by mixing 290ml Percoll with 170ml percoll mix solution (45ml 10 X PBS, 3ml 0.6N HCl, 132ml H_2O, the pH was adjusted to 7-7.2, and the mix solution was sterilized using a 0.22μm filter, Flowpore, ICN). The gradient was prepared by adding 2.5ml of 70% Percoll to a 15ml tube, then 2.5ml of 66%, 60%, and 50% Percoll were added gently over 70%, 66%, 60% Percoll, respectively. Prepared gradients were kept on ice for 15 minutes before adding cells and centrifugation.

50%, 60%, and 66% Percoll were prepared as follows;

	55%	60%	66%
70% Percoll, ice cold (ml)	21.42	24.74	28.29
HBSS, ice cold (ml)	8.58	4.26	1.71

5 - Culture conditions

Culture was carried out in HBSS/HSA supplemented with 100IU/ml penicillin (Gibco, Life Technologies Ltd, Paisley, U.K.), 100mg/ml streptomycin (Gibco),

Table 2-3

Density	Stock Percoll	Hanks/MOPS
1.055	43ml	57ml
1.060	47.1ml	52.9ml
1.065	51.4ml	48.6ml
1.072	57.2ml	42.8ml
1.077	61.5ml	38.5ml
1.080	64.0ml	36.0ml

Table 2-3 shows the preparation of working Percoll

2mM L-glutamine (Gibco), 0.075% Sodium Bicarbonate (Gibco), in a 37^0C incubator with 5% CO_2. Mouse B-cells were cultured in RPMI/ 10mg/ml HSA under the same conditions. Culture supernatants (SN) were prepared by culturing resting and activated cells in IL-4 at 100u/ml for 20 hours. After centrifugation, the supernatants of high-density B cells cultured in IL-4 at 100u/ml were collected and retained at -20^0C for assays.

6- Preparation of Fixative

Two fixatives were used in this project as follows:

(1) Glutaraldehyde, grade II, was obtained from Sigma as a 25% aqueous solution. The working solution (2.5%) was prepared by mixing 1:9 with normal saline and was stored at 4^0C. An equal volume of this solution was used to fix the cells in polarization and collagen assays.

(2) 1% paraformaldehyde (BDH) was prepared by dissolving one gram in 100ml of PBS at 60-70^0C for approximately one hour. The pH was adjusted to 7.4, and the solution stored at 4^0C. This solution was used as a fixative in immunoflourescent assays.

7- Preparation of Collagen

Type I collagen was prepared from adult rat tails. The method was based on that of Elsdale & Bard (1972), and modified by Haston & Wilkinson (1988a). Adult rat tails were collected and stored at -20^0C. 6-12 tails were thawed at room

temperature and sprayed by alcohol. The skin was stripped from the tails. The bundles of tendons were removed (each tail contains 4 bundles) and soaked with stirring, in 250ml of 3% (0.5M) acetic acid in water, overnight at 4^0C. The collagen dissolved and tropocollagen was released in the solution. If the solution was too concentrated more 3% acetic acid was added and the solution incubated at 4^0C overnight. The solution was filtered through Monofilament-Nylon filter cloth (R.Cadisch & Sons, London, UK) to remove any undissolved materials and was centrifuged at 3,000 X g for 45 minutes at 4^0C to deposit any debris. This solution was mixed with 10% NaCl (w/v) and incubated at 4^0C overnight to precipitate the collagen. The supernatant was removed and the pellet was centrifuged at 3,000 X g for 45 minutes at 4^0C. The precipitated collagen was transferred into a sterile flask and 250ml of 3% acetic acid was added, and this was incubated at 4^0C. All processes from dissolving in 10% NaCl onward were repeated once more. The collagen then was dialysed against water with several changes for three days and the pH was adjusted to 4 with HCl. This solution was aliquoted in 20ml universal tubes and stored at -20^0C until used. The concentration of collagen was determined as follows: An empty universal tube was weighed and 10ml of collagen was added to the tube. The collagen was freeze-dried overnight, and the tubes reweighed. The concentration of collagen was calculated by measuring the optical density at 230nm using a U-V spectrophotometer (LKB, Biochrom, Ultrospec 4050). The result was compared with that obtained using a freeze-dried sample from which a standard curve was drawn.

8- Preparation of collagen gels

The stock collagen solution was prepared as above at 4.8 mg/ml and stored at -20^{0}C. For assay three dimensional collagen gels were prepared at 1.5 mg/ml throughout this project. Collagen was thawed at room temperature, then placed on ice to delay the gelling time. To return it to physiological osmolarity and pH, it was diluted appropriately with 10 X HBSS, 1M MOPS and water. The gel was prepared at twice the final concentration required. The collagen gel was added to the mixture of 10 X HBSS at 1 in 10 final volume, penicillin/streptomycin at 1 in 100, L-glutamine at 1 in 100, 7.5% of sodium bicarbonate at 1 in 100, and distilled water up to the final volume. The pH was adjusted to 7.4. The solution was mixed quickly and equal volumes of appropriate locomotor stimulants were added immediately. After mixing well, 0.5ml volume of this was transferred to the wells of 24-well plates (NUNC, Roskilde, Denmark). The gels set in approximately 15-20 minutes at room temperature. The gel surface was covered by 400ml of HBSS to prevent gels drying and formation of an impenetrable surface skin (Haston et al, 1982).

9- Assessment Of Cell Viability

Before any functional studies were performed on the isolated and purified cells the viability of the suspensions were established. The most popular current viability assessment is by means of vital dye exclusion studies. Most of these techniques used to assay cell viability examine late events. Thus trypan blue was

not ideal to show apoptotic cells in culture. Therefore, morphological criteria and Kimura stain were used to identify these cells.

9-1 Trypan Blue Method

Stock trypan blue at 0.2% (w/v) in distilled water and 5 x normal saline [4.25% (w/v) NaCl] was prepared. For use, four parts of 0.2% trypan blue were mixed with one part of 5 x saline. The cell suspensions were prepared at 2-5 x 10^5 cells/ml. One part of cell suspension was diluted with one part of trypan blue/ saline solution. After mixing well, the cells were examined on chambers or slides under 40 x objective and a 10 X eyepiece lens.

Viable cells exclude the dye initially, while nonviable cells take up the dye, thereby enabling a direct visual distinction between unstained viable cells and blue-stained non-viable cells. After staining the cells, they must be counted within 3 minutes, because after that time the viable cells begin to take up dye.

9-2 Eosin Y Method

Eosin Y (BDH) was prepared at 0.2% in saline and stored at room temperature. If a precipitate forms, it could be removed by passing the solution through Whatman number 1 filter paper (Whatman International Ltd. Maidstone, UK). A cell suspension was prepared at 2-5 x 10^6 cells/ml and one part was mixed with one part of 0.2% eosin Y. Dead cells take up the dye and become reddish in colour. The advantage of this technique is that the time is less critical

than for trypan blue staining and viable cells remain unchanged even 10 minutes after staining. Since both dyes have a great affinity for proteins, it is best to eliminate serum from the cell suspension.

9-3 Morphological criteria

Phase contrast microscopy was used to investigate viability of cultured tonsillar cells especially GC B cells. After cells were cultured overnight, they were fixed with 2.5% glutaraldehyde. The dead and live cells were differentiated by their morphological appearance under a x40 objective using phase contrast microscopy.

9-4 Kimura stain

0.05% toluidine blue solution was prepared by dissolving 0.05g of toluidine blue (Merck) in 50ml of 1.8% NaCl in distilled water and 22ml of ethyl alcohol. After mixing well, distilled water was added to make the final volume 100ml. Kimura stain was prepared by mixing of 11ml of 0.05% toluidine blue solution, 0.8 ml of 0.03% light green (Merck) solution in water, 5ml of 1/15M phosphate buffer (pH 6.4), and 0.5ml of a saturated solution of saponin (Saponin white, Merck) in 50% ethyl alcohol. The 1/15M phosphate buffer was prepared at 150mM by mixing 8.0g NaCl, 1.21g dipotassium hydrogen orthophosphate (BDH), and 0.34g potassium dihydrogen orthophosphate. This was diluted in distilled water to the final concentration of 0.067M (1/15M). A saturated solution of saponin was prepared by dissolving saponin white in 50% ethylalcohol in

distilled water on a stirrer till a visible precipitate formed which no longer dissolved. The stain was filtered using Whatman filter paper. This was used to check the viability of germinal centre cells, since it was an excellent method to recognize the apoptotic cells. This method gave a ideal image of polarized, spherical and apoptotic cells in GC studies. Following fixing cells with an equal volume of 2.5 glutaraldehyde, they were washed twice in normal saline. The pellet (about 100μl), after decanting the second wash solution was mixed with an equal volume of kimura stain, following incubation for 2-5 minutes at room temperature. This was used to make slide and coverslip preparations which were studied under a x40 objective using a light microscope.

10 : Preparation of treated SRBC

10-1 Preparation of SRBCs

Whole sheep blood was obtained from SAPU (Carluke, UK). 1.5ml volumes of whole blood were layered onto 3ml volumes of separating medium - Lymphoprep (Nycomed UK Ltd, Birmingham) in 110 x 16mm conical-based tubes (McQuilkin & company, Glasgow, UK). Mononuclear cells, plasma and separating medium were removed after centrifugation at 400g for 30 min. The pellet containing SRBCs and some polymorphonuclear cells (PMN) was collected from the bottom of the tube (Böyum, 1968). SRBCs were washed three times in PBS at 1700 rpm for 5 min. 10% SRBCs were prepared by mixing 1ml of packed SRBCs in 9ml of phosphate free saline.

10-2 Preparation of Neuraminidase Treated SRBCs

GET (Gelatine, EDTA, Tris buffer) buffer was prepared by mixing 3.72g disodium EDTA $2H_2O$ (Sigma), and 6g NaCl, in one litre of distilled water. The pH was adjusted to 7.2 with 12% Tris buffer. 12% Tris buffer was prepared by dissolving 12g tris (hydroxy methyl) methylamine (BDH) in 100ml of distilled water. One gram of gelatin (Sigma) was added to the solution. The solution was stored at 4^0C after sterilization by autoclaving.

Lyophilized neuraminidase (Sigma) was reconstituted at 10U/ml in PBS. after mixing, and aliquots of 15μl were made. 5ml of PBS, 0.3ml of packed SRBCs and 15μl of neuraminidase (10U/ml) were incubated for 30 min at 37^0C with intermittent mixing. The treated cells were washed at least three times with PBS, until the supernatant became clear, and the treated cells were resuspended in GET buffer (approximately 20ml). Treated cells can be kept for a week at 4^0C. For preparing blood B-cells, the GET buffer was removed the day after and the cells were washed in Hanks to avoid possible damage of the B-cell membrane by GET buffer.

10-3 Preparation of AET-Treated SRBCs

5% AET (Sigma) was prepared by dissolving one gram of AET in 20ml of distilled water. The pH was adjusted to 8.3 with 5M NaOH (BDH).
10ml of 10% SRBCs at 1700 rpm (approximately 518g) were centrifuged for 5 minutes and the supernatant were removed. 10ml of 5% AET that

'were sterilized using a 0.22mm syringe filter, were addad to SRBC. After mixing well, cells were incubated for 15 minutes at 37^0C. Cells were washed at least five times in Hanks, and resuspended in 4ml of Hanks.

10-4 Preparation of aCD39 (AC2)-Coated SRBCs

A stock solution of chromic chloride (BDH) was prepared at 1mg/ml in sterile saline and allowed to mature for >2 months. The pH was adjusted to 5 by 1M NaOH. AC2 (aCD39, 5mg/ml) was a generous gift from Prof. John Gordon (Dept. of Immunology, University of Birmingham, UK). One ml of 10% SRBCs was centrifuged at 1700 rpm for 5 minutes and the supernatant was removed. 60μl of anti CD39 was added to the packed SRBCs and mixed well. Then 1ml 0.1mg/ml of chromic chloride in saline was added to the solution. After adding 1ml of sterile saline, it was mixed and incubated overnight at 4^0C. Antibody coating occurs immediately, but irreversible coating requires time. The cells were washed twice in Hanks at 1700 rpm for 5 min and resuspended in 4ml Hanks.

10-5 Preparation of Anti IgD-Coated SRBCs

Polyclonal Sheep Anti-human IgD was obtained from the Binding Site Ltd (Birmingham, UK) at 12mg/ml. It was diluted 1:1 in Hanks/HSA and dialysed against Hanks to remove sodium azide. The procedure is the same as mentioned above except that 50μl of aIgD was added to the packed SRBCs.

10-6 Preparation of Anti CD14-Coated SRBCs

Anti CD14 was obtained from SAPU. The concentration varied between 6-8 mg/ml. The procedure is the same as above except that 60μl of anti CD14 was used.

11 Perparation of B Cells

Four sources of B-cells were used in this project and the separation methods for each were slightly different. These sources were human blood; human tonsillar high and low density cells; GC B cells derived from the low density fraction of human tonsillar cells; and finally mouse spleen and lymph node B cells.

11-1 : Blood Mononuclear Cells From Normal Individuals

Peripheral blood B-cells, for functional studies, were prepared from either heparinized venous blood from colleagues or from buffy coats (BC), from the Blood Transfusion Centre - St Vincent Street, Glasgow - by density gradient separation (Böyum, A. 1968). Blood was collected into plastic universal tubes containing 3-4U of heparin (CP Pharmaceutical Ltd, Wrexham, UK) per 1ml of blood. 5ml volumes of blood were layered over 2.5ml of separating medium at a density of 1.077 g/ml (20^0C)- Lymphoprep - before centrifugation at 1100 rpm (approximately 220g) for 30 min at room temperature in 110 x 16mm conical-based tubes. Following removal of the interface fraction which is composed of mononuclear cells (MNC), basophils, and platelets, the cells were washed three

times with Hanks at 900 rpm for 10 min in order to eliminate the majority of contaminating platelets. Then the cells were counted in a Neubauer counting chamber to determine the concentration of cells and the percentage of monocytes and lymphocytes by Kimura stain. In contrast to blood, buffy coat was very concentrated. Care was taken to avoid overloading the gradient, which causes `streaming`(bulk sedimentation) and gradient disturbance, due to the fact that at high concentration the cells are influencing the specific gravity of the medium. Also the cells may first collect at the boundary and then massively enter the denser layer, thus causing streaming. To avoid this buffy coat was diluted to 1:10 v/v. However, an excellent result was obtained when the concentration was adjusted to 3-4 x 10^6 cells/ml with Ca++ and Mg++ -free Hanks. The mononuclear cell separation was the same as mentioned above except that 35ml volumes of diluted blood were layered onto 15ml of separating medium in 50ml tissue culture centrifuge tubes. Two methods were used to separate B cells from T cells in blood;

11-1-1 Neuraminidase Treated SRBC Method

The mononuclear cells were resuspended in Hanks and mixed at approximately 5 x 10^6 cells/ml with neuraminidase treated SRBCs in a siliconised glassware tube to prevent further adhesion of B-cells to plastic. Then an equal volume of neuraminidase treated SRBC was added to the cell suspension which was mixed well, gently inverting for 1-2 minutes by hand. Rosette formation was induced by

preincubation of the cell suspension for 15 minutes, followed by spinning at 1500 rpm (approximately 400g) for 5 min and incubation of cells on ice for 1 hour (Ownby, et al, 1983). The cells were resuspended in the same supernatant very gently. Then 6ml volumes of treated cells were layered onto 6ml volumes of separating medium - Lymphoprep. The cells were spun at 1100 rpm for 25 min in order to collect B-cells and monocytes from the interface. Human T-cells and some NK cells express cell surface CD2 antigen (Robertson et al, 1990) which binds to specific receptors on sheep erythrocytes (E) resulting in the formation of E-rosettes. E-rosette forming cells (E+) were separated from non-rosette forming cells (E-) by density gradient centrifugation. The non- rosetting cells were collected from the interface and washed twice.

To remove monocytes, the cell suspension was incubated in a plastic petri-dish at 37^0C for 1.5 Hrs. The adherent cells adhered to the dish and non-adherent cells were washed three times in Hanks and removed by gentle pipetting. Alternatively monocytes were depleted together with T-cells by using aCD14-treated SRBCs along with the neuraminidase method. Roughly 1ml of aCD14 treated cells were used per 20 x 10^6 MNCs. The procedure from then on was followed as mentioned above.

11-1-2 AET Treated SRBC Method

10 x 10^7 MNCs were mixed with 1ml of AET-treated SRBCs. After centrifugation at 1500 rpm for 3 minutes, the cells were incubated on ice for one

hour. Then cells were resuspended in the same supernatant, gently. Roughly 4ml of cell suspension (approximately 30 x 10^7 cells) were layered onto 6ml of separating medium in universal tubes and were centrifuged at 1100 rpm for 30min. The cells were collected from the interface carefully. After washing twice, the cells were mixed again with 0.5ml of AET treated SRBCs to purify further (Gordon, Guy & Walker, 1985). Finally the cells were collected as before and were prepared for assays.

11-2 Tonsil B-Cells

The tonsils that we received were removed by routine tonsillectomy from children with recurrent infections in Yorkill, Royal Hospital for Sick children, Glasgow (age range 6-15 years). Therefore they usually contained many activated B-cells and were enriched in germinal centre (GC) cells as well as containing non-GC B-cells such as recirculating cells, marginal zone cells, and antibody-forming plasma cells. The whole preparation including centrifugation steps was done on ice or at 2^0C, in order to prevent spontaneous apoptosis of cells. The tonsils were transported and then rinsed twice in HBSS/MOPS supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin to prevent bacterial growth. This medium was used for washing throughout this project. The tonsils were prepared in pairs and diced completely in medium several times in a petri-dish, and the spill was transferred to an universal tube which allowed large clumps to settle out under gravity for 2-3 min. The spill was washed twice at low speed to reduce the normal

flora (bacteria and fungi). The cells were resuspended in 20ml medium. Carefully 10ml of cell suspension were layered onto 10ml of separating medium - lymphoprep - in an universal tube and spun at 1100 rpm for 30 min. The cells were harvested from the interface and washed twice in medium. The cell suspension was prepared in 10ml of medium and was mixed with 20ml of neuraminidase treated SRBCs. The treatment followed was the same as mentioned before in the separation of blood B-cells. In contrast to blood B-cells which are mostly at a resting stage in the circulation, tonsillar B-cells are heterogenous not only in phenotype of the cells but also in density. To investigate cell interactions of different cell subsets with different chemoattractants, the cells were partially separated into distinct populations by density gradient centrifugation. Thus, the tonsil B-cells were layered on the top of a two-step discontinous Percoll gradient with specific gravity of 1.080 (65%) and 1.071 (60%), respectively. The gradients were made in universal tubes by gently overlaying 5ml volumes of 1.071 Percoll on the top of 5ml volumes of 1.080 Percoll. The B-cell population was resuspended in 10ml of medium and 5ml were layered on the top of each of two gradients. The gradients were centrifuged at 2000 rpm for 20 min to separate two major B-cell enriched fractions. High density cells (BH), which are enriched for small resting B-cells, were collected from the top of the 1.080 Percoll, while medium density cells (BM) which contain germinal centre cells were removed from the top of the 1.071 Percoll interface, but may also be distributed throughout the 1.071 Percoll as well.

11-3 Germinal Centre B-Cells

The GC B-cells were isolated by fractionation of medium-density cells (activated cells). Activated cells were resuspended in 2ml of medium, then, were mixed with 3ml of aCD39-coated SRBCs and 2ml of polyclonal aIgD-coated SRBCs. The cell suspension was mixed gently and centrifuged at 800 rpm for 3 min, followed by incubation on ice for 30 min. Then the cells were resuspended in the same supernatant and layered onto 2 x 6ml separating medium. The gradient were centrifuged at 1900 rpm for 20 min to isolate GC cells from the interface (Holder, et al, 1991). Finally, the cells were washed twice in medium and were used in assays.

11-4 Mouse B Cells

Different methods of immunization were used to study the specific locomotion response to antigens. Firstly, three mice (C57 BL/6 & BALB/c) per group were immunized using 10μg of Ova ISCOMS (0.19mg/ml) by intraperitoneal injection and mice then were killed 8, 14 or 21 days after immunization and three non-immunized mice were used as a control. In one experiment, a second challenge, 10μg of soluble antigen in saline, was given intraperitoneally. The mice were killed after 10 days by cervical dislocation. The animals were immobilized on a dissection board and the abdomen was wiped with alcohol. The skin and muscles were incised and stripped laterally to expose the peritoneal membranes. The freshly isolated spleen was removed and transferred to a 60 X 15-mm petri-dish

containing 20ml cold RPMI/HSA, then was divided at the midpoints with a 23-gauge needle. The uncut end of the spleen capsule was held and pieces of spleen were pressed against the cut end until mostly fibrous tissue remained. This expresses most of the spleen cells. The suspension was repeatedly drawn up and expelled several times through a 5-ml plastic syringe to break up cells clumps, then transferred to a 50ml centrifuge tube. After 10 minutes large clumps of debris settled out and the supernatant was transferred into a 50ml plastic tube. The cells were washed twice with cold RPMI by centrifugation at 4^0 C for 10 minutes at 300g. The cell pellet was resuspended vigorously, as clumping can occur, in 2ml of cold RPMI. The red blood cells (RBC) were removed using ammonium chloride before counting the total number of lymphocytes in the suspension of spleen cells. For lysis, 2ml of cells suspension were mixed with 8ml of lysing solution and incubated on ice for 5-10 minutes, then cold RPMI was added and centrifuged. Enrichment of B cells was achieved by complement-mediated lysis of T cells using T cell specific anti Thy-1.2 antibodies. Spleen cells were resuspended at 10^7 cells/ml in RPMI and 5mg/ml HSA containing 200 µg/ml anti Thy-1.2mAb and incubated for 60 minutes on ice. After two washes in RPMI/HSA and centrifuging for 10 minutes at 300g, the cells were resuspended at 5 x 10^6 cells/ml in RPMI/HSA containing 10% (v/v) Low-Tox rabbit complement and incubated for 60 minutes at 37^0C. The cells were washed twice in RPMI. The cell suspension (2.5ml) was layered on the top of gradients, and then spun at 1900rpm for 25 minutes at 4^0C. Cells at the 66% / 70% interface

were collected as small resting cells, whereas those at the 50% / 60% interface were large activated cells. Cells in the intermediate regions of 60% / 66% and HBSS / 50% Percoll were not used in this project. Cells were washed twice at 1200rpm for 10 minutes before running any experiments. In the second studies, CBA/BALB/c mice were divided into three groups of 8 animals. The first group were primed by interaperitoneal injection of 0.5mg of ovalbumin and $2x10^9$ heat killed *Bordetella Pertussis* (25mg of antigen was mixed with killed bacteria to a final volume of 1ml, and 0.2ml were injected per animal). The second group were primed by food pad injection of 0.5mg of ovalbumin in complete Freund`s adjuvant (antigen was prepared at 200mg/ml and mixed vigorously with an equal volume of complete Freund`s [sigma] to get a homogeneous emulsion. 0.05ml of this was injected per animal). The third group received no primary injection. 30 days after immunization, all animals were challenged with antigen (0.5mg) in incomplete Freund`s adjuvant (Bacto, Difco laboratories, Detroit, Michigan, USA). This was prepared exactly like complete Freund`s adjuvant and 0.05ml was injected per animal. At a suitable time, 4 days after challenge, the mice were killed and B cells were separated from the popliteal lymph nodes. The lymph nodes were transferred into RPMI and then mashed through a filter. The debris was removed using sterile Monofilament-neylon filter clothes. Folowing centrifugation, the T cells were eliminated as mentioned above for splenocytes.

12- Phenotyping of Cells

Different methods were used to phenotype cells which responded to chemoattractants. The Alkaline phosphatase anti-alkaline phosphtase (APPAP) technique as described by Newman and Wilkinson (1992) was used to identify responsive cells using aCD19 and aCD22, but blood B cells failed to stain with aCD19 and aCD22 was not a pan B cell marker for blood cells. Tonsillar B cells did not express markers such as sIg homogeneously. Therefore, it was difficult to separate positive from negative cells. The phenotype of the cell populations was investigated using immunofluorescence & cytological staining, to determine the purity and identity of freshly isolated and responsive cells. This was not always easy to achieve, because of the low proportion of B-cells in blood and the heterogeneity of the B-cell populations among tonsillar and spleen cells.

12-1 May Grünwald - Giemsa Method

Cells were diluted to 1 x 10^6 /ml in Hanks solution and 0.5ml volumes were poured into cytofunnels (Shandon Southern Instruments, Sewickley, P.A, U.S.A). Cells separated directly from tonsils were spun at 500 rpm in a cytocentrifuge (Shandon) for 5 min, whereas cells cultured in IL-4 were spun at 100 rpm to prevent possible damage to the cell membrane. The slides were allowed to dry completely at room temperature. The cells were fixed by dipping in absolute methanol for 4 min and dried at room temperature. The fixed cells were stained with May-Grunwald` stain (BDH) for 4 min, then washed in tap water and

immersed in water for a further 4 minutes, followed by staining the slides in Giemsa stain (BDH) diluted 1:9 in water for 30 min. Slides were then washed in tap water. After the slides were dried at room temperature, they were mounted in DPX (BDH). The slides were investigated under a X100 oil immersion objective using light microscopy.

12-2 Kimura Staining

Beside checking the viability, this method was also used to calculate the total counts of blood, tonsillar, and mouse cells. The cell suspensions were diluted 1:10 in Eppendorf tubes (Sarstendt, Nümbrecht, Germany) and mixed well. Cells were counted in an improved Neubauer chamber. Using this stain lymphocytes and monocytes had a bluish nucleus and pale blue cytoplasm whereas the cytoplasm of eosinophils were stained a yellowish colour.

12-3 Immunofluorescence Labelling

All membrane receptors can be detected easily and quantitated by using fluorescently labelled ligands or anti-receptor antibodies. Immunofluorescence was studied using either flow cytometry on a FACScan, or by microscopy. Controls for non-specific fluorescence used either FITC- or PE-labelled mouse monoclonal immunoglobulins with no known specificity, but with the same isotype as that used for phenotyping. However defining the division between positive and negative cells in a heterogeneous population of tonsillar B cells was

often difficult, especially using aIg, or with some CD markers such as CD38 and CD23. Poor expression of these markers at certain stages of B cell differentiation may explain the weak staining. Thus determination of the percentage of cells positive for these markers is less straightforward than for those for which cells express either high marker densities, or none at all, e.g., CD3 (T-cells) and CD19 (B-cells). On the other hand, when low surface marker densities cause a low level fluorescence, cell autofluorescence may set a limit on our ability to estimate specific fluorescence. Autofluorescent activity is a function of cell type and size, and seems to be related to cell constituents such as flavins and cytochromes which tend to be found in greater quantity in larger cells. Cultured and dead cells tend to be more autofluorescent than freshly separated cells (Benson, et al, 1979.). The same problem exists in immunofluorescence microscopy, but the weakly stained cells were ignored in counting.

12-3-1 FACS Staining and Analysis

The FACScan is well established as a method used to quantify and correlate multiparameter measurements on individual cells. Cells are suspended in liquid, and pass through the focused laser beam of the FACS, and produce fluorescent and scattered light. The scattered light provides extremely valuable information in flow analysis and sorting. Forward scatter (FSC) is proportional to overall cell size, but is less sensitive to internal structure. Side scatter (SSC) provides useful data on granularity of the cell. FSC & SSC allow distinction of B-cell

subpopulations among tonsillar cells, and discrimination of blood monocytes from lymphocytes. They also allow discrimination of dead and live cells, and provide a useful method to discriminate apoptotic cells from live cells. Double staining was employed in attempts to stain two antigens within the same cell populations. For example to identify germinal centre B-cells, aCD38 and aCD19 (Dako) conjugated to PE were used. The cells were stained with aCD38 , then were labelled with FITC-conjugated rabbit anti mouse immunoglobulin. After washing, the same population was stained with PE-conjugated aCD19. 10μl of all the above antibodies were used per test except for aIg antibodies, the negative control, and aCD3-aCD19, for which 5μl was used. Cells were incubated with appropriate antibodies for 20-30 minutes on ice. Freshly separated or cultured cells were stained at 5 x 10^5 cells per tube and cells which had invaded collagen gels were stained at 2 x 10^5 cells per tube. After washing with PBS/Azide, the non-conjugated antibodies were labelled with an appropriate FITC-conjugated secondary antibody for a further 20-30 minutes. FITC-conjugated rabbit anti mouse Ig (Dako) was diluted 1:50 in PBS/azide and 50μl were used in labelling each tube. Following a final washing, the cells were fixed in an equal volume of 1% paraformaldehyde in PBS. The fixed cells can be stored at 4^0C for a week. On the day of analysis, the volume of the cell populations was increased to 250μl using a 1:1 dilution of PBS and 1% paraformaldehyde. The cell suspension was then analyzed by a FACScan using the lysis II program. 10,000 events were

analyzed for freshly isolated cells, and cells which were washed from the gels. 5,000 events were scored for cells which had invaded collagen gels.

Propidium iodine (PI) was used to mark dead or apoptotic cells so that they could be distinguished from live cells. As a last step before FACS analysis, the cells were washed in medium containing about 1μg PI per ml. Several minutes exposure to the dye results in distinct labelling of the dead cells with no detectable effect on live cells. Mouse spleen cells were prepared by washing twice in staining buffer (2% FCS in saline). 3μl of appropriate antibodies were added to the 50ml of packed cells ($5x10^5$ cells each) and incubated for 30-40 minutes, followed by washing in staining buffer twice. The non-conjugated antibodies were labelled using 50μl of diluted fluorescein streptavidin for 30-40 minutes, followed by washing twice in staining buffer. The staining procedure was carried out on ice in darkness. The cell were fixed with 1% paraformaldehyde and placed at 4^0C. Cells were then washed in FACSflow and were analysed by FACScan.

Cells were initially gated by size and granularity (side scatter and forward scatter), but gating differed from one sample to another, depending on the source of samples. Positive cells were selected by gating whereby the negative control gave less than 0.5% positive staining.

12-3-2 Immunofluorescence Microscopy

This technique allows a direct phenotype visualization of polarized cells. Poly-L-lysine (Sigma, MW >200,000) was prepared at 2mg/ml in distilled water.

Slides and coverslips were immersed in absolute alcohol and were dried at room temperature and the coverslips were coated with polylysine by dipping in this solution for 15-30 minutes. One side was cleaned and allowed to dry at room temperature. Cells were fixed with 1% paraformaldehyde for 10-15 minutes, then washed once in HBSS and 5 x 10^5 cells were transferred onto the prepared coverslips to adhere for 15-30 minutes. After removal of the fluid from the top of the coverslip, one drop of Hanks was added. 10µl of FITC-labelled aCD38 (Immunotech S.A., Marseille, France), 5µl of ahIgM, or 10µl of FITC-conjugated CD3 plus PE-conjugated CD19 antibody (Dako) were added and coverslips incubated at room temperature for 15-20 minutes. The coverslips were washed by dipping into HBSS several times. The anti-human-IgM-coated coverslip was then stained with FITC-conjugated rabbit anti mouse immunoglobulin (Dako) at 1:50 dilution in PBS/azide for a further 15-20 minutes, then the coverslips were washed. Finally the coverslips were transferred to the top of slides and mounted using nail-varnish. The proportion of polarized and non-polarized cells positive for each marker was visually investigated using a X 40 phase contrast objective and X 10 eyepiece lens on a Zeiss Axioskop fluorescence microscope (Wilkinson & Higgins, 1987). 200 - 250 cells were counted per slide. The prepared slides were stable for 2-5 days at 4^0C in the dark. To study the modulation of mIgD, the high-density cells were cultured in IL-4 (20u/ml) overnight. Following washing twice in HBSS, cells were exposed to anti-IgD or HBSS and cells after appropriate time were fixed in 3.7% formaldehyde in PBS

for 15 minutes. Following washing twice in PBS, they were transferred to polylysine (2mg/ml) coated slides. The slides then were dipped into 0.2% Triton x 100 in PBS for 10 Min., and washed twice in PBS. Cells were then stained with anti-IgD (Monoclonal Ab, Dako) for 20min. washed and exposed to FITC-conjugated secondary antibody. The slides were mounted and analyzed under fluorescent microscopy.

13 Polarization Assay

The polarization assay was used to measure the shape-change response of B cells in suspension in the presence of a uniform concentration of chemotactic factor (Haston & Shields, 1984). A wide range of reagents was tested at different concentrations either alone or in combination. The polarization assays were performed in two different ways; (1) firstly in Short Term assays (<30 min. assay, immediate shape-change) on freshly isolated and cultured cells to study the effect of attractant on locomotion of cells.

(a) Freshly isolated cells (blood, high-density, low-density, and GC tonsillar B cells from human sources or B cells separated from spleen and lymph nodes of mice). B cells were suspended at $5x10^5$ cells/tube in a final volume of 0.2 ml/tube in 110 x 16mm conical-based tubes. The tubes were then transferred to a water-bath at 37^0C for 30 minutes with appropriate attractants to allow polarized morphology to develop. They were then fixed by adding an equal volume of 2.5% glutaraldehyde (Sigma) immediately and incubated for 10-15 minutes at

room temperature. Cells were washed twice using normal saline and centrifuged at 1300rpm for 10 minutes. The pellet (in about 100μl) remaining after decanting the solution was used to make slide and coverslip preparations. The proportion of cells scored as either spherical (non-motile) or polarized (motile) was counted directly using either a x40 phase-contrast objective or Kimura stain. Cells were stained for two minutes then transferred onto a cover slide and the morphology of cells were investigated under bright light using either a x40 or x100 oil immersion objective. The Kimura method shows the morphology of cells well, and is useful in the study of mixed populations. The distinction between motile, non-motile, and dead cells was usually straightforward (Fig 2-1). The non-responding cells keep their spherical shape, whereas the polarized cells showed a distinct head-tail polarity with distinct ruffling at one edge. Apoptotic cells clearly shows fragmentation of nuclei. Direct examination is more reliable than dye exclusion. 250-300 cells were counted blind and polarization was expressed as a percentage of viable cells.

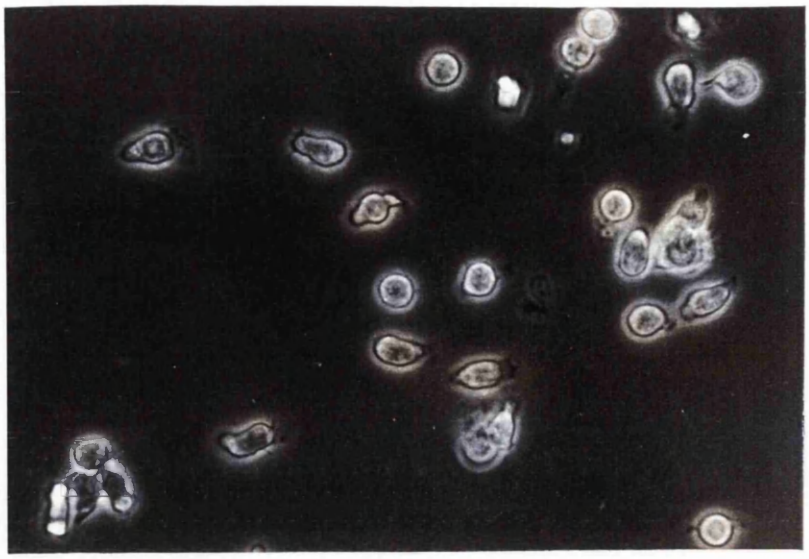
(b) Cultured cells. Cells were cultured in different reagents for 20 hours in polystyrene, round-bottom tubes (17 x 100mm) (Becton Dickinson, New Jersey, USA) at $2X10^6$ cells/ml. Following removal of the supernatant, the cells were washed twice in HBSS/HSA and exposed to different chemoattractants in various concentrations. Cells were then incubated at 37^0C for a further 30 minutes. Cells were fixed, and the procedure followed was as described above.

(2) Long Term assay (overnight culture) shows the effect of locomotor activators on locomotion. Cells were cultured in different reagents in various concentrations for 20 hours at $5x10^5$ cells/ml at a final concentration 0.5ml/tube. They were then fixed in their own supernatant using 2.5% glutaraldehyde and the percent polarized cells was calculated as described above. In time-dependent experiments, cells were cultured at $2.5x10^6$ cells/ml and after mixing 200µl of medium was harvested at appropriate intervals and fixed with an equal volume of 2.5 glutaraldehyde. They were then prepared for counting as above.

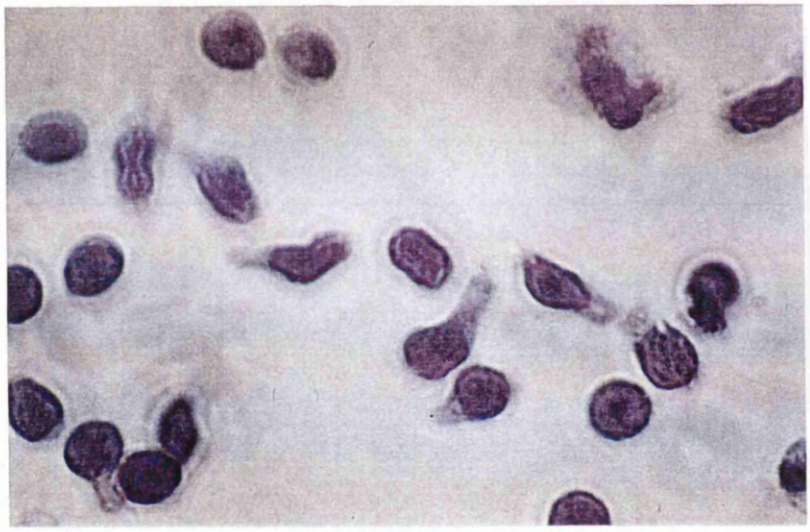
14- Collagen Gel Assay

These assays were used in the study of both high and low-density B cells and GC B cells. The following locomotor stimuli were used in high and low-density B populations; aIgD at 1µg/ml, aIgM at 1µg/ml, anti-CD40 at 1µg/ml, supernatant from IL-4 cultured B cells mixed at 1:1 with collagen, combinations of these, their appropriate isotype controls, and HBSS/HSA alone as a control. Cells that had been cultured in IL-4 (100U) for 20 hours were removed and were transferred without washing onto the pre-incubated collagen gel at 5×10^5 cells/well. Supernatant was added to gels as indicated above. Gels were incubated at 37^0C in a humidified atmosphere containing 5% CO_2 and cultured overnight (18 hours) to let the cells invade the gels. To count the percentage of invading cells, gels were then fixed with 2.5% glutaraldehyde and the numbers of cells invading the gel were counted using an inverted phase contrast microscope

Figure 2-1



a



b

Fig 2-1 Photograph showing human tonsillar B cells in overnight culture in IL-4 and IL-13.

a) morphology of polarized and non-polarized cells by Phase contrast x40 objective in IL-4. The majority of the cells are polarized.

b) morphology of polarized and non-polarized cells by Kimura stain (x100 oil immersion) in IL-13. In contrast to IL-4, fewer cells responded.

(Nikon) at 40' X magnification with an eyepiece graticule grid.

Cells on the surface of the gel in a randomly-chosen field were counted and the cells which had invaded the gel below this in the same field were counted by focusing down at different planes through the gel (Shields, et al. 1984). By these means, the percentages of cells which had invaded the gels was calculated. 200-250 cells were counted in several fields for each sample. To phenotype cells on the surface of the gel and those which had invaded, the cells on the surface of unfixed gels were removed gently and washed twice with Hanks. The gels were then overlaid with collagenase (20U/ml) at room temperature for 10 minutes to remove any remaining adherent cells from the top of the gel and washed three times with Hanks. Gels were then gently broken up by suction using Pasteur pipettes. Gels were transferred to conical tubes and incubated at 37^0C for 30 minutes with collagenase at 20U/ml (Shields et al., 1984), then the liberated cells were washed in Hanks and used for phenotyping on the FACScan. The cells were washed three times with HBSS and retained on ice. The cells washed from the gel surface and cells which were recovered using collagenase, were counted using Neubauer counting chambers. Thereby, the percentage of cells which had invaded into the gel and the proportion of dead cells in each fraction was determined using Propidium iodine and phase contrast microscopy. These cells were also used for phenotypic analysis on FACS. The same procedure was used for investigation of GC B cells except that freshly isolated cells were transferred to the top of gels containing anti-IgG,A,M (1μg/ml), anti-CD40 (1μg/ml), IL-4

(50U/ml), and combination of these/or their appropriate isotype controls, and HBSS/HSA as a control. The cells on the top of gels were cultured with or without IL-4 (50U/ml) for either 6 or 12hours. The invaded cells after 12 hours incubation were analysed for phenotype on the FACS. The cells were harvested exactly as mentioned above for high-density B cells.

15- Time Lapse Cinematograpgy

This technique was used to study cell locomotion. It was interesting to know how the B cells really move. A television camera, a time lapse video tape recorder, a television monitor, and a time / date indicator give a record of real time for analysis. The advantage of this technique is that it allows the film to be moved forward or backward in either speeded up or real time and it is not necessary to wait for the film to be developed. It also makes it possible to investigate many cells per field in each individual experiment. Several sequences of different fields were filmed over periods of 15 minutes each. FUJI super HG videotape (E-180 colour plus professional videocassette) was used for filming.

Naturally, human leukocytes including B-cells move best at or near 37^0C. To maintain this temperature during the filming, the microscope stage was kept at a constant 37^0C using a fan heater which was coupled with miniature thermistor (R S Components, Corby, UK) and a thermometer on the microscope stage. For filming purposes, a stainless steel chamber with good heat-conducting properties was used. This chamber has a central circular hole (16mm) of 0.7 mm depth

(Allen & Wilkinson 1978). 22 X 32mm coverslips were cleaned in alcohol and were sealed on one side of the hole using high vacuum grease (Dow Corning GMBH, München, Germany) to form a shallow chamber. Collagen 1.5mg/ml (0.35ml) incorporating appropriate attractants was poured into the chamber and allow to set. Gels were prepared with appropriate chemoattractants as will be discussed latter. The transparent nature of the gels allowed for direct observation of cells. The remaining space was filled up with Hanks and 5 x 10^5 high-density tonsillar B-cells were transferred to the top of the gel. To prevent evaporation of medium, a second coverslip was sealed on the top of the chamber avoiding the formation of air-bubbles. Two slides were prepared for each experiment. The chamber was transferred to the prewarmed inverted phase-contrast microscope stage (Nikon) for filming at low magnification (x 20) since a large field was preferred with enough cells for statistical sampling. Cells could be filmed immediately after the gel set while they were still on the gel surface. Alternatively they could be allowed to invade by incubating the chambers overnight in a humidified box at 37^0C. Next day, both cells within the gel and those on its surface could be filmed.

16- Pepsin Digestion of Immunoglobulins

16-1 : Preparation of 0.07M Acetate Buffer.

0.2M acetate buffer, pH = 4, was prepared by mixing 41ml of acetic acid

solution (Fisons.; 11.55ml of glacial acetic acid in one litre of distilled water) and 9ml of sodium acetate solution (BDH.; 16.4g of sodium acetate in one litre of distilled water). 0.07M acetate buffer was prepared by dissolving of 7 volumes of 0.2 acetate buffer, plus 10 volumes of 0.1M NaCl (5g NaCl in one litre of distilled water), and 3 volumes of distilled water.

16-2 Digestion of aIgD

Sheep polyvalent anti human IgD (IgG fraction) was digested with pepsin for further functional studies. The proteolytic enzyme, pepsin, cleaves IgG at the carboxyl end of the hinge region and liberates a 95,000 MW bivalent fragment, termed $F(ab`)_2$ and fragments the remaining Fc portion of the molecule to give products ranging in MW from 5,000 to 27,000 which lack antibody activity, depending on the condition of digestion. The $F(ab`)_2$ fragment contains approximately two-thirds of the original molecule, with intact antigen-binding activity. Digestion was carried out at pH 4. The concentration of aIgD was measured using a spectrophotometer (Ultrospec 4050, LKB Biochrom) at 280nm before digestion. The original concentration was 15mg/ml and 10mg/ml of HSA was added to avoid losing protein during the process. Anti IgD was dialysed against acetate buffer several times at 4^0C. The effect of pepsin on IgG is related to concentration. The optimum result is obtained at 8-10mg/ml of the antibodies. Thus the aIgD was diluted to 10mg/ml after dialysing with acetate buffer. 0.375mg of pepsin (Sigma) then was added to aIgD at ratio of 1:40 (w/w). After

mixing well, the solution was incubated at 37^0C overnight (18 hours). Digestion was terminated by adjustment of pH to 8 with 1M NaOH. The preparation was centrifuged at 3,000 rpm for 10 minutes to remove any precipitate. The supernatant was dialysed against PBS (pH 8) for several days.

16-2 Digestion of aIL-4

Anti IL-4 also was digested to abolish the possible effect of binding to Fc receptors on activation of B-cells. The same process was followed as for aIgD except that aIL-4 was diluted to 200μg/ml by acetate buffer at 1:5 and 10mg/ml HSA was added. Digestion was carried out directly by pepsin without dialysing.

17- SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

17-1 Preparation of Reagents and Buffers for SDS-PAGE.

17-1-1 Preparation of Stock Solutions

The following stock solutions were made up and used for the separating gel, stacking gel, and other working reagents:

10% SDS (Sodium Dodecyl Sulphate.; FSA Laboratory Supplies, Loughborough, UK) was dissolved in 10g per 100ml of distilled water. 0.75M Tris (BDH) was prepared by dissolving 90.9g in one litre of distilled water. The pH of the solution was adjusted to 6.8 and 8.8 using concentrated HCl. 0.86M Glycine was prepared by dissolving 32.2g of glycine (Sigma) in 500ml of distilled water.

Acrylamide solution, prepared by mixing 30g of acrylamide powder (BRL Life Technology Inc, Gaithersbury, UK), was added to 0.8g of N N`-methylene bis acrylamide (BDH) and dissolved in up to 100ml in distilled water avoiding heating. The powder dissolves slowly. This solution remains stable for four weeks, if stored in the dark at 4^0C.

17-1-2 Sample Buffer

To prepare the sample buffer, the following stock solutions were mixed to a final volume of 100ml in distilled water; 30ml of 10% SDS, 8.5ml of 0.75M Tris pH 6.8, and 10ml of glycerol (BDH). This solution was boiled for two minutes and then 10mg of bromophenol blue (Sigma) was added to the solution.

17-1-3 Well Buffer

The well buffer was prepared by mixing 10ml of 10% SDS, 33ml of 0.75 Tris pH 8.8, 220ml of 0.86M glycine, and 737ml of distilled water. The pH was adjusted to 8.3. The buffer was stored at 4^0C.

17-1-4 Gel Recipes

To run electrophoresis, two gels were prepared, (a) the separating (lower) gel and (b) the stacking (upper) gel. The separating gels were prepared and used at two different concentrations (7% & 10%). They were mixed completely and TEMED (N N N`N`-Tetramethylethyienediamine; Sigma) and ammonium

persulphate (BDH) were then added. The gels were poured onto the glass plates immediately to polymerize. The separating, and the stacking gel were prepared as shown below:

Stock solution	Separating gel		Stacking gel
	7%	10%	
Acrylamide	10ml	10ml	1.8ml
Distilled water	9.6ml	4.7ml	13ml
Tris, pH 8.8	20ml	15ml	----
Tris, pH 6.8	----	----	3ml
10% SDS	0.40ml	0.30ml	0.18ml
TEMED	20ml	15ml	9ml
Ammonium persulphate	40mg	30mg	18mg

17-1-5 Staining and Destaining/fixing solution

125ml of methanol (Fisons), 25ml of glacial acetic acid, and 100ml of distilled water were mixed well and 0.25g of coomassie brilliant blue stain (Sigma) was then added.

Destaining/fixing solution was made by mixing 30ml methanol, 30ml of acetic acid, and 240ml of distilled water.

17-1-6 Preparation of Samples

Rainbow TM protein molecular weight markers (Amersham, Arlington

Heights, Illinois, USA), at 200, 97, 69, 46, 30, 21, 14, were mixed 1:1 with sample buffer at 20mg/well. Digested aIgD or undigested aIgD were mixed 1:3 with sample buffer at 30mg per well. Digested aIL-4 and undigested aIL-4 were mixed at 1:1 with sample buffer at 3.5mg/well and 5mg/well, respectively. BSA and HSA were mixed 1:3 with sample buffer at 25mg/well, as a control.

17 SDS-PAGE ASSAYS

7% and 10%acrylamide solutions were made up as indicated before. The glass plates were attached with high vacuum grease. The glass plates were placed in vertical position and the separating gels were poured between the plates with about 2cm gap at the top of the gels. The surface of the gel was then overlaid with water or isopropanol to produce a smooth surface. After polymerisation, the isopropanol and water were removed completely and the stacking gels were poured into the glass plates on the top of separating gel. The comb was inserted between the plates. After polymerisation of stacking gel, the bottom blade was removed and the glass plates were transferred into the electrophoresis apparatus. The lower and upper tanks were filled up with well buffer to cover the gel and also to circulate the electric current. The comb was removed gently and the samples were loaded into the wells. The upper tank and lower tank were connected to the negative and positive terminal of the power supply. The system was run at a constant current of 40mA until the front dyes reached the bottom of the gel. The system was disconnected and the gel gently transferred into the

staining dish for 1-2 hours. The stained gel then was transferred into the destain dish for hours with several changes. The gels were dried in gel dryer and the samples were analysed.

18 - Autoradiography

18-1 Preparation of Emulsion :

Autoradiography emulsion type NTB2(Kodak) was obtained from IBI, Ltd(Cambridge, UK). This is actually in solid form and liquefied before use by dipping in a water bath between 43-45^{0}C . Liquefaction takes about 45-60 minutes by gentle movement under safelight condition in the dark-room. Too much agitation can lead to the formation of microscopic bubbles in the emulsion which can be difficult to remove.

18-2 Preparation of Kodak Dektol Developer:

The developer (Kodak Dektol, IBI) was diluted by adding the contents of the packet with sufficient stirring in 828 ml of pre-warmed distilled water (32-38^{0}C) until the solution became uniform. Enough water was added to the solution to make a final volume of one US quart (946ml)at 32-38^{0}C. This was diluted one-to-one with distilled water before use.

18-3 Preparation of Kodak Fixer :

The fixer (Kodak, IBI) was added to 710ml distilled water (26.5^{0}C) slowly with rapid and continuous stirring. When the powder was dissolved completely,

distilled water was added to bring the total volume to one US quart.

18 Method

Autoradiography was used to investigate the relation between expression of RNA or DNA and locomotion using [^{3}H]uridine and [^{3}H]-thymidine. Incorporation of uridine in B cells was studied in freshly isolated cells and cultured cells ; (1) resting cells isolated from tonsil were pulsed with uridine for 30 minutes at 37^{0}C by adding of 5μCi of [^{3}H] uridine per 1x10^6 cells/ml, the free uridine was removed by washing twice in Hanks, then resuspended in hanks alone, aIgD, or aIgM for 30 minutes at 37^{0}C (immediate shape-change); (2) cells were cultured in IL-4 (20 and 100U/ml), pulsed with uridine, fixed using 2.5% glutaraldehyde and washed twice; (3) cells were cultured in IL-4 (20U/ml), pulsed with uridine and washed twice. These cells were not fixed but were resuspended in different chemoattractants for 30 minutes (immediate shape-change) at 37^{0}C. At the end of this period the cells were fixed with 2.5% glutaraldehyde and washed. The fixed cells were then transferred to polylysine (2mg/ml) coated slides. The slides were dried at room temperature. Under safelight condition, these were embedded into the liquefied emulsion and allowed to drain for 4-5 seconds. The backs of the slides were wiped and they were placed horizontally to retain a uniform thickness in a light-tight (but not air-tight) box for 1-2 hours or until completely dry. The dried slides were placed into slide boxes and transferred into a dessicator containing silica gel. The dessicator was sealed with Sleek and placed in a black polyethylene bag. This was left for two

weeks at 4^0C. The exposed slides were developed by using Kodak Dektol developer for one minute under safelight. The developing process was stopped by dipping the slides into distilled water for 10 seconds and fixed by placing the slides in the Kodak fixer for 5 minutes. The slides were washed in distilled water for 5-10 minutes and dried slowly in a dust free atmosphere. These were then stained with 1:50 diluted Giemsa in water for 20 minutes, and examined using a x100 oil-immersion objective. The numbers of silver grains per cells and the percentage of cells which took up the uridine were determined. 200-250 cells were counted on each slide. To study incorporation of thymidine and locomotion, the same procedure was used. High-density B cells were studied in overnight culture. The cells were cultured in IL-4 for overnight and then pulsed with thymidine. The procedure followed was exactly as described for uridine incorporation. The same technique using uridine and thymidine was also used to study GC cells either freshly isolated or cultured with IL-4 and anti-CD40.

RESULTS

CHAPTER THREE

LOCOMOTOR PROPERTIES AND PHENOTYPIC ANALYSIS OF HIGH AND LOW DENSITY TONSILLAR B-CELLS

SECTION ONE : Phenotypic Analysis of Cell Populations

3-1-1 Tonsillar Cells

Tonsils were minced and mononuclear cells were recovered by centrifugation on Lymphoprep. The cellular components of tonsils varied from one individual to another, depending on age, type and frequency of infection. The chief cellular components of inflamed tonsils are monocytes, FDC, T-cells and B-cells. Mononuclear cells were analyzed on the FACS using CD3 as a pan T-cell marker, CD4 as a T_H cell marker, CD8 as a CTL cell marker, CD19 as a pan B-cell marker, CD14 as a monocyte and FDC marker (Schriever et al, 1989; Petrasch et al, 1990). CD14+ cells may be present on both cell types, and the B cells may also express this marker (Labeta et al, 1991), but double staining using PE-conjugated CD19 and FITC-conjugated CD14 was not detected by the FACS.

Investigation by FACS analysis showed that before purification of B-cells, the tonsillar cells in three experiments contained 53.5 ± 1.4% CD19+ cells, 40.8 ± 3% CD3+ cells, 33.5 ± 0.9% CD4+ cells, 9.5 ± 0.7% CD8+ cells, 1.1 ± 0.45% CD14+ cells, and 97.75 ± 0.25% CD45+ cells (leucogate).

3-1-2 Tonsillar B-cells

T-cells were separated using neuraminidase-treated SRBC followed by centrifugation on Lymphoprep, and the non-T cells were then harvested from the interface and analyzed by FACS. The data show that the proportion of B cells

expressing CD19 in this population in three experiments was 94.4 ± 2%. The contaminating cells were 1.77 ± 0.5 % CD3+ cells, and 4 ± 0.65 % CD14+ cells.

B-cells were found to be heterogeneous in size, density, and functional behaviour. Density gradients (60% and 65% Percoll) were used to fractionate this population. The low density cells collected from the interface of 60% / 65% Percoll were termed `activated cells'. The high density cells were harvested from the top of 65% Percoll/medium and termed `resting cells'.

3-1-2-1 Phenotyping of Freshly Isolated Tonsillar B-Cells

The two fractions of cells (resting & activated) were phenotyped directly after separation. Cells were gated by side scatter Vs forward scatter into three regions based on size as shown in Figure 3-1. Region one (R_1) represents small cells, R_2 shows larger cells, and R_3 is a combination of R_1 and R_2. Cells were analysed and the data obtained are shown in tables 3-1 and 3-2. Further analysis demonstrated that cells in R_1 and R_2 differ not only in size but also in phenotype. This analysis indicated that R_2 cells are likely to be GC B cells rather than mantle zone or virgin cells, since most of them express CD38 strongly (Fig. 3-2), in comparison with cells from the R_1 fraction, which express this receptor weakly. IgD+ cells are rare in R_2 and expression of IgM is lower on R_2 cells than on R_1 cells (Fig 3-1). As is shown in Figure 3-3, some markers such as sIgM and sIgD are expressed weakly compared with expression of CD19 or CD20. The same results were obtained from the activated fraction, i.e. R_1 cells from the activated fraction

Figure 3-1

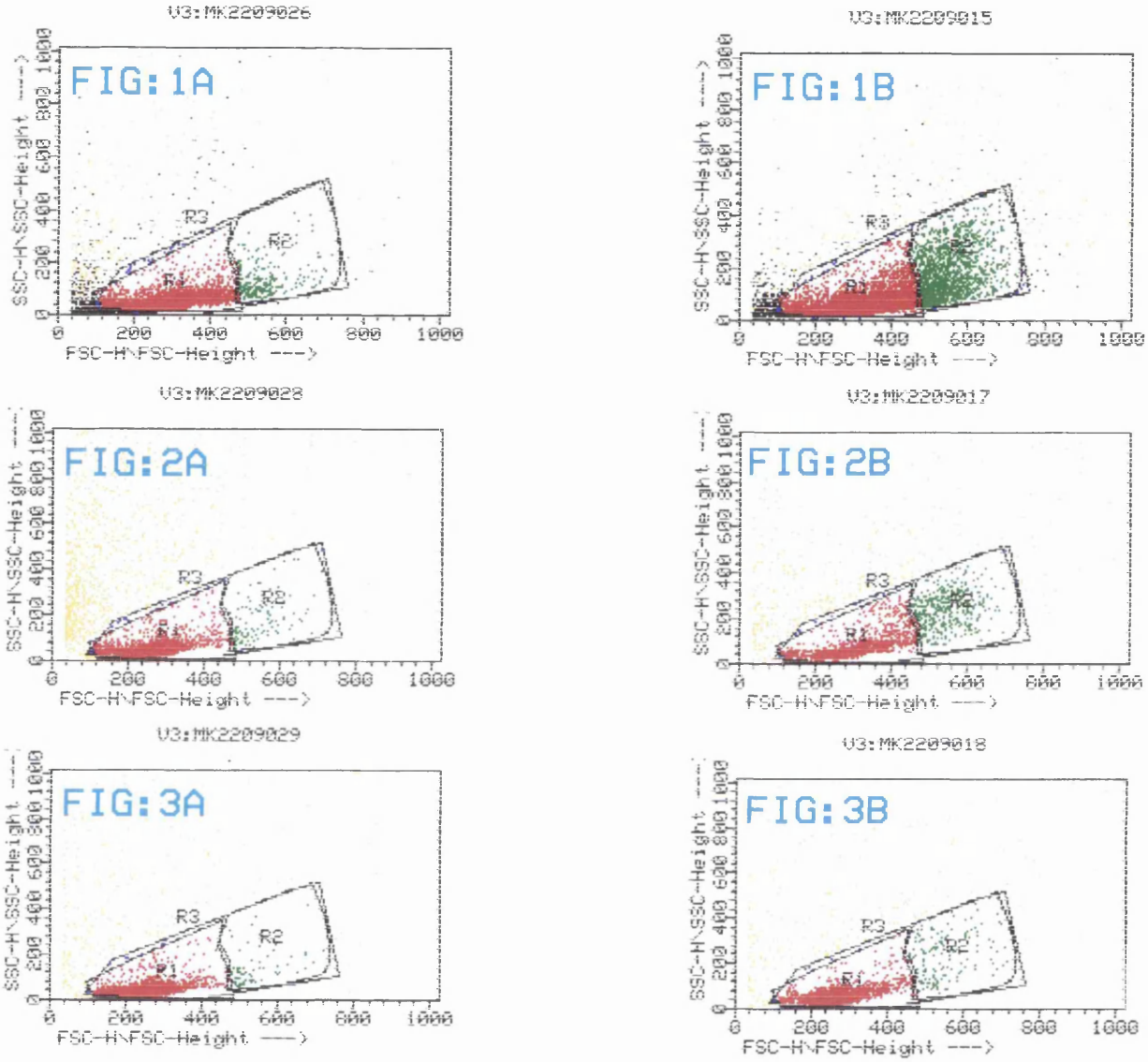


Fig 3-1 Distribution of high and low density B cells in various regions. (1a) gating of high-density cells. (1b) gating of low-density B cells. (2a) distribution IgM+ cells from high density fraction in various regions. (2b) distribution IgM+ cells from low density fraction in various regions.(3a) distribution IgD+ cells from high density fraction in various regions. (3b) distribution IgD+ cells from low density fraction in various regions

Table 3- 1

Percentage of cells positive for different markers in the resting fraction.

Cell Marker	Region one		Region two		Region three	
	Mean	Range‡	M2ean	Range	Mean	Range
CD3[10] †	1.46	0.21-3.5	0.88	0.0 - 2	1.31	0.18-3.2
CD10 [1]	9.7	9.7	24	24	4.3	4.3
CD14[2]	1.72	2.74-0.8	6.73	10.3-3.2	1.8	1.4-2.2
CD19[12]	95.9	89 - 99	95. 6	91 - 100	95. 7	89 - 99
CD20[5]	77. 9	50-93.9	83.7	38 - 99	75. 3	49-93.9
CD21[2]	85.7	81-90.3	78. 4	64-92.7	82. 5	77 - 88
CD22[1]	96	96	96.7	96.7	94.7	94.7
CD23[3]	70.7	66.2-74	85	79 - 91	63. 4	57.2-71
CD32[2]	22.1	22-22.2	25.9	26-25.6	14. 2	18.3-10
CD38[7]	71.5	45 - 89	91.5	85 - 95	63. 2	50 - 78
CD39[2]	29.3	18.6-40	19.6	15-24.3	12. 5	10-15
IgM[9]	68.5	55.7-81	29. 3	15.6-87	59. 3	38 - 79
IgD[4]	64.2	66.1-71	15.59	12.5-24	60. 1	58.7-64
IgG[2]	33.4	27-30.2	54.5	50-59	19.5	18-21
IgA[4]	20.8	18-23	34.1	31-40	15. 6	12.4-17

This table does not distinguish weakly-positive from strong-positive cells (see Fig. 3-3)

† Number in parentheses shows number of experiments.

‡ Indicated the lowest and highest of percentage events.

Table 3-2

Percentage of cells positive for different markers in the activated fraction.

Cell Marker	Region One		Region Two		Region Three	
	Mean	Range‡	Mean	Range	Mean	Range
CD3[10] †	1.31	0.0-3.6	0.29	0.0-0.4	1.12	0.0- 3
CD14 [2]	10.2	7.4-13	16.6	10.3-23	8.4	6.7-10
CD19[9]	96	91 - 98	92.7	84 - 97	95	89-98
CD20[1]	98.7	98.7	100	100	99.3	99.3
CD21[1]	91.9	91.9	81.3	81.3	89	89
CD22[2]	90. 8	84- 96.4	73.7	59.5-88	84.3	74.5-94
CD23 [2]	75.5	72-79	83.8	85-82.8	74	84-64
CD38[5]	73	65-82.8	89.5	84 - 96	74	62.5-98
CD39[2]	9.8	8.6-11	18	18.9-17	5.8	4.9-6.6
IgM[3]	73	61-80	41	35-60.6	57	29-70
IgD[3]	47.5	31.5-64	16	12 - 23	37.1	26.3-56
IgG [2]	41.3	41.6-41	44.5	51-38	32.3	37.5-27
IgA[2]	14.9	13.8-16	22	17-27	11.8	12.6-11

This table does not distinguish weakly-positive from strong-positive cells (see Fig. 3-2)

† Number in parentheses shows number of experiments.

‡ Indicates the lowest and highest percentage of events.

ND = not detected

Figure 3-2

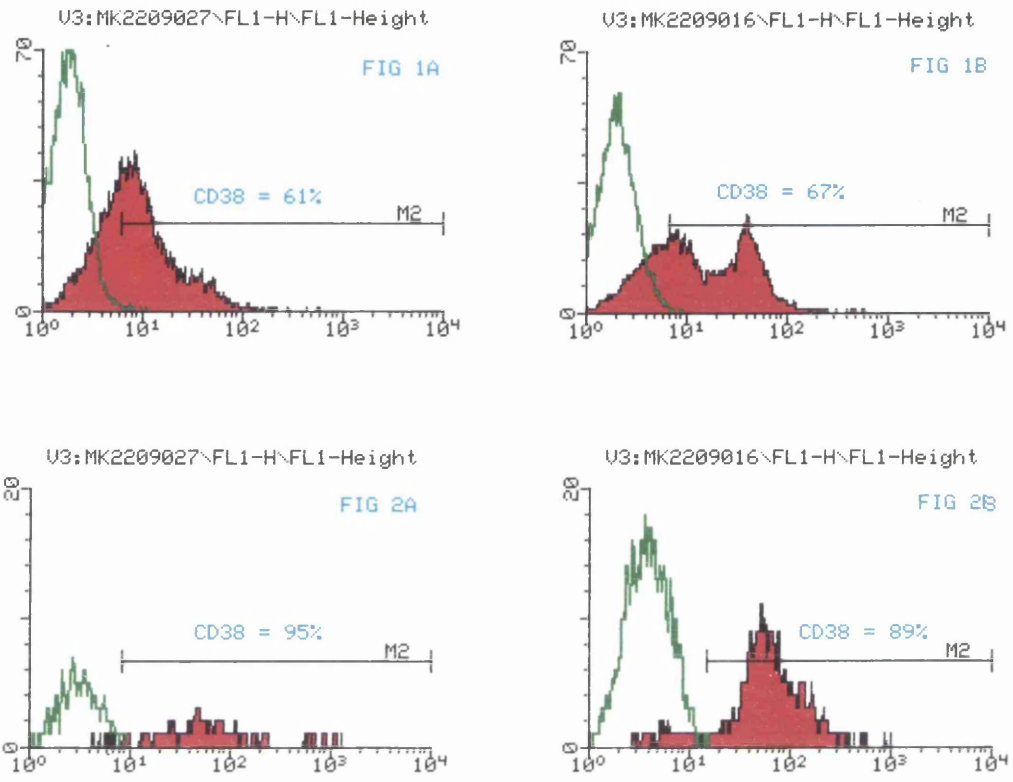


Fig. 3-2 Expression of CD38 on high and low-density fractions in region one and region two:

(Fig. 1A) Expression of CD38 in region one in high-density fraction.

(Fig. 2A) Expression of CD38 in region two in high-density fraction.

(Fig. 1B) Expression of CD38 in region one in low-density fraction.

(Fig. 2B) Expression of CD38 in region two in low-density fraction.

Figure 3-3

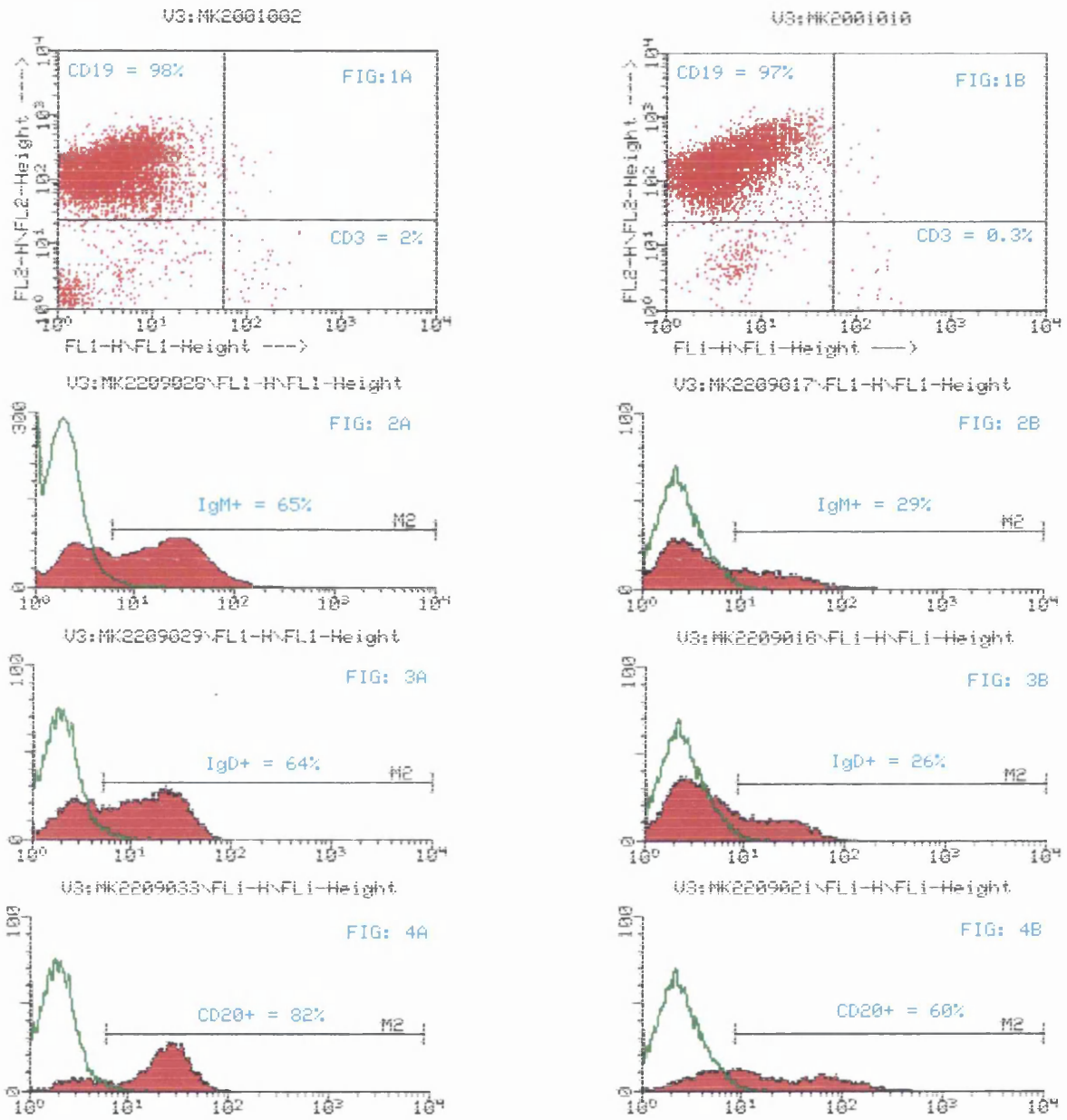


Fig 3-3 Phenotype characteristic of high and low-density tonsillar B cells. Expression of CD19+ & CD3+ (1a), IgM+ (2a), IgD+ (3a), CD20+ (4a) cells in high-density fraction. Expression of CD19+ & CD3+ (1b), IgM+ (2b), IgD+ (3b), CD22+ (4b) cells in low density fraction.

behaved like R_1 cells in the resting fraction. Table 3-1, and 3-2 show the phenotype of resting and activated B-cells enriched by density gradient from the B cell population. The values shown are the mean values for the percentage of cells positive, based on different experiments (in parentheses), with the range in different regions. Phenotypic analyses suggest that cells in region one in resting and activated fractions are similar and the latter may be activated in vivo. As a result of activation, their density may decrease and hence they settle in the low density fraction. In ten experiments, 84 ± 8% (resting cells), and 80 ± 10% (activated cells) of the total population were found in R_1. The viability of freshly isolated resting and activated cells using either trypan blue or eosin Y was determined and in all cases was more than 98%.

3-1-2-2 Phenotyping of Cultured Cells and Responding Cells

The resting and activated cells were cultured in IL-4 for 20 hours and stained and analyzed by FACS. The cell population was gated into four regions, of which region one (R_1) contained small live cells, and most probably those which were able to respond to chemoattractants. Region two (R_2) should contain larger cells. Region three (R_3) represented the whole population ($R_1 + R_2 + R_4$), and finally cells in region four (R_4) had high autofluorescence and thus were probably dead cells. As shown in Figure 3-4, after culture there were few cells in region R_2. These cells were most probably in R_4 where dead cells were located. The percentage events in two experiments for region one in the resting and activated

fractions was 68 ± 3% and 54 ± 2%, respectively. Region one cells are characteristically responsive cells in both the long-term polarization assay in IL-4 and the short-term assay (immediate shape-change) after culture of the resting and activated fraction. Most of these cells are IgM+ and IgD+. The CD23 phenotype is strongly associated with locomotor capacity. This was confirmed by analysing the invasive cells within the collagen gel. The phenotypic analysis using different markers is shown in Table 3-3 for high-density cultured cells in IL-4 after 20 hours and for low-density B cells in Table 3-4. There are differences in expression of markers in isolated high and low density cells before and after culture in IL-4. For example, the expression of sIgG in both fractions is decreased after culture in IL-4 whereas the expression of CD19 and CD20 in high density cells or expression of CD23 in low density cells is increased after culture in IL-4.

3-1-2-2-1 Collagen Gel Invasion

To phenotype locomotor cells, the cells were first cultured in IL-4 overnight, then spun to separate the cells from the culture supernatant. The cells were then layered onto top of collagen gels to which the supernatant had been added (1/2 diluted with collagen), and allow to invade. Non-invasive cells were recovered from the top of the gel, and the invasive cells recovered by digestion of the gel matrix using collagenase. To demonstrate the possible effects of collagenase on cell markers, collagenase was incubated with cells at 37^0C for 20-30 minutes at

20U/ml. The collagenase treatment had a negligible effect on expression of cell membrane markers. Morphological studies (shape-change) on the non-invasive fraction showed that 62% of cells were polarized. This finding will be discussed under time-lapse cinematograpy. For phenotypic analysis of the invasive and non-invasive cells, they were gated using the same criteria as for cultured cells. The number of events in each region is shown in Figure 3-4. Phenotypic analysis of these cells is shown in Table 3-5. The viabilities of invasive cells and those which were recovered from the top of the gel in two experiments were 86.5 ± 6.5% and 44 ± 3% respectively, for the resting cells, 62% and 8.1%, for activated cells. The percentage of invasive and non-invasive cells, either from the resting or from the activated fractions are similar to those found in R_4 of these fractions (Fig 3-4). Furthermore, most R_4 cells which had been cultured in IL-4 took up propidium iodide (PI) and the percentage of these cells was equal to percentage of dead cells in whole population. Thus most cells in R_4 are apoptotic (dead) cells. The percentage of cells in R_3 and R_4 was not scored because of autofluore scence of dead cells in R_4 . The percentage total events for invasive and non-invasive fractions for R_1 in resting fraction in two experiments were 93% and 35 ± 5%, respectively. The percentage total events for invasive and non-invasive fraction for R_1 in activated fraction were 81 ± 8% and 11%, respectively. After culture, cells that were in R2 when freshly isolated, moved to R4 (dead cell fraction).

Table 3-3

Percentage of cells positive for different markers in the resting cell fraction after overnight culture in IL-4.

Cell Marker	Region One		Region Three		Region Four	
	Mean	Range‡	Mean	Range	Mean	Range
IgM[4] †	75.9	70.5-79	58	48.7-77	31.9	22 - 42
IgD[2]	72.9	66.8-79	44.3	39.5-49	32.7	32-23.3
IgG[1]	2.4	2.4	ND	ND	ND	ND
IgA[2]	24	12 - 36	9	6 - 12	6	4 - 8
CD23[2]	91.4	89-93.9	66	65-66.9	43.7	36-55.7
CD19[1]	99.4	99.4	100	100	98.1	98.1
CD20[1]	96.4	96.4	95.2	95.2	91.2	91.2
CD38[2]	74.4	65.8-83	61.2	42.3-80	47.8	31.6-64
CD39[1]	61.4	61.4	83.6	83.6	23.2	23.2

† Number in parentheses shows the number of experiments.

‡ Indicates the lowest and highest percentage.

ND not detected

Table 3-4

Percentage of cells positive for different markers in the activated cell fraction after overnight culture in IL-4.

Cell Marker	Region One		Region Three ¶		Region Four¶	
	Mean	Range ‡	Mean	Range	Mean	Range
IgM[1] †	79	79	ND	ND	ND	ND
IgD[1]	60	60	16	16	4	4
IgG[2]	31	24-38	7	2-12	5	4-6
IgA[2]	8.5	13-4	ND	ND	ND	ND
CD23[2]	81	94-68	ND	ND	ND	ND
CD38[1]	73	73	ND	ND	ND	ND

¶ Region four (apoptotic cells) was not studied, because of autofluorescence of dead cells.

† Number in parentheses shows the number of experiments.

‡ Indicates the lowest and highest percentage.

ND not detected

Table 3-5

Percentage of cells positive for different markers in the invasive and non-invasive cell fractions from collagen gels.

	Resting	fraction	Activated	fraction
Cell Marker	Invasive	Non-invasive	Invasive	Non-invasive
CD3	0.2%	2.5%	ND	ND
CD19	97%	96%	ND	ND
CD23	80.5 ± 13.5% ¶	94%	60%	72%
CD38	43 ± 8.5% ¶	44 ± 11.5% ¶	24%	70%
IgM	74.5 ± 12.5%¶	82.5 ± 3.5%¶	66%	82%
IgD	82%	81%	75%	55%
IgG	15%	ND	19%	10%

¶ Percentage of mean is derived from two experiments.

ND not detected

Figure 3-4

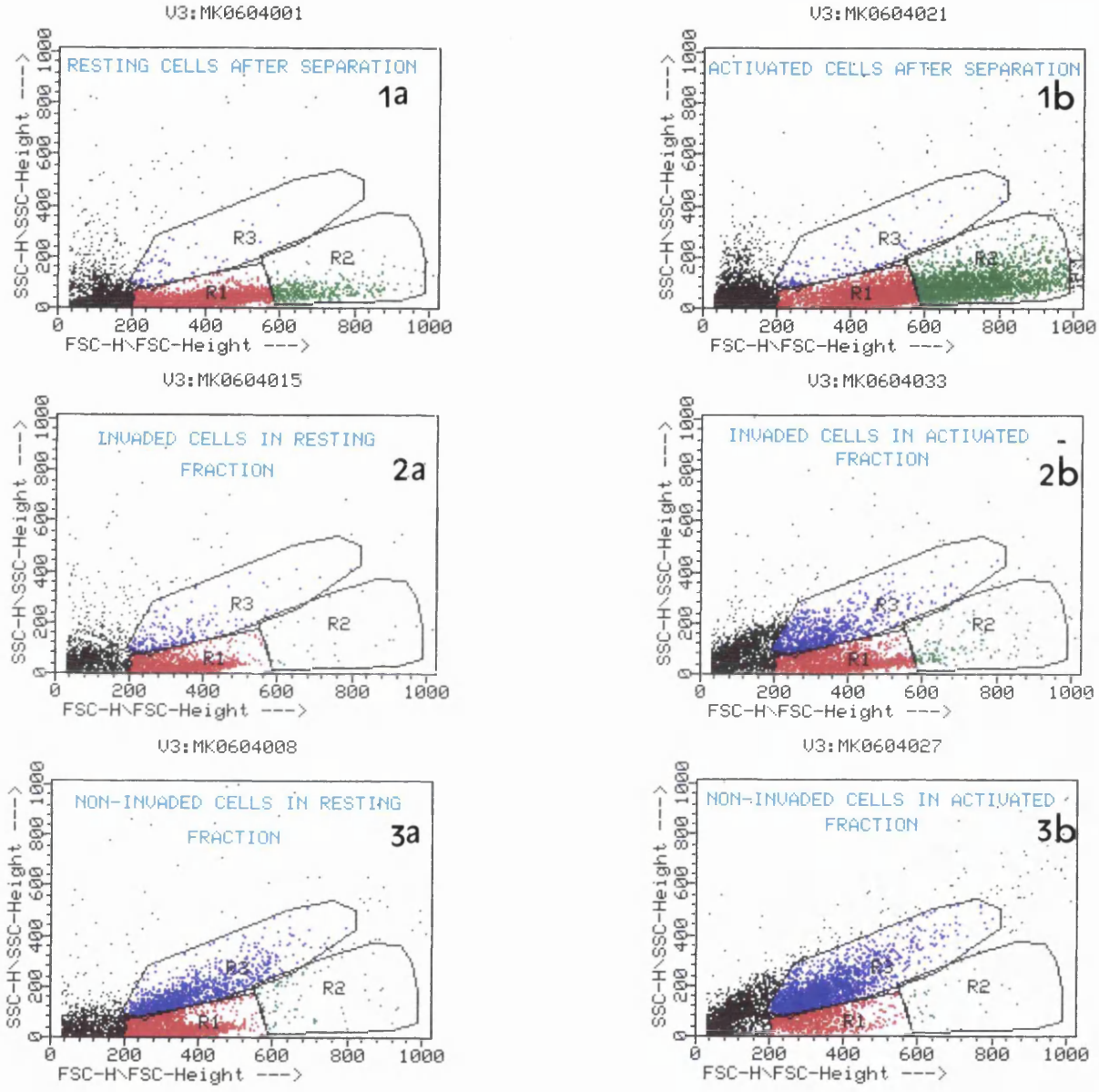


Fig 3-4 Distribution of freshly isolated, Invasive and non-invasive cells in different regions in high and low-density populations.

(1a) Freshly isolated cells in high-density fraction. (1b) Freshly isolated cells in low-density fraction. (2a) Invasive cells in high-density fraction. (2b) Invasive cells in low-density fraction.

(3a) non-invasive cells in high-density fraction. (3b)non-invasive cells in low-density fraction.

3-1-2-2-2 Polarization Assay

Fluorescence microscopy was used to investigate phenotypic characteristics of resting cells cultured in either IL-4 or medium alone (HBSS/MOPS) for 20 hours in long term polarization assays. Cells were prepared for fluorescence microscopy using anti-IgM and anti-CD38 as markers. The data obtained from cells cultured in IL-4 show that 65-86% of cells were IgM+ and 43-37% CD38+. The majority (75%) of IgM+ and 42% of CD38+ cells were polarized after culture in IL-4, whereas the responsive cells cultured in HBSS alone were 14% (IgM+) and 30% (CD38+), respectively. These data showed that IL-4, but not HBSS alone, increases the polarization of sIgM bearing cells, while the response of CD38+ cells was the same in either IL-4 or medium alone.

3-1-3 Visual Phenotyping Using May- Grünwald-Giemsa Staining

Studying of cell morphology using May-Grünwald Giemsa-stained preparations was a useful preliminary to phenotype tonsillar cells since it distinguishes large from small cells, and centrocytes with clefted nuclei were readily distinguished from small non-germinal centre cells. Cytospins were prepared from freshly isolated resting and activated fractions and from cells cultured in IL-4 for 20h. These populations contained three different subpopulations in each fraction; (1) small non-clefted cells which are mantle zone and virgin cells, (2) small clefted cells which are centrocytes, and (3) large clefted cells which are centroblasts. The proportions of cells were different in the resting

and activated fractions. Small-and large-cleaved cells underwent apoptosis when cells were cultured in IL-4, and comprised less than 10% of the whole population in each fraction. Small non-clefted cells from both fractions survived in culture, and were able to respond to chemoattractants. The data are shown in Table 3-6. A minority of CD38+ germinal centre cells with clefted nuclei were also present before culture in the resting fraction and 50% of the cells in the activated fraction were CD38+, but most of these did not survive overnight: 95% and 92% of resting and activated cells in culture after 20h were small cells with unclefted nuclei. Thus live cells from both fractions are similar not only in surface phenotype but also in morphological characteristics. The apoptotic cells mostly were destroyed during spinning in cytospin preparations, whereas they were visible in wet preparations under phase contrast microscopy or using Kimura stain. The morphological appearance of high density cells before and after culture in IL-4 is shown in Fig 3-5. In contrast to cultured cells, freshly isolated high-density B cells has a condensed chromatin of the nucleus, indicating low activation, relatively little cytoplasm, and were smaller in size. Fig 3-6 shows a photograph of low density cells before and after culture. Non-clefted small cells in both fractions have a relatively scanty cytoplasm, whereas the cultured cells were larger with more abundant cytoplasm.

SECTION TWO : LOCOMOTION ASSAYS

The locomotion of resting and activated B cells was measured using; (1)

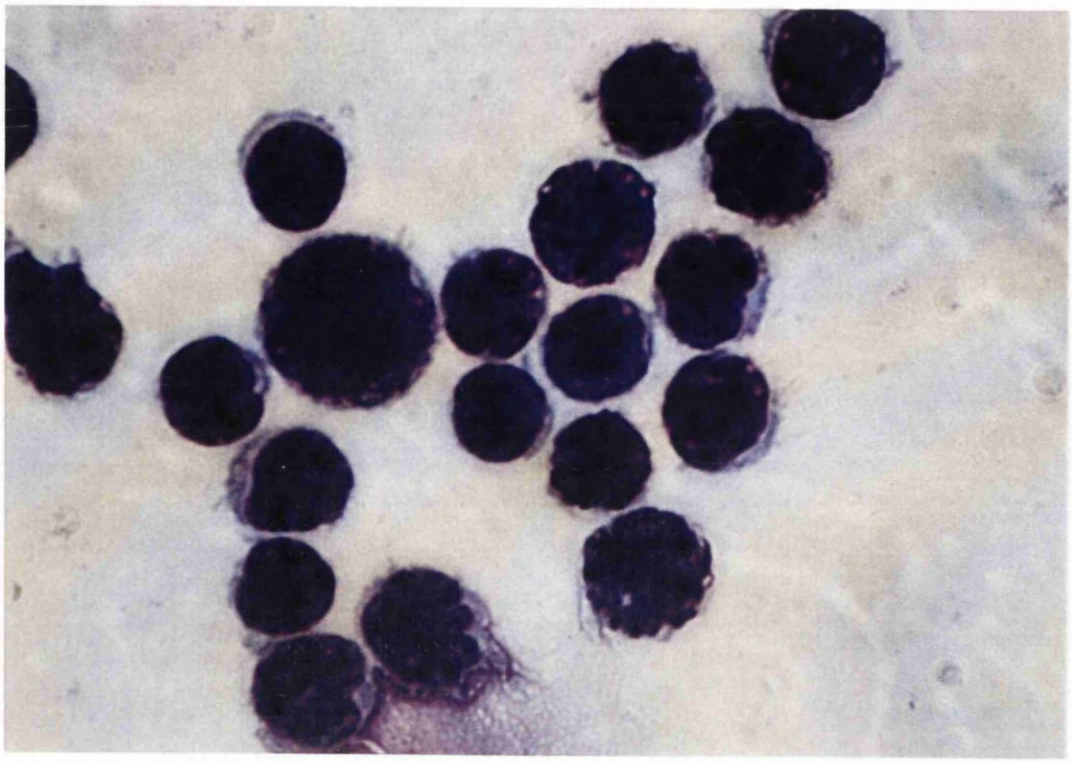
Table 3-6

Morphological differences among high and low-density B cell before and after culture.

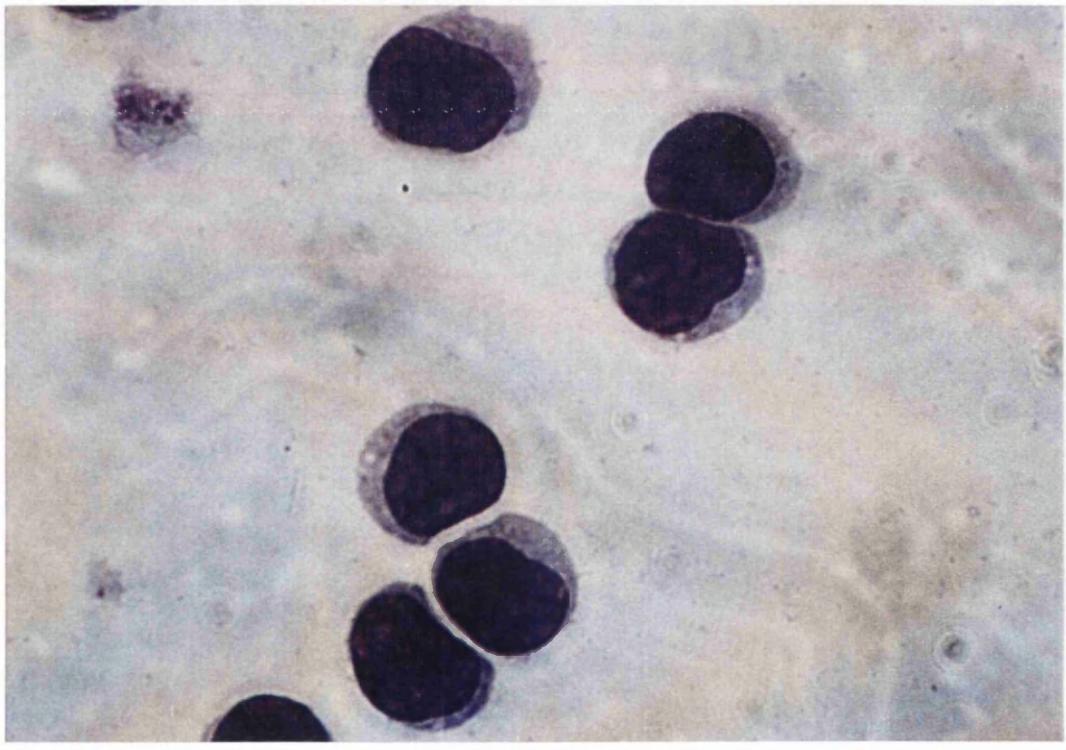
		Cell counts		
Cell fractions	Culture condition	Large clefted cells	Small clefted cells	Small non-clefted cells
High density cells	Before culture	**15.1%**	**21.3%**	**63.6%**
	After culture	**1%**	**3.5%**	**95%**
Low density cells	Before culture	**38.5%**	**20.5%**	**41%**
	After culture	**3%**	**5%**	**92%**

Table 3-6 Differentiation of resting and activated cells before and after culturing in IL-4 by morphological criteria. Following cytocentrifuge preparation, the slides were stained using May-Grunwald-Giemsa and studied at a magnification of x100. 150-200 cells were scored and the results are presented as a percentage events.

Figure 3-5



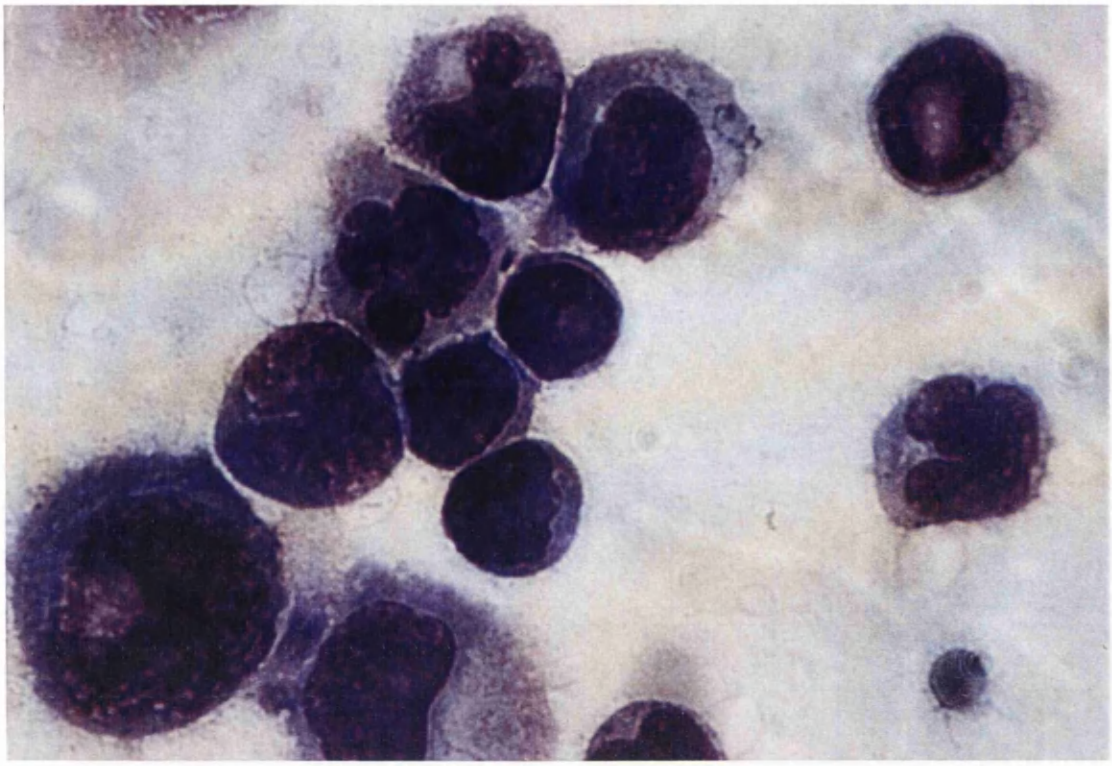
Before Culture



After Culture

Figure 3-5 Morphologic appearance of high density cells before and after culture in IL-4. Following cytocentrifuge preparation, the slides were stained using May-Grunwald-Giemsa and photographed at a microscope magnification of x100.

Figure 3-6



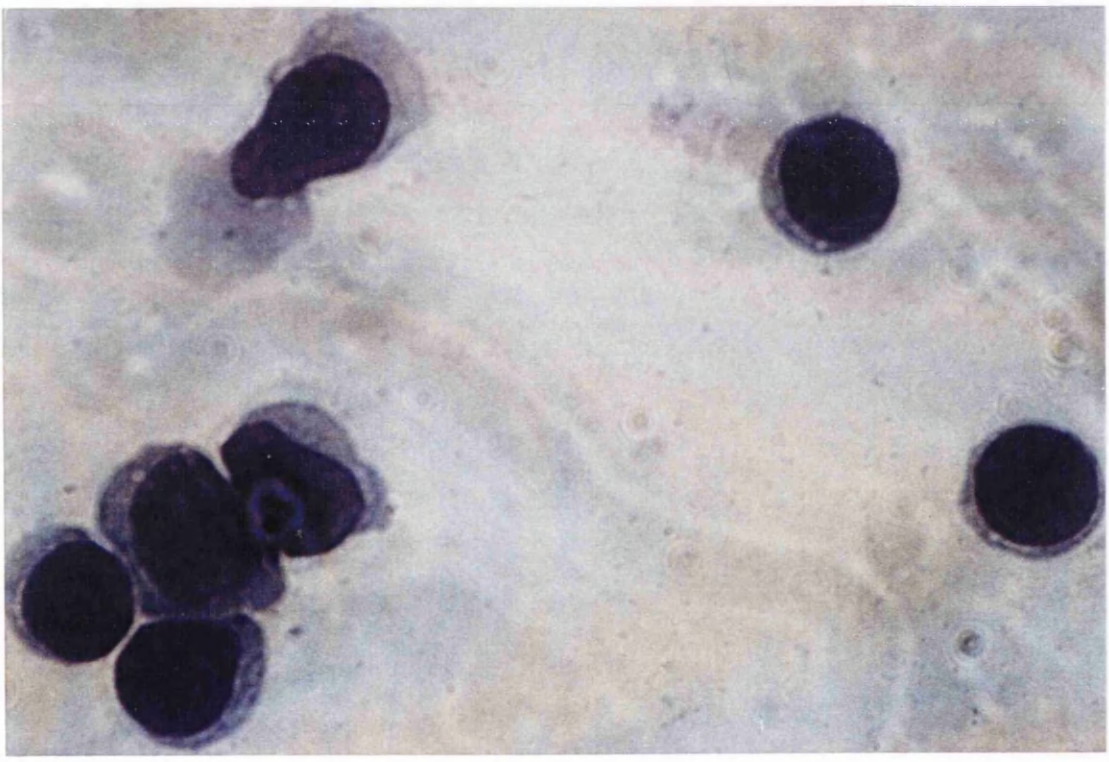


Figure 3-6 Morphologic appearance of low density cells before and after culture in IL-4. Following cytocentrifuge preparation, the slides were stained using May-Grunwalds - Giemsa and photographed at a microscope magnification of x100.

polarization assays, (a) in overnight culture which measures the gradual acquisition of locomotor capacity as the cell population becomes activated, and (b) in short-term (30 min) assays which measure the ability of the cells to respond to a chemoattractant. Short-term assays were done on cells either directly after separation or after overnight (20h) culture followed by washing. (2) Collagen gel invasion assays were used to measure the locomotion of B cells directly, and (3) time-lapse cinematography to analyse details of cell movement.

3-2-1 The Shape-Change Response to IL-4, IL- 13 & Anti-CD40 in Overnight Culture

The resting and activated cells were cultured in different concentrations of IL-4 and IL-13 for 20h. Figure 3-7 and 3-8 shows the proportion of polarized cells in IL-4 (Fig 3-7) and IL-13 (Fig 3-8) in the resting and activated fractions. The majority of cells in both fractions were IgM and IgD positive, as discussed previously. IL-4 stimulated locomotor shape-change in a high proportion of cells (30 - 40% above control). The dose response curve for IL-13 in both fractions was similar to that of IL-4 but fewer cells responded. Culture in both IL-4 and IL-13 also improved cell survival. This effect was not related to cytokine concentration. In IL-4 and IL-13, overnight survivals were 75 ± 2.7% (n = 6), and 75.5 ± 2% (n = 6), compared with 63 ± 5 % (n = 9) in medium alone without cytokines (high-density fraction). Anti CD40 also was able to enhance the locomotion response of resting tonsillar B cells in overnight culture (20h). The

response was significant compared with isotype control mouse IgG1. The dose-dependent response to anti CD40 and IgG1 is shown in Fig 3-9. Anti-CD40, like IL-4 and IL-13 enhances cell survival in a dose-dependent manner. In a high concentration of anti-CD40 (10-1μg/ml), overnight survival was 78 ± 1.8 % (n = 3). The same polarization response was observed in low-density B cells using IL-4, anti-CD40 or, more weakly, IL-13. The phenotype of responsive cells in the low-density fraction is shown in Table 3-5. The cells were mostly IgD+ and IgM+ and did not have features of GC cells. However, GC cells undergo apoptosis during prolonged culture. Cells from high and low density fractions showed similar responses in culture in IL-4, IL-13, and anti-CD40. The time course of polarization during 48h in response to IL-4 (50U) and IL-13 (1μg/ml and 10ng/ml) for the resting and activated fractions (100ng/ml) is shown in Figure 3-10 & 3-11, respectively. The slow increase in locomotor activity was most marked in IL-4, less so in IL-13, and still less in medium without cytokines. A maximum was reached after 18-20h. The same time-dependent response was observed using anti-CD40 (5μg/ml, Fig. 3-12). This development of locomotor morphology was evident after a few hours and maximal after 18-20h. At later times of culture, little further increase in the proportion of polarized cells was seen and this was much slower than would be expected using chemotactic factors which stimulate shape-change and locomotion within minutes of exposure, and the results suggests that, rather than acting as immediate stimulants, IL-4, IL-13 and anti-CD40 initiate differentiation from a non-locomotor to a locomotor phenotype.

The polarization seen after culture appears to be related to unidentified attractants released into the medium during culture rather than to IL-4 itself. Cell concentrations were also important in the percent polarization response to IL-4 and determined the amount of attractant released into the medium. Concentrations between 1.25 $x10^6$ - 10 x 10^6 cells/ml were used and the best response was seen at 2.5 x 10^6 cells/ml. The effects of specific antibodies to IL-4 and IL-13 were tested and both were shown to inhibit the capacity of their respective ligands to activate shape-change (table 3-7). Cells were incubated for 20h with IL-4 or IL-13 with or without their respective antibodies, then locomotor shape-change was scored. Percent inhibition was derived from 100 - {(% shape-change in [cytokine + ab] minus medium control/ % shape-change in cytokine alone minus medium control)x 100}. The experiments with anti-IL-4 were carried out using $F(ab`)_2$ fragments since the whole antibody itself stimulates polarization. The concentration of mouse anti-IL-4 was unknown, thus serial dilutions of antibody were used. The best inhibition (up to 61%) was observed at 1/100 dilution. These results shows that both anti-human and anti-mouse IL-4 inhibited the effect of human IL-4. The cells were cultured with IL-4 (20u) and anti IL-13 (200ng-4ng/ml) to check for possible cross-inhibition. Anti-IL-13 not only had no effect but enhanced locomotion, except at 200ng which gave a partial inhibition up to 17%. Based on observation that engagement of CD40 may be required to activate the B cell receptor for activation-related functions (Banchereau et al, 1994), experiments were performed to investigate the effect of combining IL-4, anti-

CD40, and anti-IgM as locomotor activators in overnight culture. The results together with those for the anti-CD40 isotype control IgG1 are shown in Fig 3-13. IL-4 (20u/ml) together with anti-CD40 (1μg/ml) stimulated polarization of more cells than either alone. A combination of IL-4 with anti-CD40 and anti-IgM (1μg/ml) stimulated polarization of still more cells (60-70% of the total viable population). In the absence of IL-4, anti-CD40 + anti-IgM did not induce significantly more polarization than anti-CD40 alone. Mouse IgG2a, the isotype control for anti-IgM had no effect on polarization (data not shown). Anti-IgD also was used in the same combination with IL-4, anti-CD40 or with appropriate controls and a slightly higher response was seen (one experiment, data not shown). Resting B cells showed no polarization response to IL-5 (n = 1) between 4-200 U/ml, IL-6 (n = 1) between 4-200 U/ml, IL-7 between 0.16-800 U/ml (n = 3), IL-15 (n = 2) between 1μg-100pg/ml and sCD23 (n = 2) between 10μg-100pg/ml, and finally IL-1α (n = 1) between 50ng-10ng/ml after 20h culture (data not shown). There was no response when these cells were cultured with anti-IgM (n = 3) and anti-IgD (n = 2), or antigens such as protein A (n = 2), *Pneumovax II* (composed of 23 different Polysaccharides) between 10mg-10pg (n = 1), or heat inactivated *Staphylococcus aureus* (cowan A strain) between 1.5×10^7 and 1×10^7 bacteria/ml (n = 1), except with anti-IgM at 1μg/ml which give a small response (12 ± 2%, n = 3). Anti-Ig caused cell death after overnight culture (20h) in a dose-dependent manner. This was more significant using anti-IgM rather than anti-IgD. Cell death become apparent at a concentration of 1μg/ml and reached

Figure 3-7

Polarization to IL-4

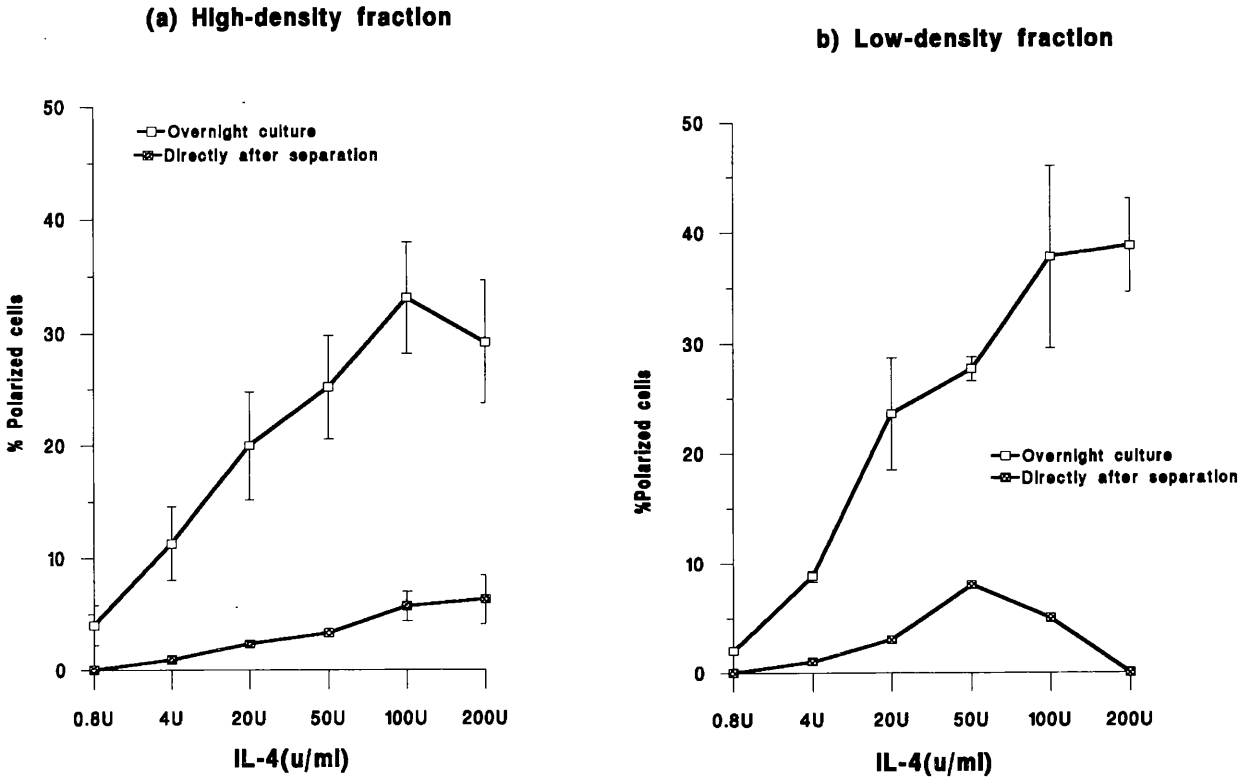


Figure 3-7 Polarization of high-density (Resting) and low-density (Activated) tonsillar B cells to IL-4 (a) Dose responsive curve of high-density B cells, (b) dose responsive curve of low-density B cells. The data are expressed as (test-control) values.

Upper curves: Cells cultured overnight in IL-4 (20H) and fixed in their own supernatants. (Left Fig.) high-density cells (mean ± SEM, n=6). Polarization in medium alone was 20.6 ± 4.8 % : P values: test cf. cytokine-free control; IL-4 20 & 50U/ml P : <0.05> 0.01 : 100 & 200U/ml P : <0.01 . (Right Fig) Low-density cells (mean ± SEM, n=3) polarization in medium alone was 29.5 ± 4.2. P value for 100 & 200U/ml P <0.05>0.03.

Lower curve: 30 min. assay (immediate shape-change) Cells exposed to IL-4 directly after separation (a) High-density B cells (mean ± SEM, n = 2) Polarization in medium alone was 6.4 ± 1.3. Differences are not statistically significant using Student T-test. (b) Low-density B cells in one experiment. Polarization in medium alone was 15.5.

Figure 3-8

Polarization to IL-13

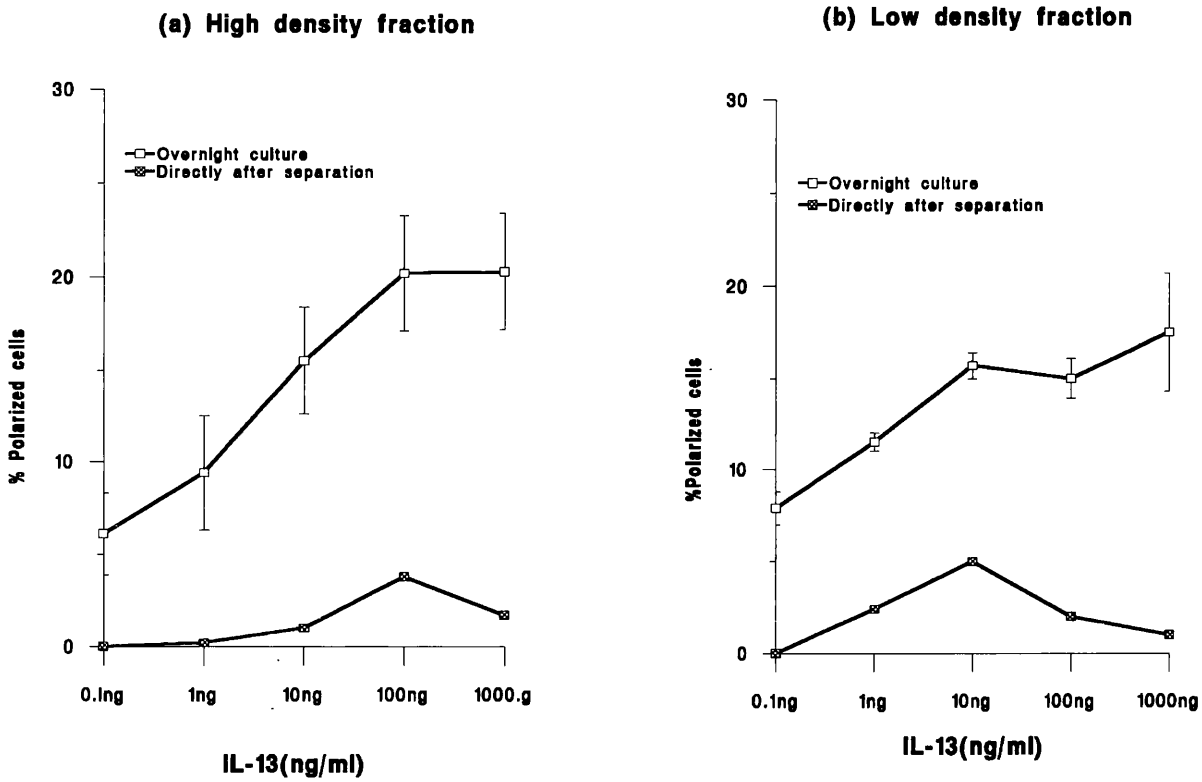


Figure 3-8 Polarization of high-density (Resting) and low-density (Activated) tonsillar B cells to IL-13 (a) Dose responsive curve of high-density B cells, (b) dose responsive curve of low-density B cells. The data are expressed as (test-control) values.

Upper curves: Cells cultured overnight in IL-13 (20H) and fixed in their own supernatants. (Left Fig) high-density cells (mean ± SEM, n=6). Polarization in medium alone was 20.0 ± 2.6 % : P value: test cf. cytokine-free control; IL-13 >10ng/ml P : <0.05> 0.01. (Right Fig) Low-density cells (mean ± SEM, n = 2) polarization in medium alone was 27.8 ± 3.8. Differences are not statistically significant.

Lower curve : 30 min. assay (immediate shape-change) Cells exposed to IL-13 directly after separation (a) High-density B cells (mean ± SEM, n = 2) Polarization in medium alone was 6.4 ± 1.3. Differences are not statistically significant using Student T-test. (b) Low-density B cells in one experiment. Polarization in medium alone was 15.5.

Figure 3-9

Polarization to anti-CD40 & its isotype control mIgG1

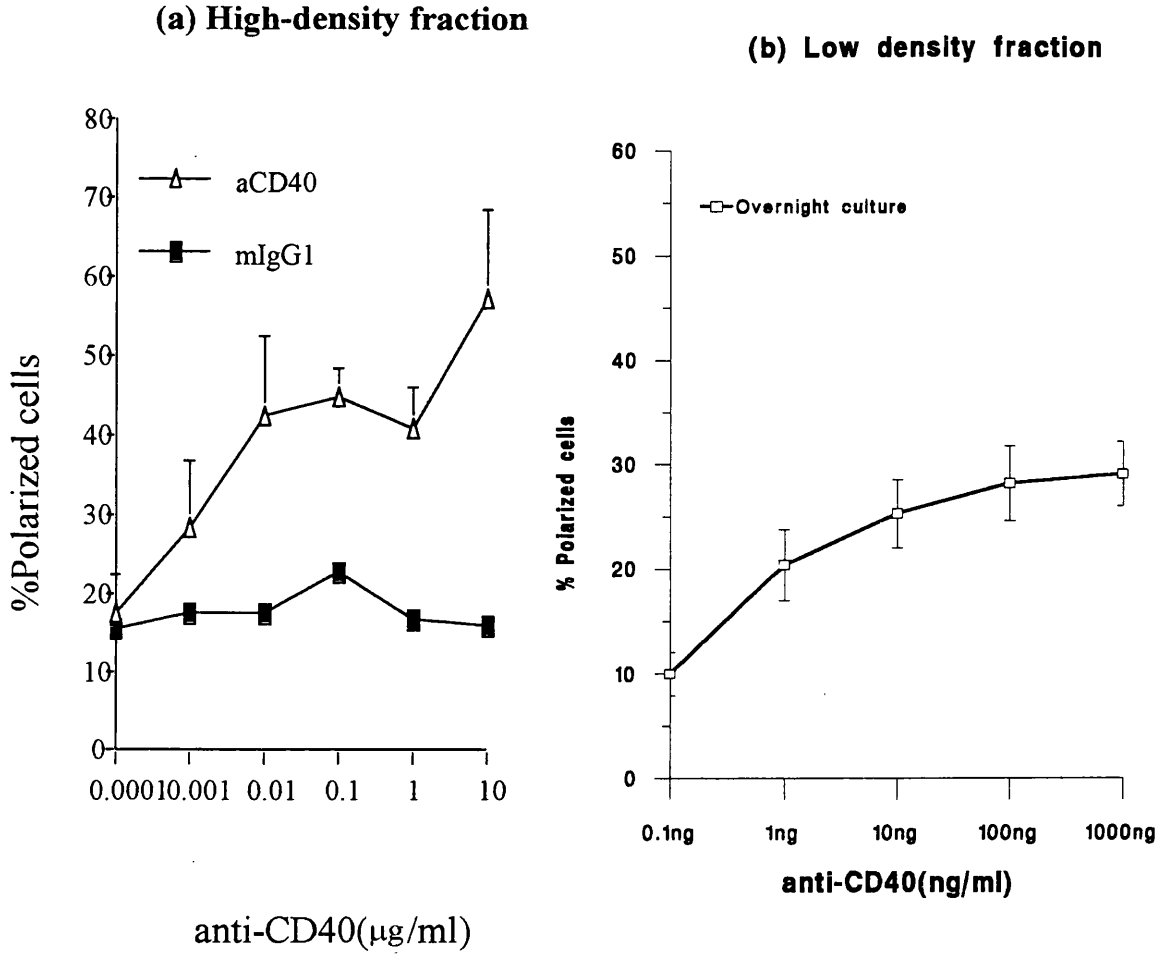


Figure 3-9 Polarization of high-density (Resting) and low-density (Activated) tonsillar B cells to anti-CD40 (a) Dose responsive curve of high-density B cells, (b) dose responsive curve of low-density B cells. The data are expressed as (test-control) values.

Upper curves: Cells cultured overnight in anti-CD40 (20H) and fixed in their own supernatants. (Left Fig) high-density cells (mean ± SEM, n = 3). Polarization in medium alone was 21.6 ± 2.2 % . P : <0.01; anti-CD40 at > 0.01μg/ml cf. medium control. B) Low-density cells (mean ± SEM, n = 2). Polarization in medium alone was 30.9 ± 0.1 %. There was not enough data for estimation of P value.

Lower curve : Isotype control mouse IgG1. Result from a single experiment in high-density fraction.

Figure 3-10

Time course polarization to IL-4

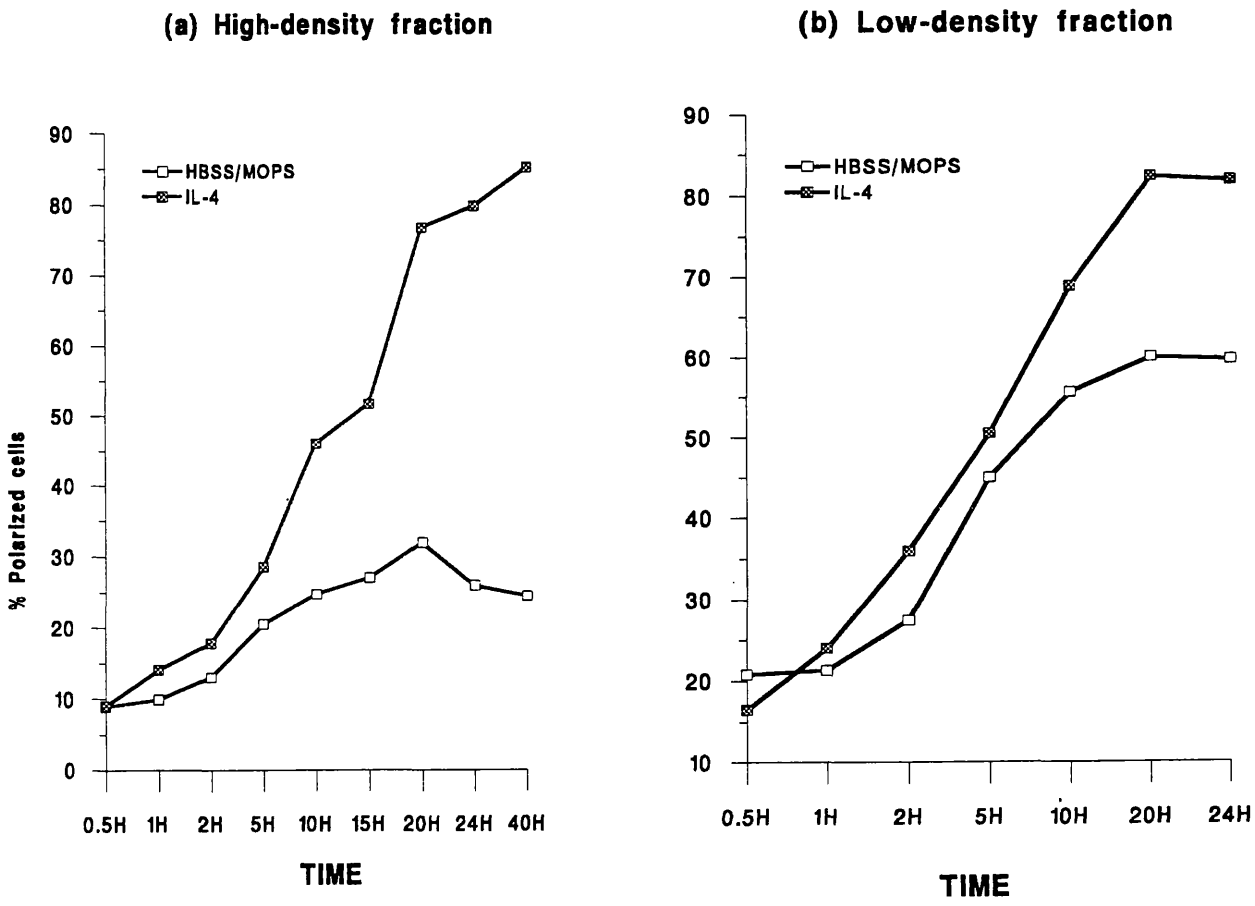


Figure 3-10 Time-course for polarization of high-density and low density B cells cultured in IL-4 (50U/ml) together with medium alone without cytokine. Data from one experiment.

Figure 3-11

Time course polarization to IL-13

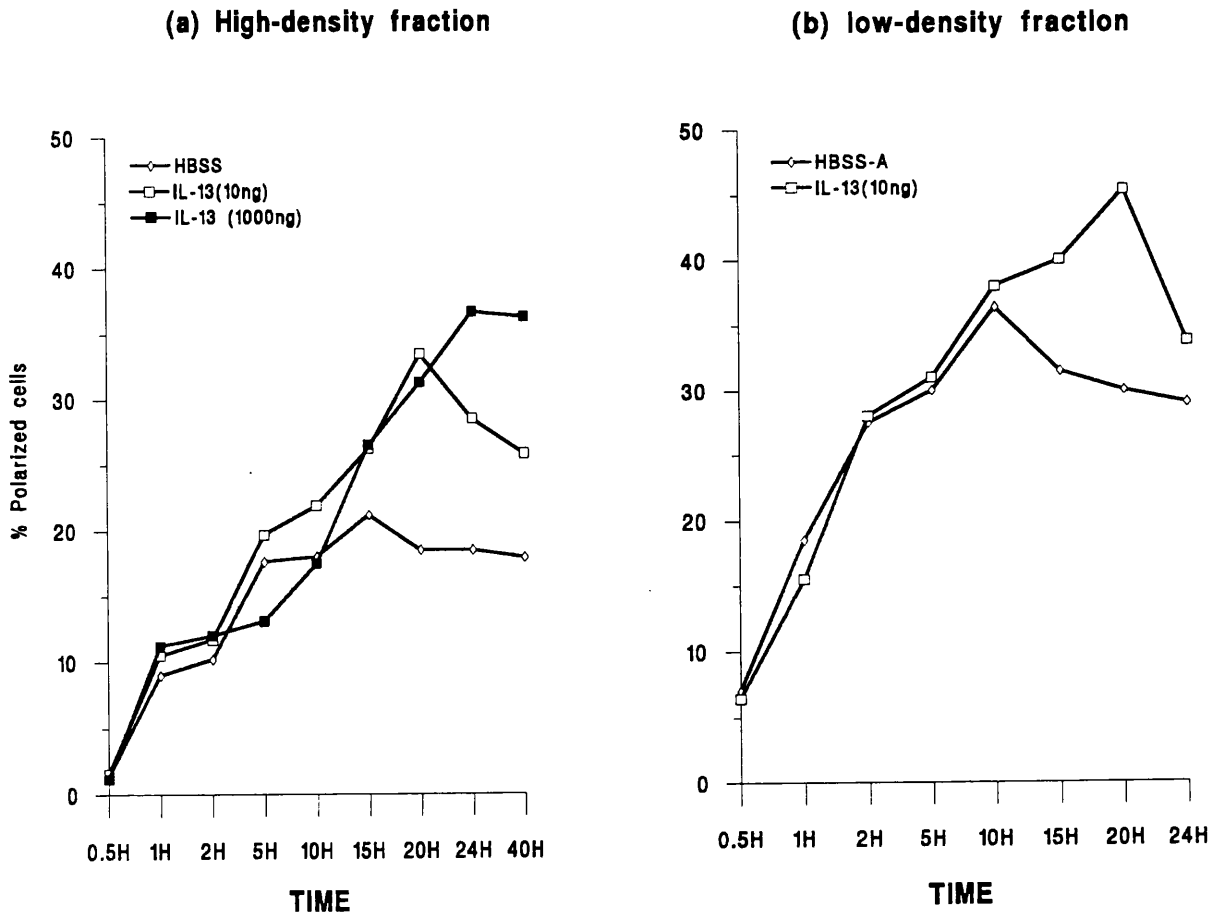


Figure 3-11 Time-course for polarization of high-density and low density B cells cultured in IL-13 together with medium alone without cytokine. Data from one experiment.

(a) High-density B cells in IL-13 (1μg/ml and 10ng/ml), (b) Low-density B cells (100ng/ml).

Figure 3-12

Time course polarization to anti-CD40

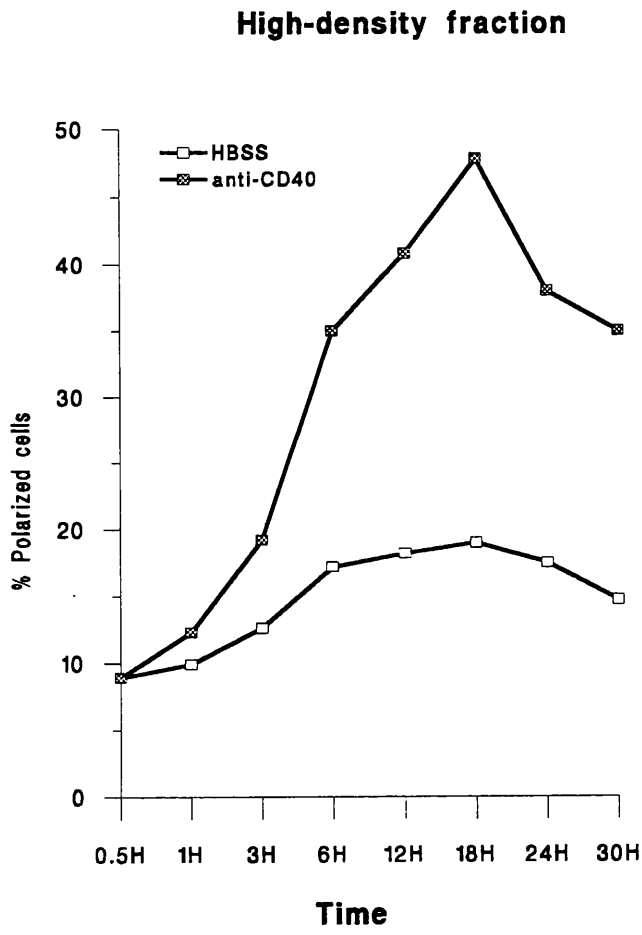


Figure 3-12 Time-course polarization of high-density B cells cultured in anti-CD40 (5μg/ml) together with medium alone without antibody. Data from one experiment.

Table 3-7

Inhibition by antibody of locomotor activation by IL-4 & IL-13.

% inhibition after 20h culture in

IL-4 (100U/ml)

anti-human IL-4	%inhibition	mouse anti IL-4(11B11)	%inhibition
4 (μg/ml)	100	1/5	7.5
2	95	1/25	14.5
1	42	1/50	33
0.5	2	1/100	61
		1/500	25
		1/1000	11

IL-13(100ng/ml)

aIL-13 (ng/ml)	%inhibition
200	90%
40	89%
20	62%
16	54%
8	34%

IL-13(10ng/ml)

aIL-13(ng/ml)	%inhibition
200	100
40	89
20	58
16	54
8	21

Figure 3-13

Multiple effect of IL-4, anti-CD40, and anti-IgM in overnight culture

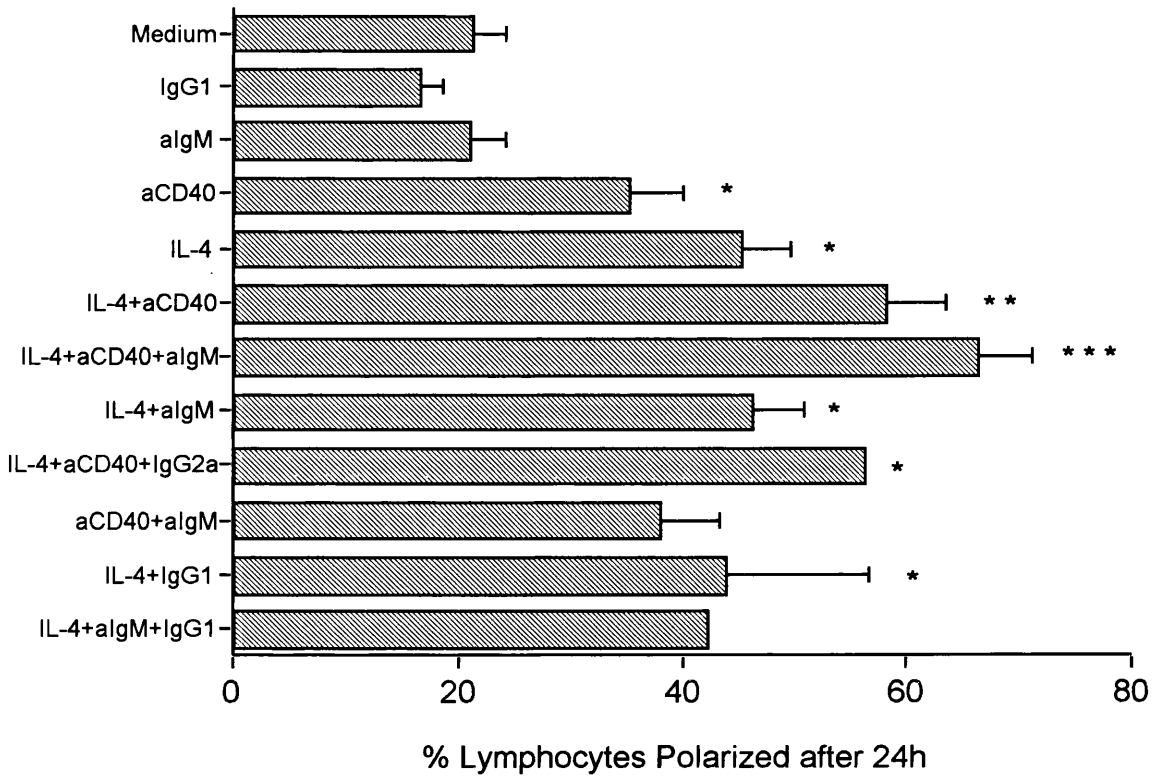


Figure 3-13 Overnight polarization of high density tonsil B cells in combination of IL-4 (20U/ml), anti-CD40 (1μg/ml), and anti-IgM (100ng/ml) or mouse IgG1 (100ng/ml). Mean ± SEM for 7-9 experiments. Where no standard error bar is shown, N <3: t-test: *P : <0.01 cf. medium control; ** P : < 0.01 cf. both medium control and anti-CD40; *** P <0.01 cf. medium control, IL-4, anti-CD40 or anti-IgM alone. Mouse IgG2a was used as an isotype control for anti-IgM and gave no activity (data not shown)

up to 85% at 10μg/ml. Induction of cell death by anti-IgM could be overridden by co-culturing with either IL-4 or anti-CD40. Although anti-IgM by itself had no significant effect on the locomotor response in a long term assay, anti-IgM plus IL-4 in appropriate concentration enhanced the response compared with IL-4 alone (Fig 3-14). There was no response using mouse IgG2a, mouse IgG1, or sheep Ig between 10mg-1ng/ml which were used as controls for the antibodies. LPS also was tested and gave no effect at concentrations between 10μg-10ng/ml (n = 2). TNF-α neither polarized the B cells nor had any effect on polarization induced by IL-4 when they were co-cultured. In contrast to TNF-α, another T_{H1} cytokine, IFN-γ, inhibited polarization induced by IL-4 but itself had no effect on B cells in a long term assay (n = 3, Fig 3-13A). The inhibitory effect of IFN-γ on the response to IL-4 was slightly enhanced by culturing the cells for one hour in IFN-γ before adding IL-4 (one experiment, data not shown). No polarization was observed when activated cells were cultured in IL-5 (n = 1), IL-6 (n = 1), IL-7 (n = 3), sCD23 (n = 3), anti-IgM (n = 3), anti-IgD (n = 2) at the same concentration as resting cells except with anti-IgM at 1μg/ml (14 ± 3). It was concluded that IL-4, and IL-13, but not other B cell cytokines tested, confer locomotor capacity on B cells during a 24h period so that the cells change shape in response to attractants present in their own supernatants. This was true for anti CD40 as well. However, overnight culture experiments do not provide evidence about which attractants cause B cells to change shape and to migrate towards a gradient source immediately (<30 min) following stimulation.

Figure 3-14

Polarization using IL-4 and anti-IgM in overnight culture

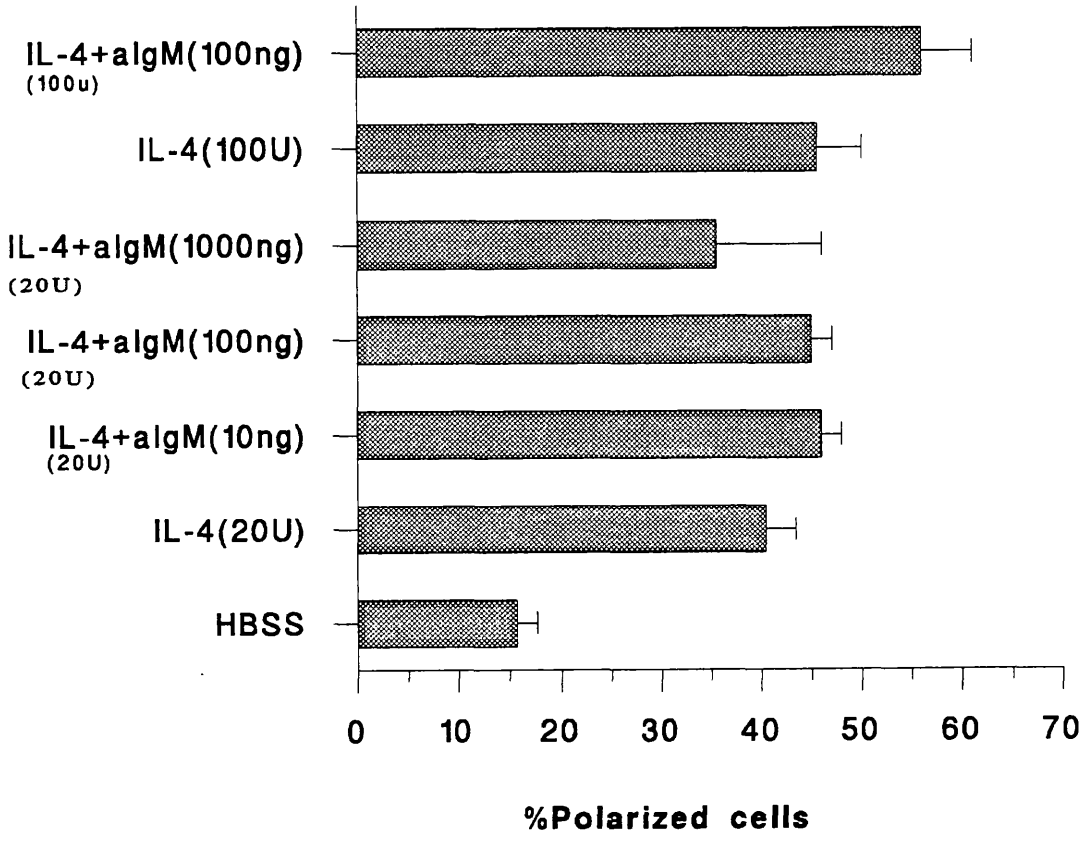


Figure 3-14 Polarization of high-density (Resting) tonsillar B cells to IL-4 + anti-IgM fixed in their own supernatants after overnight culture (20H) (mean ± SEM, n = 3). P values: test cf. cytokine-free control; IL-4 20U/ml plus 10-100ng/ml of anti-IgM P : <0.04> 0.01 : IL-4 100U/ml plus 100ng/ml of anti-IgM P : <0.01> 0.003.

Figure 3-13A

Inhibition effect of IFN-γ on polarization

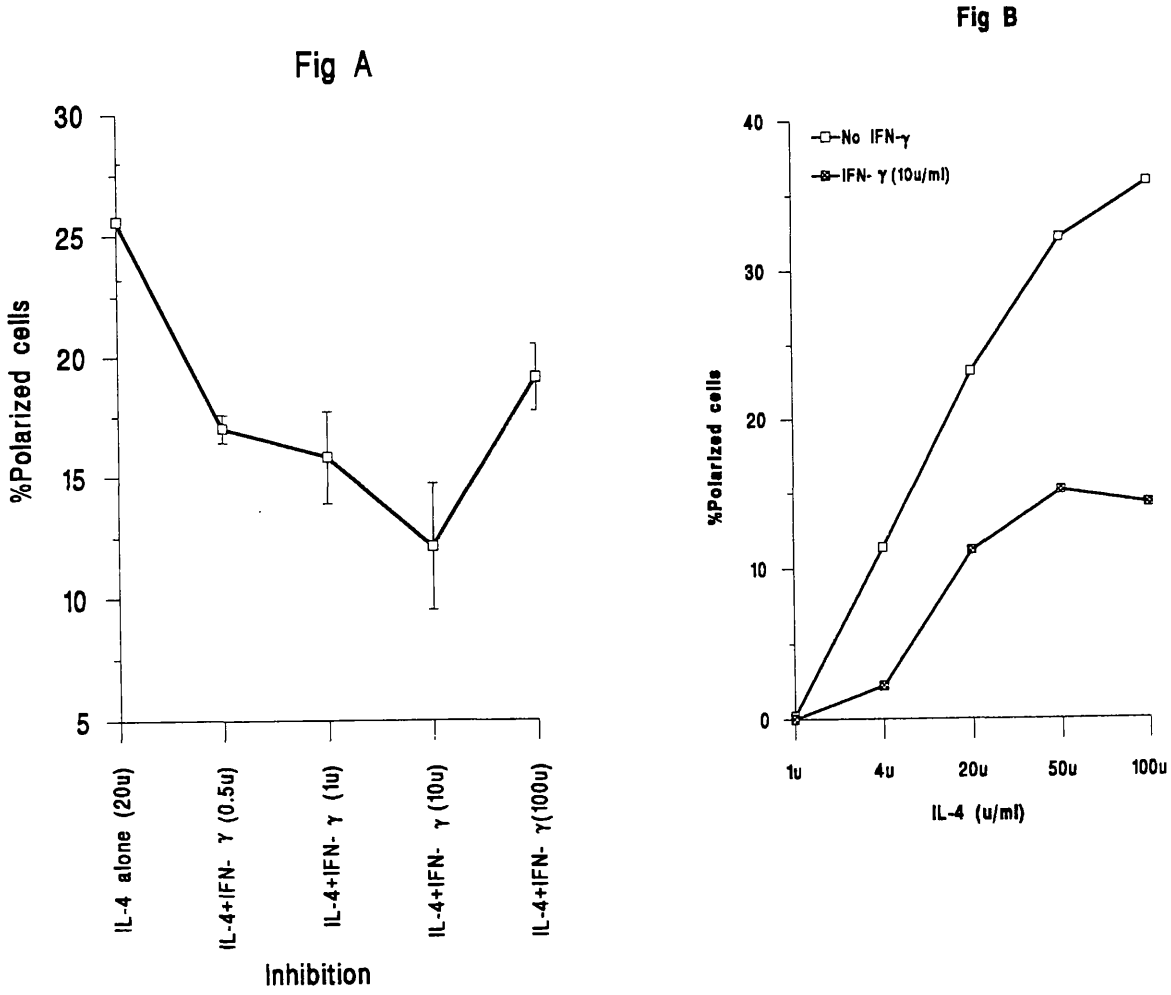


Figure 3-13A Effect of IFN-γ on polarization induced by IL-4. The resting cells were cultured with IL-4 and IFN-γ. The data are expressed as (test-control) values. (a) inhibitory effect of different concentrations of IFN-γ on IL-4 (20U/ml) (mean ± SEM, n = 3). Polarization in medium alone was 20.6 ± 0.6 %. The percent inhibition was 38.2 ± 2.7%; 51 ± 4.9%; 25.5 ± 2.3% for 1U, 10U, and 100U of IFN-γ, respectively . P values: test cf. cytokine control; IFN-γ 1 & 10U/ml P : <0.03> 0.05.

(b) Inhibition curve using 10U/ml of IFN-γ and different concentrations of IL-4 (n = 1). The percent inhibition was 100, 81, 52, 53, 40% for IL-4 1, 4, 20, 50, 100U/ml, respectively. Percent inhibition was calculated as before (Table 3-7).

3-2-2 The Response of High and Low-density B Cells to Chemoattractants in Short-Term Shape-Change Assays.

3-2-2-1 Response to Cytokines, to Anti-CD40, and to Supernatants from Cultured Cells.

In addition to studying the gradual effects of locomotor activators on locomotion of B cell in overnight culture, we also studied the immediate (<30 minutes) effects of attractants on B cell polarization and locomotion, using either cells direct from the tonsil or cultured B cells. This provides valuable information about the response of cells before and after culture to chemoatractants. Cultured cells were washed twice to remove the attractants released into the medium during culture. After washing, most of the cells rounded up and could be retested against freshly added attractants. Thus, the supernatant medium was removed from B cells cultured in IL-4 or anti CD40, and the cells were washed to allow them to regain spherical morphology. They were then re-exposed for 30 minutes either to pure cytokine, to anti-CD40, or to their own supernatants. B cells that had been cultured in IL-4 and anti-CD40 showed rapid shape change in their own supernatants (Figure 3-15), and to a lesser extent in supernatants from B cells to which no cytokine had been added. They showed no response to either supernatants of T cell cultured in IL-4 or to supernatants of B cells cultured in FCS 25% alone. These cells were also tested for immediate shape change in IL-4 and IL-13. A small response was seen when cells that had been cultured in IL-4

(20u) and IL-13 (100ng/ml) were re-exposed to an equivalent amount of IL-4 and IL-13 for 30 minutes {3.2 ± 1.5 (n = 6) and 2.2 ± 0.2 (n = 2), respectively}. The best response was seen using IL-4 (100U/ml), and this was below 10%. Cells were cultured in anti-CD40 for 20 hours then washed and tested for a short-term response to IL-4. These cells gave no response to IL-4. The supernatants from IL-4 and IL-13 cultures but not IL-4 and IL-13 themselves, induced shape-change of cells within 30 minutes, suggesting that supernatants contain attractants released by the cells themselves in response to culture with cytokine. This finding was supported by addition of anti-IL-4 to these supernatants, which caused only partial inhibition of their activity (Table 3.8). Fig 3-7 (lower curve Fig 3-7a) shows that the immediate response of freshly isolated resting cells was considerably lower than that of cells from long-term culture in IL-4 (bottom curve compared with top curve).

Overall, these results demonstrate that IL-4 does not account for the response of pre-cultured cells to the supernatants. No response was observed when cells freshly isolated from the tonsil were exposed to these supernatants. This finding suggests that in order for freshly isolated cells to become capable of locomotion, they require to be cultured in locomotor activators such as IL-4, IL-13, and anti-CD40. Cells pre-cultured in IL-4, but not cells directly from the tonsil (not shown), responded by immediate polarization in response to anti-CD40. Fig 3-15A shows the dose response of B cells which had been cultured in IL-4 overnight, then washed, exposed to anti-CD40 and its isotype control IgG1 in a

short term assay. Anti-CD40 had no effect on pre-culture resting cells in anti-IgD (1μg/ml for 30 min.). These results shows that anti-CD40 acts both as a long term activator (Fig 3-9) and as a chemoattractant (Fig 3-15A) for B cells. No response was seen in short-term assays to a number of other cytokines viz. IL-7 between 0.5-400U (n = 5), MIP-1α, MIP-1ß, IL-8, IL-2, IL-15, RANTES all tested at between 1μg-1ng/ml (n = 1), or sCD23 tested at between 1μg-100pg/ml (n = 7). No IL-2R was detected when cultured cells were stained using anti-IL-2R antibodies (CD25 & CD122). B cells purified directly from the tonsil were also tested against IL-4 and IL-13 in short term assays. In contrast to the cultured cells, these failed to respond. There was no response with other cytokines such as IL-2, IL-7, IL-8, IL-15, MIP-1α, MIP-1ß, and sCD23 at the same concentrations which were used before on cultured cells.

3-2-2-2 Effect of Treatment of Cells with Anti-Immunoglobulins on Polarization

The majority of B cells in the resting fraction were sIgM+, sIgD+ , and since B cell shape-change on exposure to anti-Ig may provide a model for a locomotor response to antigen (Ward et al, 1977; Gray et al, 1994), the effect of anti-IgM and anti-IgD was tested in short-term assays. A proportion of high-density B cells both before and after culture in IL-4 showed a shape-change dose-response in the presence of anti-IgM (Fig. 3-16) but did not respond to the isotype control mouse IgG2a (data not shown). The response was more marked after overnight culture

Figure 3-15

Polarization to supernatants

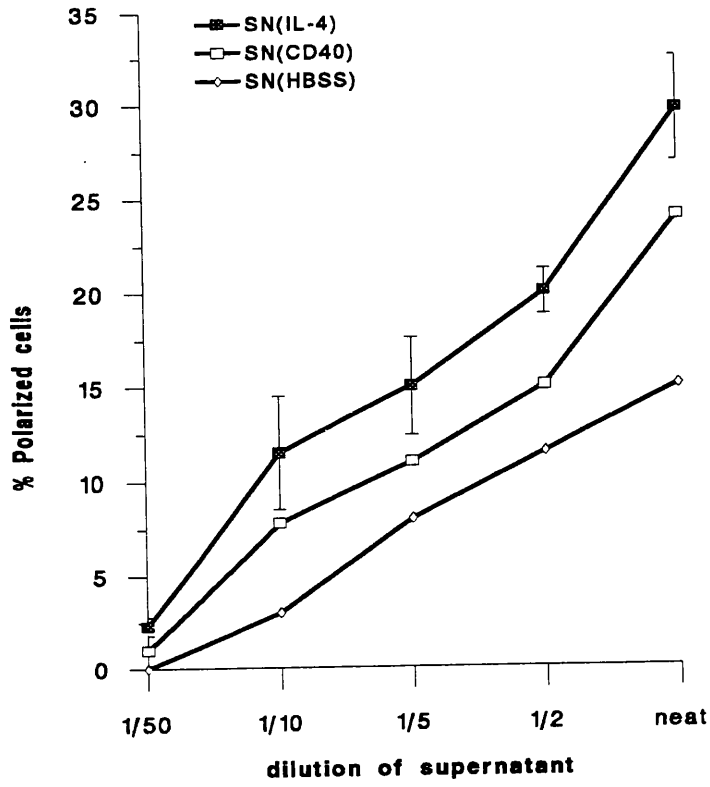


Figure 3-15 Cells were cultured in IL-4, anti-CD40, and HBSS alone, washed, and resuspended in dilutions of their own supernatants. (Upper curve) Dose response curve for cells cultured overnight in IL-4(20U/ml), and exposed to supernatants derived from overnight cultures of B cells in IL-4 (100U/ml). (mean ± SEM, n = 3). Control values (medium alone : Polarization in medium was 32.9 ± 3.3 %.) have been subtracted. Neat supernatant cf. control: P <0.02. (Middle curve) Dose response curve of cells cultured in anti-CD40 (100ng/ml) to its own supernatants. Polarization in medium alone was 30% (n = 1). (Lower curve) Dose response curve of cells cultured in HBSS alone to its own supernatants. Polarization in medium alone was 17% (n = 1).

Figure 15A

30 Min. assay to anti-CD40

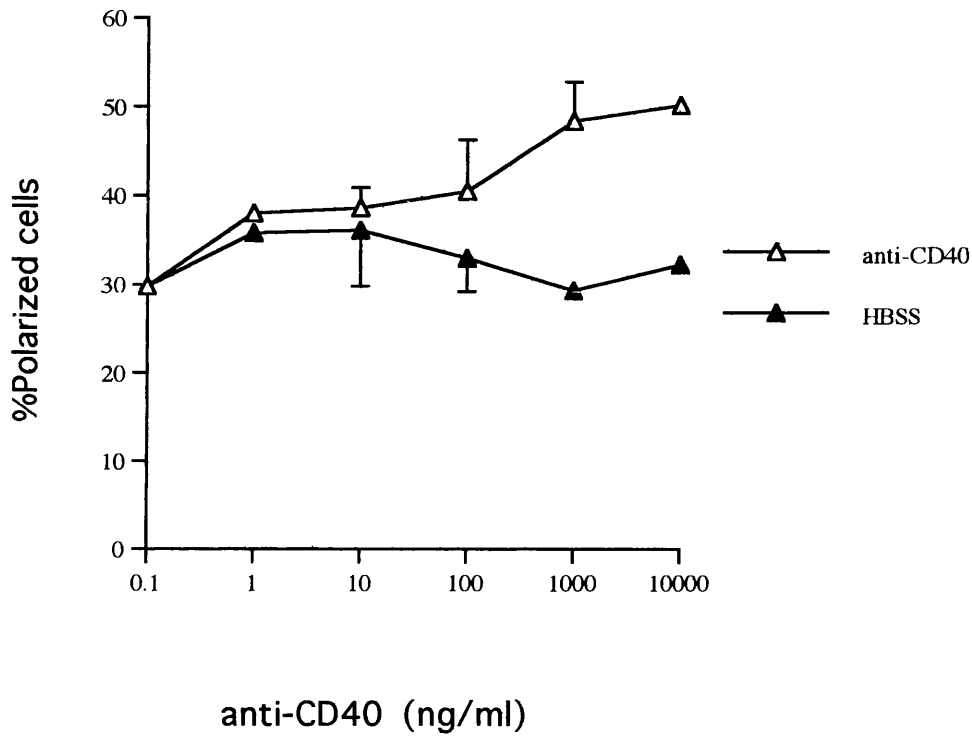


Fig. 15A Response to anti-CD40 and its isotype control IgG1 of B cell after overnight culture in IL-4 (20u/ml). Mean ± SEM, n = 3. P <0.01 Vs isotype control. Cells direct from the tonsil showed no response to anti-CD40 (data not shown).

Table 3-8

Inhibition of locomotor activation of supernatants by aIL-4.

anti-human IL4 (μg/ml)	% Inhibition
4	24
2	34
1	24
0.5	0

Resting cells were cultured in IL-4 (20 & 100U) overnight and the cells cultured in IL-4 (20U) were then washed twice and re-exposed to supernatants from cultures in IL-4 (100U) alone or to the same supernatants plus anti-IL-4 in 30 minutes assay. The percent inhibition was calculated as mentioned for table 3-7 .

Figure 3-16

Polarization to anti-IgM & anti-IgD

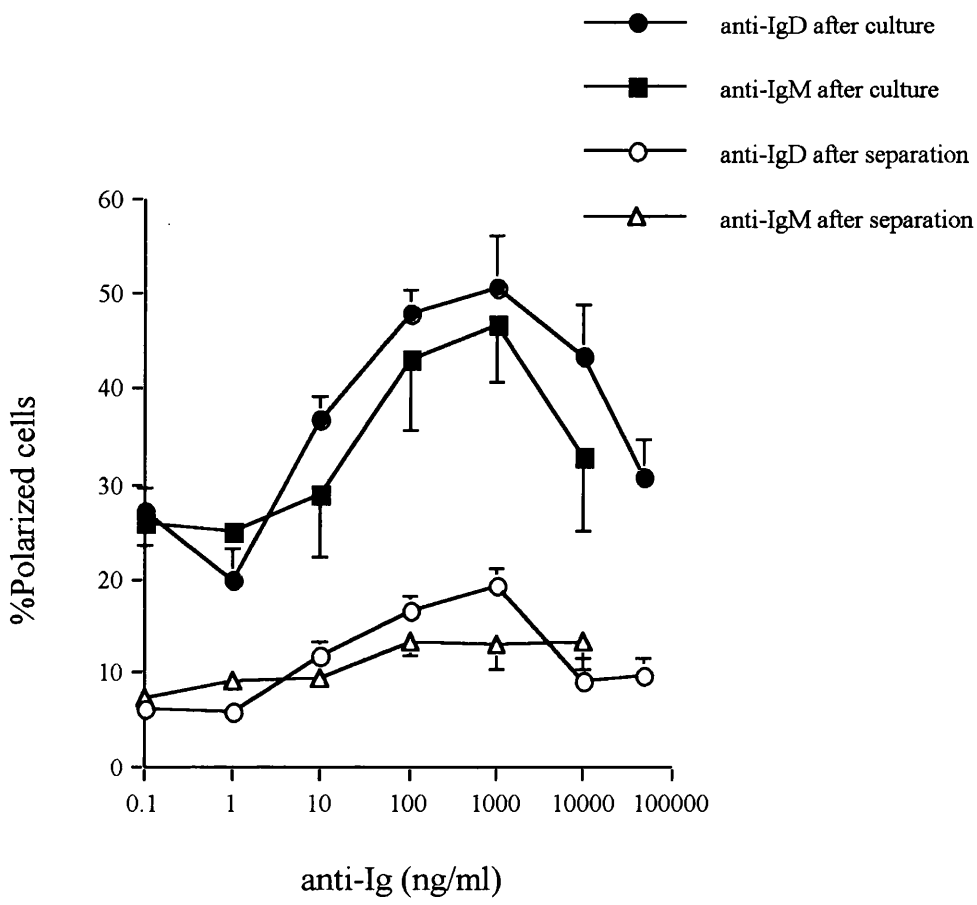


Figure 3-16 Dose response of B cells to anti-IgM and anti-IgD in a 30 minute shape-change assay. Open symbols: cells direct from tonsil; Filled symbols : cells after overnight culture in IL-4 (20U/ml) (mean ± SEM, n = 4). Control values (medium alone : cells direct from Blood, 7.5 ± 0.7 % polarized cells; cells after overnight culture, 23.8 ± 1.2 %) have been subtracted. All P values (test compared to control) at 100ng/ml and 1μg/ml < 0.05.

in IL-4 than in cells direct from the tonsil. The IL-4 cultured B cells were also tested for shape-change in the presence of polyclonal sheep anti-human IgD (Fig. 3-16). These responses were stronger than that with anti-IgM and a similar response was seen using monoclonal mouse anti human IgD. These results suggest that anti-IgM and anti-IgD act as a chemoattractants for B cells and that anti-IgD has a stronger effect than anti-IgM. A time-course study showed that polarization in response to anti-IgD was almost fully developed in 10 minutes (Fig 3-17) which is characteristic of a response to a chemoattractant and contrasts with the slow polarization shown in Fig 3-7, 3-8, 3-9. After 60 minutes, the proportion of polarized cells began to decrease slowly to baseline levels. Control sheep IgG had no effect on shape-change. The high-density B cells were cultured in IL-4 (20u/ml) overnight. Following washing twice, they were exposed to anti-IgD (monoclonal Ab, Dako) or HBSS alone. Cells were then analysed; firstly after 30 min. when the response is maximum and then after 7h when the response has returned to baseline. Fluorescent microscopy analysis showed that cells after 30 min. in anti-IgD were strongly positive compared with its isotype control FITC-conjugated mouse IgG1. Cells were cultured in anti-IgD and HBSS alone for 7h. Those exposed to HBSS alone showed strong fluorescent activity for surface IgD compared with IgG1 isotype control, whereas those cultured in anti-IgD were absolutely negative. However cells permeabilized in 0.2% Triton x 100 failed to show any fluorescent activity inside the cells. This result was confirmed by FACS (Fig. 3-17A). Thus B cells are capable of recognizing anti-Ig as a chemoattractant

and culture in IL-4 increases the proportion of cells responsive to anti-Ig. Table 3-9 shows data obtained from the combination of anti-IgM and anti-IgD in the immediate shape-change response (30 minutes assay). These experiments do not show an additive effect of these antibodies used at high concentration, but at lower concentrations, the polarization response was slightly higher than that seen with either antibody alone. This difference in locomotion may be due to the increase in total anti-Ig concentration rather than to a combined effect. The same results were seen with cells pre-cultured in IL-4 (data not shown). However, combination of a supernatant from IL-4 cultured cells with either anti-IgM or anti-IgD increased the percentage of responsive cells (Fig 3-18). No further response was observed by adding anti-CD40 to the supernatant. However the response slightly decreased (data not shown). A polyvalent sheep antiserum against IgG,M,A also induced immediate shape-change in B cells (Fig. 3-19). Control sheep IgG had no effect on shape-change. Thus B cells are capable of recognizing anti-Ig as a chemoattractant and culture in IL-4 increases the proportion of cells responsive to anti-Ig. To confirm this, high-density B cells were cultured either in 25% FCS, anti-CD40 (1μg/ml), IFN-γ (10u/ml), and or HBSS/MOPS alone. Following washing, they were exposed to anti-IgD. The results are shown in Fig 3-20. Following culture in FCS, cells showed no response to any of the above attractants (data not shown). This suggests that cells cultured in HBSS alone might be activated through adhesion to the plate surface, though less so than cells cultured in IL-4. Thus overnight culture in IL-4 not only increases the

Figure 3-17

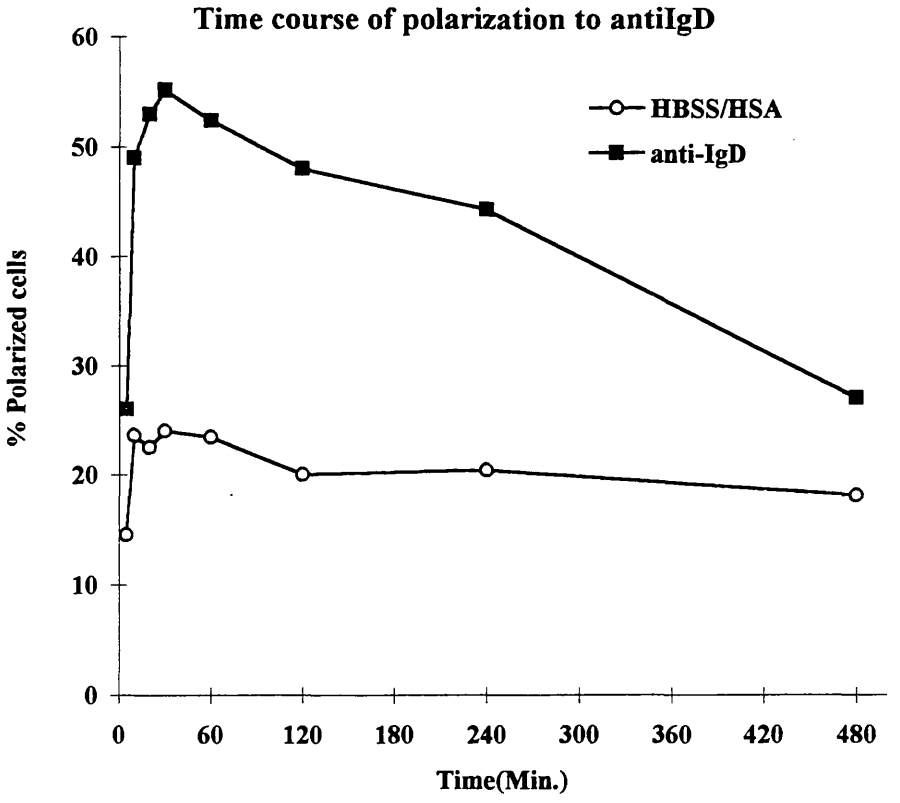
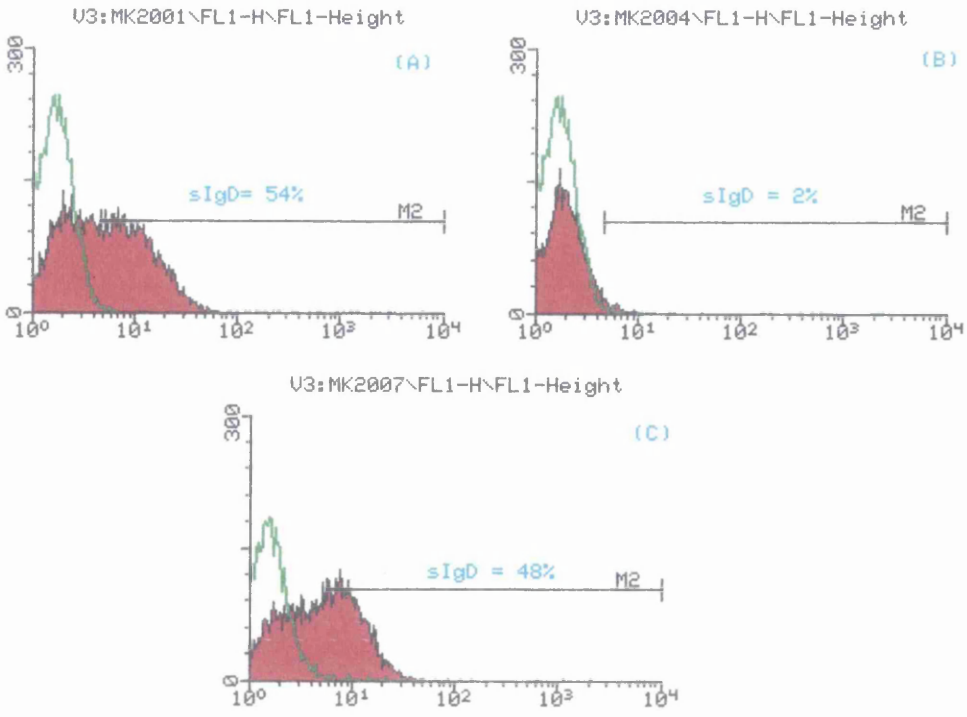


Figure 3-17 Cells were cultured in IL-4 (20u/ml) overnight, washed ans retested against anti-IgD (1000ng/ml) or HBSS-HSA.

Figure 3-17A



Cells were cultured in IL-4(20u/ml) overnight, washed and exposed to anti-IgD (1μg/ml) and HBSS alone. (a) Cells exposed to anti-IgD after 30 Min. and stained. (b) Cells exposed to anti-IgD after 7h. and stained. (c)) Cells exposed to HBSS alone after 7h. and stained.

Table 3-9

Effect on polarization of anti-IgM and anti-IgD

	Hanks/MOPS	aIgD(1μg)	aIgD(100ng)	aIgD(10ng)
Hanks/MOPS	6.9 ± 0.6(3)	21 ± 8.3(3)	19.2 ± 9(3)	12 ± 4.2(3)
aIgM(1μg)	7.8 ± 2.5(2)	21.5 ± 1.4(2)	26.1 ± 15.4(2)	18.8 ± 3.1(2)
aIgM(100ng)	11.1 ± 3.6(2)	ND	ND	21.7 ± 11.9(2)
aIgM(10ng)	10.9 ± 2.6(2)	16.5 ± 0.4(2)	16.4 ± 2.2(2)	16 ± 2(3)

Table 3-9 Polarization assays using combinations of anti-IgM and anti-IgD. The freshly isolated high-density B cells were exposed to these attractants for 30 minutes. The percent polarizations are presented as mean ± SEM. The numbers in parentheses show the number of experiments.

ND = not detected

Figure 3-18

Polarization to supernatant and anti-Igs

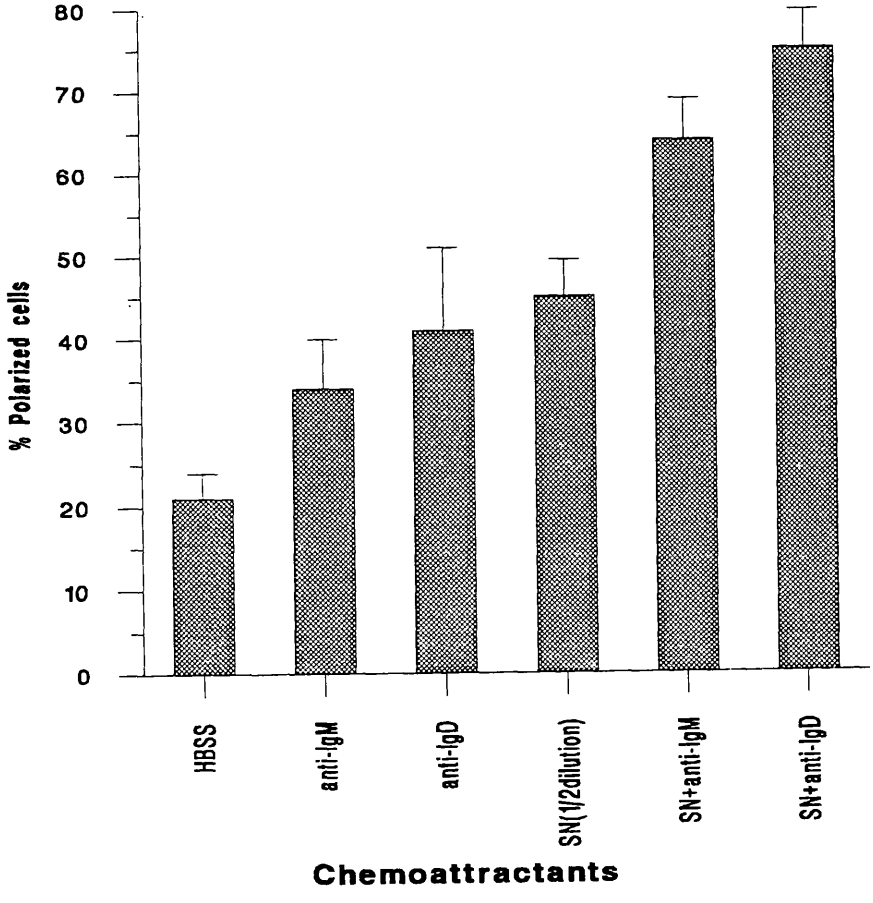


Figure 3-18 Cells cultured overnight in IL-4 (20U/ml), washed, and resuspended in 1/2 dilutions of supernatants from overnight cultures of B cells in IL-4 (100U/ml) alone and in combination with anti-IgM and anti-IgD. (mean ± SEM, n = 2).

Figure 3-19

Polarization to anti-IgG,A,M

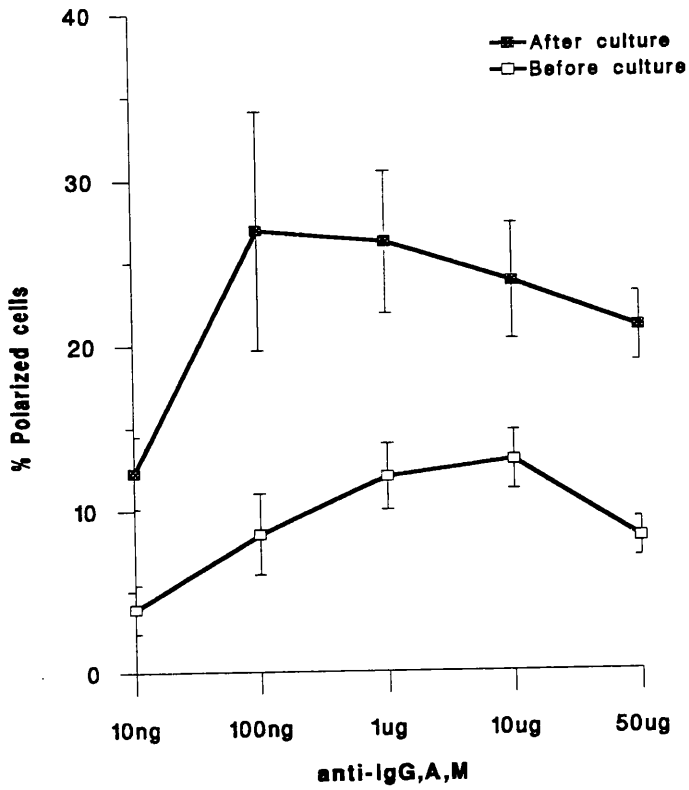


Figure 3-19 Polarization responses to different concentrations to anti-IgG,A,M in a 30 minutes shape-change assay. (a) Cells direct from tonsil (mean ± SEM, n = 3), Control values (medium alone : Polarization in medium alone was 6.8 ± 0.7 %) have been subtracted.: P value: test cf. medium alone; anti-IgG,A,M >1μg/ml P : 0.03, anti-IgG,A,M 10μg/ml P <0.001.

(b) Cells cultured overnight in IL-4 (20U/ml), washed, and retested to anti-IgG,A,M (mean ± SEM, n = 4). Polarization in medium alone was 24.5 ± 1.9%. (mean ± SEM, n = 4). Control values have been subtracted. All P values (test compared to control) at 10ng/ml and 50μg/ml were < 0.05> 0.01.

Figure 3-20

Effect of anti-CD40 & IFN-γ on polarization to anti-IgD

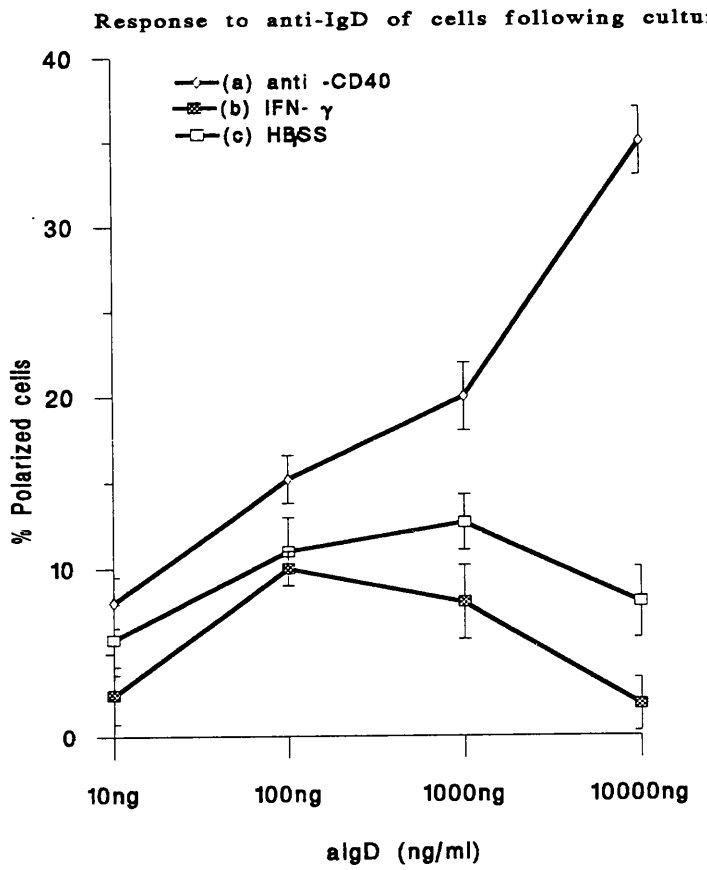


Figure 3-20 Polarization responses of B cells to anti-IgD in a 30 minute shape-change assay. The high-density B cells were cultured overnight in either anti-CD40, IFN-γ, or HBSS alone for 20h, washed and retested. Control values (medium alone : Polarization in anti-CD40, IFN-γ, and medium alone was 24 ± 0.7 %, 10.5 ± 0.5%, 12 ± 3.6%, respectively, mean ± SEM, n = 2) have been subtracted.

(a) Cells were cultured in anti CD40 (1μg/ml): P value: test cf. medium alone; anti-IgD >10μg/ml P : 0.01. (b) Cells were cultured in IFN-γ (10U/ml). (c) cells were cultured in HBSS. Polarization response in either HBSS or IFN-γ was not significant.

locomotion of resting cells but also increases the responsiveness of the cells toward the above attractants. It is interesting that cells cultured in IFN-γ after overnight culture did not respond to anti-IgD as a chemoattractant and the proportion of polarized cells was even lower than that found in cells cultured in HBSS alone. Therefore, summarizing the results from these experiments, it can be concluded that those stimuli (IL-4 or anti-CD40) which push the cells from G_0 to G_1 also increase their responsiveness toward anti-Ig.

3-2-3 Assessment of the Purity and Activity of $F(ab`)_2$ Fragments of anti-IgD

Following the observation that mouse IgG1, but not IgG2a is recognized by the human FcγRII (Van de Winkel & Anderson, 1991), sheep anti human IgD was digested by pepsin to remove the Fc fragment. $F(ab`)_2$ fragments were prepared to control for Fc receptor-mediated activation of locomotion. This was tested in the polarization assay and as seen in Fig 3-21, the $F(ab`)_2$ dimer was slightly less active than the whole antibody. In terms of titre, $F(ab`)_2$ dimer was equivalent to whole antibody. In parallel experiments, the Fc receptor was blocked with either hIgG (1μg/ml) or anti- CD32(1μg/ml). The data show that blocking of the Fc receptor by hIgG decreased the shape-change response slightly. Different concentrations of hIgG had no effect (10μg-10ng, data not shown), whereas anti-CD32 polarized the cells (Fig 3-22). These data suggest that in contrast to anti-CD32, hIgG blocks the Fc receptor on B cells without having an effect on the

locomotion response. It was reported previously that IL-4 abolishes the effect of Fc receptor on activation of B cells (O`Garra et al, 1985). Thus, the cells were cultured in HBBS and then exposed to anti-IgD and digested anti-IgD, but as is shown in Fig 3-22, HBSS causes some cell activation alone, and is therefore not an entirely satisfactory control.

Following digestion of sheep anti IgD and anti human IL-4 with pepsin, the protein was layered on SDS-PAGE electrophoresis to determine molecular weights and efficiency of digestion. 7% and 10% separating gels were used. When the concentration of separating gel was decreased, these changes resulted in better resolution of the protein bands. The calibration curves are depicted in Fig 3-23 and photographs of the gels are shown in Fig 3-24. In the gels illustrated in Fig 3-24 the bands for anti-IgD (30μg/ml), digested anti-IgD (30μg/ml), anti human IL-4 (15μg/ml), and digested anti IL-4 (10μg/ml) were compared with markers of known molecular weight. Bovine serum albumin (BSA) and human serum albumin (HSA) were used as controls of known molecular weight. The molecular weights of the protein bands were calculated from the molecular weight markers. An approximately linear curve was obtained using known molecular weight markers and known controls (BSA & HSA). This curve shows that anti-IgD and anti-IL-4 (IgG fraction) are located at 155 Kd and the digested fragment of these antibodies at 105 Kd (Fig 3-23). No band was detected at 150 Kd in digested fragments from either anti IgD or anti IL-4.

Figure 3-21

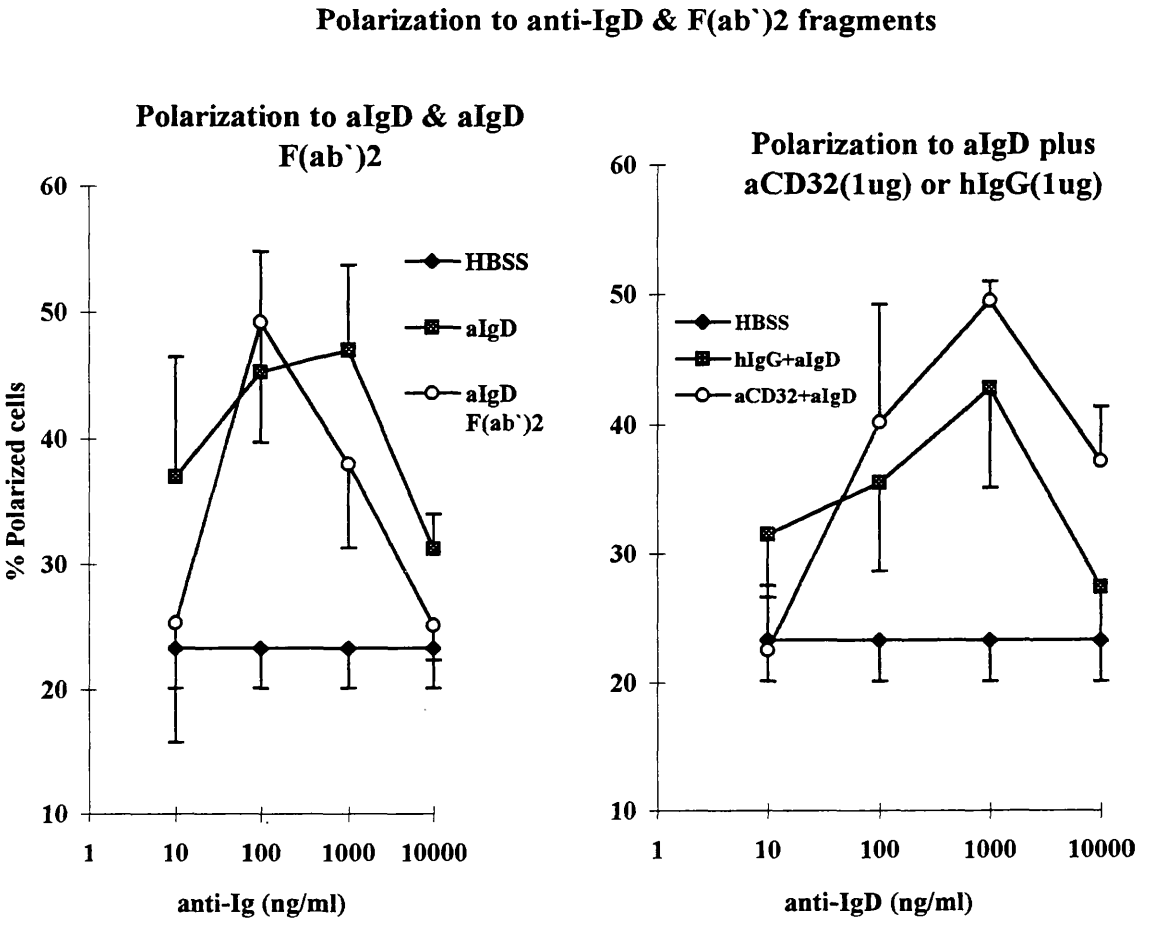


Figure 3-19 Polarization response of B cells cultured overnight in IL-4 (20u/ml). washed, and retested in whole aIgD or aIgD F(ab`)2 fragments (Left), or in a combination of either hIgG(1ug/ml) + aIgD or anti-CD32 (1ug/ml)+ aIgD (Right). Control values (polarization in medium controls for human IgG and anti-CD32 were 25.3 ± 3% and 27.5 ± 7%, respectively, mean ± SEM, n = 3)
All part of same experiment. aIgD in Left hand graph is a control for the curves in the Right hand graph.

Figure 3-22

Polarization to anti-CD32

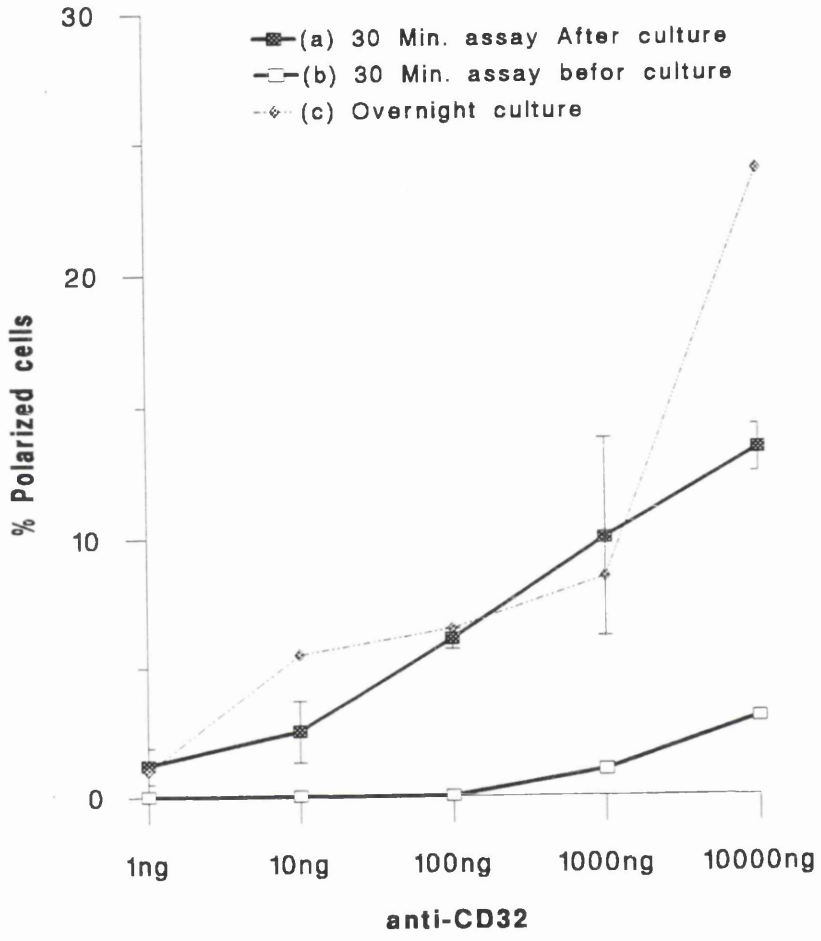
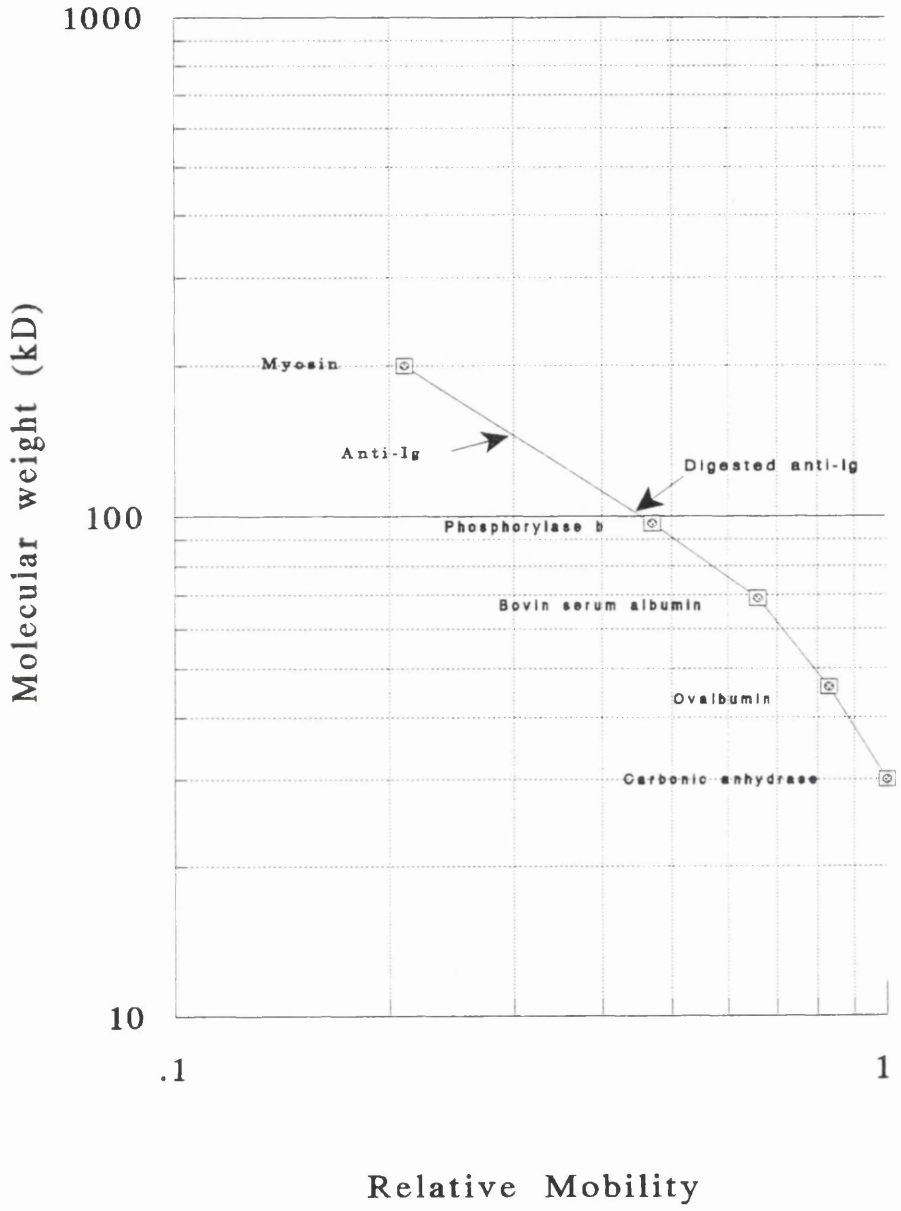


Figure 3-22 Immediate shape-change and overnight Polarization of high-density B cells to anti-CD32 (a) Dose response of cells cultured overnight in IL-4 (20U/ml), washed, and retested in anti-CD32 (mean ± SEM, n = 2). Control values (medium alone : Polarization in medium alone was 20.5 ± 1.8%). have been subtracted (b) Dose responsive curve of freshly isolated cells in one experiment. polarization in medium alone was 6.6%. (c) Cells were cultured overnight in anti-CD32(20H) and fixed in their own supernatants in one experiment. polarization in medium alone was 23%.

Figure 3-23

Calibration curve for antibodies



Calibration curve using Rainbow marker, RPN756. Undigested anti-Ig was detected at 155KD and the bands for digested form were precipitated at 105KD. No bands for digested antibody were detected at 155KD.

Figure 3-24

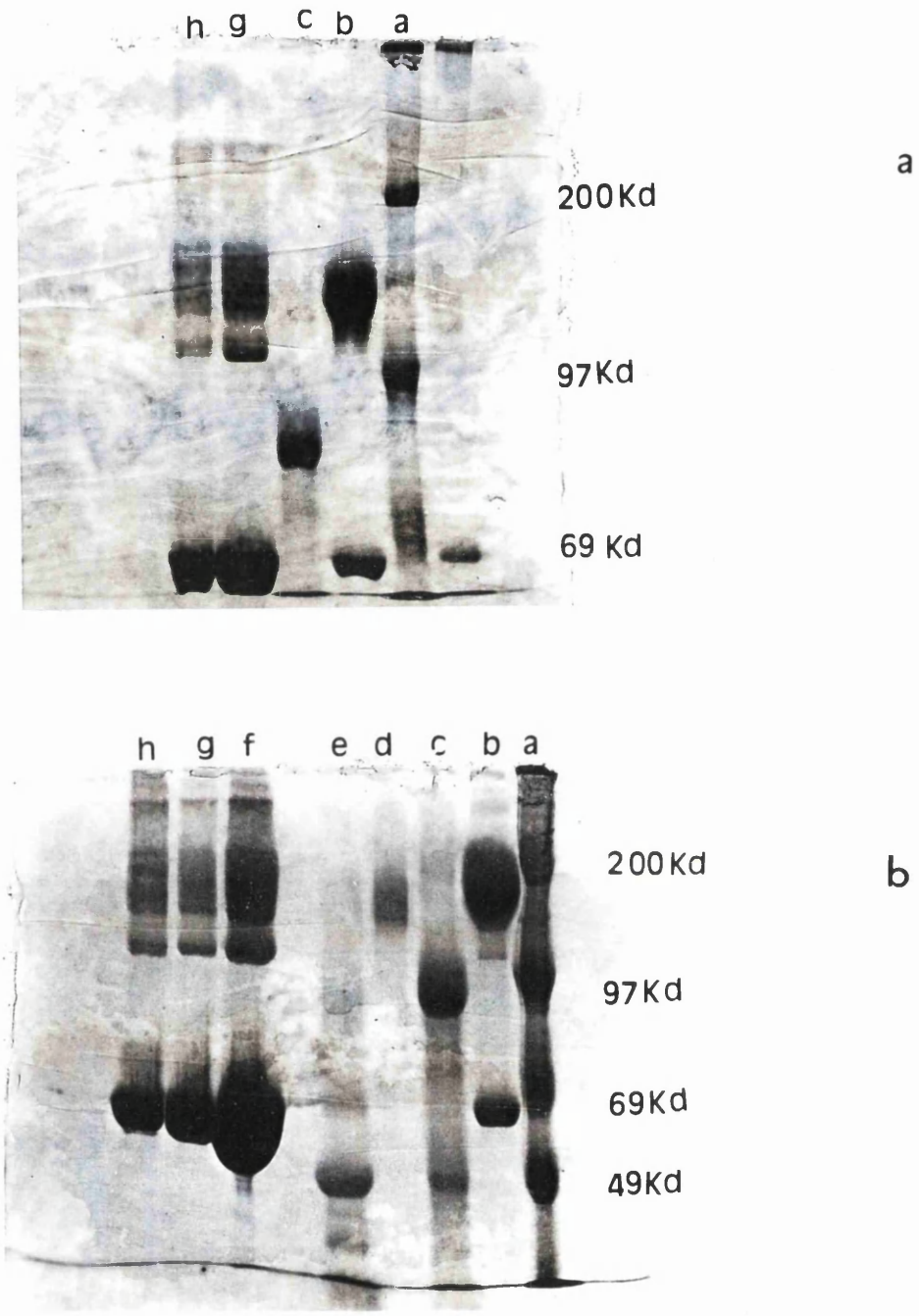


Fig. 3-24 SDS-PAGE analysis of antibodies used to study B cell locomotion. Various protein loads of (a) molecular weight markers, (b) anti-IgD, (c) digested anti-IgD, (d) anti -IL-4, (e)digested anti-IL-4, (f) supernatant of resting cells after overnight culture in IL-4, (g) BSA, and (h) HSA. Gels were stained using Coomassie brilliant Blue R-250. Different concentration of separating gels were used. Fig A : 7% separating gel; Fig B : 10% separating gels .

3-2-4 Locomotion into Collagen Gels

While measurement of shape-change is a reliable correlate of locomotion, the assay does not measure locomotion itself. To do this, we overlaid lymphocytes on a three-dimensional collagen gel incorporating attractants and allowed them to invade the gel . Firstly, B cells cultured in various ways were placed on collagen gels (1.2mg/ml) to which no attractant had been added. Of cells cultured in medium alone, 27.1% invaded the gel; of IL-4 cultured cells, 38.1%; and of IL-13 cultured cells, 42.7%. Secondly, cells that had been cultured overnight in IL-4 were placed on gels containing culture supernatants or purified antibodies such as; anti-IgM, anti-IgD, anti-CD40, their appropriate isotype controls or combinations of these agents. The cells then were allowed to invade for a further 18 hours. These gels were denser than those used above (collagen 1.5mg/ml) and consequently fewer cells invaded the gels in the medium control. The number of cells invading gels incorporating any of the stimuli was greater than that invading gels containing medium alone and was much greater when stimuli were combined (Table 3-10). The gel invasion assay selects the locomotor population and demonstrates clearly that small resting sIgM+ B cells not only change shape in response to anti-IgM, anti-IgD, and anti-CD40, but also show invasive locomotion in response to these antibodies and to attractants released into the supernatant during culture. In similar experiments, low density B cells (activated cells) were also overlaid on collagen gels made of culture supernatants (1/2 dilution), combination of supernatant + anti-IgM (1μg/ml)+ anti-CD40(1μg/ml), and HBSS-

MOPS alone. After 18 hours incubation 35.2% of cells had invaded gels contaning supernatants, 33% of cells invaded gels containing anti-IgM, and 23,7% of cells had invaded into the gels containig HBSS/HSA. These cells were not GC B cells, since most expressed sIgD (Table 3-4) and also GC B cells undergo apoptosis during culture.

3-2-5 Time-Lapse Cinematography

Following the observation that cells with motile morphology were present on top of collagen gels after overnight culture, time-lapse videomicroscopy was used to study the locomotion of responsive tonsillar resting B cells placed on the upper surface of gels containing attractants. Different source of attractants such as anti-IgM and the supernatant from resting cells cultured in IL-4 were incorporated into the gels. Freshly isolated resting cells were layered over gels containing anti-IgM (1μg/ml). After 30 minutes incubation, 12% of cells showed motile morphology but did not show translocation because the cells were not adherent to the gel. In the next experiments, the resting cells were cultured in IL-4 overnight and, after washing, cells were layered on the top of a collagen gel containing culture supernatant (1/2 dilution, 1.5mg/ml). The preparation was filmed following incubation for 30 minutes. Obervation of locomotion indicated that 57.2% of cells showed motile morphology, but these cell remained sessile because they did not adhere to the gel. No invasion was seen within 30 minutes either of the freshly isolated population or in the cells precultured in IL-4, probably because this is too

Table 3-10

Invasion of collagen gels by IL-4 cultured tonsillar B cells

Attractant	% cells invading the gel (mean ± SEM) (collagen 1.5mg/ml)	
HBSS-HSA	18.5 ± 2.7	(n=6)
anti-IgM (1μg/ml)	35.0 ± 4.2	(n=3)
anti-IgD (1μg/ml)	40.3 ± 2.9	(n=3)
anti-CD40 (1μg/ml)	32.2 ± 3.6	(n=3)
SN (1/2 dilution)	36.5 ± 4	(n=3)
anti-IgM + anti-CD40	35.6 ± 3.8	(n=4)
anti-IgD + anti-CD40	36	(n=1)
SN + anti-IgM + anti-CD40	49.5 ± 3.8	(n=2)
IgG1 (1μg/ml)	17.7 ± 2.1	(n=2)
IgG2a (1μg/ml)	19.5	(n=1)
sheep Ig (1μg/ml)	16.6	(n=1)

P for anti-IgD and combination of SN+anti-IgM+anti-CD40 vs HBSS-HSA <0.01: P for SN, anti-IgM, anti-CD40 and anti-IgM + anti-CD40 vs HBSS-HSA <0.05>0.01.

SN = supernatant of high-density B cells cultured in IL-4 (100U/ml) harvested after 20h.

early for significant invasion to be evident. The gels were then incubated for 15 hours and cells from the top of the gels as well as those which had invaded were filmed. The data shows that 40.3% of cells on the top of the gels were motile, and most of them were adherent to gel. Many cells invaded the gel overnight (proportions are given in Table 3-10), and filming of these cells showed continual shape change and movement within the gel matrix. Gel invasion is therefore dependent on the cells being able to make attachment to the gel surface.

3-2-6 Polarization Related to Growth Measured by Uridine Incorporation

It was demonstrated previously that high density B cells cultured in IL-4 for 20 hours (long term assay), not only increase their capacity to respond to chemoattractants, but also gradually acquire motile morphology (shape change). These cells also show an immediate response to their own supernatants and to polyclonal activators such as anti-IgM and anti-IgD. Here, we studied the relation between shape-change and growth by measuring [^{3}H]-uridine incorporation by resting B cells direct from the tonsil and after culture in IL-4. Table 3-11 shows the results of autoradiography after [^{3}H]-uridine incorporation. 200-250 cells per sample were scored. There were very few labelled cells (lightly labelled cells $\leq$ 10 grains per cell 8.3 $\pm$ 4.1%, and heavily labelled cells $\geq$10 grains per cell 5.5 $\pm$ 0.35%) before culture. The small proportion of these cells that polarized in short term assay, either spontaneously or in response to anti-IgM and anti-IgD

showed greater uptake of uridine than the cells that did not respond. The number of grains was increased after cells were cultured in IL-4 (20u/ml or 100u/ml) (lightly labelled cells ≤ 10 grains per cells 33.5 ± 0.5%, and heavily labelled cells ≥ 10 grains per cells 44.8 ± 2.3%). After culture overnight in IL-4, the proportion of heavily labelled cells had increased to about 40%. The proportion of polarized cells showing heavily uridine labelling after culture was higher (70 - 80%) than the proportion among the spherical cells or in the whole population. This was also true for the polarized cells responding to anti-IgM, anti-IgD, or to culture supernatants as chemoattractants. Analysis of the results from immediate shape-change assays using either freshly isolated cells or cells cultured in IL-4, demonstrated that heavily labelled cells had more capacity to respond to chemoattractants than lightly labelled or non-labelled cells. Therefore, these experiments demonstrated that the responsive cells are mostly among the heavily labelled population. This was concluded from the response of cells to different concentrations of anti-IgD, anti-IgM, and supernatant compared with medium alone. In a parallel experiment, high density cells were cultured overnight in IL-4, 20 and 100u/ml, then was pulsed with [3H]-thymidine and slides were prepared for microscopic analysis. The results obtained shows that there was no thymidine labelled cells among them. The results of autoradiography are shown in Table 3-11a and 3-11b. These findings suggest that the cell population activated for locomotion and for responses to chemoattractants are also the cells most active in RNA, but not DNA synthesis. The autoradiographes show a relation between

Table 3-11a

Autoradiography:[3H]-uridine incorporation by spherical and polarized B cells

30 minute assay on cells direct from tonsil:-

Source of cells and stimuli	% polarized cells in population	Percent of cells showing >10 grains per cell		
		Spherical	Polarized	All cells
HBSS/HSA	9.2	3	25	5.1
aIgM (100ng/ml)	12.7	1.5	31	5.2
aIgD (1μg/ml)	15.9	1.6	31	6.9

For short term assay on cells from the tonsil, cells were pulsed with uridine for 30 minutes, then washed twice and exposed to different polarizing stimuli for 30 minutes.

Long term assay: Cells cultured of overnight

IL-4 (20u/ml)	33	22	79.6	40
IL-4 (100u/ml)	37.6	10	78	38

In long term assay cells were cultured in IL-4 for 20h, then pulsed with uridine, fixed, and washed twice.

Table 3-11b

Autoradiography:[3H]-uridine incorporation by spherical and polarized B cells

30 minute assay on cells cultured in IL-4:-

Source of cells and stimuli	% polarized cells in population	Percent of cells showing >10 grains per cell		
		Spherical	Polarized	All cells
HBSS/HSA	16.2	37.6	75	43.7
aIgM (100ng/ml)	18.6	38.1	85.2	46.9
aIgD (1μg/ml)	27.5	26.7	86.3	43.1
aIgD (10ng/ml	16.8	32.7	73	55.1
SN (neat)	37.8	20	74.6	41
SN (1/10 dilution)	26.3	31	62.2	39.2

Cells were cultured in IL-4 (20u/ml) for 20 hours, pulsed with uridine, washed and retested with above attractants for 30 minutes

SN : Supernatant from high-density B cells cultured in IL-4 (100u/ml)

Figure 3-25

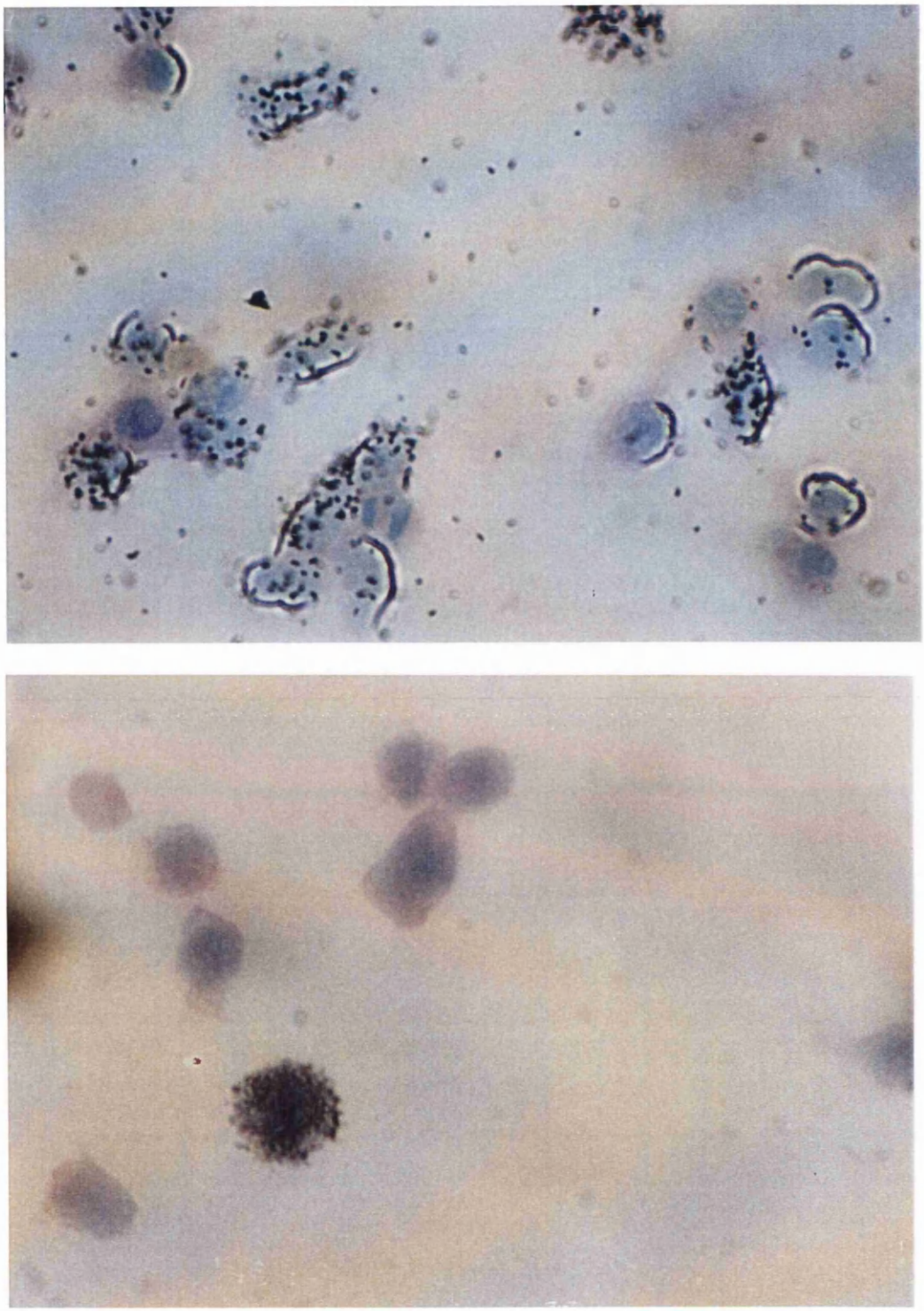


Fig 3-25 Upper Fig. Autoradiographs showing [3H]-uridine incorporation in high-density B cells after overnight culture in IL-4. High-density B cells did not incorporate [3H[-thymidine. Lower Fig. Autoradiographs showing [3H]-thymidine incorporation in GC B cells after overnight culture in IL-4 and anti-CD40. Note that the cells with high grain count for uridine but not for thymidine are also the polarized cells.

locomotion and [^{3}H]-uridine but not [^{3}H]-thymidine incorporation (Fig. 3-25).

SECTION THREE : Discussion

Previous studies suggested that resting lymphocytes have a poor locomotor capacity, but that this capacity can be enhanced by a period of culture with growth activators such as mitogens or antigens (Wilkinson, 1990; Wilkinson, 1986). These studies mainly concerned T cells, and in the present study, a similar approach was adapted to define (a) stimuli that confer locomotor capacity on B cells in overnight culture, and (b) chemoattractants for B cells achieved within 30 minutes. The locomotor properties of a subpopulation of small, high density, sIgM+, sIgD+ B cells from tonsil, a phenotype which suggests that they belong to the recirculating population are reported here. A small minority of these cells showed locomotor activity when tested directly after separation, but their locomotor capacity was considerably enhanced by a period of overnight culture in IL-4, anti-CD40 or IL-13. Using the definitions in the introduction to this project, IL-4 and IL-13 are good activators of locomotor capacity, while anti-IgM and anti-IgD are chemoattractants. It was reported earlier that IL-4, an early activation signal for B cells, would induce locomotor capacity in resting cells during overnight culture (Wilkinson & Islam, 1989; Clinchy et al, 1991). It is reported here that IL-13 and anti-CD40 behaved similarly. Also the present study defines both the phenotype of the responding cells and the importance of cytokines as activators of locomotor activity in B cells more precisely. Both IL-4 and IL-13 are cytokines whose capacity to activate B cells is well documented

(McKenzie et al, 1993; Minty et al, 1993; Howard et al, 1982; Isakson et al, 1982; Paul & Ohara, 1987). The activities of the two cytokines are very similar, but IL-4 has been shown to be the more potent of the two (Punnonen et al, 1993; Defrance et al, 1994). These observations of the superior capacity of IL-4 over IL-13 as an activator of B cell locomotion are consistent with differences between these cytokines in other properties. IL-4, IL-13 and CD40L act early in B cell differentiation. Thus, it was conjectured that acquisition of locomotor capacity was an early event which accompanied the transition from G_0 to G_1. For further progress through the cell cycle, other cytokines are necessary. Both the present study on tonsil B cells and the previous studies on blood B cells demonstrate that early signals appear to be the crucial ones for activating the locomotor capacity of B cells. Cytokines acting later in B cell maturation did not activate locomotion in this project or in those of others (Wilkinson & Islam, 1989; Clinchy et al, 1993), even using B cells that had already been cultured in IL-4.

Much evidence has accrued recently to suggest that full activation of B cell differentiation and proliferation requires multiple signals working through different receptors (Brines and Klaus, 1993; Gordon et al, 1989; Van den Eertwegh et al, 1993). Ligation of CD40 with anti-CD40 induces B cell proliferation (Gordon et al, 1988, Clark et al, 1989; Wheeler et al, 1993) and the antibody mimics the action of the natural CD40L present on T cells early after activation (Banchereau et al, 1994). The available evidence therefore strongly implicates CD40 as a pivotal molecule in regulation of B cell locomotion.

Blocking the CD40-CD40L interaction inhibits the development of memory B cells (Gray et al, 1994). Early gene expression and later proliferation of B cells are enhanced by culture in combinations of anti-CD40 or of CD40L with IL-4 (Gordon et al, 1989; Clark et al, 1989). Activation signals in B cells are also generated through engagement of the antigen receptor either by antigen or by anti-Ig. Locomotor stimulation by anti-Ig has been observed in B cells (Schreiner et al, 1975; Ward et al, 1977), and CD40 has been reported to lower the threshold for stimulation of B cells by anti-Ig (Wheeler et al, 1993).

This finding suggests that, like the proliferation response, the capacity for locomotion is optimal after B cells have been stimulated through more than one receptor. More locomotor cells were present after culture in both anti-CD40 and IL-4 than in either alone. Overnight culture in anti-IgM or anti-IgD alone did not increase the proportion of locomotor cells but addition of anti-IgM to anti-CD40 and IL-4 gave maximal locomotor activation. Since anti-Ig by itself does not induce the locomotion response and keeping in mind that surface Ig is the only receptor that transmits Ag-specific signals to B cells, it can be postulated that anti-Ig (Ag) stimulation provides the initial signal that renders Ag-specific B cells more receptive for the subsequent helper signals anti-CD40 and IL-4 or IL-13.

B cells have receptors for both IL-4 (Zola et al, 1993) and IFN-γ (Valente et al, 1992). In contrast to the previous finding on blood cells (Wilkinson and Islam, 1989) IFN-γ did not induce a locomotion response in tonsillar B cells in overnight culture, neither were the supernatants from IFN-γ-cultured cells active. Possible

explanations are as follows; Possibly the IFN-γ used in the earlier study was not 100%. Secondly, the purity of tonsil B cells was much higher than that from blood and non-B cells might have been responsible for the earlier response either by releasing locomotor activators for B cells or by themselves responding to IFN-γ. Here, IFN-γ, a T_{H1} type cytokine, was able to inhibit the locomotion induced by IL-4 in B cells. This finding suggests that B cells might receive two different signals from these two cytokines: a positive signal through the IL-4R and a negative signal through IFN-γR. The result reported here directly demonstrates that IFN-γ is one of the physiological factors that can influence the locomotion of B cells induced by IL-4. Inhibition by IFN-γ of the response to IL-4 together with down-regulation of the immediate shape-change response of IFN-γ cultured cells to anti-IgD suggests that the suppression of the T_{H2} type response by IFN-γ might be initiated at the beginning of the immune response even before the cells enter G_1 cycle, keeping in mind that both cytokines act as an early stage of B cell differentiation (Defrance et al, 1987; Boyd, 1987). IL-10, a T_{H2} type cytokine, is another cytokine which has been reported to inhibit the locomotion response of B cells to IL-4 (Clinchy et al, 1994). This result is consistent with a model in which the inhibitory effect of IFN-γ on B cell locomotion would adversely affect the T_{H2} but not the T_{H1} type of immune response..

In a normal individual, each B cell clone possesses an unique receptor for antigen. Given this frequency, an attempt to detect a specific locomotor response using antigen to stimulate the B cell antigen receptor may be unrealistic in this

model. The majority of studies in this project have been performed using polyclonal activators such as anti-IgD or anti-IgM to 'mimic' the effect of antigen. The effect of anti-IgM and anti-IgD as chemoattractants is of interest and relates to observations made during the 1970s. It was reported that B cell lines showed chemotactic responses (Russell et al, 1975) and that lymphocytes from the lymph nodes of mice migrated into filters in response to gradients of the immunizing antigen but not towards control proteins (Wilkinson et al, 1977). In retrospect, it seems certain that only B cells could have responded in such a way to soluble antigen. Observations were made at about the same time that non-isotype specific anti-Ig could cause capping accompanied by uropod formation in mouse B cells and that mouse and rat B cells would migrate into filters towards anti-Ig (Schreiner & Unanue, 1975; Ward et al,1977). The cultured cells responded better than cells prior to culture to attractants present in their own supernatants as well as to anti-IgM, anti-IgD, and to the combinations of anti-Igs with their own supernatants. Experiments using supernatants of cultured cells in IL-4 or anti-CD40 in locomotion of B cells may suggest an of autocrine effect on B cell locomotion following activation in T-dependent immune responses. This finding is consistent with others showing that under appropriate conditions, B cells may produce their own regulatory molecules for growth (Gordon, Gay & Walker, 1985). Both anti-IgM and anti-IgD proved to be good attractants for B cells, with a more powerful effect on IL-4 cultured cells than on those direct from the tonsil. In all of our experiments, anti-IgD was a stronger attractant than anti-IgM and this

may suggest a differences in behaviour between these two mIgs. The difference in the locomotion response using anti-IgD and anti-IgM is consistent with several differences in behaviour between these two antibodies (Luxembourg and Cooper, 1994). This is possibly because mature resting B cells generally express a much higher level of mIgD than mIgM (Havran et al, 1984). Anti-CD40 was not an attractant for resting cells direct from the tonsil, but in contrast to IL-4 and IL-13, anti-CD40 induced immediate shape change in B cells that had been activated by overnight culture in IL-4 following by washing. CD40L is a T cell surface molecule which might not be expected to form chemotactic gradients. However, cultured T cell lines may release soluble CD40L (Armitage et al, 1992), and soluble CD40L can activate B cells (Lane et al, 1993). There are also reports that lymphocyte locomotion can be activated not only by gradients of soluble molecules but also by surface contact. For example, L selectin-dependent polarization of lymphocytes on contact with high endothelial cells has been demonstrated in vitro (Harris & Miyasaka, 1995). Possibly binding to CD40L on the surface of T cells activates shape change in the B cells which are then capable of migrating towards other attractants.

The effect of anti-Igs on locomotion was observed both in polarization assays and in collagen gel assays. The response to both was dose-dependent, optimal at 100ng-1μg/ml of antibody, and less apparent at higher concentrations (Fig 3-16). The polarization response to anti-IgD was maximal within 30 Min. (Fig 3-17), and gradually dropped after one hour (Fig. 3-17A). This type of dose response is

frequently seen with chemoattractants which, at high concentrations, bind rapidly to the receptors all over the cell, preventing the cells from polarizing efficiently, whereas at lower concentrations, cells can integrate a head-tail polarization response by detecting non-homogenities of ligand binding (Mckay et al, 1991; Tranquillo et al, 1988). It is likely that the locomotor response to anti-Ig mimics a response to antigen. Gradients of soluble antigens probably exist in vivo and may attract B cells. Taken together, the earlier findings and those reported here support the scenario outlined earlier, in which recirculating B cells can show chemotactic responses to antigen but migration of B cells to foci of such antigens would not on its own lead to proliferation in the absence of T cell-derived signals. Locomotor activity associated with clustering of B cells with T cells and accessory cells may be induced by a combination of signals from native antigen, either in solution or present on the surface of accessory cells in clusters with T cells, with signals (e.g. through CD40L) generated on contact with helper T cells bearing processed antigen. Anti-CD40 and to a lesser extent IL-4 and anti-Ig induce homotypic aggregation which seems to be important in B cell contact and facilitates the exchange of autocrine factors (Björck et al, 1992). This observation may be related to expression of LFA-1/ICAM-1 adhesion molecules by activation of B cells through mIg and CD40 receptor (Barrette et al, 1991; Dang and Rock, 1991) and interaction of CD23 and CD21 (Björck et al, 1993a).

The kinetic studies on locomotor activators and chemoattractants clearly demonstrate that in contrast to attractants, B cells gradually responded to locomotor

activators. Freshly isolated cells are mostly in the G_0 phase of cycle and require time to transit from G_0 to G_1 and to respond to attractants. Attractants may be gradually released into the supernatant during culture, but their nature has not been determined. Cells cultured in cytokines such as IL-4, then washed responded strongly to their own supernatants but not to the cytokine with which they had been cultured. In mixed populations of spherical and polarized B cells after culture in IL-4, the locomotor cells showed increased uridine uptake, but not thymidine uptake, compared to the spherical cells. These cells also increased in size. This finding strongly suggests that the locomotor subpopulation represents the cells that have moved into the G1 phase of growth, as was reported earlier for cultured T cells (Wilkinson, 1986).

The findings reported here suggest that efficient B cell locomotion towards attractants such as anti-immunoglobulin requires a population that has been activated to progress from G_0 into the early stage of the G_1 phase of cell cycle. The signals for this activation are products of T cells (IL-4, IL-13, and agents that bind CD40) which themselves are expressed as a result of contact with antigen. Recirculating lymphocytes are mainly resting G_0 cells and these experiments do not address the question how such cells migrate from the blood to lymphoid tissue. The signals for this step, both adhesive and locomotor, may be generated by contact with appropriate molecules on high endothelial cells (Harris & Miyasaka, 1995). It is more likely that the locomotor signals studied here come into play once B cells meet antigen and that they are T cell-dependent. After

activation, B cells would leave the recirculating pool and follow the migratory pathways which lead to germinal centre formation and the production of memory cells. It will be interesting to investigate how the locomotion of B cells at later stages of maturation is directed.

CHAPTER FOUR

LOCOMOTOR PROPERTIES AND PHENOTYPIC ANALYSIS OF GERMINAL CENTRE B CELLS

SECTION ONE : Phenotypic Analysis

4-1-1 Germinal Centre B Cells

Following from the observations of locomotor responses of high and low density B cells described in the previous chapter, it became clear that most of the responsive cells in both the high density and low density B cell fractions were IgD+ and IgM+. These studies were extended by isolating GC B cells from the low density fraction using anti-CD39 (AC2) and anti-IgD coated sheep red blood cells (SRBCs). The non-rosetting CD39- IgD- GC cells were then isolated from the rosetting (CD39+ IgD + fraction) using Lymphoprep density gradient separation. The identity of GC B cells was determined on the basis of their phenotypic characteristics. These cells mostly co-express CD19 and CD38 strongly. Investigation on FACScan analysis in three experiments show that this fraction contained 81 ± 1.7 % CD19+, CD38+ cells and 3.4 ± 0.33 % CD14 bearing cells. To eliminate CD14 bearing cells, this fraction was treated with anti-CD14 coated SRBCs and GC cells were harvested from the interface fraction after centrifugation on Lymphoprep.

4-1-2 Analysis of Freshly Isolated GC B Cells

The majority of cells found in mature GC are of two types: (1) Centroblasts expressing little or no smIg which made up about 20-35 % of the whole population of GC cells based on autoradiography using [^{3}H]-thymidine and analysis of cytospin

preparations. These are large cells with clefted nuclei that proliferate within the dark zone; (2) Centrocytes, which form a non-dividing progeny, are mostly located in the light zone and may express IgA, IgG, or CD38 strongly. In this population, membrane IgD is no longer present and small number of cells express IgM (Holder et al, 1993a; Holder at al, 1991). These are smaller in size and might still have clefted nuclei. The morphology of freshly isolated GC B cells is shown in Fig. 4-1. Since these cells express CD38 strongly, double staining using anti-CD38 and anti-CD19 was applied to identify them. The phenotypic characteristics of GC B cells using different markers are shown in Table 4-1. Morphological examination of GC cells cultured in IL-4 and anti-CD40 showed that these reagents not only increase the motility and survival but also morphologically differentiate them towards a distinct type when compared with the starting population. The cultured cells (which are centrocytes, because centroblasts undergo apoptosis in culture period) became larger with a central nucleus and more cytoplasm, and they had almost lost the nuclear cleft which was more prominent in freshly isolated cells. Fig. 4-2 shows GC B cells on FACS, and expression of CD38 on GC was compared with that on high-density and low-density B cell fractions. Expression of this marker on high-density B cells is low and low-density cells express this marker heterogeneously, while GC B cells show homogenous expression. The majority of cells co-expressed CD38 and CD19 strongly and expression of IgD was almost absent. Despite their size differences the GC cells, in contrast with high and low density cells, showed a homogenous phenotype. All separation was carried out at 4^0C to prevent apoptosis

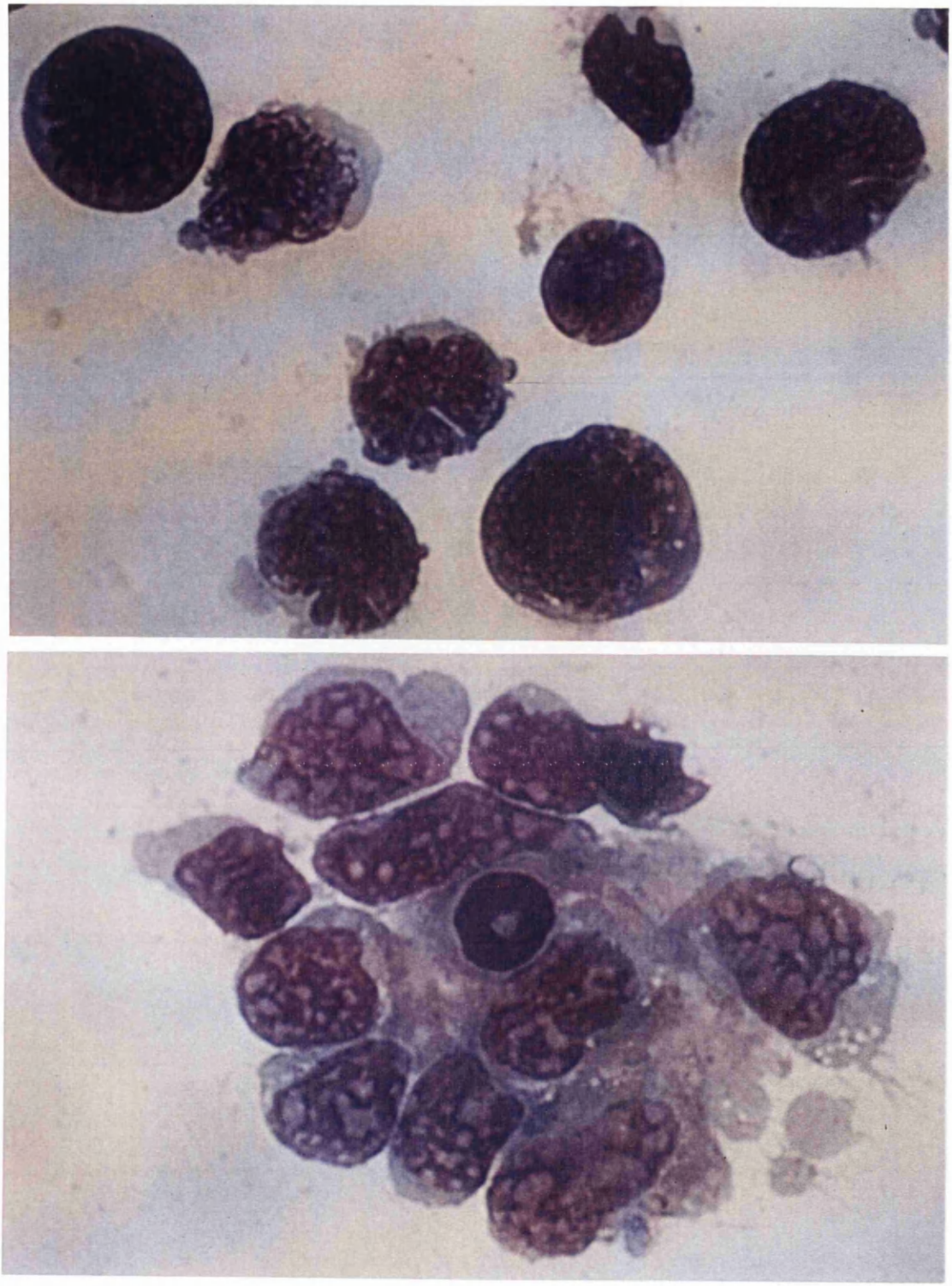
Table 4-1

Phenotype characteristic of freshly isolated GC B cells

Cell Markers	Mean	Range
CD38 - CD19 (4)	83 ± 2.1	90 - 78
CD3(2)	0.75 ± 0.25	1- 0.5
CD10(2)	10 ± 3	13 - 7
CD14(2)	2 ± 1	3 - 1
CD19(4)	97.3 ± 0.5	97 - 95
CD20(4)	88.3 ± 1.9	92 - 85
CD21(3)	86 ± 0.6	87 - 85
CD22(2)	86.5 ± 0.5	87- 86
CD23(3)	67 ± 3.5	73 - 61
CD32(2)	21.5 ± 10	32 - 11
CD38 (4)	88.8 ± 2.3	97 - 85
CD69(1)	93	93
IgA(2)	36 ± 1	35 - 37
IgG(2)	13 ± 4	17 - 9
IgM(3)	15 ± 6	21 - 3
IgD(3)	1.5 ± 0.2	2 - 1

The GC B cells were prepared by negative selection using anti-CD39 and anti-IgD, following depletion of CD14 bearing cells using anti-CD14 coated sheep red Blood cells. Numbers in parentheses show the number of experiments. Range shows high and low events.

Figure 4-1



a

b

Fig 4-1 Morphology of germinal centre (GC) cells before and after 12 hours culture. Following cytocentrifugation, preparations were stained using May-Grunwald-Giemsa and photographed at a microscope magnification of x100. (a) Freshly isolated cells. (b) cultured cells in IL-4(50u/ml) and anti-CD40 (1μg/ml).

Figure 4-2

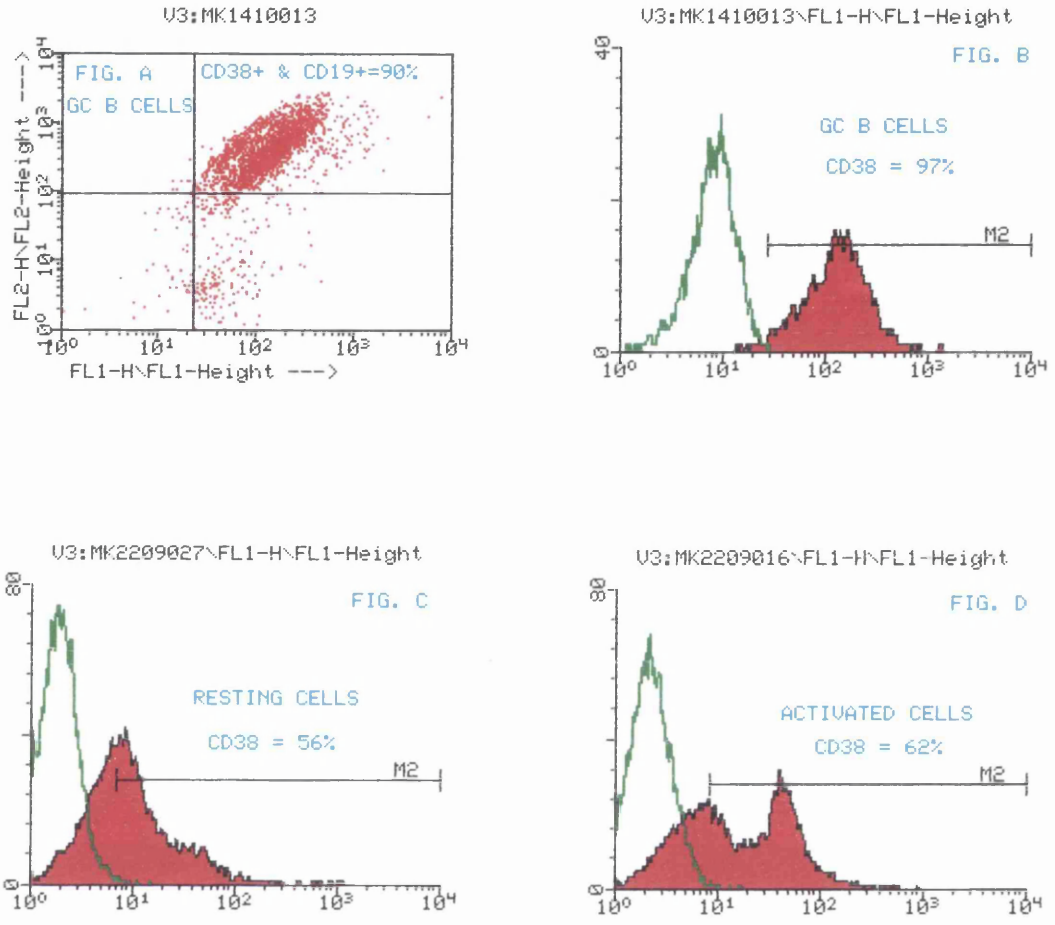


Fig 4-2 Analysis of germinal centre cells on FACS. (Fig. A) Expression of CD19+ & CD38+ in germinal centre B cells. There is strong expression of CD38+ in GC B cells (Fig. B) compared with high-density B cells (Fig. C) and low-density B cells (Fig. D).

and the viability was determined frequently and was more than 97% in freshly isolated cells. Since trypan blue only shows late events of cell death, it was not ideal for studying death in GC cells. Thus viability of GC B cells was determined by either phase contrast microscopy using a x40 objective or by Kimura stain in wet preparations using a x40 or x100 objective. This method gives good visualization of both apoptotic cells and polarized cells in either normal light or phase contrast and was ideal to study the morphology of cells. A photograph of these cells is shown in Fig 4-1.

4-1-3 Characteristics of Cultured GC B Cells

GC cells were cultured in IL-4 (50u/ml) and anti-CD40 (1μg/ml) for twelve hours. Following washing, the cells were stained with different markers. Two distinct regions were identified on the FACS of which region one (R1) represents the live population and region two (R2) the apoptotic population with high autofluoresence (dead cells). The data obtained from two experiments show that 54 ± 4 % cells strongly express CD38 and CD19. Percentage positive cells for other markers were as follows : CD20 (86 ± 1 %), CD21 (65 %), CD23 (50.5 ± 4.5 %), IgM (16 ± 6 %), IgG (25%), and IgA (25%). The low expression of CD38 on cultured cells compared with freshly isolated cells might be due to the effect of the incubation period on expression of this marker and also, since it was difficult to separate dead and live cells completely on FACS, the high autofluoresence activity

of dead cells increased the negative background. It has been reported that GC B cells undergo apoptosis when they are cultured in vitro (Liu at al, 1989) and death rate was related to the culture conditions. It was found (see below) that a six-hour culture period was enough for inducing a locomotor GC population and the characteristics of these cells were therefore defined. Firstly cytospins from freshly isolated cells and from cells cultured in different media were examined. The results obtained (Table 4-2) indicate that the large cells with centroblast features, are absent after culture and have undergone apoptosis. Secondly results from autoradiography analysis (Table 4-6) demonstrate that 15-20% of freshly isolated cells took up [3H]-thymidine and this drops to 4-5% in cells cultured in IL-4 (50U/ml) in combination with anti-CD40 (1μg/ml) for six hours. The apoptotic cells are large and appear to be centroblasts which are still in cycle and have not received survival signals in vivo or in vitro. To study the responsive cells further, they were cultured in HBSS alone, in IL-4(20u/ml) + anti-CD40 (1μg/ml), or in IL-4 (50u/ml) + anti-CD40 (1μg/ml) in time course assays. The cells were then fixed in 1% paraformaldehyde and stained using anti-CD38 (Table 4-3). The results show that the responsive cells in all media strongly express CD38. The proportion of these cells is in agreement with results obtained from wet preparations in polarization assays. Investigation by FACS analysis of the cells which had invaded collagen gels after 12 hours revealed that after collagenase treatment 65% of the invasive cells were positive for CD38+, CD19+ and 75% positive for CD23. The morphology of freshly isolated and cultured cells is shown in Fig 4-1. Fig 4-3 shows the morphological features of

Table 4-2

Percentage of GC cells in different morphologies

Slide preparation	Large clefted cells	Small clefted cells	Small non-clefted
Freshly isolated GC cells	34.5 ± 4.5¶	54 ± 6¶	11.5 ± 1.5¶
GC cells cultured in HBSS	10	51	37
GC cells cultured in anti-CD40 (1μg/ml)	12	59	29
GC cells cultured in IL-4 (50u/ml)	10.5 ± 0.5¶	55 ± 6¶	34.5 ± 5.5¶
GC cells cultured in IL-4 + anti-CD40	15	62	23

¶ mean of two experiments

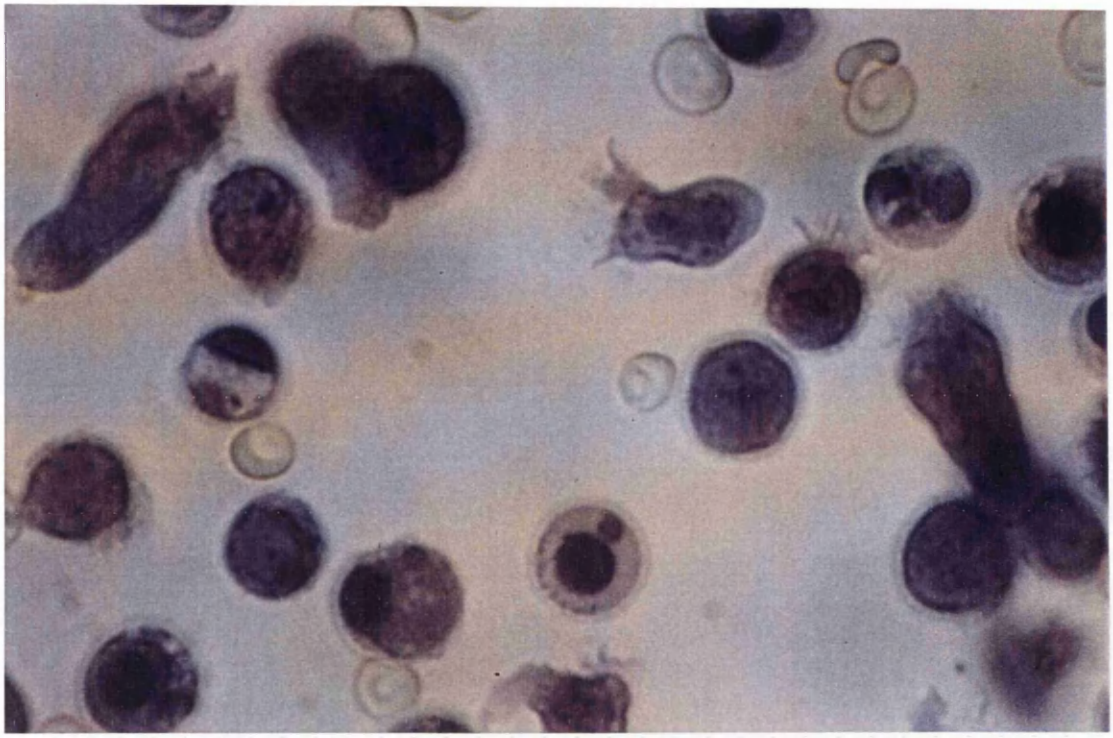
Table 4-3

Effect of culture conditions on CD38 phenotype of polarized cells

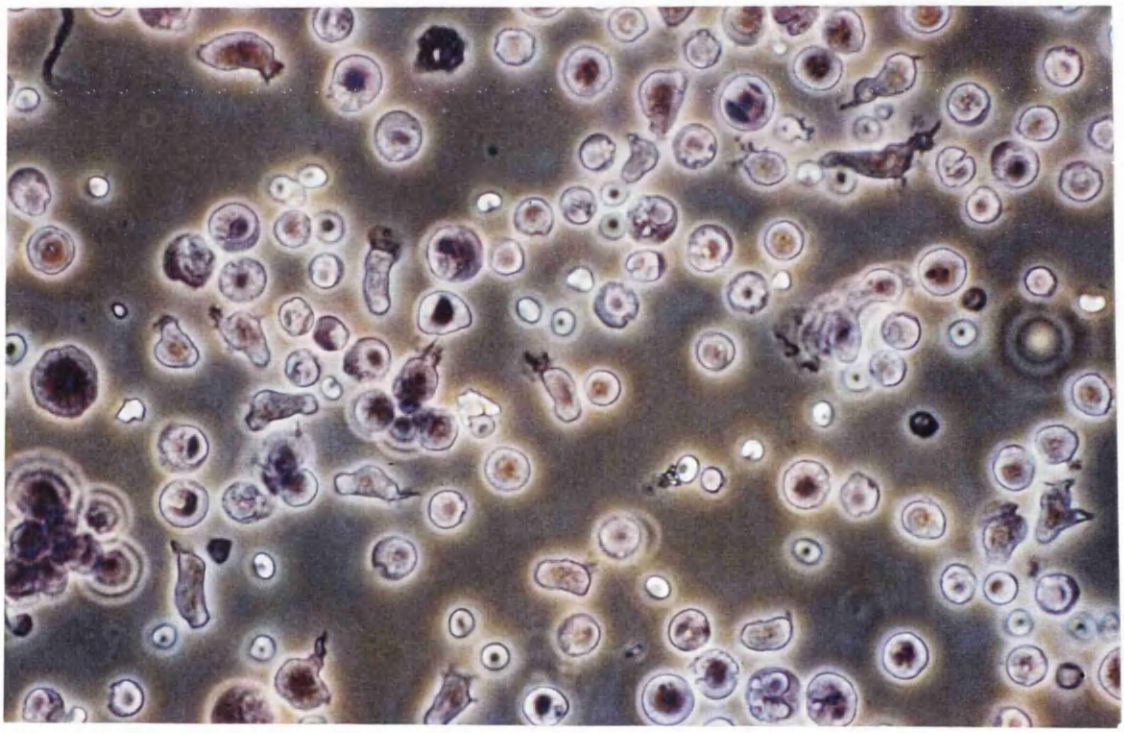
	% CD38 + polarized cells		
Culture condition	3H	6H	12H
HBSS alone	10	18.7	25.9
IL-4(20u/ml) + aCD40	15.3	29.2	52.5
IL-4(50u/ml) + aCD40	21.9	32.1	55.5

The GC B cells were cultured in different media for 3, 6, 12 hours, then they were then fixed in paraformaldehyde. The cells were stained with anti-CD38 and studied under flourescence microscopy using a x40 objective. 150-200 cells were counted and percent CD38+ polarized cells are shown in this table.

Figure 4-3



a



b

Fig 4-3 Morphological features of polarized, spherical, and apoptotic cells after culture. Following Kimura stain preparation, the slide was photographed at a microscope magnification of x100 and x40. (a) GC cells morphology after 18 hours in IL-4 and anti-CD40 showing polarized cells, spherical cells, and apoptotic cells with fragmentation of nuclei which is the hallmark characteristic of apoptotic cells at x100 using light microscopy. (b) Phase contrast preparation of same sample at x40.

spherical, polarized, and apoptotic GC cells.

SECTION TWO : LOCOMOTION ASSAYS

The locomotion of GC B cells was studied as described in the previous chapter, using polarization assays, collagen invasion assays.

4-2-1 Time-Dependent Responses of GC B Cells

According to the model introduced by Liu et al (I989), the GC cells undergo apoptosis unless they receive two signals through FDC, and through T cells. Isolated GC B cells underwent rapid and spontaneous apoptosis when they were cultured in vitro (Liu et al, 1989). This self destruction could be arrested by cross-linking of the surface antigen receptors on GC B cells with immobilized anti-Ig and additional survival signals were provided by engaging surface CD40 on T-cells (Liu et al, 1989). IL-4 together with anti-CD40 also enhanced GC B cell survival by delaying spontaneous apoptosis in GC cells. In preliminary experiments, the GC B cells were cultured with different concentrations of IL-4 (20, 50, and 100U/ml) in combination with anti-CD40 (1μg/ml) in overnight culture. The results obtained showed that IL-4 at 50U/ml (the optimal dose) not only enhanced the locomotor response of GC cells but also increased their viability. Neither IL-4 nor anti-CD40 alone induced an immediate shape-change response in freshly prepared cells. Therefore, a time course assay was used to study the gradual effects of these

reagents and others for 24 hours. The data obtained are shown in Fig 4-4, 4-5, 4-6, 4-7. The percent polarization response was analysed as a proportion of the live cell population, and of the total (live + dead cells) population. 400-600 cells were scored for each test. Data are presented in the upper figures (4-4, 4-5, 4-6, 4-7), showing counts of the percent polarized cells in the whole population in the absence of rescue signals. The locomotor response is evident after three hours and reaches a maximum after six hours, then dropping gradually because an increasing proportion of the cells became apoptotic. In contrast to this finding, in the presence of reagents which can promote the survival of the cells the response curve remains constant. The percentage difference between the viability in IL-4 (50U/ml) plus anti-CD40 (1μg/ml) with or without anti-IgG,M,A (1μg/ml) on the one hand and HBSS alone on the other parallels the percentage difference in the proportion of polarized cells. This may suggest that the survival and locomotion signals are identical and those cells that receive the survival signal become motile while those cells which are not able to respond by polarization undergo apoptosis spontaneously. The data suggest that CD40, especially together with IL-4, is of importance not only in delay of apoptosis but also for induction of locomotion in GC B cells . The viability of cells in HBSS alone is the same as in IL-4, anti-IgG,M,A, a supernatant derived from overnight culture of high density cells in IL-4, or combinations of these reagents as shown in Fig 4-4, 4-5. This suggests that a distinct population survived and those may receive the survival signal in vivo. A slow increase in locomotor activity was seen in these populations compared with HBSS alone in the live fraction (more than

40% difference with control). This was stronger within 18 hours in the supernatant derived from high-density cells than in IL-4. In contrast to combination of IL-4 and anti-CD40, combinations of other substances such as supernatant derived from high-density cells, anti-IgG,A,M, with either IL-4 or anti-CD40 did not rescue the GC. Once again, this finding demonstrates the importance of IL-4 and anti-CD40 in locomotion and survival of GC B cells. Anti-CD40 alone could rescue a small proportion of cells from death, but maximal rescue was seen in the combination of anti-CD40 with IL-4 and /or anti-IgG,M,A (up to 30-40%). Multiple activation not only rescued the cells from apoptosis but also promoted the locomotor response of the GC population. As in the previous chapter anti-CD40, anti-Ig, and IL-4 induce homotypic aggregation and this was more significant in GC B cells. The effect of these reagents on aggregation of GC cells was studied by culturing these cells in HBSS, IL-4 (50u/ml), anti-Ig (1μg/ml), anti-CD40 (1μg/ml), and in combinations of these reagents for 12 hours. The cells were fixed and the numbers of clusters were counted per field using an inverted microscope (x40 objective). Clustering was seen by three hours of culture and the size, and number of clusters depended on the culture period and the condition of culture. The data obtained revealed that IL-4, anti-Ig and anti-CD40 alone induce homotypic aggregation which was marked in anti-CD40, less so in IL-4, and still less so in anti-Ig and medium alone. An interesting finding was that aggregated GC B cells were alive and mostly non-motile whereas dead cells were mostly among the separated population. Combinations of anti-CD40 and anti-Ig with or without IL-4 did not promote clustering, but

Figure 4-4

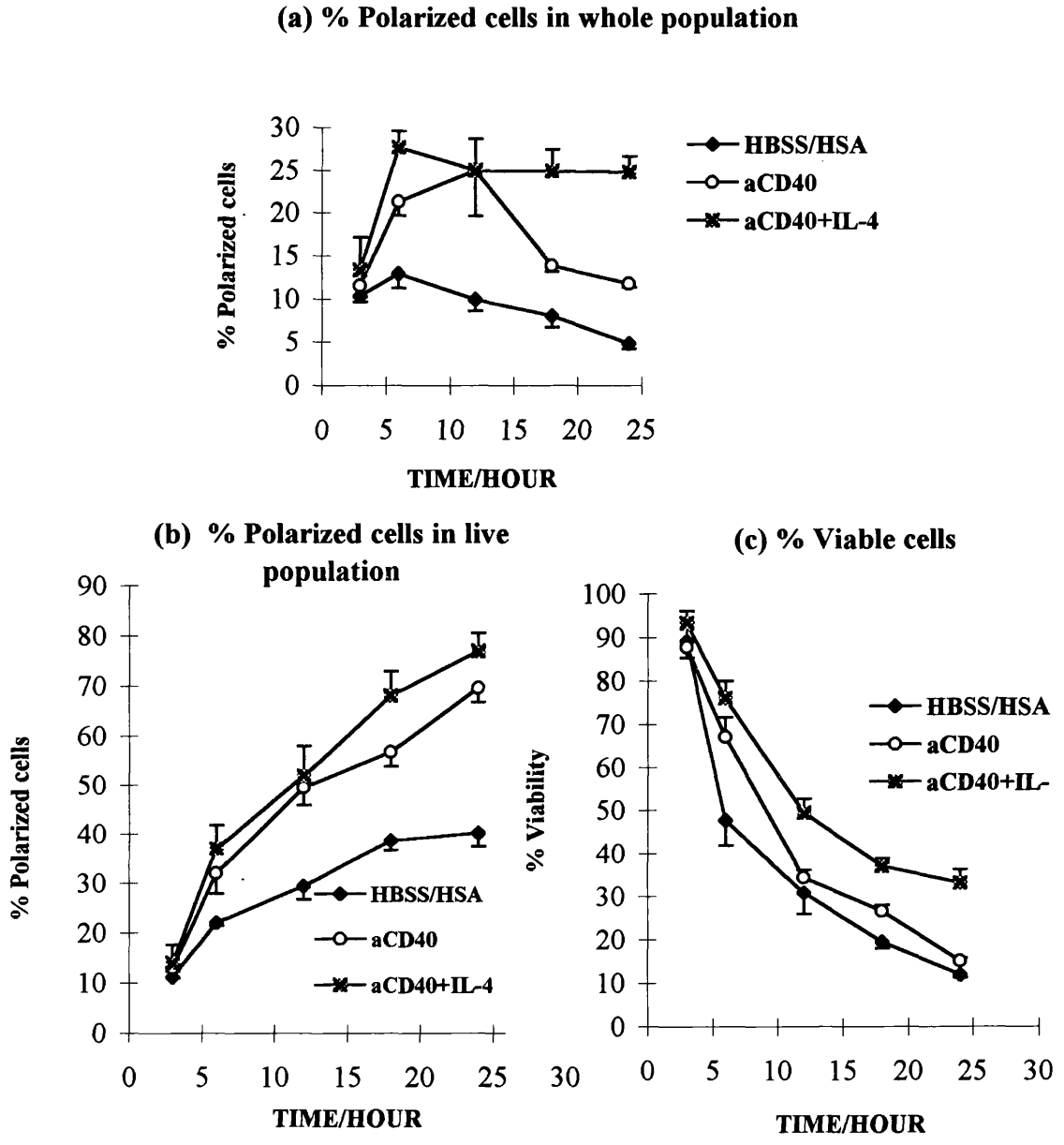


Figure 4-4 Time-course for polarization of GC B cells cultured in anti-CD40 (1mg/ml) (mean ± SEM, n = 4), anti-CD40 (1mg/ml) and IL-4 (50u/ml) (mean ± SEM, n= 4). and HBSS alone (mean ± SEM, n= 4).. (a) time curve for polarization of GC B cells as % of cells in whole population (dead and live cells), (b) time curve for polarization of GC B cells as % of cells in live population, (c) Viability of GC B cells. P values: test cf. reagents-free control; polarization assays: using anti-CD40 after 12,18,24h in live population P: <0.003> 0.007: after 6,12,18,24h in whole population P: <0.002> 0.007: polarization assays: using anti-CD40+IL-4 after 6,12,18,24h in live population P: <0.004> 0.005: after 6,12,18,24h in whole population P: <0.002> 0.005: P: for Viability; after 6,12,18,18,24h P: <0.007>0.02.

Figure 4-5

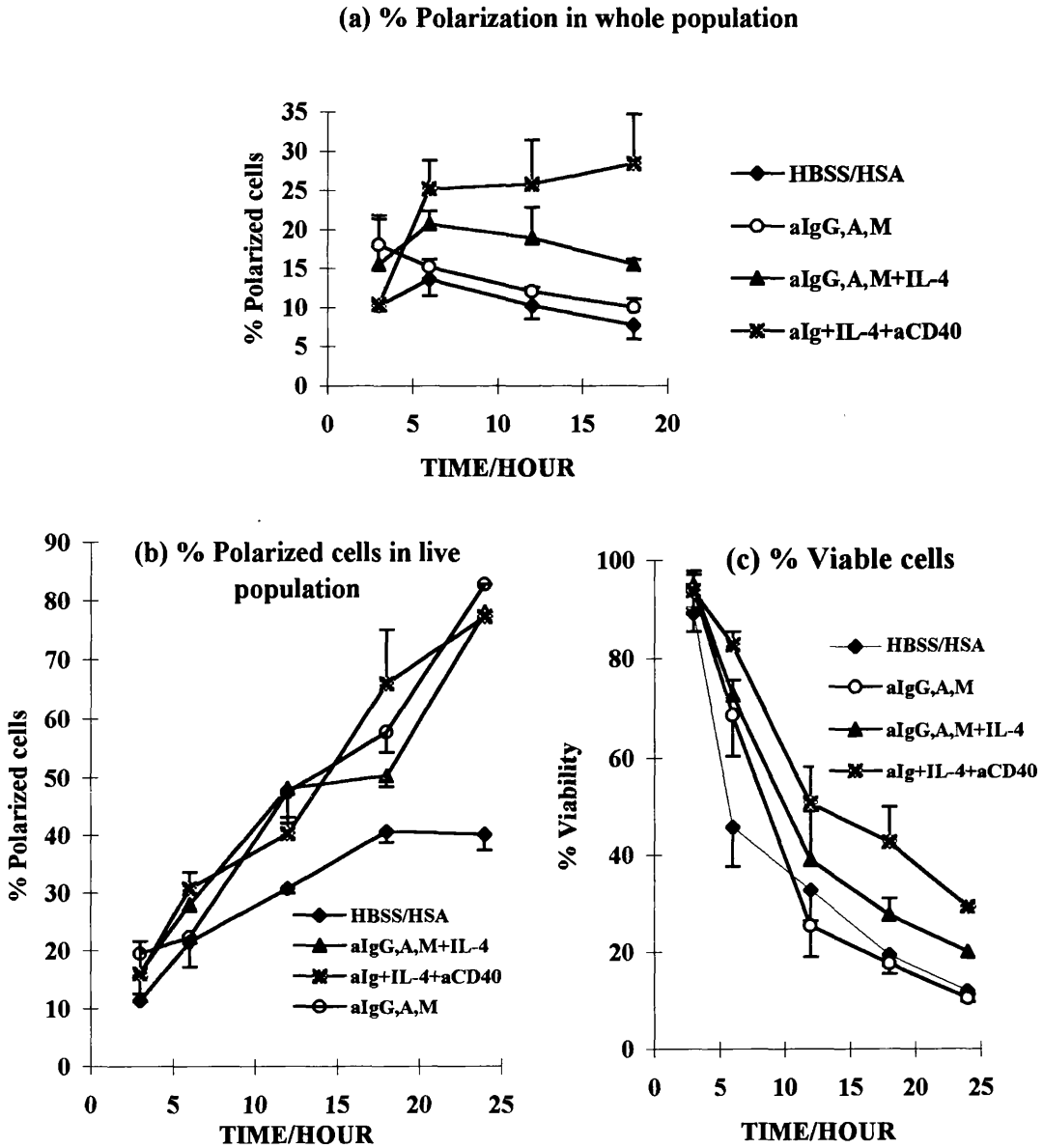


Figure 4-5 Time-course for polarization of GC B cells cultured in anti-IgG,A,M (1μg/ml) (mean ± SEM, n = 3), anti-IgG,A,M (1μg/ml) and IL-4 (50u/ml) (mean ± SEM, n= 2). anti-IgG,A,M (1μg/ml) + anti-CD40 (1μg/ml) and IL-4 (50u/ml) (mean ± SEM, n= 3), and HBSS (mean ± SEM, n= 3). (a) time curve for polarization of GC B cells as % of cells in whole population (dead and live cells), (b) time curve for polarization of GC B cells as % of cells in live population, (c) Viability of GC B cells. P values: test cf. reagents-free control; polarization assays: using anti-IgG,A,M after 12,18,24h in live population P: <0.007> 0.07: P value for polarization in whole population and for viability was not significant: using anti-IgG,A,M+IL-4 after 12,24h in live population P: <0.04> 0.03: P value for polarization in whole population and for viability was not significant: using anti-IgG,A,M+IL-4+anti-CD40 after 12,18,24h in live population P: <0.007> 0.05: after 6,12,18,24h in whole population P: <0.05: P: for Viability; after 6h P: = 0.005.

Figure 4-6

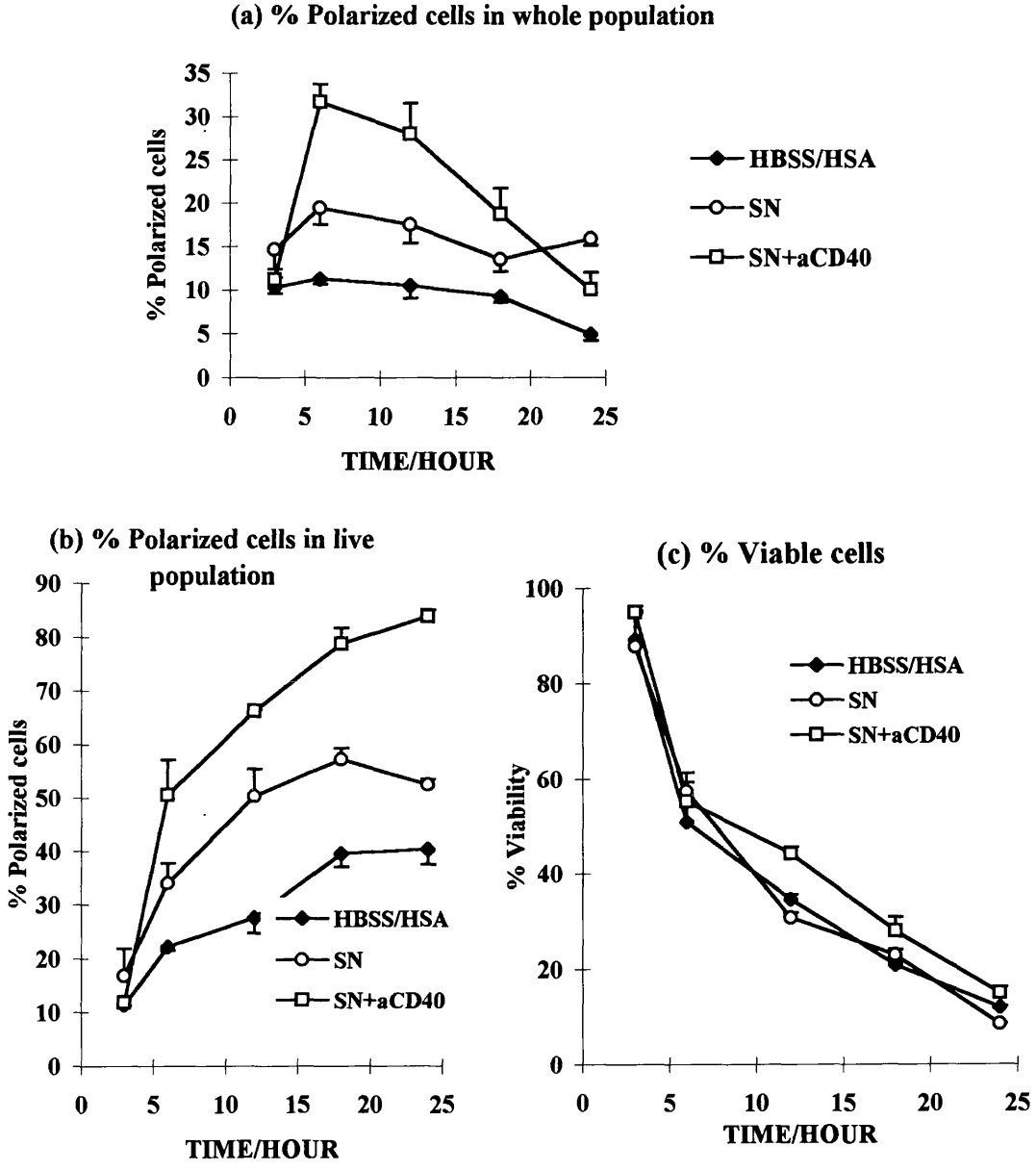


Figure 4-6 Time-course for polarization of GC B cells cultured in supernatant derived from high-density cells cultured in IL-4 (mean ± SEM, n = 3), supernatant + anti-CD40 (1mg/ml) (mean ± SEM, n= 2), and HBSS alone (mean ± SEM, n=3). (a) time curve for polarization of GC B cells as % of cells in whole population (dead and live cells), (b) time curve for polarization of GC B cells as % of cells in live population, (c) Viability of GC B cells. P values: test cf. reagent-free control; polarization assays: using anti-supernatant alone after 12,18h in live population P: <0.03> 0.01: after 6,12h in whole population P: <0.05: P value for for viability was not significant: using supernatant + anti-CD40 after 12,18,24h in live population P: <0.005>0.009: P value for polarization in whole population and for viability was not significant.

Figure 4-7

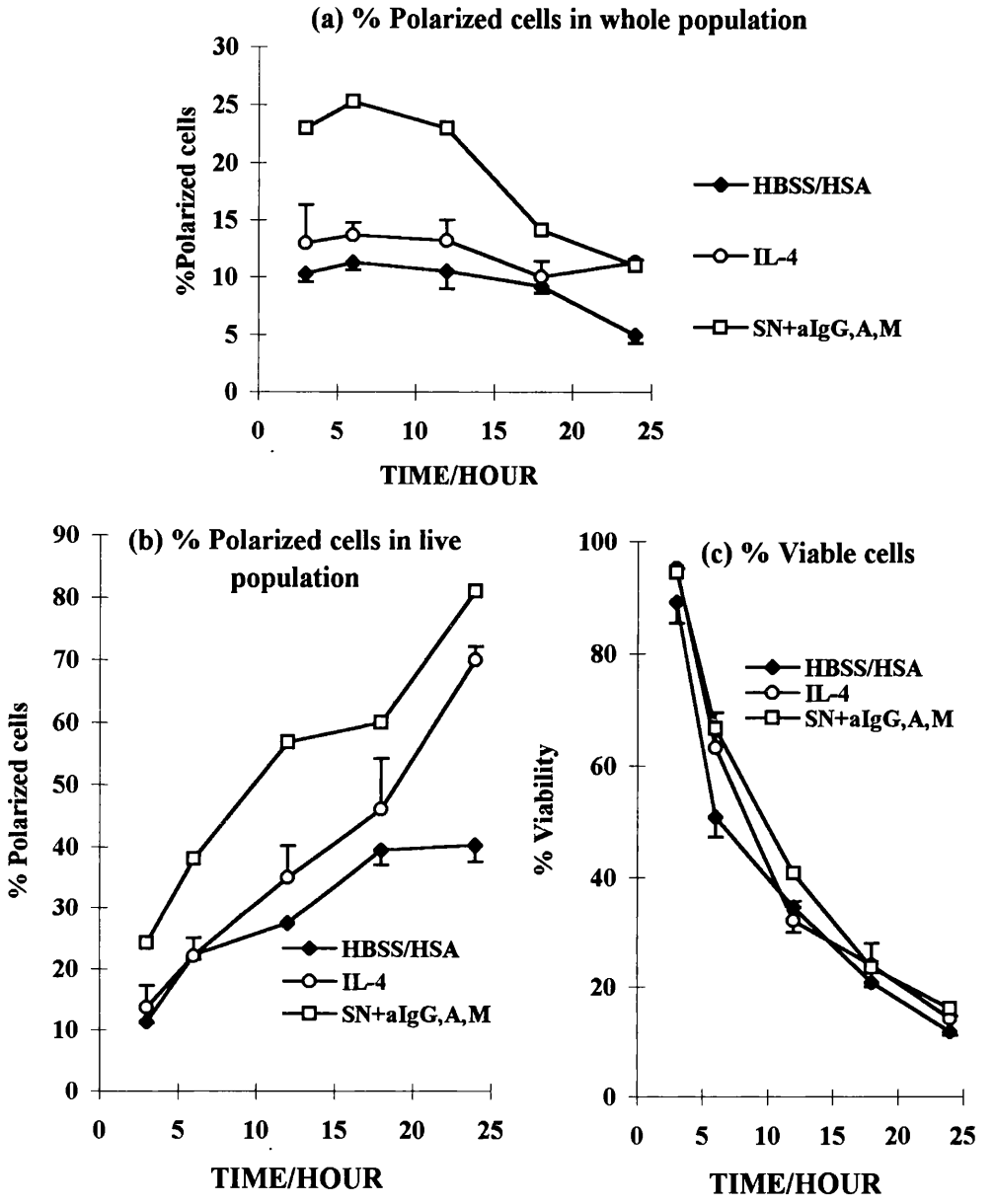


Figure 4-7 Time-course for polarization of GC B cells cultured in IL-4 (50u/ml) (mean ± SEM, n = 3), supernatant derived from high-density cells cultured in IL-4 supernatant + anti-IgG,A,M(1μg/ml) and IL-4 (50u/ml) (mean ± SEM, n= 1), and HBSS alone (mean ± SEM, n= 3). (a) time curve for polarization of GC B cells as % of cells in whole population (dead and live cells), (b) time curve for polarization of GC B cells as % of cells in live population, (c) Viability of GC B cells. P values: test cf. reagent-free control was not significant except for IL-4 after 24 hours in live population (P 0.05)

Table 4-4

Number of clusters per field in overnight culture

	Number of clusters	
Medium	<10 cells	>10 cells
HBSS alone	1.6	0.7
Anti-IgG,A,M(1μg/ml)	2	3.7
IL-4 (50u/ml)	2.2	7.9
Anti - CD40 (1μg/ml)	1.1	12.3
IL-4 + anti-IgG,A,M	1.1	10.4
Anti- CD40+anti-IgG,A,M	2.3	11.9
IL-4 + anti-CD40 + anti-IgG,A,M	0.4	11.5

Table 4-3 GC B cells were cultured in different stimuli for 12 hours at $2x10^6$ cells per ml. Fifteen fields per sample was scored and percentage cluster was calculated.

< 10 cells = Cluster is made of more than 5 and less than 10 cells

>10 cells = Cluster is made of more than 10 cells

combinations of IL-4 and anti-Ig enhanced aggregation slightly (table 4-4).

4-2-2 Immediate Shape-Change Assays (short term assays)

Besides investigating the time course of activation of GC cell locomotion, the immediate (<30 min) effect of attractants on GC B cells was also investigated. This was carried out exactly as described in chapter three. Observation of freshly isolated GC B cells demonstrates that the small cells (centrocytes) are mostly in G_0 and are not able to respond to attractants properly unless cultured. Monoclonal mouse antibodies against human IgM, IgG, IgA, and polyclonal sheep anti-human IgG,M,A were used as chemoattractants which mimic the function of antigen. These antibodies acted as attractants for GC B cells. but, the antibodies which were active were different from those active on resting B cells. Freshly isolated GC B cells were exposed to different concentrations of these anti-Igs (Fig 4-8 and 4-9). With either anti-IgM or anti-IgG,A,M, the best response was seen using high doses of antibody (1μg/ml). On the other hand, high concentrations down-regulate the polarization response to anti-IgG or IgA and the best response was revealed at a lower concentration (1ng/ml). This finding may suggest that a low concentration of antigen is required for initiation of the locomotion response in IgA and IgG bearing cells. No responses were seen using isotype control sheep Ig, mouse IgG1 and IgG2a. IL-4 alone at different concentrations (4-200U/ml), had no effect on the immediate response but combinations (100u/ml) with different concentrations of anti-Igs promote the locomotion response slightly (n = 2, data not shown). The

effects of locomotor activators in promotion of polarization were investigated. GC B cells were cultured in these reagents for either 6 or 12 hours. Firstly GC B cells were cultured in HBSS, anti-CD40, or IL-4 for six hours. Following washing in HBSS, cells were exposed to anti-Igs and medium alone. Cells cultured in medium alone were not able to respond to anti-Igs, except that anti-IgG,A,M at 1μg/ml gave a partial response compared with medium alone (11 ± 1.8, n = 2). The immediate effects of anti-Ig on cells cultured in IL-4 or anti-CD40 alone for 6 hours are shown in Fig 4-8, 4-9, 4-10. Once again GC B cells respond to anti-IgA and anti-IgG at low concentration whereas the responses were significant at high concentrations using anti-IgG,A,M. Anti-IgA and anti-IgG had the same result on cells cultured in IL-4 for 6 hours (data not shown). To study further the effect of incubation time in promotion of responsiveness of cells, the GC B cells were cultured in IL-4 for 12 hours. Following washing, cells were exposed to anti-Igs. Fig. 4-8 shows the curve for anti-IgG,A,M and Fig 4-9 shows the curve using anti-IgM (n = 1). As is shown in Fig 4-9, increasing the incubation period does not enhance the polarisation response to anti-IgM. No significant response was observed using anti-IgG and anti-IgA. This may suggest that IL4 alone might not have any effect on IgA and IgG bearing cells which had already switched their sIgs or that these cells might undergo apoptosis during the period of incubation .

Based on the finding that stimulation of B cells through more than one receptor promotes the locomotor response of high-density B cells, a combination of IL-4 and anti-CD40 was used to investigate the effect of locomotor activators on GC B cells.

The data are shown in Fig. 4-11. Cells bearing IgG, and especially those bearing IgA responded at low concentrations whereas at high concentrations (>100ng/ml) the response was negative, compared to controls (data not shown). Variation of response in these experiments was due to different proportions of positive cells in the different samples (Table 4-1). Exposure to anti-CD40 did not induce any immediate response in cells cultured in either HBSS, anti-IgG,A,M, or IL-4.

Since high-density B cells released unknown attractants in overnight culture, GC B cells cultured in CD40 with IL-4 after six hours were re-exposed to their own supernatant, following washing. No response was observed towards these supernatants, but the cells responded to supernatants derived from high-density B cells cultured in IL-4. GC B cells cultured for 12 hours responded better than those cultured for 6 hours (Fig. 4-12). This result is in agreement with our finding that IL-4 cultured GC B cells after 12 hours gave better responses to anti-Igs than cells which were cultured for six hours (Fig 4-8 and 4-9). Anti-CD32 also induced a polarization response in cells cultured in anti-CD40 with IL-4 or in HBSS alone (Fig. 4-12). This difference is correlated with the previous finding that GC cultured in HBSS alone after 6 hours could not promote the locomotor capacity of these cells toward anti-Igs. Thus, anti-CD40 plus IL-4 not only enhanced the survival but also promoted the locomotor response of GC cells to attractants and this effect was higher than for cells cultured in either IL-4 or CD40 alone. Cells cultured in IL-4 and anti-CD40 responded better than those cultured in HBSS alone. The response curves to CD32 and anti-Igs (anti-IgA and anti-IgG) are similar. Both attractants

Figure 4-8

30 min. Polarization assay of GC B cells to anti-IgG,A,M before and after culture in IL-4

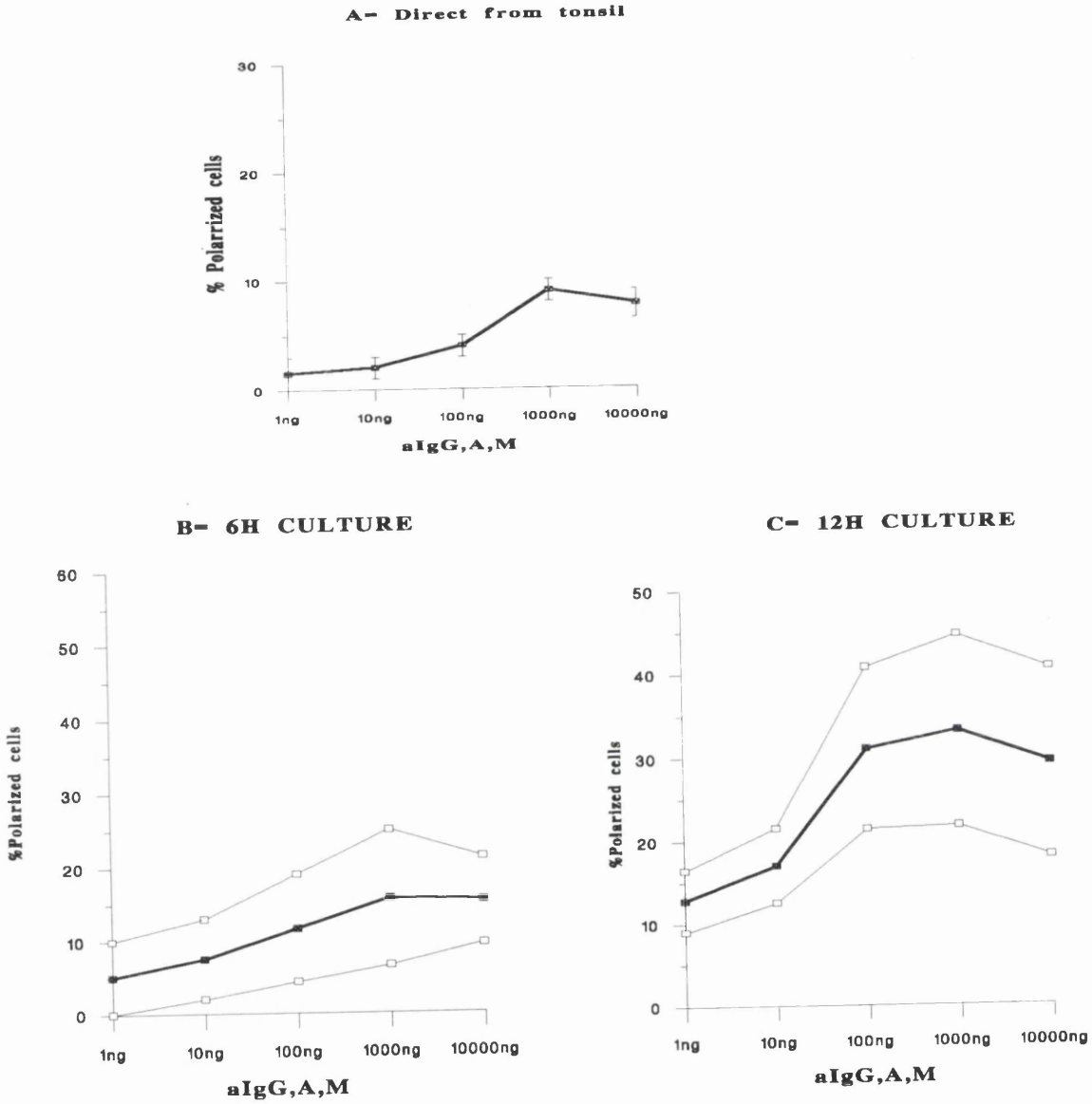


Figure 4-8 Dose response curves of GC B cells in a 30 minute shape-change assay before and after culture for 6 and 12 hours culture in IL-4 (50u/ml) to anti-IgG,A,M. (a) dose response curve after separation (mean ± SEM, n = 2). Control values (medium alone : Polarization in medium for HBSS was 7.9 ± 0.1 %) have been subtracted.(b) dose response curve after 6 hours culture, Since the proportion of cells was varied in different samples, the upper and lower curve show two different experiments and the middle curve shows the mean (thick line). Control values (medium alone : Polarization in medium was 24 ± 5.5 %) have been subtracted; (c) dose response curve after 12 hours culture, Since the proportion of cells was varied in different samples, the upper and lower curve show two different experiments and the middle curve shows the mean (thick line). Control values (medium alone : Polarization in medium was 28 ± 12%) have been subtracted.

Figure 4-9

30 min. Polarization assay of GC B cells to anti-IgM before and after culture in IL-4

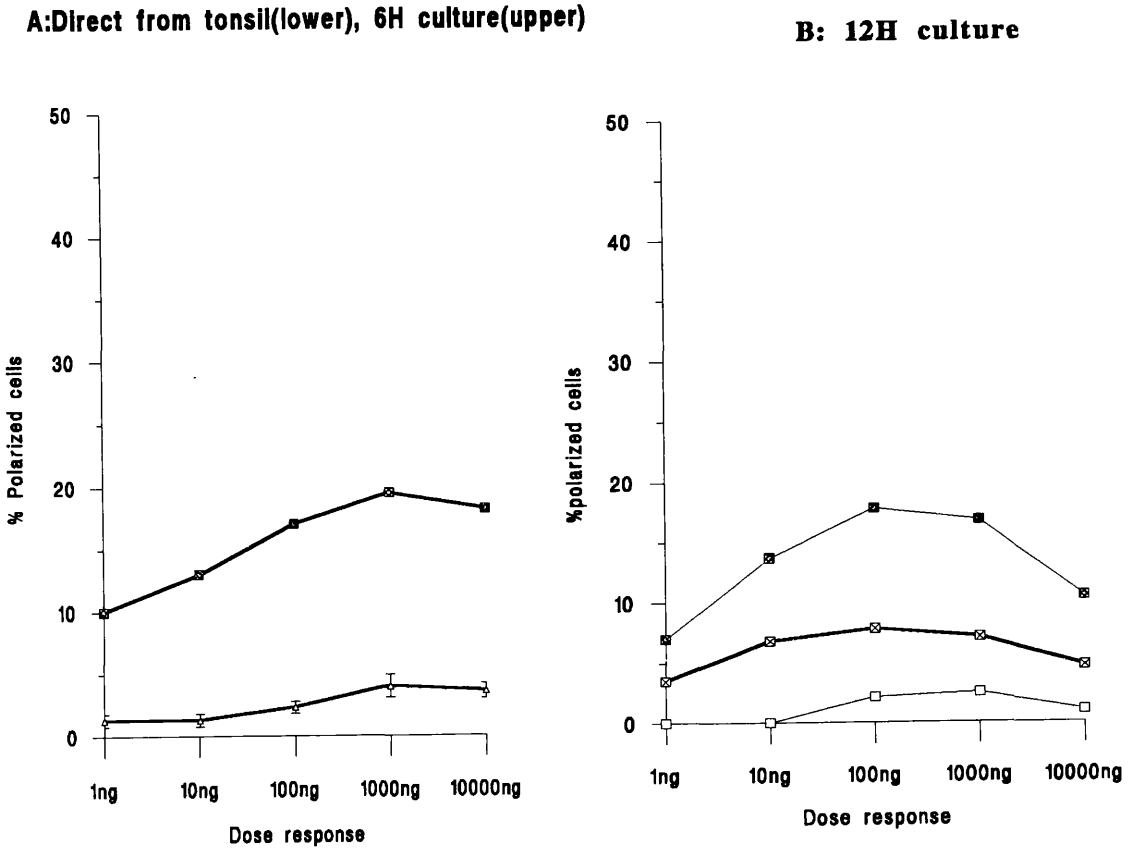


Figure 4-9 Dose response curves of GC B cells in a 30 minute shape-change assay before and after 6 and 12 hours culture in IL-4 (50u/ml) to anti-IgM. (a) dose response curve before and after culture. Filled symbols : cells after 12 hours culture (n = 1). Open symbols: cells direct from tonsil (n = 2). Control values (medium alone : Polarization in medium for culture cells was 35 and for freshly isolated cells was 7.9 ± 0.1) have been subtracted. (b) dose response curve after 6 hours culture, Since the proportion of cells was varied in different samples, the upper and lower curve show two different experiments and middle curve shows the mean (thick line). Control values (medium alone : Polarization in medium was 24 ± 5.5 %) have been subtracted.

Figure 4-10

30 min. Polarization assay of GC B cells to anti-Ig before and after culture in aCD40

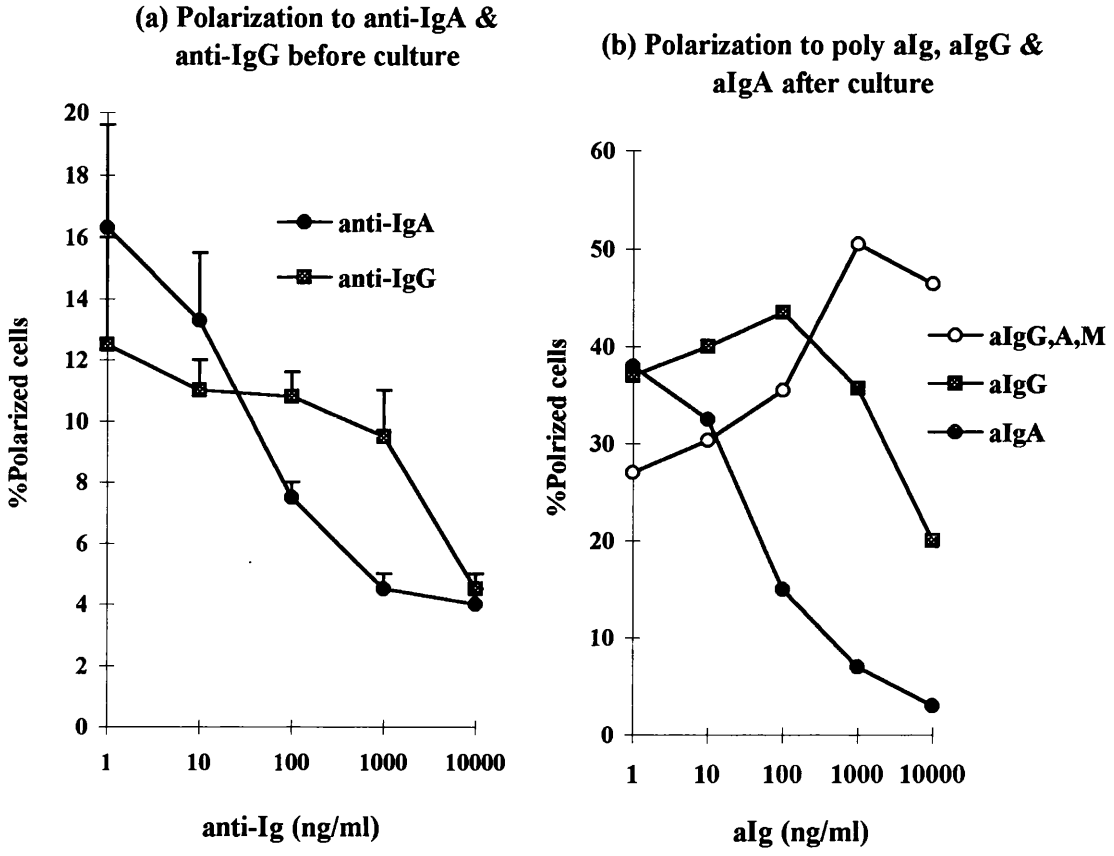


Figure 4-10 Dose response curves of GC B cells in a 30 minute shape-change assay before and after 6 hours culture in anti-CD40 (1mg/ml) to anti-IgG,A,M, anti-IgG and anti-IgA. (a) dose response curve after separation to anti-IgG (mean ± SEM, n = 2) and anti-IgA (mean ± SEM, n = 2). Control values (polarization in medium alone was 8+1). (b) dose response curve after culture in anti-CD40(1mg/ml) to anti-IgG,A,M (anti-IgG and anti-IgA (n =1). Polarization in medium alone was 27%.
Note : Difference in scale on Y-axis between the two groups.

Figure 4-11

30 min. Polarization assay of GC B cells to anti-Ig after culture in IL-4 & aCD40

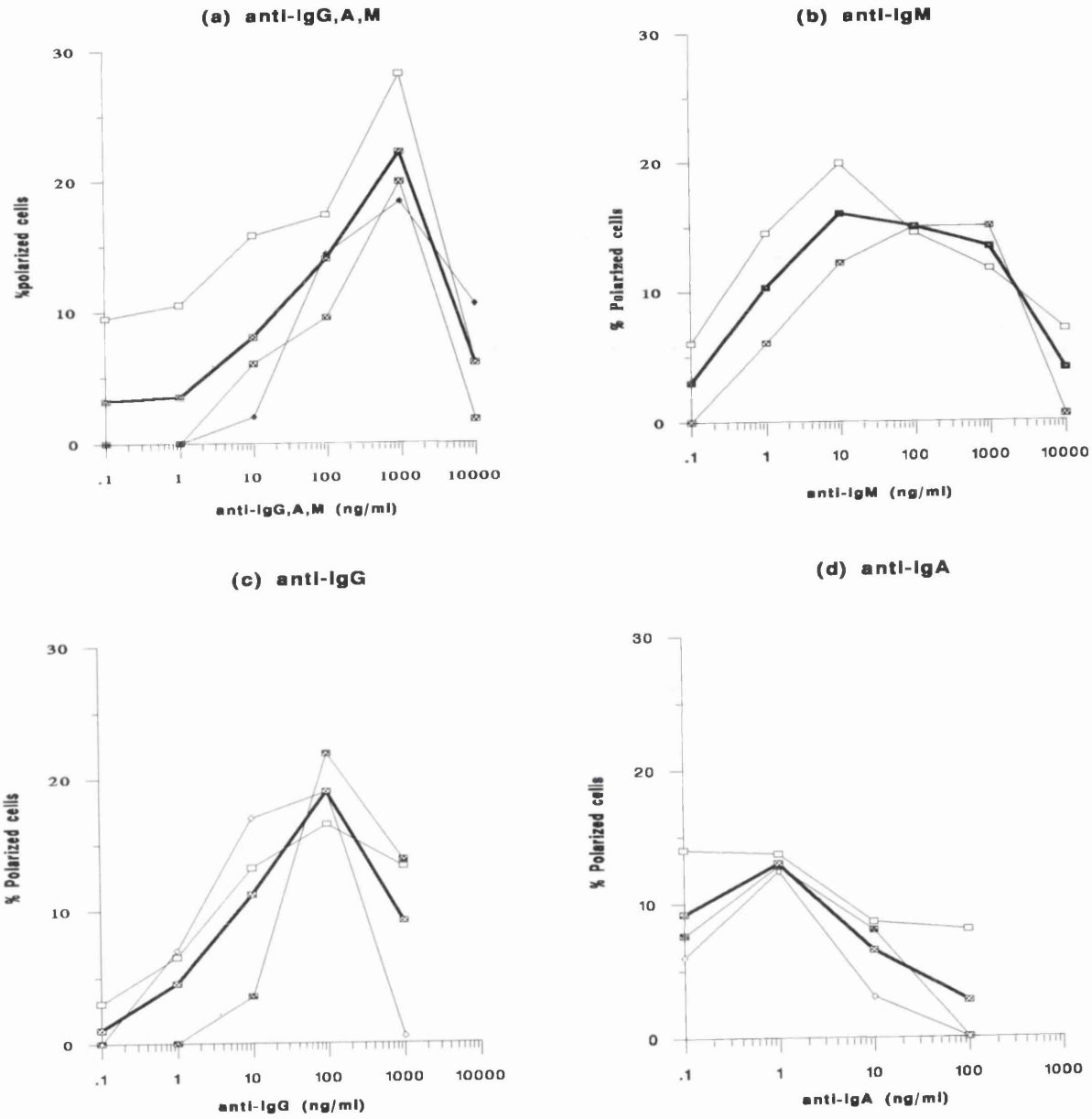


Figure 4-11 Dose response of GC B cells to anti-Ig in a 30 minute shape-change assay after cells were cultured for six hours in anti-CD40 (1μg/ml) and IL-4 (50u/ml). (a)dose response curve to anti-IgG,A,M (n =3); P values (test compared to control) at 100ng/ml and 10ng/ml were < 0.05. (b) anti-IgM (n =2); P values (test compared to control) at 100ng/ml and 1μg/ml were < 0.4> 0.02. (c) anti-IgG (n =3); P values (test compared to control) at 100ng/ml was < 0.08, and (d) anti IgA (n =3), P values (test compared to control) at 1ng/ml was < 0.04. Control values (medium alone : Polarization in medium alone for HBSS alone was 32 ± 2.3%) have been subtracted. Since the proportion of cells was varied in different samples, the thin curve shows the single experiments and the thick line represents the mean.

Figure 4-12

30 min. Polarization assay of GC B cells to anti-CD32 and supernatant after culture

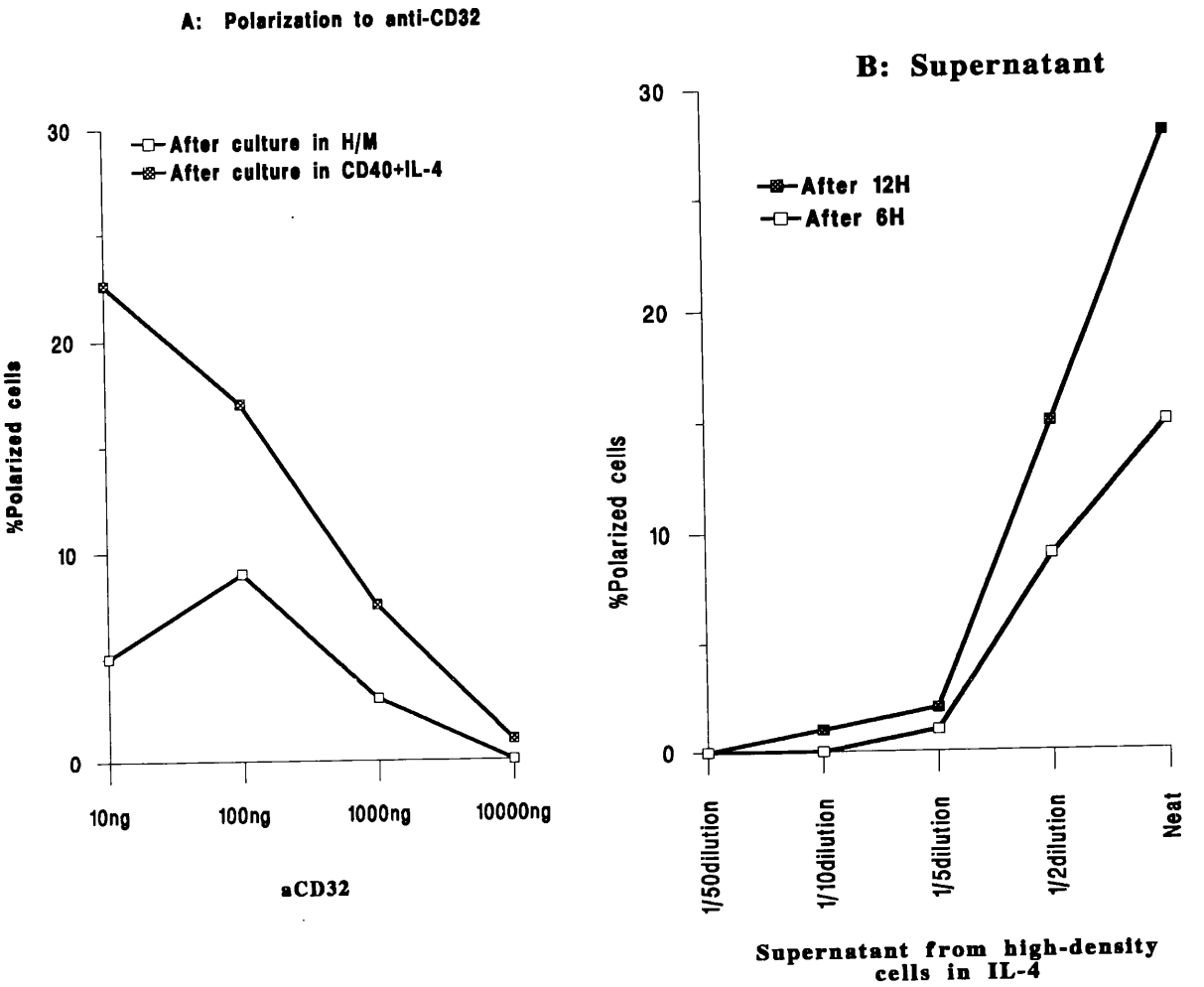


Figure 4-12 Dose response of GC B cells in a 30 minute shape-change assay. (a)dose response curve to anti-CD32 after culture in IL-4 (50u/ml) and anti-CD40 (1μg/ml)(Filled squares) and HBSS alone(Open squares) after six hours. Control values (medium alone : Polarization in medium for cells cultured in IL-4+ anti-CD40 39%, and for HBSS (25%, n = 1) have been subtracted (b) Dose response curve to supernatant derived from high-density cells cultured in IL-4 after GC B cells were cultured after 12 hours (Filled squares) and after 6 hours (Open square). Control values (medium alone : Polarization in medium for cells cultured in IL-4 after 12 was 25% and after 6hours was 19 %, n = 1) have been subtracted.

gave better responses at low concentration and this is in contrast to our findings using anti-IgM and anti IgG,A,M with GC cells and also to the finding with high-density cells. Higher concentrations of attractants were needed in the latter cases(figure 3-16, 3-20, 3-21 previous Chapter). This may suggest; Firstly that somatically mutated cells (sIgG or sIgA bearing cells) are more sensitive than virgin cells, or, secondly, that anti-IgG and anti-IgA at high concentration give a negative signal to GC cells. This negative response may be due to the Fc fragment of the antibody, since anti-CD32 gave a better response at low concentration with GC B cells than it gave with high-density cells which respond to high concentrations of anti CD32. Variation in responses to anti-Igs as chemoattractants for cultured cells may reflect the different proportions of cells positive for these sIgs in the isolated population. The polarization response towards anti-Igs is especially significant bearing in mind that the proportion of GC cells bearing sIgA, sIgG, and sIgM is not high on separated cells. There was no response to sCD23 between 1μg - 10ng/ml (n = 2), to anti-CD21(n = 1), to IL-2 between 1μg - 1ng/ml(n = 2), and to IL-7 between 400 - 0.5u/ml (n = 2).

4-2-3 Locomotion of GC B Cells into Collagen Gels

Isolated GC B cells were overlaid on three dimensional collagen gels (1.2mg/ml) into which various reagents or medium alone were mixed. Firstly cells in medium alone on the top of the gels were cultured with different attractants in the gels. Secondly cells were cultured in IL-4 (50u/ml) on the top of the gels with different

Table 4-5 A

Invasion of collagen gels by GC B cells after 6 Hours

Attractants	% cells invading the gels (mean ± SEM) (collagen 1.2 mg/ml)	
	Cells above gel in HBSS alone	Cells above gel in IL-4 (50/ml)
HBSS-HSA	13 ± 1†	12.2 ± 0.8†
IL-4 (50u/ml)	21.9 ± 3.1†	12.7 ± 2.3†
anti-CD40 (1μg/ml)	18.4 ± 0. 7†	21.5 ± 0.5†
Mouse IgG1 (1μg/ml)	16.1	11.6
IL-4 + anti-CD40	10.2	16.1
IL-4 + mouse IgG1	8.5	10.2

ND = not detected

† = Mean + SEM of two experiments

Table 4-5 B

Invasion of collagen gels by GC B cells after 12 Hours

Attractants	%cells invading the gels (mean ± SEM) (collagen 1.2 mg/ml)	
	Cells above gel in HBSS alone	Cells above gel in IL-4 (50/ml)
HBSS-HSA	17.4 ± 1.4†	16.6 ± 0.9†
IL-4 (50u/ml)	37.5 ± 4.5†	30.3 ± 1.3†
anti-CD40 (1μg/ml)	36.6 ± 1.4†	40.8 ± 1.3†
anti-IgG,A,M (1μg/ml)	41.8 ± 3.8†	40
Mouse IgG1 (1μg/ml)	ND	18.5 ± 2.5†
IL-4 + anti-CD40	30	23
IL-4 + mouse IgG1	25.3	17

This and the previous table show that the optimal time for reading collagen gel assays is different between 12 and six hours.

ND = not detected

† = Mean + SEM of two experiments

attractants in the gels. The percent invasion was measured at six and twelve hours of incubation. The results shown in table 4-5 indicated that cells invaded gels containing attractants to a greater extent than gels containing medium alone. However the percent invasion was lower when anti-CD40 was combined with IL-4 in all cases. These differences may be due to cluster formation induced by these reagents. However, the number of clusters induced was identical in these reagents in long term incubation (after 12 hours, Table 4-4). Cells invaded better when no IL-4 was added to the top of the collagen gels. The proportion of cells that invaded the gels within twelve hours was greater than within six hours.

4-2-4 Polarization Related to Growth Measured by Uridine and Thymidine Incorporation

Centrocytes are derived from centroblasts in secondary follicles after several cell divisions. Centrocytes do not divide, and re-enter the G_0 stage. G_0 cells respond poorly to attractants but do respond when they move into G_1. We therefore investigated the relation between stage of cell cycle and locomotion. To study this, [^{3}H]-uridine and [^{3}H]-thymidine incorporation were measured in freshly isolated GC B cells directly after separation and culture in IL-4 (50u/ml) and anti-CD40 (1μg/ml). The data demonstrate that 30 ± 2.8 % of freshly isolated cells take up uridine and 15.6 ± 4 % take up thymidine. The result of autoradiography after [^{3}H]-uridine and [^{3}H]-thymidine incorporation is shown in Table 4-6 and 4-7. The positive cells took up thymidine very strongly and were heavily labelled after

incorporation. These cells were among the large population whereas those that took up uridine were distributed in both large and small cells. The interesting finding is that the cells that took up thymidine were not polarized. This finding suggests that centroblasts are not able to recognize attractants but that they are able to do so when they develop into centrocytes. The numbers of cells which were labelled with thymidine decreased to 4 ± 0.5% after culture in IL-4 and CD40 for six hours, whereas, the proportion of uridine labelled cells increased significantly. The proportion of polarized cells showing uridine labelling after culture was higher than the proportion among spherical cells or among the whole population. This was also the case for polarized cells responding to anti-Igs including anti-IgM, anti-IgG, anti-IgA, and anti-IgG,A,M, in contrast to the few percent of thymidine labelled cells which did not respond to attractants. This finding clearly reveals that the large cells (centroblasts) are not the responsive population and that they undergo apoptosis during the incubation time. A photograph of uridine and thymidine labelled cells is shown in Fig 3-25.

Table 4-6

Autoradiography: [^{3}H]uridine incorporation by spherical and polarized GC B cells 30 minutes assay direct from tonsil in:-

Source of cells and stimuli	Percent of polarized cells in population	Percent of cells showing >10 grains per cell		
		Spherical	Polarized	All cells
HBSS/HSA	10.9	13	32	12.4
IL-4(50u/ml) + anti-IgG,A,M (1μg/ml)	13.5	6.5	39	11
IL-4 (50u/ml) + anti-CD40 (1μg/ml)	11.6	11	46	16
IL-4 + anti-CD40 + anti-IgG,A,M	13.6	10.8	40	16

30 minutes assay on GC B cells cultured in IL-4(50u/ml) + anti-CD40(1μg/ml)in:-

Source of cells and stimuli	Percent of polarized cells showing grains	Percent of cells showing >5 grains per cell	
		Spherical	Polarized
BSS/HSA	54	49.1	50.9
anti-IgG,A,M (1μg/ml)	63	32	68
anti-IgM (1μg/ml)	69	31	69
anti-IgG (100ng/ml)	56	44.5	55.5
anti-IgA (10ng/ml)	57	44.2	55.8

Table 4-7

Autoradiography[^{3}H]Thymidine incorporation by spherical and polarized GC B cells 30 minutes assay direct from tonsil in:-

Source of cells and stimuli	Percent of polarized cells in population	Percent of cells showing >10 grains per cell		
		Spherical	Polarized	All cells
HBSS/HSA	10.3	16.6	0	14.5
IL-4(50u/ml) + anti-IgG,A,M (1μg/ml)	14.4	18.5	0	16.3
IL-4 (50u/ml) + anti-CD40 (1μg/ml)	11.4	18.6	0	15.7
IL-4 + anti-CD40 + anti-IgG,A,M	15.4	18.4	0	15.6
30 minutes assay on GC B cells cultured in IL-4(50u/ml) + CD40(1μg/ml)in:-				
HBSS/HSA	ND	3	0	ND
anti-IgG,A,M (1μg/ml)	ND	5.7	0	ND
anti-IgM (1μg/ml)	ND	4	0	ND
anti-IgG (100ng/ml)	ND	4	0	ND
anti-IgA (10ng/ml)	ND	4	0	ND

Freshly isolated GC B cells were pulsed with thymidine and then exposed to attractants for 30 min., fixed and studied under light microscopy. 200-250 cells were counted for each sample. Since it was difficult to recognize apoptotic cells (Giemsa counter-stain should be diluted in order to see the grains), the cells without grains were not counted in cells after culture.

4-2-5 Discussion

It was shown in the previous chapter that small high-density B cells acquired locomotor capacity following activation by anti-CD40, IL-4 and IL-13 and transit from the G_0 to the G_1 phase. These cells were then able to recognize the anti-Igs as an attractant. The fluorescent phenotyping analysis clearly demonstrated that the responsive cells were sIgM and sIgD positive. These cells after activation translocate from T areas into the follicles and form the GC where the rapid antigen-driven proliferation takes place and a few high affinity variants selectively develop into memory cells or plasma cells (Liu et al, 1989 & 1992; Maclennan, 1994). Following the study of high-density B cells which represent cells which have not recently met antigen, it was interesting to compare this with locomotor properties of GC B. Centrocytes, like the high-density B cells, are not in cycle and mostly are in the G_0 phase. Thus, the same protocol was applied to study their behaviour. As before, IL-4 and anti-CD40 were used as locomotor activators and anti-Igs as attractants. The cells directly after separation from tonsil responded poorly to stimuli, but the proportion of responsive cells increased following culture in IL-4 and anti-CD40. The results presented here indicate that IL-4 in combination with anti-CD40 not only promotes the survival but also increases the locomotor capacity of GC B cells. Since GC T-cells express both IL-4 and the CD40 ligand (Buch et al, 1993;Buch et al, 1992; Liu et al, 1992; Holder at al, 1991) it seems plausible that GC T cells can send appropriate signals for locomotion to B cells. Anti-CD40 and to lesser extent IL-4 also induced

homotypic aggregation which is reported to be important for B cell contact and proliferation by autocrine factor exchange (Björck, 1993b). The cultured cells were next tested in short-term assays for their response to anti-Igs. All anti-Igs such as anti-IgG,A,M, anti-IgM, anti-IgG, and anti-IgA were demonstrated to have chemoattractant activity for centrocytes. The most interesting finding was that centrocytes responded to anti-IgG and anti-IgA at very low (ng/ml) concentrations in contrast to anti-IgM. This finding suggests that cells capable of high affinity binding may recognize antigen at very low concentration. Cells cultured in IL-4 and anti-CD40 were more capable of locomotion than freshly isolated cells. This finding suggests that for activation of locomotion in the secondary response T cell-derived signals are required. The result obtained from autoradiography clearly revealed the high death rate among the centroblasts. The percentages of thymidine labelled cells dramatically dropped within 6 hours and these cells had almost disappeared after 12 hours of culture. The most interesting finding was that the cells that took up thymidine were not polarized. This finding suggests that centroblasts are not able to recognize attractants but that they are able to do so when they develop into centrocytes. This is consistent with the observation that the highest death rate is in the border between the dark zone where proliferation occurred and the light zone where most centrocytes are located (MacLennan, 1994). Therefore, those centrocytes which still can not recognize the immune complex on FDCs because of failure in affinity maturation will die. There are many observations that cells of many types, when undergoing mitosis, stop

moving, because the cytoskelton is needed for the events of cell division and cytokinesis.

CHAPTER FIVE

LOCOMOTOR PROPERTIES AND PHENOTYPIC ANALYSIS OF BLOOD B CELLS

SECTION ONE: Phenotypic analysis of cell population

5-1-1 Mononuclear Cells

Mononuclear cells were separated from healthy individuals on lymphocyte separating medium. They were then analysed on FACS using CD3 as a pan T cell marker, CD19 as pan B cell marker, CD14 as a pan monocyte marker, and CD45 as a general leukocyte marker. The data obtained from cells freshly isolated from blood showed that the mononuclear fraction contained 65 ± 5.6% (n = 5) CD3+ cells, 6.8 ± 0.7% CD19+ (n = 5) cells, 8.5 ± 0.5% CD14+ (n = 2) cells and about 96% of the cells within the lymphocyte gate expressed CD45.

5-1-2 Blood B Cells

Two methods were used to eliminate T cells using either neuraminidase-treated SRBCs or AET-treated SRBCs followed by centrifugation on separating medium. After trying both systems for purification of B cells the AET-treated SRBC protocol was selected because it gave a higher yield of blood B cells. Analysis of the interface fraction from separating medium showed that 29.7 ± 3.3% of the cells carried CD14. Monocytes were then eliminated using either CD14 treated SRBCs or by incubation at 37^0 C in a tissue culture dish for 1-2 hours, followed by removal of non-adherent cells. In contrast to tonsillar B cells, purification of B cells was difficult, because of the low percentage of B cells in the blood

mononuclear fraction,. The viability of the isolated cells was determined before each experiment using eosin Y and was greater than 98%. Cells were then analysed on FACS and the data obtained are presented in Table 5-1. After purification, some CD3+, CD14+, CD56+ cells were still present as well as some negative cells which were not identified. These negative cells might be B cells which lost their CD19 receptor during the separating process. For example, it was shown that lysing buffer decreases the expression of CD19 or in the neuraminidase protocol, that the number of negative cells was higher than in the AET protocol. The result given here demonstrates that CD19 is the best marker for B cells. The number of CD19+ cells is equal with the sum of Lambda+ and Kappa+ cells in a given experiment (Table 5-1). B cells in blood do not express other B cell markers such as CD20, CD21, CD22 in high percentage (Table 5-1).

5-1-3 Phenotyping of Responsive Cells

Fluorescence microscopy was used to identify the phenotype of polarized cells. The cells were cultured in IL-4 and IL-13, then were stained with FITC-conjugated CD3 and PE-conjugated CD19. The result obtained is presented in Table 5-2. The phenotypic analysis revealed that IL-4 and IL-13 had no effect on polarization of T cells and most of the polarized cells were B cells.

SECTION TWO: Locomotion Assays

Most of the locomotion assays in blood B cells were carried out using the

Table 5-1

Phenotype characteristic of freshly isolated blood B cells

Cell Markers	Mean	Range
CD3[(16)†]	8.9 ± 1.9	2-22
CD14[(2)]	10.6 ± 2	4-16
CD19 [(16)]	37.3 ± 4.4	16-69
CD20[(2)]	20 ± 6 [(61.5 ± 7.5) ‡]	14-26
CD21[(1)]	4[(16)]	4
CD22[(5)]	13.7 ± 2 [(28.8 ± 7) ‡]	9-22
CD45[(1)]	97	97
CD56[(6)]	4.5 ± 1.8	2-13
Lambda chain [(2)]	15 ± 3 [(29.5 ± 6.5) ‡]	12-18
Kappa chain [(2)]	15 ± 2 [(29.5 ± 6.5) ‡]	13-17

† Number in parentheses shows number of experiments.

‡ number in parentheses shows the population of cells in these experiments which were CD19+.

Table 5-2

A : Phenotyping of cells responsive to cytokines in purified B cell fraction

Source of stimuli	percent polarized cells using fluorescence microscopy		Percent polarized cells in normal wet preparation
	CD3+ cells	CD19+ cells	
HBSS/HSA	8.3	31.8	22.8
IL-4 (50u/ml)	8.5	42.2	36
IL-4 (100u/ml)	12.1	47.7	38.7
IL-13 (1μg/ml)	4.6	39.7	36.7

B = Phenotyping of cells responsive to cytokines in whole mononuclear fraction

Source of stimuli	percent polarized cells using fluorescence microscopy		Percent polarized cells in normal wet preparation
	CD3+ cells	CD19+ cells	
HBSS/HSA	9.7	15	12.5
IL-4 (50u/ml)	10.6	22.5	14.2
IL-4 (100u/ml)	11.9	30.3	17.8
IL-13 (100ng/ml)	8.7	24.4	22.1
IL-13 (1μg/ml)	7	39.7	17.4

polarization assay in overnight culture or using the 30 minute assay before and after culture. In a preliminary experiment to find a suitable medium, the B cells were cultured in RPMI or HBSS alone in overnight culture. RPMI gives a very high polarization on its own compared with HBSS. The proportions of cells polarized in overnight culture were as follows; 46.2% in RPMI/HSA, 33% in RPMI/FCS, 23.6% in HBSS/FCS, and 18.3% for HBSS/HSA. Thus, blood B cells were cultured in HBSS/HSA and other reagents were added to this to determine the specific effect of stimuli on locomotion.

5-2-1 Polarization assays

Firstly, the mononuclear fraction was exposed to different concentrations of IL-4 and IL-13 in overnight culture. The polarization response was then investigated in normal wet preparation and phenotype of the cells was studied by fluorescence microscopy using FITC-conjugated CD3 and PE-conjugated CD19. 200-250 cells were scored for each and the results are presented in Table 5-2B. Secondly, the B cells were then purified and exposed to various concentrations of IL-4, IL-13, and anti-CD40 in overnight culture. IL-4 and IL-13 induced polarization at about 17% above the control. Anti-CD40 induced significant polarization compared to its isotype control mouse IgG1. The dose response curve for anti-CD40 was similar to that for IL-4 and IL-13 (Fig 5-1). There was no response to IL-4, IL-13 and anti-CD40 in a 30 minute assay on cells separated freshly from blood. B cells were then cultured in IL-4 (20u/ml) in overnight

culture to study the behaviour of cells after culture. Following washing, they were exposed to cytokines or anti-CD40 in a 30 minute assay to see if the cytokines or anti-CD40 acted as chemoattractants. No response was seen (n = 2). IL-7 at between 1u-200u/ml and sCD23 at between 100pg-1μg/ml did not induce any polarization response in 30 minute assays either before (n = 1) or after overnight culture in IL-4 (20u/ml) (n = 2). This was also true for overnight assays using IL-7 and sCD23 at different concentrations (n = 2).

Freshly isolated B cells gave no response to different concentrations of anti-IgM or anti-IgD at between 10ng-10μg/ml in 30 minute assays. B cells responded poorly to anti-Igs in overnight culture (Fig 5-2), but in contrast to high-density tonsillar cells, the viability of cells was more than 92% even at high concentration. Blood B cells were then cultured in IL-4 (20u/ml) overnight, washed and exposed to anti-IgM and anti-IgD (Fig 5-2). The polarization induced by anti-IgD was higher than with anti-IgM. Cells cultured in IL-4 were also re-exposed to their own supernatant after washing and about 28% of cells were polarized above the control value.

5-2-2 CONCLUSION

The effect of IL-4, IL13, anti-CD40, and anti-Igs on high-density tonsillar B cells was described in chapter three and it was therefore of interest to know whether these reagents had the same effects on blood B cells. To address this question, blood B cells were separated and exposed to these stimuli under the

Figure 5-1

Polarization of blood B cells to cytokines and anti-CD40

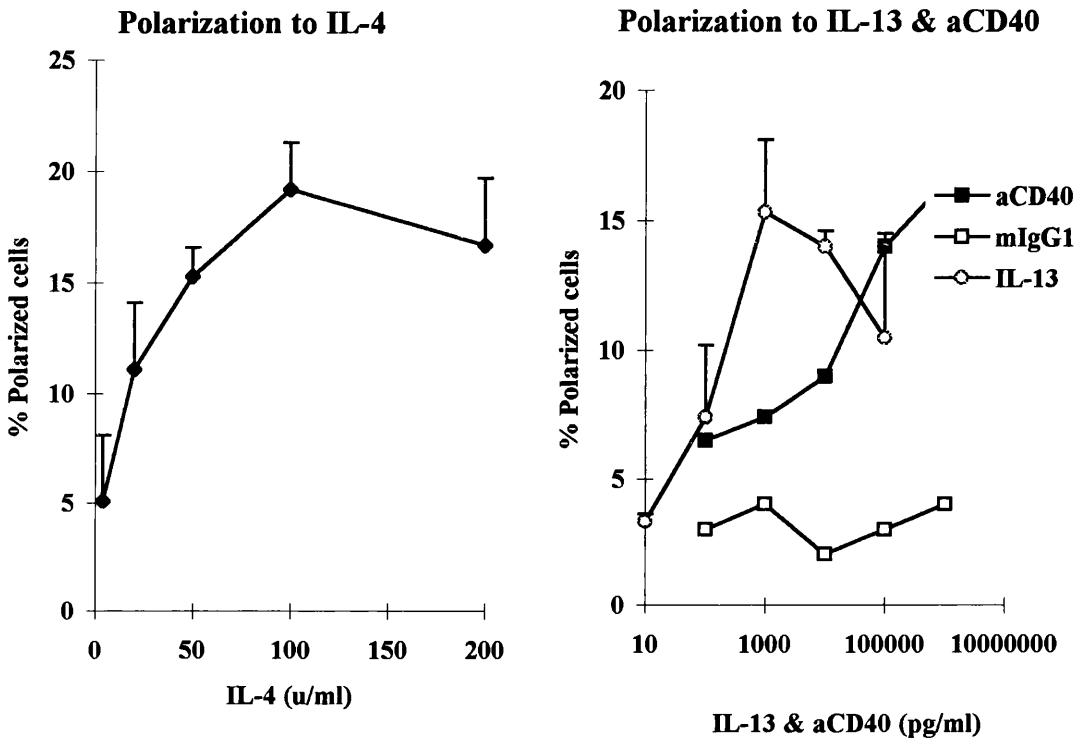


Figure 5-1 Dose response curves of blood cells to IL-4 (mean+SEM, n = 2), IL-13 (mean+SEM, n = 2), and anti-CD40 (n = 1) in overnight culture.
Left figure: Dose response curve to IL-4. Control value (polarization in medium alone was 26.5 ± 4. 5%) has been subtracted.
Right figure: Dose response curves to IL-13 and anti-CD40. Control values (polarization in medium alone to IL-13 was 26.5 ± 4.5% and to aCD40 was 31%) have been subtracted.

Figure 5-2

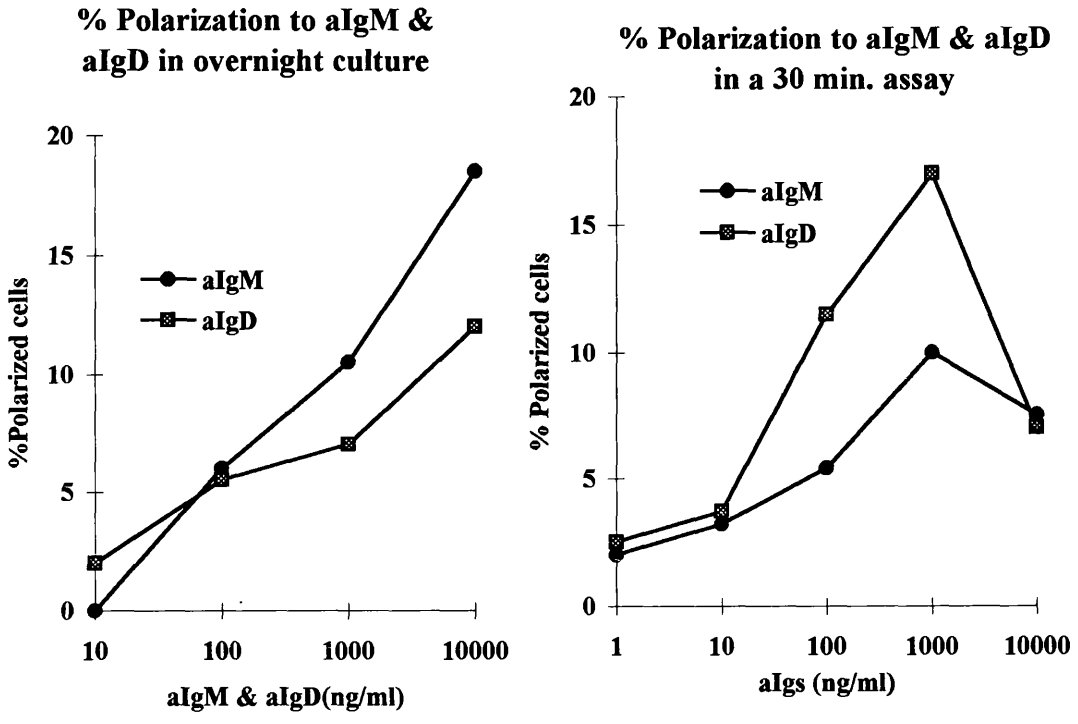


Figure 5-2 Dose response of blood B cells to anti-IgM & anti-IgD.
Left figure. In overnight culture. Control value (Polarization in medium alone was 13.5) has been subtracted.
Right figure in a 30 minute assay. Cells were cultured in IL-4(20u.ml) for 20H, washed and retested. Control value (polarization in medium alone was 4.5) has been subtracted.

same conditions as described for the tonsil cells. The percent response of blood B cells to all stimuli was lower than that of high-density tonsillar cells. Freshly isolated blood B cells did not response in a 30 minute assay to IL-4, IL-13, anti-CD40, or anti-Igs. However, like tonsil B cells, they polarized overnight in response to IL-4, IL-13 or anti-CD40, and cells that had been cultured in IL-4 responded to anti-IgD in a short-term assay. The percentage of blood cells responding to these agents was lower than that of high-density tonsil B cells. Two possibilities can explain these differences; Firstly, blood B cells were not pure and were contaminated with other cells and this decreased the specific response of B cells to these stimuli. Secondly, blood B cells most probably are at G_0 phase of cycle, whereas 5-6% of high-density tonsillar B cells were heavily labelled with uridine and about 6-9% of cells were lightly labelled (less than 10 grains per cells, data not shown).

The death rate in blood B cells using anti-Igs such as anti-IgD and specially anti-IgM at as high as 10μg/ml was not significant compared with that in high-density tonsillar B cells. This may be due to signals delivered through the physical contact between B and contaminating cells (e.g. Monocytes or T cells) during the culture period, since B cells in tonsils were completely pure and were unable to receive such signals from T cells unless co-cultured with IL-4 or anti-CD40.

CHAPTER SIX

THE LOCOMOTOR RESPONSE OF MOUSE B CELLS TO ANTIGEN

6-1 INTRODUCTION

The response of B cells to pathogens and other foreign substances is mediated through the clonally distributed membrane-bound immunoglobulins (mIg) on their surface, which are receptors for specific antigens. Cross-linking of these mIgs of resting B cells by antigens or anti-Ig antibodies generally activates B cells to enter the G_1 phase of the cell cycle. The other proliferative signals are normally provided by helper T cells. The nature of the response of B cells to antigen depends upon the differentiation state of the B cell and the nature of additional signals delivered by helper T cells. In the studies reported in previous chapters the role of mIgD and mIgM in locomotion of resting B cells was shown and the similar role for mIgA, mIgG, and mIgM in a distinct type of mature B cell, the germinal centre B cell, locomotion was also shown. Contact with these mIgs is necessary to prevent programmed cell death (Liu et al, 1989). The locomotor responses of B cells to anti-Ig strongly suggest that it would be important to look at specific locomotor response to antigens. To do this, a standard immunization procedure was used employing antigen together with adjuvant. This required a change of species, from human to the mouse. The rationale for this study was based on the following findings that 1) Human and rat B cells can recognize anti-Ig as chemoattractants (Komai at al, 1995; Ward et al, 1977). It was also reported that mouse B cells recognize antigen as attractants (Wilkinson et al, 1976). 2) The development of germinal centres and recruitment of B cells into them, is antigen-dependent (Coico at al, 1983). 3) Primed B cells accumulate in the germinal

centre after 3-4 days of challenge. This investigation is still at an early stage, and the results reported here are those of preliminary experiments.

6-2 Splenocytes

C57 BL/6 and BALB/c mice were immunized by peritoneal injection using ovalbumin and ISCOMs. The immunized animals together with control animals (three animals per group) were killed 8, 15, and 21 days (10 days after challenge) after primary immunization or 10 days after challenge. The cells were harvested from spleen and lymph nodes. T cells were eliminated by lysis using monoclonal anti-Thy-1.2 antibody with low-tox rabbit complement as described in chapter two. The splenocytes but not lymph node B cells were then layered on a three-step discontinuous Percoll gradient to separate high and low density B cells. The phenotypic characteristic of the cells obtained is shown in Table 6-1. F4/80 is a marker for mouse activated macrophages and showed that these cells were mostly located at the top of 1.055 Percoll gradients, so the high or low-density fraction does not include this marker.

6-3 Polarization Assays

6-3-1 30 minute Polarization Assay Directly After Separation

Both high and low-density B cell fractions were exposed to different concentrations of ovalbumin ranging between 10ng-1mg/ml (n = 2), and mouse rIL-4 ranging between 0.8u-100u/ml (n = 2). There was no response to ovalbumin

Table 6-1

Phenotypic analysis of splenocytes on FACS

Sample	Immunization period	Percent positive cells in whole mononuclear fraction					Percent positive cells in purified B cell fraction		
		CD3+	CD4+	CD8+	Ig+	F4/80+	CD3+	Ig+	F4/80+
Sample One	Control	43	24	16	49	ND	3	90	ND
	8 days	31	15	11	45	ND	2.4	78	ND
Sample Two	Control	36	ND	ND	49	ND	2	90	ND
	8 days	41	ND	ND	47	ND	2	90	ND
	challenged	36	ND	ND	47	ND	2	88	ND
Sample Three	control	47.5	30.7	17.6	43	10	4.6	86	6
	8 days	46	20	14	48	9	3.5	88	20
	14 days	45	24	13	48	7	3.4	91	15
	challenged	45	27	14	48	7	3.6	91	10

ND = Not detected

in either immunized mice, non-immunized mice or in challenged mice. Clinchy et al (1991) have reported that rIL-4 induces a significant increase in the number of polarized cells within 30 minutes using purified B cells. On the contrary, both small high-density and large low-density B cells gave no response to mouse IL-4 within 30 minutes in both immunized and non-immunized mice, but did respond in overnight culture. This finding is consistent with our pervious findings (Komai et al, 1995; Wilkinson & islam, 1989) which showed that IL-4 had a gradual effect on human B cell locomotion and acts as a locomotor activator, but not as a chemoattractant.

6-3-2 Overnight Culture

The mononuclear fraction and the high-density B cell fraction were cultured overnight in the presence of ovalbumin (10μg/ml), IL-4 (20u/ml), anti-Ig (polyclonal), or combinations of ovalbumin and IL-4 or in RPMI/HSA. The percent polarization responses are shown in Table 6-2. In contrast to human high-density B cells, IL-4 did not induce a significant response in purified mouse B cell populations, but did so in mixed populations (T & B cells). This finding suggests that additional signals may be required for such a response and these signals are probably provided by T cells via physical contact during the culture period. The result becomes more significant bearing in mind that about 50% of the cell populations carry sIg. T cell may also be required for the response of mononuclear cells towards the polyclonal anti-Ig in these studies, because

Table 6-2

Percent polarization of splenocytes in overnight culture

Time of harvesting of cell after Immunization	Stimuli	Percent polarized cells	
		Mononuclear fraction	B cell fraction
After 8 days	Ovalbumin(10μg/ml)	23.5	20
	IL-4 (20u/ml)	38	20±3.2✶
	anti-Ig (1μg/ml)	25	30
	Ovalbumin+IL-4	29.3	30
	RPMI	18	14±1✶
After 15 days	Ovalbumin(10μg/ml)	22.6	24
	IL-4 (20u/ml)	37.5	25
	anti-Ig (1μg/ml)	25.9	33
	Ovalbumin+IL-4	27	25
	RPMI	21	18
No Immunization	Ovalbumin(10μg/ml)	22.4	15
	IL-4 (20u/ml)	40	21.7±3.1✶
	anti-Ig (1μg/ml)	36	27
	Ovalbumin+IL-4	27	24
	RPMI	20	12.7±1.2✶

✶ Mean of three experiments

viability of B cells purified from the T cells in overnight culture was about 25-30% in the high-density fraction, whereas the proportion of viable cells increased to more than 90% in mononuclear fractions containing T cells. This finding suggests that physical contact between cells in mixed populations not only increases the proportion of responsive cells but also can abolish the negative signals induced by anti-Ig and antigen.

6-3-3 30 Minute Polarization Assay After Overnight Culture

High-density B cells from the immunized and non-immunized mice were cultured in IL-4 (20u/ml) overnight, washed and retested with different concentrations of ovalbumin, polyclonal anti-Ig, anti-IgM, and in their own supernatants. The results obtained from three experiments are shown in Fig 6-1, 6-2 and Fig 6-3. In the different groups, identical symbols are used to denote each individual experiment to show the dose response curves to antigen.

Taken together, B cells derived from immunized mice, specially those taken 8 days after immunization, responded better than those from control mice. However, the response was significant in only one study. High-density B cells cultured in IL-4 from the immunized mice (8 days) responded better to anti-Ig and anti-IgM than those from control mice. High-density B cells were also cultured in IL-4 (20u/ml), washed twice and re-exposed to their own supernatants. 23% of B cells derived from 8 days immunized, or challenged mice responded to their own supernatants (the percent polarized cells in medium alone was subtracted),

Figure 6-1

Polarization to ovalbumin after 8 days immunization

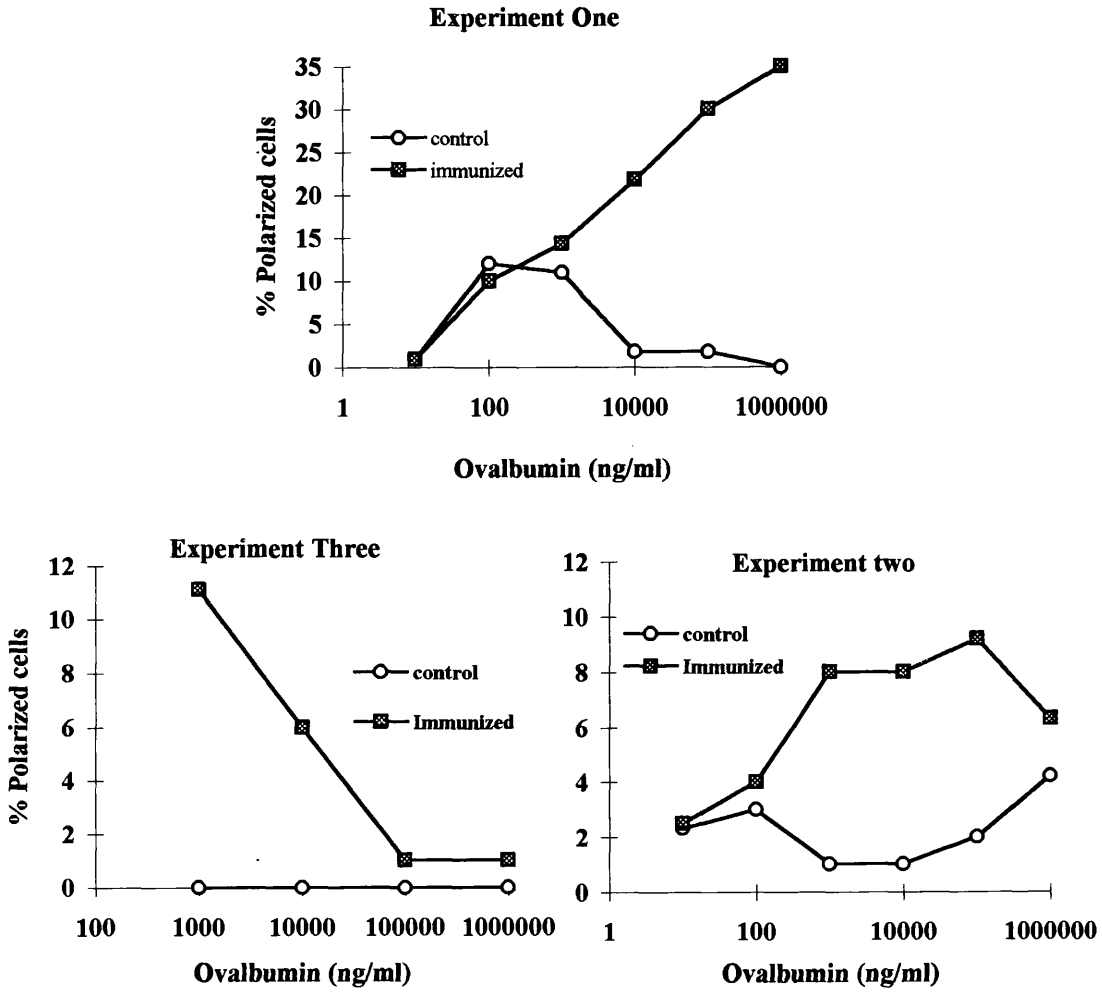


Fig 6-1 Dose response Polarization curves to ovalbumin of B cells taken after 8 days immunization and from non-immunized mice. Control values have been subtracted. Polarization in medium alone was 25 ± 2%.
Note: Difference in scale on Y-axis between three groups.

Figure 6-2

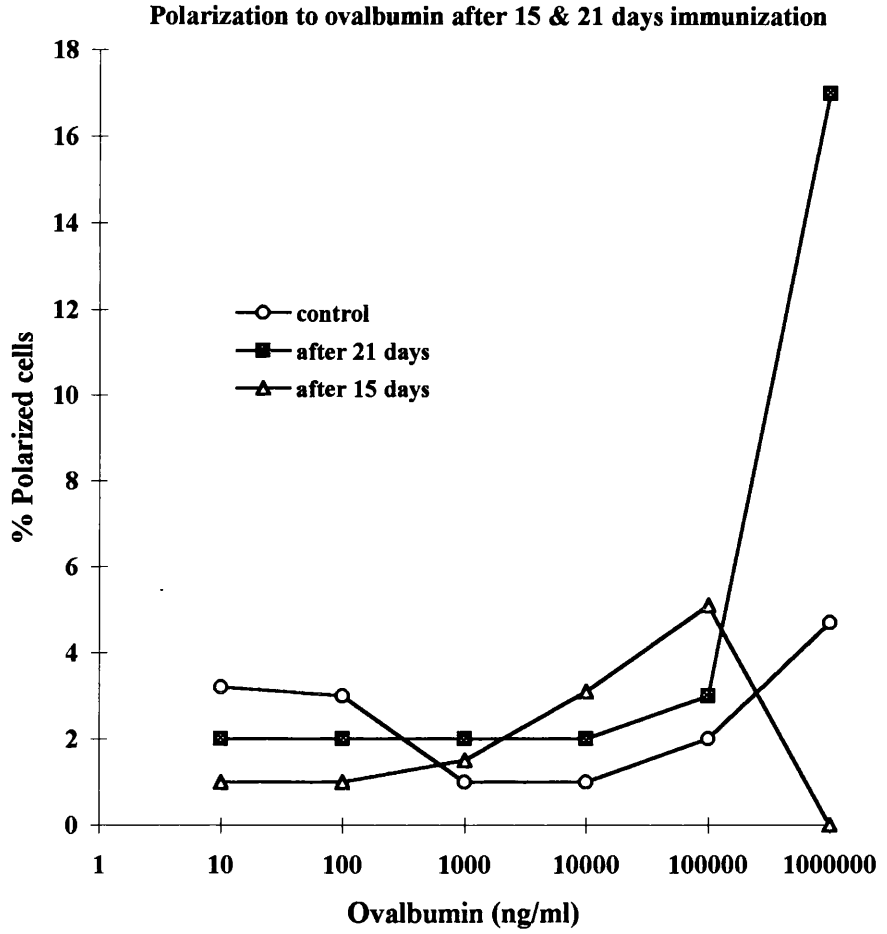


Fig 6-2 Dose response curves to ovalbumin of B cells taken from control mice and from mice 15days and 21 days after immunization. Control value has been subtracted. Polarization in medium alone was 26.5.

Figure 6-3

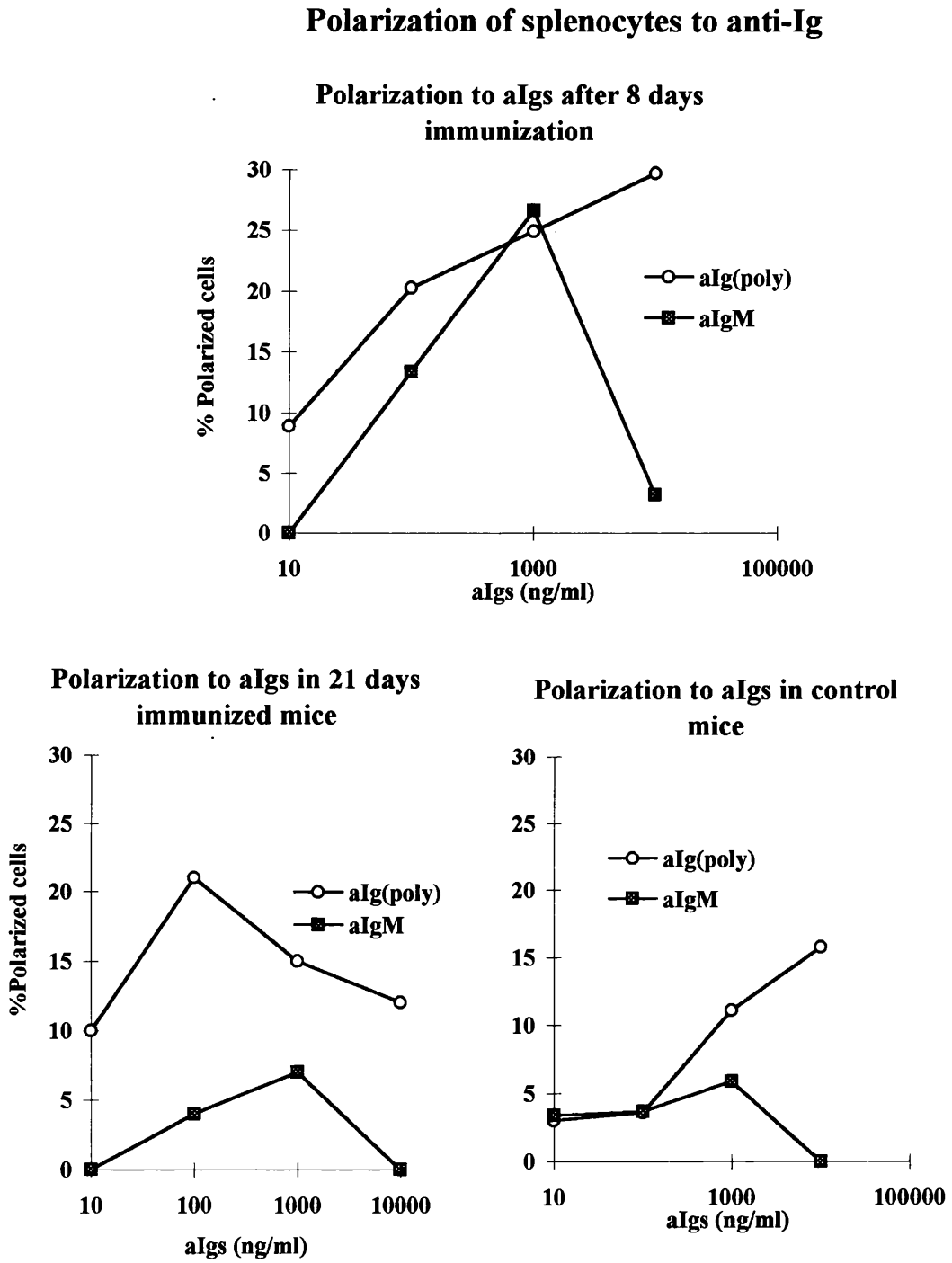


Fig 6-2 Dose response curves to anti-IgM (Squares, n = 1) & anti-IgG,A,M (Circles, n = 1) . Control values have been subtracted. Polarization in medium alone was 23 ± 2%.

in contrast to 16.5% for B cells derived from non-immunized mice.

In a parallel experiment, CBA/BALB/c mice were divided into three groups (6-8 animals per group). One group was immunized with ovalbumin and complete Freund adjuvant by food pad injection. Another group was immunized using killed *Bordetella pertussis* by intraperitoneal injection. After 30 days these two groups together with control mice were challenged with antigen and incomplete Freund by food pad injection. The mice were killed after 4 days and B cells were purified from lymph nodes. High-density B cells were cultured in IL-4 overnight, Following washing twice, they were exposed to different concentrations of ovalbumin. There was no response to antigen in all cases. This was also true for cells isolated directly from the lymph nodes. Since most primed cells leave the germinal centre within 3-4 days after the challenge, in most of the experiments reported above, the high-density B cells fraction was used. However, results in chapter four suggested that isotype switching of tonsillar germinal centre B cells made them more sensitive to low dose of anti-Ig. Thus, the experiments described above should be repeated using the low-density fraction or using purified germinal centre B cells.

6-4 Discussion

In previous chapters, we used anti-Igs that may mimic the function of antigen in vivo and demonstrated that high-density tonsillar B cells, blood B cells, and GC B cells can recognize anti-Igs as an attractant. Therefore, the antigen, ovalbumin,

was used in various experimental systems in mice to study specific antigenic response in vitro.

Coico et al (1983) reported that after a soluble antigen injection, the germinal centre reaction was initiated within 4 days and reached a peak of development by day 10. Antigen re-injection on day 10 reactivates their development and finally the proliferation of germinal centres wanes by day 14. It was tried to use the same model to investigate specific B cell locomotor responses. The best response was observed 8 days after primary immunization. Mice immunized for 15 days or challenged mice did not respond significantly.

MacLennan et al (BSI, March, 1995) reported that 3-4 days after challenge is the best time to get specific B cells in germinal centres, and after 4 days specific B cells leave the centres. Therefore, the B cells were purified from the lymph nodes after 4 days. However, there was no specific locomotor response to the antigen administered.

The results obtained so far are very preliminary and are not impressive. Further work is needed to stabiles a response. There may be several reason why our preliminary investigation was unsuccessful. Firstly, we looked at high-density B cells, while the specific B cells might be among larger cells in the low-density fraction as suggested by the earlier study (Wilkinson et al, 1976). Secondly, it is also quite important to know the proportion of specific B cells in the whole population. Thirdly, other factors such as amount of antigen and route of

immunization should also be considered and, more investigation is required to address the question about the behaviour of antigen-specific cells.

CHAPTER SEVEN

GENERAL DISCUSSION

The results reported here revealed the effect of cytokines and physical contact in B cell locomotion. Taken together several interesting points emerge from these data: (1) Interactions of IL-4, IL-13 and anti-CD40 increase the locomotor capacity of resting B cells (IgD+ & IgM+ cells) from the high-density fraction and of activated B cells (IgD+ & IgM+, non-germinal centre cells) derived from the low-density fraction. Small resting B cells in G_0 phase show little locomotor capacity unless they enter G_1. This is also true for germinal centre B cells. (2) Overnight culture in IL-4 and most probably in anti-CD40 and IL-13 causes cells to enter the G1 phase of growth, and induces a locomotor phenotype in the resting B cells when they can recognize attractants such as anti-IgM and anti-IgD. This induction is maximal using combinations of stimulants; e.g. IL-4 + anti-CD40 + anti-Ig. IFN-γ suppresses this function. Circulating blood B cells show the same behaviour as high density tonsil B cells towards these reagents.

Further works on germinal centre B cells revealed that in contrast to centroblasts, centrocytes are able to recognize anti-Igs as attractants, and especially after 6-12 hours culture in IL-4 and anti-CD40, they respond to anti-Igs (anti-IgG,A,M or anti-IgG and anti-IgA) more strongly than freshly isolated cells. Centrocytes respond to anti-IgA at very low concentrations compared with the response to anti-IgM in germinal centre B cells or in resting B cells.

(3) Culture in anti-Ig (anti-IgM, anti-IgD and anti-IgG.M.A) alone not only did not activate the B cells to become a locomotor population but also, in high concentration, caused apoptosis in resting cells due to hyper cross linking, (4)

There is a direct relation between viability of high-density cells and germinal centre cells and locomotion. Those reagents which induce locomotion can also increase the viability.

This project was limited in time and there is still more to do. Some unanswered questions are listed below. The most important is to highlight the specific antigenic-locomotor response. Polyclonal B cell activators such as LPS are reported to induce locomotion of mouse B cells (Clinchy et al, 1991), but these workers did not directly show antigen-specific locomotor responses. Such responses can be compared in immunized and non-immunized mice. Further studies are required for antigen-specific locomotion, which has only been studied here in high-density cells, and it is required to look at preactivated populations, i.e. large G_1 cells, which may be more sensitive to signals through sIg. More work on the method of immunization and the time of harvesting cells, as well as comparisons of the primary and secondary responses is needed. Anti-CD32 enhances locomotion, so can locomotion be signalled through FcR? Experiments are needed to test this. Since B cells especially centrocytes, interact with immune complexes expressed on FDCs and these complexes play an important role in rescuing of high affinity B cells in germinal centres, immune complexes might also be important stimulants of locomotion. Accessory molecules such as CD20 (IF5) that are reported (Golay et al, 1985) to cause resting B cells to enter into G1 phase might be important in locomotor activation of B cells. The other molecules such as CD19, CD21 and CD23 which have also been reported to be important in

cross-linking of BCR in differentiation of B cells might also be important in B cell locomotion. More work is required on cytokine control on locomotion of B cells. IFN-γ, a T_{H1} cytokine inhibits activation by IL-4, a T_{H2} cytokine. What do other cytokines do, e.g. T_{H1} cytokines such as IL-2?. The work with IFN-γ needs to be expanded. For example, does anti-IFN-γ block the inhibitory effect of IFN-γ?. Finally the relation between locomotion and clustering needs more study. Our preliminary data suggest that aggregated GC B cells are live whereas isolated cells died when stimulated with anti-CD40 and IL-4. This preliminary result may suggest that cell aggregation induced by anti-CD40 and IL-4 inhibits cell death. It was also true that most of the aggregated cells were non-motile. What is the relation of locomotion and clustering and what is the relative time-course of the two events? Does locomotion precede clustering or vice versa?. What cells are present in the cluster? FDCs? or T cells? The answers to these questions are not known, but investigation of the relation between clustering and locomotion should be straightforward.

One other question that needs to be answered is whether anti-Ig induces a true chemotactic response in B cells, i.e. a directional locomotor response, rather than simply inducing the cells to migrate randomly. Random locomotion would not be an effective mechanism for cell accumulation at a site of antigen deposition. This can be studied using an orientation assay (Zigmond, 1977) or a checkerboard assay (Zigmond & Hirsch, 1973).

The migration of lymphocytes including B cells through the body is an effector function that facilitates the regulation of immune responses. In vivo, during the T dependent immune response a considerable amount of B cell movement takes place within the lymphoid tissues. The mechanisms controlling this B cell traffic are largely unknown. However, directed locomotion is almost certainly necessary to permit the close physical contact between antigen-specific T and B cells during the T dependent immune response.

How might in-vitro observations in this project relate to the events occurring in the microenvironment of lymphoid tissue in which memory B cells develop? What are the locomotor attractants that regulate each of these steps? B cells recirculate by crossing HEV (in lymph nodes but not in spleen). These recirculating cells are G_0 small resting cells. What regulates the locomotion of these G_0 cells? Are there specific adhesion molecules for HEV on the B cells as there are on T cells (e.g. L-selectin)? Following from this, the important migrations of B cells may be those in lymphoid tissues. As the T cells become activated by APCs, and depending on whether they take the T_{H1} or T_{H2} pathway, they secrete cytokines such as IL-4, IL-13 and express CD40L. It is possible that T cell signals via IL-4 and CD40 ligand assist in directing this movement in GC B cells. The cytokines and CD40 ligand induce the locomotion of B cells in addition to their other effects related to the recognition of antigen by the B cell population. As a result of this, appropriately activated B cells will migrate towards the site of antigen deposition. Since germinal centre formation is T cell dependent, it is

proposed that a combination of signals from antigen, CD40L and T cell-derived cytokines such as IL-4 will induce locomotion in germinal cells allowing B cells to traverse the follicles and to mature into plasma cells or memory cells. After a massive proliferation of B cells in the dark zone, anti-CD40 + IL-4 activation was initially proposed to account for the B cells in the apical light zone of germinal centre (Holder et al, 1991). Therefore it was not surprising that cultured GC B cells in anti-CD40 + IL-4 responded to anti-Ig more strongly than cells direct after separation. In this compartment, CD40 triggering induced rescue of the B cells from apoptosis (Liu et al, 1989) and therefore play a role in generation of memory B cells. During this process, IL-4 could be secreted by activated T cells (Buch et al, 1993). The effect of anti-CD40 on locomotion of resting B cells and germinal B cells is consistent with other findings that CD40L is important in initiation and termination of the T dependent response (Clark and Shu, 1990; Liu et al, 1989). Why do antibody forming cells move out of the germinal centre and why are the memory cells concentrated in the marginal zone (MacLennan, 1994)? These questions remained to be answered. The results reported here, revealed that anti-Igs play a vital role in locomotion of B cells. The locomotor properties of other B cell activators such as LPS (only in the mouse, as LPS does not work in man), or T independent antigens, or other cell surface molecules, e.g. CD20 have not been studied. Is antigen the most important chemoattractant for B cells? Are the chemokines chemoattractants for B cells? The latter cytokines probably induce T cells to move into inflammatory sites in large numbers. On the other hand, it is

possible that inflammatory attractants are not important for B cells, because once they have made antibody, it is the antibody and not the B cell that acts locally. All these points remain to be investigated.

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