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Natural Killer Cell Evolution : Cellular and Molecular Studies on *Xenopus*.

by

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B.Sc. (Hons.)

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A Thesis submitted in accordance with
the requirements for the degree of
Master of Science.

University of Durham.
Department of Biological Sciences.

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09 MAY 1997

For my Mum and Dad with love

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Declaration

No part of this thesis has been previously submitted in support of an application for an M.Sc. degree or equivalent qualification at the University of Durham, or any other university or institute of higher education.

Chromium release microcytotoxicity assays were performed in collaboration with Mrs. Trudy Horton.

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Abstract

The presence of natural killer cells at lower evolutionary levels was investigated in the amphibian *Xenopus laevis*. Chromium release microcytotoxicity assays revealed that fresh splenocytes from early-thymectomised *Xenopus* displayed significant spontaneous cytotoxicity against allogeneic B₃B₇ thymic tumor cell targets, unlike those from control *Xenopus*, suggesting that 'NK-like' activity is greater in thymectomised (T cell-deficient) animals. Addition of Concanavalin A-derived active supernatants to splenocytes from a thymectomised animal caused a significant increase in cytolytic activity, but had no effect on cells from a control animal. This finding of enhanced cytotoxicity was indicative of lymphokine-activated killing in *Xenopus*, and supported the concept that tumour cell lysis was mediated by NK - like cells.

Attempts were made to enrich the splenocytes for natural killer cells through the selective depletion of other lymphocyte subsets, using the techniques of 'panning' and 'magnetic bead' separation following monoclonal antibody labelling of cells. On comparison of the two techniques, it was found that both were able to deplete a splenocyte culture of B cells to the same extent, but that magnetic sorting produced far superior results for depletion of T cells. Optimum conditions for magnetic sorting were determined, and used to generate 'purified' populations which were tested for their cytolytic activity. Such preliminary investigations suggested that natural killer-like activity in *Xenopus* is likely to be mediated by a 'non-T / non-B' lymphoid subset.

Finally, preliminary work was undertaken into the development of 'phage display' technology for the generation of single chain F_v antibody fragments (ultimately against NK cell surface antigens). PCR amplification of the V_H and Kappa chains was attempted on RNA extracted (using various methods) from Carboxypeptidase Y-injected-, B₃B₇-injected-, and unimmunised mice. Following RNA extraction under optimum conditions, Kappa chains were successfully amplified from experimental spleens, but the heavy chains still require more development.

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Abbreviations

ADCC	antibody dependent cellular cytotoxicity
ALC	anti light chain
α mm	α -methyl mannoside
APBS	amphibian phosphate buffered saline
ASN	active supernatant
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CD	cluster designation
cDNA	complimentary DNA
CO_2	carbon dioxide
Con A	concanavalin A
CPM	counts per minute
CPY	carboxypeptidase Y
CSN	control supernatant
$^{\circ}\text{C}$	degrees centigrade
DEPC	diethyl pyrocarbonate
DG	sn-1,2-diacylglycerol
DNA	deoxyribonucleic acid
dNTP	dioxyribonucleoside triphosphate
DPM	degenerations per minute
DTT	dithiothreitol
E : T	effector : target
$\text{F}_{(\text{ab})}$	antibody-binding portion of immunoglobulin
FACS	fluorescence activated cell sorting
F_c	constant portion of immunoglobulin
FCS	fetal calf serum

Fd	V _H + C _{H1} (first constant domain of heavy Ig chain)
FITC	fluorescein isothiocyanate
F _V	variable domains of immunoglobulin
g	relative centrifugal force
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
INF	interferon
IP ₃	inositol-1,4,5-triphosphate
Kb	kilobases
kDa	kilo Dalton
L15	Leibovitz-15 medium
LAK	lymphokine activated killing / killer
LGL	large granular lymphocyte
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MPC	magnetic particle concentrator
mRNA	messenger RNA
NaN ₃	sodium azide
NCAM	neural cell adhesion molecule
NCC	nonspecific cytotoxic cells
NK	natural killer
NKC	NK gene complex
NKR	natural killer cell receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohaemagglutinin
PI	phosphoinositide
PKC	protein kinase C
PMA	phorbol myristate acetate

PTK	protein tyrosine kinase
rIL-2	recombinant interleukin 2
RNA	ribonucleic acid
scFv	single chain Fv fragment
SI	stimulation index
sIg	surface immunoglobulin
SN	supernatant
T _c	cytotoxic T cell
TCGF	T cell growth factor
TCR	T cell receptor
TNF	tumour necrosis factor
Tx	thymectomised
UV	ultraviolet
V _H	variable domain of heavy chain of Ig
V _L	variable domain of light chain of Ig
μCi	microCurie
2°	secondary
³ HTdr	tritiated thymidine
⁵¹ Cr	⁵¹ chromium

Chapter 1

General Introduction

1.1 Natural Killer Cells - Characterisation and Functions

Natural Killer cells represent a unique population of lymphocytes that are capable of spontaneous cytotoxicity, and are thus believed to form a first line of defence within the immune system. They were initially recognised for their tumouricidal activity but are now believed to be important in destroying virally-infected cells too. Since they require no prior sensitisation to lyse their targets, and are not genetically restricted, they provide an excellent means of first line defence. Further, their effect is usually very rapid - NK cell subsets requiring assistance from accessory cells usually reach maximum cytolytic activity within fourteen hours, while those independent of accessory cell assistance can achieve maximum killing in only four hours (Howell and Fitzgerald-Bocarsly, 1991). However, NK cells do not generate immunological memory like T- and B- cells, nor do they possess a clonally distributed receptor (Brooks *et al*, 1983), which suggests that evolutionarily they existed before T and B cells, providing a multi-specific primitive immune system (Janeway, 1989).

Numerous studies (in rats, mice and humans) have revealed that this natural killer activity is typically associated with large granular lymphocytes (LGLs), containing a high cytoplasm to nuclear ratio, distinct azurophilic granules (Luini *et al*, 1981), and a kidney-shaped nucleus (O'Shea & Ortaldo, 1992). However, heterogeneity does exist amongst NK cell populations, both morphologically and functionally (Hercend *et al*, 1983), and this is likely to represent different stages of NK cell differentiation/activation. (Allavena & Ortaldo, 1984). Thus, the reactivity



of an entire NK population is likely to represent the sum of the reactivities of all the different NK populations present.

Characterisation of the cell surface antigens has demonstrated that NK cells are phenotypically distinct from both B- and T- cells, since they express neither surface immunoglobulin nor T cell receptor / CD3 complex. Instead, they are characterised by the presence of both CD16 (immunoglobulin Fc receptor, FcγRIII) and CD56 (Leu-19), although both CD16⁺ CD56⁻ and CD16⁻ CD56⁺ variants have been observed. Other cell surface antigens observed include the myelomonocytic-related marker CD11, and the T-cell-related markers CD2 and CD8, all of which may be important in target recognition and the subsequent activation of the NK cell.

Natural Killer cells can thus be defined as follows (Taken from O'Shea & Ortaldo, 1992) :

"NK cells are CD3⁻ T cell receptor (alpha/beta, gamma/delta)⁻ large granular lymphocytes. They commonly express markers such as CD16 and NKH-1 (Leu 19) in humans and NK-1.1/NK-2.1 in mice. They mediate cytolytic reactions that do not require expression of Class I or Class II MHC molecules on the target cells.

Certain T lymphocytes which are either alpha/beta⁺ or gamma/delta⁺ may express, particularly upon activation, a cytolytic activity that resembles that of NK cells. These T lymphocytes should not be termed NK cells. They could be termed either T lymphocytes displaying 'NK-like' activity or 'non-MHC requiring' cytotoxicity."

A large proportion of the NK activity is found in peripheral blood, where NK cells account for approximately 10 - 15 % of the lymphocyte population. However, LGLs (and the associated cytotoxicity) have also been observed in the lungs, liver

and spleen (where they are located predominantly in the red pulp - Witte *et al*, 1989), and to a lesser extent in the bone marrow and lymph nodes. It is interesting that different lymphoid organs contain phenotypically different NK cell subsets (Witte *et al*, 1989), but that they are similarly distributed in a number of animal species, since this provides clues to a possible differentiation pathway.

NK cells are believed to have several important roles in the immune system, including **the regulation of haematopoiesis, an involvement in transplantation** (possibly exerting anti-graft effects early after transplantation, but more importantly, providing protection against malignancies and opportunistic infections until B- and T - cell functions have completely recovered - Hogan in "The Natural Killer Cell" Chapter 5), **combating bacterial infection** (they are attracted by bacterial products to the site of inflammation, where they can both directly phagocytose the bacterial cell, and produce a variety of cytokines which act on other effector cells to mediate the inflammatory response), **combating viral infection**, and **possessing strong tumouricidal activity**.

A great deal of research is currently taking place into these last two functions since tumours and viruses (especially the human immunodeficiency virus (HIV) which causes AIDS (acquired immunodeficiency syndrome)) pose an enormous threat to the health of society. It is possible that NK lysis of virally-infected cells is mediated via several mechanisms :- NK cells are first activated by, and proliferate to, either viral glycoproteins, cytokines or virus-induced type 1 interferon (INF α, β), they may then undergo direct cell-mediated lysis, or secrete cytokines which either inhibit virus replication or activate other mechanisms of natural immunity. It is further possible that, in the later stages of infection, NK cells can lyse virally-infected cells which have been coated with antibody, via the ADCC (Antibody - dependent cellular cytotoxicity) mechanism. (Welsh & Vargas-Cortes, in "The Natural Killer Cell" Chapter 4). Further research is thus necessary to assess the relevant contribution of

each of these mechanisms, and hence which could be exploited for the highest therapeutic benefit.

1.2 Origin of NK cells - developmental relationship between NK and T Cells.

Numerous studies over the last two decades have demonstrated that NK- and T-cells share a number of common features, suggesting that they may be derived from a common progenitor. Furthermore, since NK cells are involved in the two earliest phases of mammalian host defence against infection (innate resistance and the early interferon-inducible phase that is largely antigen non-specific), while T-cells are involved in the last, highly antigen-specific phase that generates immunological memory, it is also likely that cytotoxic T-cells evolved from NK cells, probably through the acquisition of a clonally-distributed receptor (Janeway, 1989). Indirect evidence for the developmental relationship between NK- and T-cells comes from studies in immunodeficient patients: one patient was observed to have normal myeloid and B-cells but no T- or NK-cells (suggesting that there is a common T-cell-NK-cell progenitor distinct from totipotent lymphoid progenitors), while another patient had normal T- and B-cells but no NK cells, suggesting that at least the later stages of NK- and T-cell differentiation are separate from each other (Lanier, Spits *et al*, 1992).

More direct evidence has come from the study of the cell surface markers present on T- and NK-cells, both fetal and adult. The most distinguishing feature between the two cell types is that T-cells express a T-cell receptor (either $\alpha\beta$ or $\gamma\delta$, but usually the former), while NK cells do not - otherwise their expression of surface antigens is remarkably similar (providing further evidence that they are related). NK cells are usually classed as membrane $CD3^- CD56^+$, and T cells as either $mCD3^+ CD5^+ CD4^+ CD8^-$ or $mCD3^+ CD5^+ CD4^- CD8^+$. Both cells express CD2, CD7, CD18, CD16, CD29, CD38, CD44 and CD45RA, and both lose expression of CD34 (which is considered to be a marker of immature progenitor cells of several lineages) as they mature.

Although NK cells do not express the CD3 complex (consisting of γ , δ , ϵ and ζ chains), which is associated with the TCR and is involved in signal transduction in T-cells, it has been shown that they may express certain of the individual subunits. Lanier, Chang *et al* (1992) have demonstrated that resting adult NK cells, freshly isolated from human peripheral blood, contain CD3 ϵ transcripts in their cytoplasm, but not CD3 γ or δ . In-vitro activation of these NK cells causes the expression of the CD3 ϵ mRNA into CD3 ϵ proteins which, seemingly, are restricted to the cytoplasm. Since there are no γ or δ proteins present, there is no formation of a CD3 complex. In comparison, fetal NK clones from human liver express both CD3 ϵ and CD3 δ (and in some cases CD3 γ as well) in the cytoplasm, and are therefore capable of forming CD3 δ,ϵ complexes (and low levels of CD3 γ,ϵ in those clones expressing CD3 γ). However, these complexes are again restricted to the cytoplasm, indicating that they are unable to be transported to the cell surface. Although CD3 ζ has been detected on the surface of peripheral blood NK cells (Anderson *et al*, 1989) and has been found to be identical to the T cell CD3 ζ (Moingeon *et al*, 1990), there has been no evidence of CD3 ϵ,ζ complexes in either fetal or adult NK clones (Lanier, Chang *et al*, 1992).

It is not yet known whether the fetal CD3 γ , δ , ϵ^+ and the adult CD3 ϵ^+ NK cells represent different NK cell subsets (the latter replacing the former after birth), or whether they represent the developmental progression of a single cell subset. (In this case, it would imply that CD3 expression is down-regulated as the NK cell differentiates, possibly due to the inavailability of a TCR heterodimer for it to combine with). The function of these cytoplasmic CD3 subunits is also unknown (if they even have a function at all - it may be that they are simply inadvertently expressed by a promiscuous transcriptional factor during the activation of the NK cell).

These results suggest, therefore, that the T-cell-NK-cell progenitor may express CD3 γ δ , ϵ and ζ subunits, and that their fate depends on the signals that they receive. For example, those cells destined for the T-lineage pathway presumably receive signals for homing to the thymus and rearrangement of TCR genes. On entry to the thymus, these cells proliferate extensively, rearrange their TCR genes to generate a vast diversity of TCR molecules, and begin to express low levels of both CD4 and CD8. They then undergo education via a process of positive and negative selection to ensure that only self-tolerant cells can exit to the periphery. This selection involves the close interaction of thymocytes with the non-lymphoid cells of the thymus, including macrophages, epithelial cells and dendritic cells, which express high levels of self MHC proteins (both class I and class II). Thymocytes whose TCRs recognise and respond to self MHC are positively selected to survive, since these are potentially useful, but those which respond too strongly (by recognition of both the self MHC and the self peptide it is presenting) are destroyed (negative selection) because they are harmful. Any thymocyte that does not recognise self MHC is useless and dies naturally within about three days. Depending on whether the thymocyte TCR binds to a class I or class II MHC molecule, the selected cell will up-regulate CD8 expression and become a cytotoxic T-cell, or up-regulate CD4 expression and become a helper T-cell respectively (von Boehmer & Kisielow, 1991).

Lanier *et al* (1992) have suggested that T-cell-NK-cell progenitor cells (in the fetal liver or bone marrow) that fail to receive such signals, or that lack a thymic microenvironment, will, by default, proceed along the NK cell developmental pathway, with the resultant expression of CD56, loss of CD34, and acquisition of cytolytic activity. However, it is also possible that positive signals are necessary for the progenitor cells to enter the NK differentiation pathway.

Interestingly, fetal NK cells have been shown to be capable of most of the functions recognised in mature NK cells (Lanier, Spits *et al*, 1992), including non-MHC restricted cytolysis, antibody dependent cellular cytotoxicity, response to

interleukin-2, and production of regulatory cytokines (including gamma-interferon and tumour necrosis factor α). This would indicate that immunocompetent NK cells develop before T-cells during fetal ontogeny (which is consistent with their provision of an early, first line form of defence).

Although NK cells have been observed at a very low frequency in the thymus (<0.1% of cells, with the phenotype $mCD3\epsilon^- NK1.1^+$ in mice, and $mCD3\epsilon^- CD56^+ CD16^{+/+}$ in humans), they develop normally in athymic nude mice, and thus represent a thymus-independent lymphoid lineage. Consequently, it is postulated that the T-cell-NK-cell progenitor differentiates into T-cells in a thymic environment, and NK cells in an extrathymic environment.

Evidence for this includes work by Poggi *et al* (1993) who have demonstrated that triple negative $CD3^- CD4^- CD8^-$ embryonic liver cells (at 6 - 8 weeks of gestation) can be induced to differentiate into either T- or NK cells (expressing functional receptor molecules) depending on the culture conditions used. When cultured with phytohaemagglutinin (PHA) and recombinant interleukin 2 (rIL-2), they gave rise to $CD3^+$ lymphocytes expressing either $\alpha\beta$ or $\gamma\delta$ TCR, but when cultured with irradiated H9 cells and rIL-2, they gave rise to $CD3^- CD16^+ CD56^+$ cells with strong cytolytic activity. Although the results do not determine whether T- and NK cells are derived from the same precursor, they do demonstrate that the fetal liver does contain T-cell / NK cell progenitors, and that there could be an extrathymic developmental pathway.

Further evidence was provided by Rodewald *et al* (see Lanier, Spits *et al*, 1992) who demonstrated that, in mice, day 14 $CD16^+$ (Fc γ RIII) fetal thymocytes differentiated into mature T-cells when injected intrathymically, but differentiated into $NK1.1^+$ NK cells with NK and ADCC activity when injected intravenously and treated with IL-2. However, although the results suggest a common $CD16^+$ T-cell-NK-cell progenitor, it is not proven. (Interestingly, when mature adult mouse NK

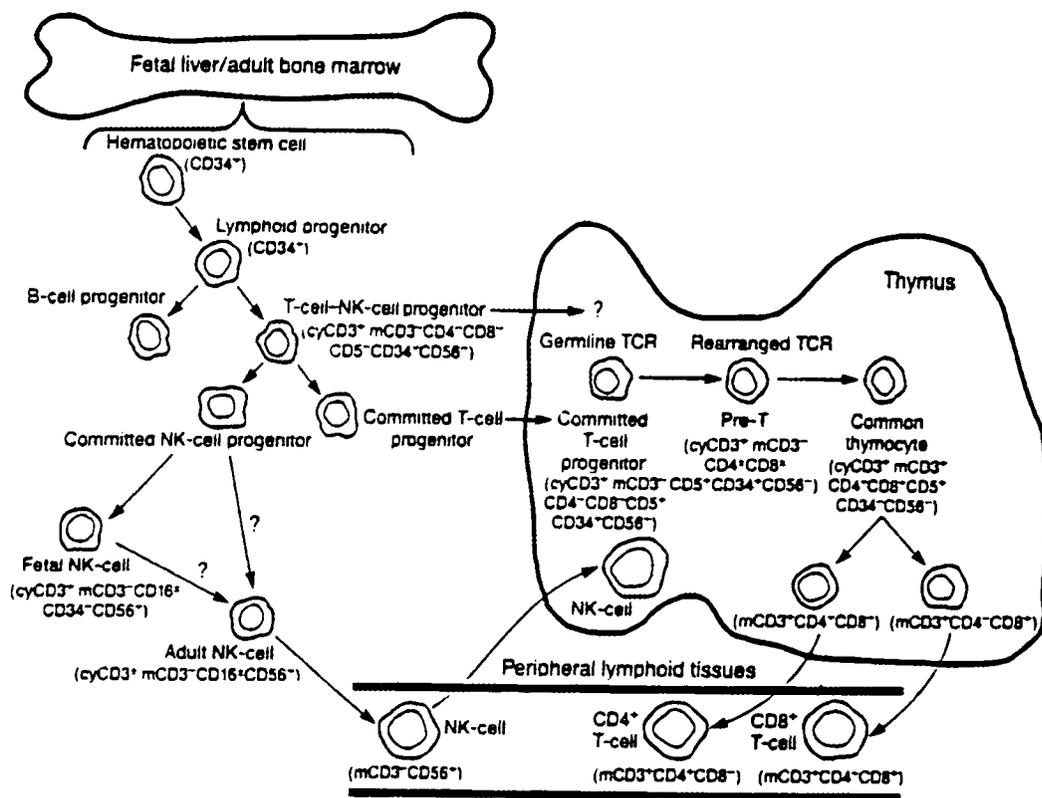
cells were injected intrathymically, they were unable to generate a T-cell lineage, implying that committed NK cells are void of any T-cell progenitor activity).

Similarly, Brooks *et al* (1993) have shown that the majority of day 14 fetal thymocytes from mice can differentiate into lymphocytes with many characteristics of mature NK cells if exposed briefly to IL-4 + PMA (phorbol myristate acetate) then treated with IL-2. Consequently, these cultured fetal thymocytes have the phenotype Ig⁻, Thy1⁺, CD2⁻, CD3⁻, CD4⁻, CD5⁻, CD8⁻, CD44⁺, CD45⁺, CD45R⁺, Ly6A⁺, HSA⁻, MHC-I⁺, MHC-II⁺, F4/80⁻, NK1.1⁺, asialo-GM1⁺, express powerful cytolytic activity against NK-sensitive targets, and secrete various cytokines. (Likewise, studies in humans have shown that treatment of fresh thymocytes with rIL-2 results in the induction of NK activity, and that this activity can be correlated with the appearance of the NKH1 antigen on a sub-population of cells - Michon *et al*, 1988). In addition, and perhaps somewhat surprisingly, it has also been observed (Brooks *et al*, 1983) that mature cytotoxic lymphocytes are capable of non-specific cytolytic activity, including perforin-mediated cytotoxicity, after stimulation with interleukin-2 (Milanese *et al*, 1986). In fact, it seems that they have differentiated into cells indistinguishable from splenic NK cells, exhibiting an increase in size and granularity, the loss of antigen-specific cytolytic activity, and a change in the cell surface molecules. It is therefore proposed that NK cells belong to the T-cell lineage, and in particular, may be very closely related to cytotoxic cells.

All this evidence (together with the study of lymphocyte phenotypes in both the fetal and adult thymus) has led Lanier, Spits *et al* (1992) to propose the following hypothetical scheme for the differentiation of T and NK cells (see Figure 1.1). From this scheme, it would appear that the very early stages of lymphocyte development are identical, and that the most likely phenotype for a T-cell-NK-cell progenitor is CD2⁻ CD3⁻ CD4⁻ CD8⁻ CD5⁻ CD7⁺ CD19⁻ CD33⁻ CD34⁺ CD56⁻. At this point the two pathways then diverge and the progenitor is committed to the T or NK cell lineage, differentiating in the thymus or extrathymically respectively.

More recently, Sánchez *et al* (1994) have confirmed this T cell / NK cell relationship by identifying a progenitor in the human thymus which is capable of giving rise to both T and NK cell lineages. This progenitor is a CD34⁺ Triple Negative ([TN] CD3⁻, CD4⁻, CD8⁻) thymocyte which has been shown to have both a high NK cell clonogenic capacity, and the ability to generate both mature CD3⁺ T cells and CD56⁺ CD3⁻ NK cells in murine fetal thymic organ culture (mFTOC). It is hypothesised that in the thymic microenvironment, the majority of these progenitors will gradually lose their CD34 and acquire CD2, CD5, CD28, CD38, and CD1, at which point they will have become irreversibly committed to the T cell lineage (as in Figure 1.1). However, a small percentage will differentiate into mature NK cells, indicating that although NK cells represent a thymic-independent cell line, they are capable of developing inside the thymus too. As yet, the immunological significance of this is unknown.

Figure 1.1 : Hypothetical scheme of NK cell and T cell differentiation (Taken from Lanier, Spits *et al*, 1992)



1.3 NK Cell specificity - molecular events involved in target recognition and biochemical activation.

NK cells are known to recognise and lyse a variety of tumour and virally-infected cells, but the mechanism by which they do this is not yet fully elucidated. Since physical contact between the natural killer cell and its target is necessary for killing to take place, it seems likely that lysis is mediated through specific interactions between cell surface molecules expressed on the two cell types. In other words, there are believed to be receptors on the surface of NK cells, distinct from both surface immunoglobulin on B cells and T cell receptors on T cells, which interact with ligands on target cells to regulate killing. Recent studies have proposed several potential receptors (many of which may enhance adhesion), and have suggested that there may be structures involved in both activation and inhibition of the cytolytic activity.

One of the earliest-discovered mechanisms by which NK cells act is that of antibody-dependent cellular cytotoxicity, or ADCC, whereby effector cells are able to lyse immunoglobulin (Ig) - coated targets through their expression of a receptor for the constant (Fc) portion of the immunoglobulin. Such a receptor has been identified in NK cells - it is a transmembrane, phosphatidylinositol glycan-linked molecule (Simmons & Seed, 1988) of approximately 50 - 70 kDa (Lanier, Ruitenberg & Phillips, 1988). It has been shown to bind IgG complexes with low affinity and has been termed FC γ RIII or CD16. (O'Shea & Ortaldo, 1992). The discovery that it is co-associated with the CD3 ζ homodimer on the membrane of human NK cells (Lanier *et al*, 1989; Anderson *et al*, 1990), and the knowledge that CD3 ζ is involved in signal transduction after binding of the TCR in T cells, has led to the suggestion that CD16 is a functional NK membrane receptor which allows activation of the NK cell lytic pathway through the CD3 ζ .

Similarly, CD2 is also thought to function as an adhesion/activation molecule in NK cells (Scott *et al*, 1989). It is a 50 - 58 kDa glycoprotein belonging to the Ig gene superfamily, and is believed to enhance adhesion by binding to its ligand LFA3 (another adhesion molecule, expressed on several cell types). Furthermore, redirected lysis assays have demonstrated that it has signal transducing functions, and may also operate via CD3 ζ (Anderson *et al*, 1989). However, it is unlikely that either of these molecules (CD16 or CD2) play a crucial role in natural killing, since NK cells which lack them still exhibit cytotoxicity (Nagler *et al*, 1989).

Other interactions which may promote adhesion include LFA1 (CD11a - an integrin gene superfamily member) on the NK cell with ICAM-1 (a 90 kDa transmembrane protein with homology to neural cell adhesion molecule NCAM) on the target cell, and CD56 (a 175 - 185 kDa glycoprotein that has been identified as an isoform of NCAM - Lanier, Testi *et al*, 1989) on the NK cell with its ligand on the target cell.

However, there are other putative natural killer cell receptors (NKR) which are potentially more important, and these have been discussed in several reviews (Hofer *et al*, 1992; Yokoyama, 1993; Yokoyama & Seaman, 1993). One of these is the rodent NKR-P1 antigen, which is believed to be responsible for NK cell activation in spontaneous cytotoxicity. NKR-P1 is a 60 kDa, disulphide-linked homodimer which is expressed by all rat NK cells, possibly in multiple, related forms. It is a type II integral membrane protein with an extracellular carboxy terminal and a single transmembrane domain, and has significant homology with members of the C-type lectin superfamily (Giorda *et al*, 1990). Since several members of this superfamily (which are frequently cell surface receptors) bind to specific carbohydrates (in a Ca²⁺-dependent manner), it is suggested that the NKR-P1 ligand is also a carbohydrate, although as yet no such ligand has been identified. (Given that NKR-P1 belongs to a family of homologous genes, with each NK cell expressing

more than one isoform, it is possible that the ligand is also polymorphic, or that there is a different ligand for each isoform).

Evidence that NKR-P1 is an activation receptor comes from redirected lysis assays (where a mAb binds to the effector cell via its $F_{(ab)}$ regions, and to the target cell via its Fc region, thus bridging the two cells and stimulating the receptor) and from direct stimulation of the NK cell with a mAb that recognises NKR-P1 (mAb 3.2.3 - Chambers *et al*, 1989). Both of these result in tyrosine phosphorylation, the activation of phosphoinositide turnover, an increase in intracellular calcium, and the resultant degranulation by the NK cell, all of which are biochemical events observed when an NK cell contacts their natural targets. Similar studies have been made in both mice and humans, and it has been found that NK cells in these species also express activation receptors, termed NK1.1 and NKG2 respectively. These display similar characteristics to NKR-P1, and similarly exist in several isoforms.

Another receptor family identified on NK cells, that shows homology to the C-type lectin supergene family, is represented by the mouse Ly-49, a dimeric glycoprotein (Yokoyama & Seaman, 1993). Again this is a type II integral membrane protein with an extracellular carboxy domain, and is encoded by a member of a family of highly-related genes, but in this instance, the receptor is believed to act as an inhibitor of NK cell cytolytic activity since NK cells expressing Ly-49 are unable to lyse normally-susceptible tumour targets. This has been correlated with the observation that there is an inverse relationship between MHC class I expression on target cells and their susceptibility to NK cell lysis, to suggest that the ligand for Ly-49 is MHC class I (Hofer *et al*, 1992).

Although NK cell-mediated lysis is known to be non-MHC restricted, several groups have demonstrated that mutant target cell lines which are deficient in MHC class I expression are more susceptible to NK cell-mediated lysis than the parental strains. For example, H-2 deficient variants of the RBL-5 lymphoma (which is

normally NK-resistant and highly malignant) have severely reduced tumourigenicity and are NK-sensitive (Kärre *et al*, 1986). Furthermore, numerous studies have shown that reconstitution of MHC class I expression, through various molecular techniques, results in the restoration of NK-resistance (Yokoyama & Seaman, 1993; Ljunggren & Kärre, 1990). This effect is, however, quantitative, and there is a minimum level of MHC class I expression necessary for protection against lysis to be provided (Kärre *et al*, 1986; Ljunggren & Kärre, 1990; Storkus *et al*, 1989).

Two mechanisms have been proposed to account for the inhibitory effect of MHC class I on NK cell activity (Ljunggren & Kärre, 1990). The first is that the MHC antigen 'masks' the normal stimulatory signal on the target cell, such that recognition and activation is blocked in the presence of MHC (thus removal of MHC allows lysis to occur), and the second is that there is a receptor on the NK cell which specifically recognises the MHC antigen and relays a 'negative' signal into the cell. Various studies have suggested that the latter model is more likely.

It is postulated that Ly-49 represents the receptor family involved in 'recognising' the presence/absence of MHC class I molecules, since Ly-49⁺ cells do not lyse H-2^d and H-2^k targets, but Ly-49⁻ cells do. In particular, it is thought that Ly-49 interacts with the $\alpha 1/\alpha 2$ domains of the MHC molecule (since it is these domains that have been shown to provide the 'protective' function against natural killing - Storkus *et al*, 1989), suggesting that the peptide-binding cleft may be involved (Chadwick *et al*, 1992; Storkus *et al*, 1992), and further, that the MHC haplotype of the target cell is involved in the specificity. This inhibitory influence of the Ly-49/MHC class I interaction seems to be dominant over all stimulatory signals, allowing a tolerance to self cells.

The genes encoding both Ly-49 and NKR-P1 have been characterised, and have been found to represent gene families that are genetically linked (Yokoyama &

Seaman, 1993). In mice they have been mapped to chromosome 6, and in humans to chromosome 12, and in both cases are separated by approximately 0.4 centiMorgans (about 500kb). This genetic region has been termed the NK gene complex (NKC), and is predicted to encode a number of related genes of varying homology, all of which may be functionally important in NK cell activity. Consequently, different NK cells may express different combinations of these various receptors and thus may exhibit different target specificity, which would account for the observed heterogeneity in NK cell populations.

Irrelevant of which of these receptors plays the most important role in natural killing, the ultimate consequence of engagement of a stimulatory receptor must be the triggering of the cytolytic machinery, with secretion of granule contents (such as serine esterases), target lysis, and production/secretion of regulatory cytokines (which may involve alteration of gene expression). However, the biochemical steps leading to these events are, as yet, only poorly understood.

In comparison with activation of T cells through the TCR (which is likely to be analogous to activation of NK cells through the putative NKR), it is believed that transmembrane signalling in NK cells occurs through phosphoinositide turnover (Chow & Jondal, 1990), with generation of the second messengers inositol-1,4,5-triphosphate (IP₃) and sn-1,2-diacylglycerol (DG) (Windebank *et al*, 1988). IP₃ then mobilises intracellular Ca²⁺ from endoplasmic reticulum stores, while DG activates protein kinase C (PKC - a major intracellular serine/threonine kinase), both resulting in cell activation (Leibson *et al*, 1990). In addition, it is thought that this second messenger activation system may be further regulated by another second messenger system such as cAMP (Windebank *et al*, 1988).

Numerous studies (reviewed in O'Shea & Ortaldo, 1992) have shown that exposure of NK cells to tumour targets or antibody-coated targets, or direct stimulation of FcγRIII, results in an increase of phosphoinositide (PI) turnover and a

rise in the intracellular calcium levels (Cassatella *et al*, 1989), supporting the suggestion that the NKR is coupled to PI hydrolysis and PKC activation. This increase in calcium levels (caused by both intracellular release and extracellular influx) is further believed to be critical in the production of cytokines (such as IFN γ and TNF) which play an important role in part of the natural killer cells mode of action (Cassatella *et al*, 1989). (Interestingly, virally-infected cells do not induce PI turnover or calcium release, which suggests that these targets may activate a different biochemical pathway, but, as yet, this is unknown). However, there may also exist some sort of autoregulation by PKC, such that PI turnover coupled to one type of surface receptor may be inhibited (allowing 'desensitisation' to prolonged target exposure), without blocking the second messenger cascade induced by engagement of a different membrane receptor (Leibson *et al*, 1990).

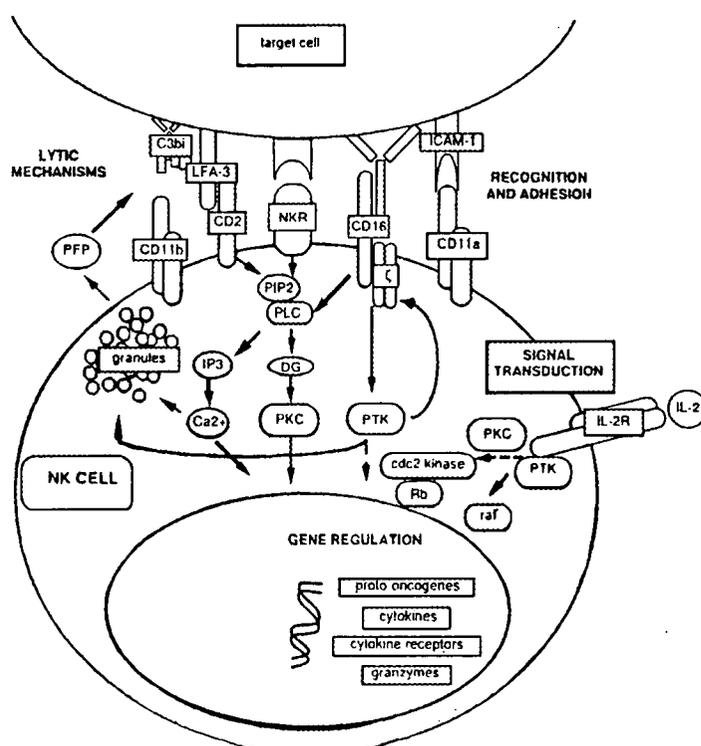
In addition to the serine/threonine kinase (PKC), it appears that signalling may also occur via a protein tyrosine kinase (PTK). O'Shea *et al* (1991) have demonstrated that stimulation of NK cells via Fc γ RIII (either with antibody-coated cells or anti-Fc γ RIII mAb) induces phosphorylation of the ζ chain, a known substrate of PTK in T cells. Since ζ has been shown to be physically associated with Fc γ RIII (Lanier *et al*, 1989; Anderson *et al*, 1990), this would suggest that Fc γ RIII is therefore coupled to a PTK, and has a similar signalling mechanism to that observed in T cells via the TCR and the lck and fyn PTKs. (NK cells have, in fact, been shown to express lck and fgr PTKs - see O'Shea *et al*, 1991).

It is feasible that signalling in NK cells occurs via a cascade of kinases, including both protein tyrosine kinases and serine/threonine kinases. For example, NK cells possess an IL-2 receptor (of which the p75 glycoprotein is the structure primarily responsible for activation by IL-2 - Phillips *et al*, 1989; Tsudo *et al*, 1987) which appears to be coupled to a PTK, but whose function is also blocked by PKC inhibitors. (Stimulation of NK cells by IL-2 significantly broadens the range of targets that the cell can lyse, in a phenomenon known as Lymphokine Activated

Killing or LAK, Mason *et al*, 1990. Furthermore, IL-2-stimulation appears to act synergistically with stimulation of FcγRIII (CD16) to induce optimum levels of activation antigens on the cell surface, and promote optimum NK cell proliferation - Harris *et al*, 1989).

There are clearly many interactions and biochemical events involved in the activation of NK cell cytolytic activity (some of which are schematised in Figure 1.2), and much information still to be obtained about them. Since natural killer cells potentially provide a highly important function in tumour and viral immunity, it is appropriate that a thorough and detailed investigation into their mode of action is made, particularly into their molecular interactions, as this may lead to crucial therapeutic developments.

Figure 1.2 : Schematic representation of the postulated molecular and biochemical events occurring between an NK cell and its target. (Taken from O'Shea & Ortaldo, 1992)



1.4 Phylogeny of NK-like cells

Studies on other vertebrates have further suggested that there is a common evolutionary origin between NK and T_c cells, since various common features have been identified in different species. For example, Göbel *et al* (1994) have characterised a candidate NK cell in chickens. These cells are splenic granular lymphocytes which are both thymus- and bursa- independent. They exhibit no T cell receptor gene rearrangements, nor surface expression of either TCR or CD3, although they do express cytoplasmic CD3 (similar to that observed in mammalian NK cells). Since these chicken LGLs (large granular lymphocytes) are also able to bind chicken IgG, it is further possible that they possess an FcγRIII homologue, which may allow them to perform ADCC. Avian NK cells also express CD8 markers (in the form of αα homodimers) and IL-2 receptors (especially following culture in IL-2-rich supernatant), but do not express CD4, MHC class II or immunoglobulin. Additionally, Göbel *et al* (1994) have demonstrated that these NK-like cells exhibit spontaneous cytotoxicity against an established NK cell tumour target, all of which suggests that they are the mammalian NK homologue.

In fish, Evans *et al* (1988) have identified a monoclonal antibody (5C6) which binds to a receptor on the surface of fish nonspecific cytotoxic cells (NCC - analogous to human natural killer cells), inhibiting their ability to lyse target B-lymphoblastoid cells (by blocking recognition between the effector and its target). It is probable that the mAb 5C6 recognises a receptor involved in the activation of fish NK cells, inducing increased levels of Ca²⁺, increased expression of membrane receptors, and increased cytotoxicity (Evans *et al*, 1990), similar to that observed in mammalian NK cells. Furthermore, NK-like cells in teleost fish have been shown to lyse human cell lines sensitive to human NK cell lysis (Harris *et al*, 1991), and that both human and fish killing of these lines can be blocked by mAb 5C6, suggesting

that fish and human NK cells may use similar evolutionarily conserved cell surface receptors to recognise the same target antigens.

In amphibians (which occupy a crucial position in the evolution of both modern birds and mammals), natural killer cell activity against tumours has been demonstrated in several anuran species, in both the spleen, peripheral blood and bone marrow (Ghoneum *et al*, 1990). These anuran NK cells were found to be similar to mammalian NK cells in both their cell structure and intracellular changes after binding to their target, and in their response to various inhibitory drugs. Antibody-dependent cellular cytotoxicity (one of the characterising features of NK cells, and possibly one of the oldest methods whereby vertebrates reject non-self tissue) has also been observed in *Xenopus*, against antibody-coated chicken red blood cells (Jurd & Doritis, 1977). Horton, Horton & Varley (1989) have demonstrated cytotoxicity of adult *Xenopus* splenocytes against MHC class I-negative tadpole target cells, which could represent NK cell-mediated lysis due to lack of MHC expression, eg. via an Ly-49 homologue. (However, since these larvae do express MHC class II, such killing may be caused by T cell recognition of class II instead.)

1.5 The Immunobiology of *Xenopus*

Numerous immunobiological studies have established that the South African clawed toad, *Xenopus laevis*, is an ideal animal model for investigating vertebrate immunology (Horton, 1994). It is an entirely aquatic anuran amphibian which is easy to maintain and breed in the laboratory, and, since its larvae are free-living and thus free of maternal influences, is easily experimentally manipulated. For example, thymectomy can be performed at an early developmental stage, which is effective at eliminating T cells (Gravenor *et al*, 1995). Such thymectomised (Tx) *Xenopus* are of obvious relevance to studies designed to probe NK cells at this level of evolution.

Xenopus is particularly valuable because of the remarkable immunological resemblance it has to higher vertebrates, possessing all the basic features of the sophisticated immune system found in mammals. For example, *Xenopus* possesses several lymphoid organs including a thymus (with a cytoarchitecture that resembles that of other jawed vertebrates), spleen (differentiated into red and white pulp), gut associated lymphoid tissue (GALT), liver and kidney (Plytycz & Bigaj, 1983., Katagiri & Tochinnai, 1987). There is a division of lymphocytes into B and T cells (Manning, Donnelly & Cohen, 1976) (including helper and cytotoxic functions), and there are three classes of immunoglobulins - IgM, IgY and IgX, analogous to the mammalian IgM, IgG and IgA respectively. *Xenopus* is also the most primitive vertebrate to have a defined major histocompatibility complex (MHC), known as the XLA (*Xenopus* leucocyte-associated antigens), which encodes class I, class II, and class III - like molecules. Furthermore, in-depth analysis of the *Xenopus* immune system is facilitated by the availability of genetically defined strains, isogenic clones, labelled cellular markers, and an increasing number of monoclonal antibodies to *Xenopus* cell surface markers (Horton *et al*, 1995; Du Pasquier, Schwager & Flajnik, 1989).

With respect to studying natural killer cell activity, anuran amphibians are particularly useful since they are known to develop both benign and malignant tumours (sometimes viral-associated), which, as already stated, represent one of the major targets of NK cells. Moreover, their response to chemical carcinogens is analogous to that in mammals, and the tumours are generally similar to those found in other vertebrates (Anver, 1992), such that they provide a useful comparative model for the tumouricidal activity of NK cells.

The occurrence of a spontaneous lymphoid tumour in the thymus of a *Xenopus* male of the inbred family ff in Basel (Du Pasquier & Robert, 1992), and the subsequent characterisation of several *Xenopus* thymus tumour cell lines (Robert, Guiet & Du Pasquier, 1994) has provided appropriate target cells on which such studies can be made. Certain *Xenopus* tumour cells (eg. the ff-2 and B₃B₇ cells - both derived from ff inbred *Xenopus*) are unable to survive if transplanted to histocompatible control or allogeneic adults, even after day 20 larval thymectomy. However, the same tumours *could* survive if transplanted to histocompatible (ff) larvae. In contrast, tumours (eg. 15/0) derived from LG15 clonal animals *are able to survive* in LG15 adults as well as in LG15 larval hosts, but fail to survive in ff *Xenopus* hosts (Robert, Guiet & Du Pasquier, 1994). Since the LG15-derived tumour cells, and also the B₃B₇ cells, fail to express MHC proteins, their rejection by certain hosts could well be effected by NK-like cells against non-MHC-encoded target antigens. Fortunately, now that these tumour cell lines are established in our laboratory, the question of whether NK-like activity is shown towards these cells can be tested experimentally by *in vitro* cytolytic assays.

Chapter 2

Cellular Investigation into the Proliferative Responses and Cytotoxicity exhibited by *Xenopus* Splenocytes, and their Enrichment by Cell Separation Techniques

2.1 Introduction

One of the defining characteristics of mammalian natural killer cells is that they are able to spontaneously lyse certain tumour cell targets without prior sensitisation. The initial goal of this project was, therefore, to identify such spontaneous cytotoxicity in *Xenopus laevis*, to determine whether there were 'natural killer' cells present at this level of evolution. These experiments have involved chromium release microcytotoxicity assays (following the protocol of Horton *et.al.*, 1989) which assess the ability of *Xenopus* splenocytes to spontaneously lyse ⁵¹Cr-labelled *Xenopus* B₃ B₇ tumour targets. Secondly, since IL-2 is known to activate mammalian and avian NK cells, and increase the range of targets that are susceptible to NK lysis, in a phenomenon known as Lymphokine Activated Killing (or LAK), a further goal of the project was to investigate whether such a cytokine-enhanced killing phenomenon occurred in *Xenopus*. LAK activity has been explored following

in vitro stimulation of *Xenopus* splenocytes with putative *Xenopus* IL-2. Mammalian IL-2 cannot be used since there is evidence that it fails to stimulate *Xenopus* lymphocytes *in vitro* (Watkins & Cohen, 1987).

In order to achieve these goals, my research began with a series of experiments attempting to generate active supernatants from *Xenopus* splenocytes by *in vitro* stimulation with the T cell mitogen Concanavalin A. Supernatant activity was initially assessed in tritiated thymidine ($^3\text{HTdR}$) assays using splenocytes or splenic lymphoblast targets. Since *Xenopus laevis* lymphocytes are known to produce a 'T-cell-growth-factor' or 'interleukin-2' when they are stimulated by T cell mitogens (Watkins & Cohen, 1987; Haynes & Cohen, 1993), it is likely that the proliferative activity observed in these culture supernatants is due to the presence of IL-2 in them. Thus, supernatants that were stimulatory in terms of $^3\text{HTdR}$ incorporation were then tested for their ability to potentiate spontaneous cytotoxicity of *Xenopus* lymphocytes towards ^{51}Cr -labelled tumour targets. In other words, mitogens were being used to generate IL-2-rich supernatants, which could then be utilised to treat *Xenopus* splenocytes in an attempt to increase either their level of cytolytic activity or the range of targets that were susceptible to their killing.

The next phase of the work investigated whether splenocytes could be enriched for natural killer cells, such that higher cytolytic activity against tumour cells could be observed. Enrichment would also be useful for later molecular work, when concentrated, purer NK populations were needed for generation of mAbs to NK cell surface antigens for characterisation of NK receptors. Cell enrichment is possible through the selective removal of other lymphocyte populations, and two techniques were attempted in this study. My initial series of experiments concentrated on the cell separation technique of "panning" (modified from that described by Wysocki & Sato, 1978, see also Bleicher & Cohen, 1981). In this method, a sterile plastic petri dish is incubated overnight with anti-mouse Ig such that the whole bottom of the dish is covered with antibody (see Figure 2.1a). Splenocytes are then incubated with the

mouse monoclonal antibody that recognises a cell surface structure specific for the cell population to be depleted, such that the target population becomes coated in antibody (Figure 2.1b). When these splenocytes are swirled in the petri dish, binding takes place between the anti-Ig on the dish and the Ig (mouse mAb) coating the cells, thus 'adhering' the target population to the dish base (Figure 2.1c). 'Non mAb-coated' splenocytes do not bind to the anti-Ig, and remain free in the supernatant as 'non-adherents'. Consequently, when the supernatant is removed, these non-adherent cells are removed too (Figure 2.1d), producing a cell population that should be free of the target cells. (Adherent cells are freed into solution by gently scraping them from the petri dish base with a sterile scraper).

Following on from these 'panning' experiments, and in an attempt to improve upon them, a second method of *Xenopus* spleen cell enrichment was employed - that of magnetic bead cell separation. This technique appeared a novel, and promising, approach to enrich for non-T/non-B lymphoid cells. The principles of separation are the same as those used in panning, i.e. coating the target cells in the appropriate mouse monoclonal antibody (Figure 2.2a), and then binding these to a solid surface through the interaction with anti-mouse Ig (Figure 2.2b). However, in this technique, the solid surface is a magnetic bead rather than the plastic petri dish. The beads used in this study were Dynabeads® M-450, already coated with sheep anti-mouse IgG. MAb-coated splenocytes are mixed with the Dynabeads® and incubated for a sufficient length of time to allow binding to occur between the anti-mouse Ig and the mouse mAb (Figure 2.2b). To achieve separation, this mix is then placed into a 'Magnetic Particle Concentrator' (MPC) which has a magnet on one side, such that the superparamagnetic beads are attracted to that side, pulling the target cells with them (Figure 2.2c). Similar to the panning technique, the supernatant containing the non-target cells (the non-adherents) can easily be pipetted off and kept aside as a 'target-cell-depleted' population. The adherent (or target) cells can be removed from the beads either by enzymatic action or overnight culture, but in either case the

sample must be placed in the MPC to remove the magnetic beads from the cell suspension before the splenocytes can be used in further assays.

Both techniques can, therefore, be used to produce either a 'pure' population of target (mAb-coated) cells (i.e. the adherents), or a mixed population depleted of the target cells (i.e. the non-adherents). Given that natural killer cells constitute only a small portion of the total lymphocyte population (and thus may prove difficult to pull off enough as target cells), it was considered that the more likely technique of enriching a cell population for NK cells would be to deplete the splenocyte culture of other large populations of cells. This was thus attempted with various monoclonal antibodies to both T cells and B cells. Evaluation of the purity of each population, and hence how successful the separation technique had been, was facilitated by the spectrum of monoclonal antibodies that are now available to *Xenopus* lymphocyte antigens, and their identification through flow cytometry. Furthermore, having separated the various populations, a further goal of the project was to determine the cytolytic potential of each cell population, in an attempt to identify the lymphoid subset responsible for the cytolytic activity.

Finally, the information obtained from this study provided a sound and thorough basis on which further goals could be set regarding a molecular investigation into the function, surface antigens and biochemical activation of natural killer cells (see Chapter 3).

Figure 2.1 : Diagrammatic Representation of Cell Enrichment by 'Panning'.

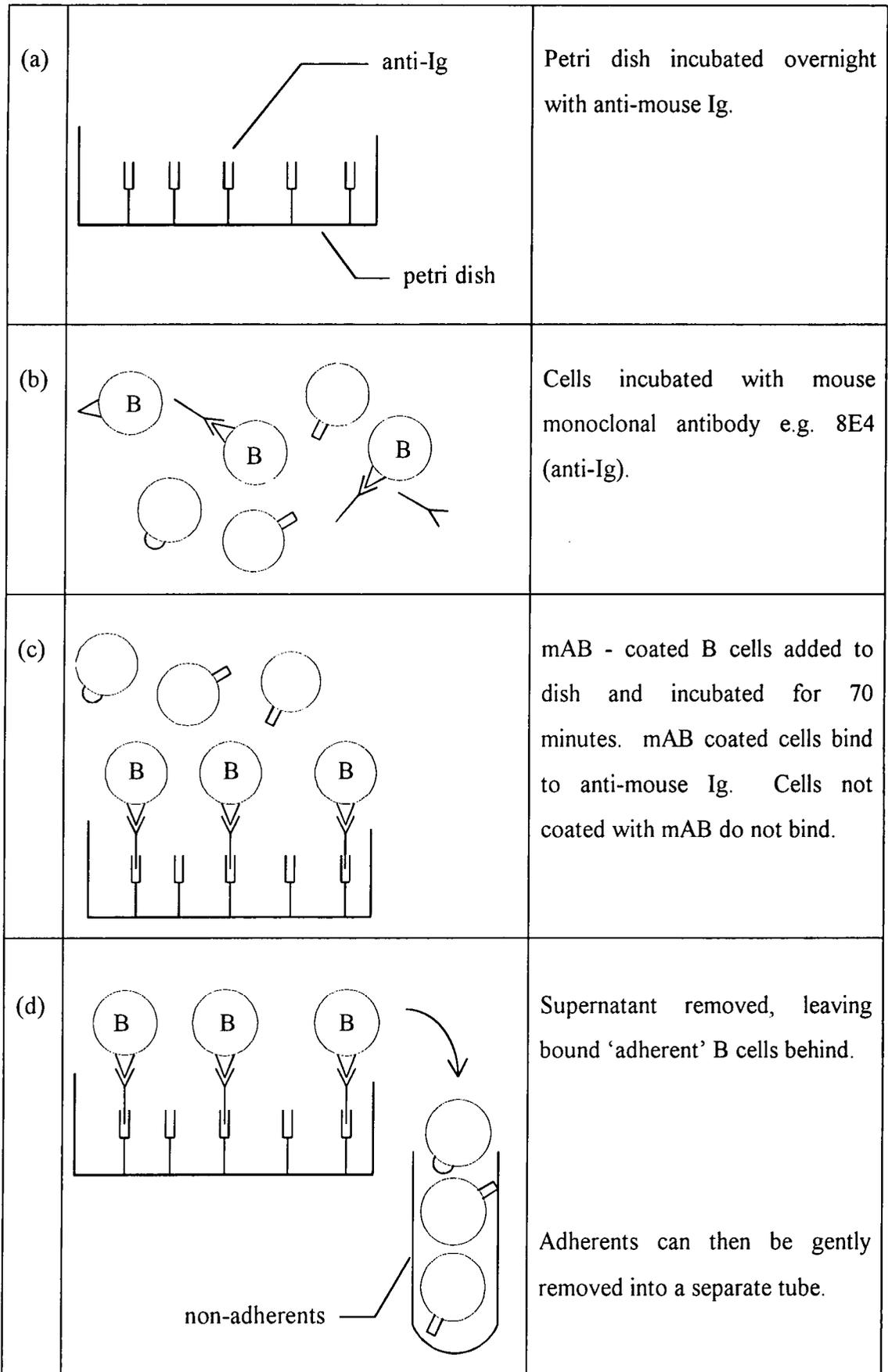
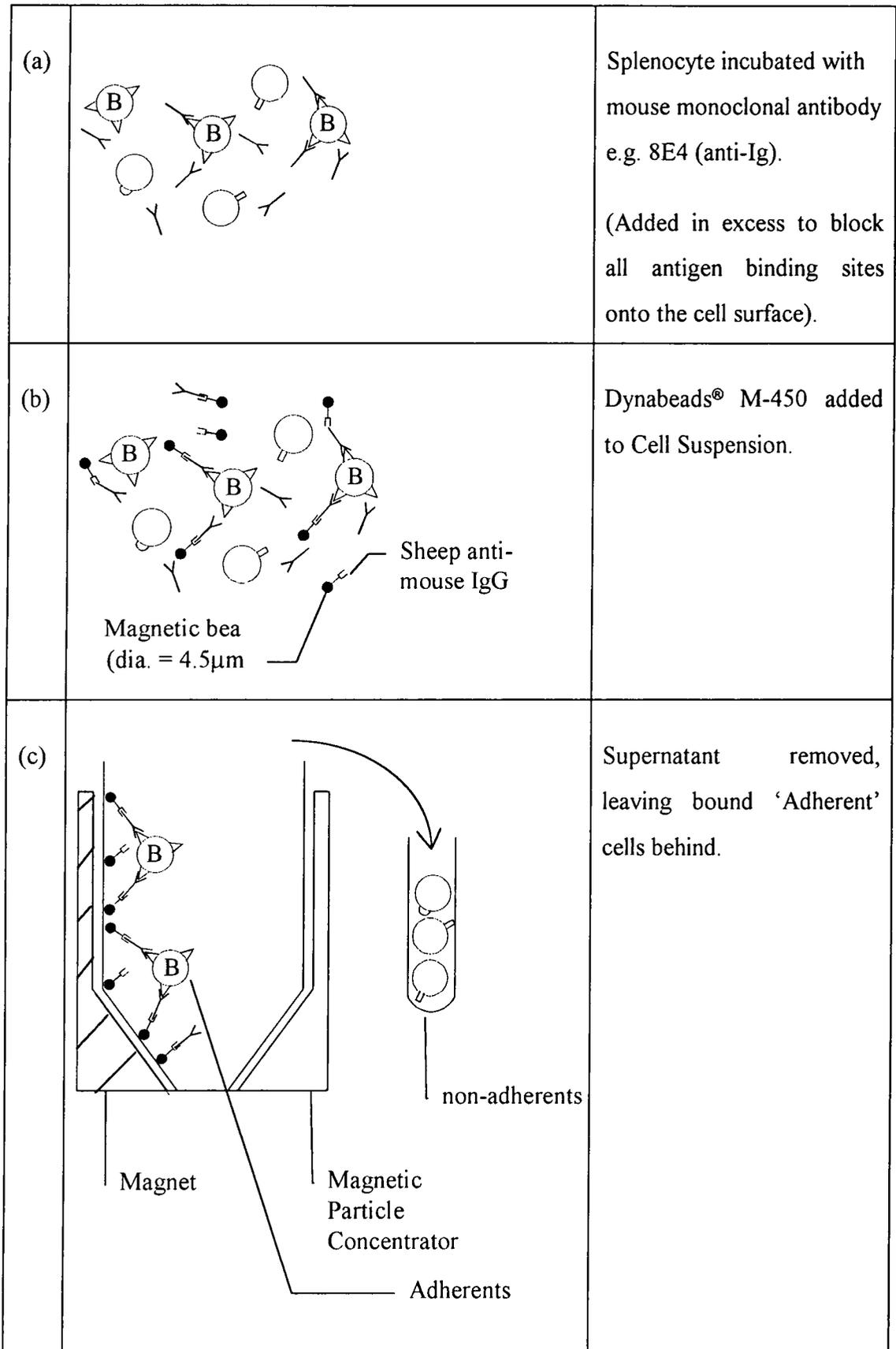


Figure 2.2 : Diagrammatic Representation of the Indirect Technique of Cell Separation using Magnetic Beads.



2.2 Materials and Methods

2.2.1 Animals and Rearing

Outbred *Xenopus laevis* were bred in the laboratory and reared at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in dechlorinated water. As soon as embryos developed into tadpoles, and until their metamorphosis, they were fed with nettle powder. Toadlets were then initially fed with bloodworms but gradually progressed to minced mammalian heart, supplemented with pellets (Diet N° 1, Blades Biologicals). Adults were also fed with minced mammalian heart and supplemented with Diet N° 2 pellets. Ages of animals used are given in Results section.

2.2.2 Early Thymectomy

Xenopus tadpoles were mainly thymectomised (Tx) at 6-7 days/stage 47-48 (Nieuwkoop & Faber, 1967), but a few thymectomies were also carried out on 35 day old / stage 56/7 larvae. All thymectomies were performed by Dr J. D. Horton. Thymectomy was performed by electromicrocautery as previously described (Horton & Manning, 1972), and the absence of a thymus was checked by dissection of adults on killing.

2.2.3 Preparation of Splenocyte Suspensions

Animals were over-anaesthetised in MS222 (3-aminobenzoic acid ethyl ester [Sigma]) and then sprayed with 70% alcohol. In a laminar air flow hood, their spleens were then dissected out using forceps and micro-scissors and placed in a small, sterile petri-dish containing 2ml sterile culture medium (see 2.8.1) or panning medium (see 2.4). The spleen was then teased apart with a pair of tungsten needles, under a dissecting microscope, to break open the organ and release the cells into suspension. This suspension was then transferred to a 5ml plastic sterile test tube (Falcon), and the petri-dish washed out with a further 2ml medium to collect any remaining cells (also added to the tube). After thoroughly mixing, the suspension

was allowed to settle for 30 - 40 seconds (to remove any large pieces of tissue) before transferring the supernatant suspension to a fresh 5ml tube. This was then centrifuged at 300g for 10 minutes, the supernatant discarded, and the pellet resuspended in a known volume of culture medium. Lymphocytes were counted using a haemocytometer and adjusted to a concentration of 1 million cells per ml.

2.2.4 Panning (Lymphocyte Enrichment)

2.2.4.1 Preparation of the panning petri-dish

An aliquot (30 μ l of anti-IgG, or 100 μ l of anti-IgM) of anti-mouse Ig (10 μ g/ml, Vector) was added to 10ml of carbonate/bicarbonate buffer (pH 9.8) and poured into a petri-dish (Sterilin, 90mm \times 15mm). This was gently swirled to ensure that the whole of the bottom of the dish was covered, and then it was left overnight at 4°C

The buffer was then tipped away, the bottom covered with 'blocking' buffer (carbonate/bicarbonate buffer containing 1% BSA), and left to incubate at room temperature for 30 minutes. The dish was washed free of blocking buffer by thoroughly swirling with 'panning' medium (amphibian phosphate buffered saline [APBS], 0.1% BSA), and then the bottom was covered with panning medium until ready for use.

2.2.4.2 Panning procedure

Splenic lymphocytes were resuspended in panning medium for cell counting. Approximately 1ml of the lymphocyte suspension was removed into a sterile tube and labelled as 'non-panned'. The remainder was then aliquoted into tubes containing ca. 1ml each (ie. 1×10^6 lymphocytes) and centrifuged at 300g for 10 minutes. Supernatants were removed and each pellet was resuspended in 100 μ l of the appropriate diluted mouse monoclonal antibody (either of IgM or IgG isotype), then left to incubate for 30 minutes at 4°C.

Unbound antibody was removed by washing the cells twice with 2ml panning medium and centrifuging at 300g for 10 minutes. After the final centrifugation, supernatants were discarded and each pellet (of mAb-coated cells) was resuspended in 1ml panning medium (If there were less than 6 tubes, extra medium was added to the appropriate number of tubes to give a total volume of 6ml). Ensuring that they were thoroughly mixed, the cells (usually between 3 and 10 million) were gently pipetted onto the anti-mouse Ig-coated dish, swirled, and then left to incubate at 4°C for 70 minutes (including a gentle swirl after 40 minutes).

Non-adherent cells were removed by gently pipetting off the supernatant into a sterile universal (S.H. Scientific), and washing twice by swirling with 4ml panning medium (each time removing the washings into the same universal). The adherent cells were finally removed by gently scraping the plate with a cell scraper (Greiner) and removing the medium into a fresh sterile universal. Again two washes were made, scraping each time, and retaining the washings.

Both universals were then centrifuged at 300g for 10 minutes, the supernatants removed and the pellets resuspended in 1ml panning medium. Cells were counted using a haemocytometer.

2.2.4.3 Preparation of panned cells for flow cytometry or tritiated thymidine incorporation

Non-panned, non-adherent and adherent populations were centrifuged (300g, 10 minutes), the supernatants discarded and the pellets resuspended in bicarbonated L15 culture medium (see 2.6.1) at 1 million leucocytes per ml. Cells were then cultured overnight in a 24-well flat-bottomed plate (1ml per well) prior to flow cytometry, or used directly in a ³HTdR assay.

2.2.5 Magnetic Bead Enrichment of Lymphocytes

2.2.5.1 Preparation of splenocytes

Splenic lymphocytes (adjusted to a concentration of 1 million cells per ml in culture medium) were dispensed in 1ml aliquots into sterile tubes and centrifuged at 300g for 10 minutes. Supernatants were removed and pellets were resuspended in 0.5ml of the appropriate monoclonal antibody (made up in sterile amphibian phosphate buffered saline [APBS] + 1% fetal calf serum [FCS], pH 7.4 [= APBS/FCS]), before being incubated on ice for 45 minutes. (Some control cells were set aside, these were resuspended in bicarbonated culture medium and transferred to a 24-well, flat-bottomed plate for overnight culture).

Experimental cells were then washed twice with 1ml APBS/FCS and resuspended in 1ml APBS/FCS for counting. After centrifugation at 300g for 10 minutes, each pellet was resuspended in 0.5ml APBS/FCS and transferred to a separate sterile eppendorf (Greiner).

2.2.5.2 Preparation of beads

The immunomagnetic beads used were Dynal® M-450 Sheep anti-mouse IgG Dynabeads®. (These are monosized, superparamagnetic, polystyrene beads with affinity purified sheep anti-mouse IgG covalently bound to the surface. They are supplied as a suspension containing 4×10^8 beads/ml in PBS pH 7.4 with 0.1% HSA and 0.02% NaN₃).

After calculating how many beads would be required for the entire experiment, the contents of the stock vial were resuspended, and the appropriate volume transferred to a sterile eppendorf. This was placed in the Magnetic Particle Concentrator (MPC) for 1 minute and the supernatant removed to remove the sodium azide. The beads were then washed twice with 1ml APBS/FCS (removing the supernatant in the MPC), resuspended in APBS/FCS at the original volume, and

adsorbed with *Xenopus* serum at 1: 20 dilution for 40 minutes on ice (to remove any non-specific anti-*Xenopus* reactivity).

2.2.5.3 Adsorption of antibody-coated cells onto beads

The appropriate volume of washed and pre-adsorbed beads were added to the mAb-coated splenocytes in eppendorfs, and rotated at room temperature for 30 minutes. 0.5ml APBS/FCS was then added to each eppendorf, and the samples were thoroughly resuspended before being placed into the MPC for 2 minutes to collect the beads and adherent cells ('adherents'). Supernatant cell suspensions were transferred to sterile tubes and labelled as 'non-adherents'. Adherent cells and beads were washed with 1ml APBS/FCS, the washings being added to the 'non-adherent' tubes. The pellet of adherent cells and beads was then resuspended in 1ml bicarbonated culture medium. All non-adherent cells were thoroughly resuspended and counted, centrifuged at 300g for 10 minutes, and also resuspended in 1ml bicarbonated culture medium. All cells (including an aliquot of 'non-beaded' splenocytes) were then transferred to a 24-well, flat-bottomed plate for overnight culture.

2.2.5.4 Preparation of cell populations for flow cytometry

To remove the beads from the positively-selected cells, after overnight culture 'adherent' samples were transferred to eppendorfs, placed in the MPC for 1 minute, and the supernatant suspension removed to tubes. The beads were washed with a further 1ml of FACS medium, placed in the MPC, and the supernatant suspension added to the appropriate tubes. Non-beaded, 'non-adherent' and 'adherent' splenocyte populations were then centrifuged at 300g for 10 minutes, and cell pellets resuspended in 1ml FACS medium for counting. Finally, each sample was adjusted to 1×10^6 leucocytes/ml in FACS medium, ready for flow cytometry (see 2.6).

2.2.5.5 Preparation of cell populations for ⁵¹chromium release assays

For experiments where a chromium release assay was to be performed on the resulting populations, bead-sorted samples were resuspended in 1ml B₃B₇ tumour medium, before being cultured overnight. The next day, some cells were removed for flow cytometry (see 2.6), and the rest were serially diluted with B₃B₇ tumour medium in 96-well, round-bottomed plates, to act as effector cells in 6 hour ⁵¹Cr-release killing assays against B₃B₇ *Xenopus* thymus tumour cells(see 2.9).

2.2.6 Flow Cytometry

Splenocytes taken directly *ex vivo* or after overnight culture were washed and resuspended at 1×10^6 leucocytes/ml in APBS containing 0.1% BSA and 0.1% NaN₃ (FACS medium). 200µl samples (2×10^5 cells) were aliquoted into the appropriate wells of a 96-well, round-bottomed plate. The plate was then centrifuged (300g, 10 minutes) and the supernatant discarded before resuspending each pellet in 50µl of the appropriate monoclonal antibody (mAb) and incubating on ice in the dark for 20 minutes.

Any unbound antibody was then removed through two cycles of washing with FACS medium and the pelleted cells were resuspended in 50µl of the secondary FITC-conjugated antibody (a 1 in 20 dilution of FITC-labelled F(Ab)₂ fraction of rabbit anti-mouse immunoglobulin (DAKO), that had previously been adsorbed with 5% *Xenopus* serum and centrifuged at 3000g for 5 minutes to remove any debris), and incubated on ice in the dark for 20 minutes. This 2° FITC Ab allows the visualisation of unconjugated supernatant mAbs and amplifies the signal of purified FITC-conjugated mAbs. Cells were then washed twice with FACS medium containing mouse serum (1 in 80) to remove any unbound FITC-conjugated anti-mouse antibody.

Finally, the cell pellets were resuspended in 50µl of the appropriate PE-conjugated mAb, left on ice in the dark for 20 minutes, and then washed twice with

FACS medium. Cells were then resuspended in 400µl of FACS medium and left on ice in the dark until analysed on the flow cytometer (Coulter XL, MCL).

(If for any reason the cells needed to be kept at this stage, 400µl of formaldehyde fixative (1.0%) was added (final concentration 0.5%) and fixed cells stored at 4°C until FACS analysis could be carried out).

2.2.7 Monoclonal Antibodies

2.2.7.1 mAbs used for FL1 (FITC) fluorescence

All the mAbs (except 2B1) were mouse hybridoma supernatants and therefore required FITC-conjugated anti-mouse IgG for visualisation. (The mAbs are IgG isotype, except for AM22 and 5C6 which are IgM isotype).

- CT3 - this is directed against the chicken CD3 antigen and should therefore not react with *Xenopus* cells. It is used as a negative control to determine the level of background fluorescence on the FITC channel. (Chen *et. al.*, 1986).
- 8E4:57 & 10A9 - both mAbs recognise surface immunoglobulin M (sIgM) class on *Xenopus* B cells. (Langeberg *et.al.*, 1987; Hsu & Du Pasquier, 1984).
- ALC mix - this 'anti-light chain' mix is a mixture of three mAbs (1E9, 409B8, 13B2) which individually recognise different light chain epitopes of the surface immunoglobulin on *Xenopus* B cells. (Hsu *et.al.*, 1991).
- AM22 - recognises the *Xenopus* CD8 equivalent (eg. on cytotoxic T cells). (Flajnik *et.al.*, 1990).
- 2B1 - recognises the *Xenopus* CD5 antigen. (Jurgens *et.al.*, 1995).

- 5C6 - putative anti-NK cell mouse mAb raised against catfish NK-like cells (Evans *et.al.*, 1988).

2.2.7.2 mAbs used for FL2 (PE) fluorescence

The following mAbs (IgG isotypes, except for F17 which is IgM) were all directly conjugated to the phycoerythrin (PE) fluorochrome (prepared and donated by Southern Biotechnology, Birmingham, Alabama): -

- Ig - mouse IgG₁ (DAKO). It is used as a negative control to determine the level of background fluorescence on the PE channel.
- 2B1 - recognises the *Xenopus* CD5 antigen. (Jurgens *et.al.*, 1995).
- F17 - recognises the *Xenopus* CD8 equivalent (eg. on cytotoxic T cells). (Ibrahim *et.al.*, 1991).
- D8 - recognises surface IgM antigen on *Xenopus* B cells. (Ibrahim *et.al.*, 1991).

2.2.8 *In vitro* Studies on Splenocytes

2.2.8.1 Culture medium

The basic culture medium used was Leibovitz-15 (L-15) medium (+ L-glutamine) (Gibco), diluted to amphibian osmolarity (220 mosm) with autoclaved double distilled water, and supplemented with 0.01M HEPES buffer, 2mM 2-mercaptoethanol (BDH), amphotericin B (2.5µg/ml) and penicillin (50iu/ml) streptomycin (50µg/ml) sulphate. It was further supplemented with heat inactivated (decomplemented) fetal calf serum (FCS; batch 1973, APP) to give a final concentration of 1% FCS. All media were prepared aseptically in a laminar air flow

hood and were filter-sterilised using 0.22µm filters (Gelman). Medium was stored at 4°C until ready for use. 0.01M sodium bicarbonate (Gibco) was added as a buffer for cell culture in the CO₂ incubator.

2.2.8.2 Generation of active supernatants using Con A

1ml splenocyte suspensions (1×10^6 lymphocytes per ml) were dispensed into each well of a 24-well, flat-bottomed cell culture plate (Greiner). Active supernatants were generated by adding freshly-prepared Con A dilutions to each well, to give 'in-well' concentrations of 1µg/ml or 2.5µg/ml. Control supernatants were generated using 1ml of splenocytes cultured with 100µl medium instead of mitogen.

After incubation for 48 hours at 26°C ± 1°C in 5% CO₂, cells were gently resuspended to remove any clumping, then centrifuged at 300g for 10 minutes. Culture supernatants were gently removed by pipetting and transferred to clean tubes (Falcon). Both experimental and control supernatants were treated with 0.1M α - methyl mannoside (αmm, Sigma) to neutralise any remaining Con A. Finally, these supernatants were filtered through 0.2µm filters (Gelman) and stored in freezing cryovials (Nunc) at -20°C until use.

2.2.8.3 Tritiated thymidine assays

Splenocytes were adjusted to 1×10^6 cells/ml in bicarbonated culture medium, then 100µl aliquots were dispensed into the appropriate wells of a 96-well, flat-bottomed tissue culture plate (Greiner). Cultures were set up in triplicate and stimulated with either 50µl of active supernatant (ASN) or control supernatant (CSN), 10µl of Con A [Sigma] (freshly prepared at various concentrations in bicarbonated medium), or 12.5µl of LPS (0.5mg/ml, Sigma). An additional 50µl medium was added to the CSN/ASN-stimulated wells, and a control triplicate was set up containing 100µl cells and 100µl medium. Cells were cultured for 48 hours at 26°C ± 1°C (in 5% CO₂) before being pulsed with 1µCi tritiated thymidine (³HTdR, Amersham, Sp.Act = 5Ci/mmol) per well. After a further 18 hours, cells were

harvested, and counted on a Packard scintillation counter which counts the amount of tritiated thymidine incorporated into the cells that are in S phase during the pulse period. Counts are given as DPM (disintegrations per minute).

Stimulation Indices were calculated as follows:

$$\text{Stimulation Index (SI)} = \frac{\text{DPM Stimulated Cells}}{\text{DPM Control Cells (medium-treated)}}$$

2.2.9 Chromium Release Assays

B₃B₇ tumour cells were adjusted to a concentration of 1×10^6 cells/ml in tumour medium and cultured overnight in the presence of ⁵¹chromium at 100μCi/ml (specific activity 350 - 600mCi/mg Cr) to label the tumour cell targets. The next day the tumour cells were washed three times in tumour medium, resuspended at a concentration of 5×10^5 cells/ml in tumour medium, and incubated for ½ - 1 hour in a humidified incubator (27°C ± 1°C, 5% CO₂) to reduce spontaneous release of ⁵¹chromium. (They were washed again before use).

100μl of these ⁵¹Cr-labelled tumour cell targets (ie. 5×10^4 cells) were then added to 100μl effector cells (control, ASN-treated or separated splenocyte populations) serially diluted to give effector : target (E:T) ratios of 40:1 → 5:1 in 96-well, round-bottomed plates. The plate was then centrifuged at 300g for 2-3 minutes before being incubated in a humidified incubator (27°C ± 1°C, 5% CO₂) for 6 hours.

After this time, the contents of all the wells were gently pipetted and then the plate was centrifuged at 300g, for 10 minutes. 100μl supernatant was removed from each well, transferred to Titertek tubes, and the amount of ⁵¹chromium that had been released into the supernatant was measured on a Packard gamma counter.

Minimum levels of spontaneous chromium release were determined by mixing 100μl labelled tumour targets with 100μl B₃B₇ tumour medium, whilst maximum

levels of specific chromium release were determined by mixing 100µl labelled tumour targets with 100µl sterile distilled water and freeze-thawing three times.

Percentage of specific ⁵¹chromium release was calculated according to the following equation:

$$\% \text{ specific } ^{51}\text{Cr release} = \frac{\text{CPM Experimental SN} - \text{CPM Minimum SN}}{\text{CPM Maximum SN} - \text{CPM Minimum SN}} \times 100$$

2.3 Results

2.3.1 Generation of Active Supernatants by use of Con A stimulation

Note that in all the proliferative assays involving tritiated thymidine, the standard deviations were no greater than 11.7%. (Results not shown on individual graphs).

2.3.1.1 Reactivity to Con A (³HTdR incorporation)

An initial experiment was set up to determine the optimal Con A dose for inducing ³H thymidine incorporation of *Xenopus* splenocytes using L15 culture medium (Fig. 2.3). It was found that Con A at 'in-well' concentrations of 1µg/ml and 2.5µg/ml were both capable of stimulating splenocytes from control *Xenopus*, although 2.5µg/ml produced the highest stimulation. This is in agreement with Ho (1992). Stimulation indices varied widely between individual experiments, from 10 to 77, but on each occasion the highest stimulation index was given by Con A at 2.5µg/ml. The varied SI probably reflects differences in the animals used, maybe in health or age, and is unavoidable when using outbred animals. Con A at 'in-well' concentrations of 0.1µg/ml and 10µg/ml both appeared to produce little or no splenocyte stimulation

In order to check the T cell dependence of Con A stimulation, a brief investigation of Con A responses in thymectomised *Xenopus* was carried out. Con A is able to minimally stimulate (SI = 4) splenocytes from late-thymectomised (stage 56/57, 35 days old) *Xenopus*, but not those from early (stage 48, day 7) thymectomised frogs (Fig. 2.4).

As a result of the above experiments, Con A at 'in-well' concentrations of 1 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ were used to generate 48-hour active supernatants from control splenocytes. These supernatants were termed ASN 1 and ASN 2.5 respectively. When testing ASNs for their ability to stimulate other splenocytes, it is essential that there is no residual Con A from the original production of the ASN, since if Con A persists, it would not be certain whether any observed stimulation was due to the mitogen itself or a cytokine emerging in the ASN. Consequently, the effectiveness of α -methyl-mannoside (α -mm) at removing Con A activity was examined. It was found that 0.1M α -mm completely abrogates induced ^3H thymidine incorporation seen with Con A alone (Fig. 2.4).

2.3.1.2 Proliferative responses to active supernatants

Con A-derived ASNs generated in this study had various stimulatory capabilities, probably reflecting the different extent to which the cells were stimulated by the Con A in the original production of the ASN:

Cells which had been pre-cultured in Con A for 48 hours and then in L15 medium for 48 hours (ie. lymphoblasts), when further treated (for 48 hours) with either ASN 1 or ASN 2.5, were not induced to incorporate more $^3\text{HTdR}$ than background cultures (ie. Con A pre-cultured cells that had been further treated with medium rather than ASN for 48 hours). Interestingly, these background cultures display significantly elevated DPM, probably due to stimulation by the Con A during the initial pre-culture period. (Fig. 2.5, Table 2.1). In contrast, those cells that were pre-cultured in L15 only for 96 hours exhibited significant stimulation, (with indices of 4.7 and 6.1) when stimulated with ASN 1 or ASN 2.5 respectively.

Control supernatants (CSNs - supernatants generated from cells cultured in L15 alone ie. no Con A) had no stimulatory effect on any of the three cell populations

(as expected). In fact, the $^3\text{HTdR}$ incorporation was significantly lower than the background DPM in the Con A pre-cultured cells, suggesting inhibition. (See Fig. 2.5, Table 2.1).

When the above lymphoblast populations were left for a further 5 days of pre-culture (ie. 48 hours in Con A then 7 days in L15), the same ASN 2.5 was then stimulatory (Fig. 2.6), giving stimulation indices of 4.9 and 3.3 for cells pre-cultured with $1\mu\text{g/ml}$ and $2.5\mu\text{g/ml}$ respectively (Table 2.2). This was undoubtedly due to DPM of Con A- pre-cultured cells returning to comparable levels to splenocytes pre-cultured in medium alone.

Further batches of active and control supernatants were generated - one batch was derived from control *Xenopus* (termed C.CSN, C.ASN 1 and C.ASN 2.5), and one batch was derived from late Tx (stage 56/57) *Xenopus* (termed Tx.CSN, Tx.ASN 1 and Tx.ASN 2.5). Both batches of ASNs were able to stimulate fresh splenocytes (ie. tested directly *ex vivo*) from both control and early-thymectomised animals (Fig. 2.7). The ASN 2.5 from both control and Tx donor gave the highest stimulation in each case. Splenocytes from control and early-thymectomised animals responded with similar stimulation indices (see Table 2.3).

2.3.2 Tumour Cytotoxicity Mediated by *Xenopus* Splenocytes

2.3.2.1 Splenocytes *ex vivo*

As a background from which the effect of ASNs on the ability of *Xenopus* splenocytes to lyse B₃B₇ thymus tumour cells could be determined, several experiments were performed assessing the killing activity of unstimulated (ie. without ASN) splenocytes. Such spontaneous lysis was assessed in $^{51}\text{Chromium}$ release assays using B₃B₇ tumour cell targets (a *Xenopus* thymus tumour cell line obtained

from Louis Du Pasquier and established in our laboratory - see Du Pasquier *et al*, 1992).

In these initial experiments, effector cells (control splenocytes, control thymocytes, and Tx splenocytes) were removed from the animal and assayed directly (ie. no culture period) at various E:T ratios. The results, summarised in Table 2.4, demonstrate that control splenocytes were unable to display significant (ie. >10% specific release) lysis of B₃B₇ tumour targets, with maximal specific ⁵¹Cr release being only 6%. Thymocytes also induced no lysis of B₃B₇ cells, and were therefore useful as controls. In comparison, splenocytes from thymectomised animals did exhibit significant spontaneous lysis, with percentage lysis reaching as high as 17%. (Neither control nor Tx splenocytes were able to lyse the human tumour cells K562 in 6 hour ⁵¹Cr release assays - results not shown).

2.3.2.2 Effect of ASNs

From this basis, experiments were performed to determine the effect of ASNs on specific cytotoxicity. Thus, splenocytes from both control and thymectomised animals were cultured at 5×10^6 cells/ml for 48 hours in either medium or ASN, before being recounted, washed, and utilised as effectors in ⁵¹Chromium release assays. Figure 2.8 illustrates the results of this experiment, and shows that in all cases there is a correlation between E:T ratio and percentage lysis, with more effector cells leading to more killing.

The addition of ASN to the control splenocytes appears to have no effect on their killing ability, since the two control samples (cultured in medium or ASN) exhibit almost identical percentage lysis at all E:T ratios. (Highest percentage lysis observed was at an E:T ratio of 45:1, with medium- and ASN- treated effectors displaying 48% and 50% lysis respectively - Table 2.5). In comparison, the addition of ASN to splenocytes from a thymectomised animal had a noticeable effect, significantly increasing the percentage lysis observed at all E:T ratios. For example,

at an E:T ratio of 45:1, Tx splenocytes cultured in medium alone exhibited only 14% lysis, whereas those cultured with ASN exhibited an increase to 50% lysis. Interestingly, the medium-cultured Tx splenocytes in this experiment exhibit killing levels which are comparable to those observed with Tx splenocytes tested directly from the animal (14% lysis at 45:1 for cultured Tx cells - Table 2.5- compared with 16% lysis at 58:1 for fresh Tx cells - Table 2.4). In other words, culturing them for 48 hours in medium does not affect their killing ability. The requirement for Tx (but not control) splenocytes to be cultured in ASN to become effective killers of the tumour cells is interesting. The difference may be explained, in some way, by the lack of T cells in Tx frogs and the consequential need to supply T cell - derived cytokines.

Figure 2.9 shows the proportion of T cells in both control and Tx splenocytes cultured in tumour medium for 48 hours, as determined by flow cytometry. While control cells contain 65% 2B1⁺ cells, Tx cells contain only 22% 2B1⁺ cells. Furthermore, the fluorescence intensity of 2B1 staining of the Tx cells was significantly less than on control cells. The CD8 marker monitored by F17 has gone from the Tx spleen.

The induction of anti-tumour cytotoxicity in control cells following 48 hour culture in medium alone was confirmed in an additional experiment. Here, control splenocytes were taken fresh from an animal - some were assayed directly in a ⁵¹Chromium release assay; the rest were cultured in either medium or ASN for 48 hours and then assayed. The results are shown in figure 2.10. As expected, the fresh splenocytes exhibited very little killing activity - at an E:T ratio of 40:1, the percentage lysis was only 2%. When the same splenocytes were then cultured with medium for 48 hours, the amount of killing had increased to 22% (at 40:1). Although higher percentage release has been observed in previous experiments under similar conditions, this is still a significant increase. Incubation of cells with ASN

also resulted in an increase in percentage lysis from that observed with fresh cells, but only to the level of that observed with medium-cultured cells.

2.3.3 Phenotypic and Functional Studies on Spleen Cell Populations Following Panning

In the flow cytometric data presented, markers in both single and dual colour analyses were set up so that background staining registered at the 2% level. Dual fluorescence staining provides a more in depth characterisation of cell populations.

All experiments were performed on 7 - 10 month old control animals from the same batch. Non-panned splenocytes were nearly always found to have approximately 20 - 30% B cells and 60 - 70% T cells (of which about one-third are CD8⁺ T cells). (Fig. 2.11)

2.3.3.1 Panning with anti-Ig mAbs

Initial experiments were carried out on controls, using the mAb 8E4 for panning which binds to surface immunoglobulin (sIg) on B cells.

Figure 2.12 illustrates the results from one of the better separations using 8E4, and demonstrates that panning has enriched the non-adherent population for 2B1⁺ cells (T cells) from 73% to 84%, while 10A9⁺ cells (B cells) have been depleted from 19% to 3%. (Non-adherent cells have also been enriched for AM22⁺ [T_C] cells from 25% to 31%, as expected - results not shown). Although the adherent population is enriched for 10A9⁺ cells (64%, B cells), it is contaminated with quite a high proportion of 2B1⁺ cells (17% T cells).

In a different panning experiment using 8E4 to achieve B cell removal, a $^3\text{HTdR}$ assay was performed on all three populations (non-panned, non-adherents [T cell enriched], and adherents [B cell enriched]) after stimulation with Con A and LPS, to determine whether cell separation affects the functionality of the different lymphocytes (Fig. 2.13, Table 2.6). Non-panned splenocytes proliferated in response to both Con A and LPS, with a larger response to Con A (SI = 48.6) than LPS (SI = 9.80), reflecting the higher proportion of T cells to B cells. The non-adherents (T cell enriched) responded to Con A, but showed only minimal stimulation by LPS (SI = 1.40). The low SI of the non-adherent cells to Con A (SI = 4.87) was in part due to the high background DPM of these cells. (Compare with the non-panned cells which have a low background DPM and thus a SI to Con A of 48.6). From the stimulation indices (Table 2.6), the adherent splenocytes fail to respond significantly to LPS. However, the actual counts for LPS are very high (40150 DPM), suggesting that the stimulation is masked by the very high background counts (31430 DPM) for the adherent cells. This may well be due to anti-IgM stimulating the B cell population to proliferate (See Schwager & Hadji-Azini, 1985).

A final panning experiment was set up to examine the ability of a mix of anti-light chain mAbs (ALC) to remove B cells. The flow cytometric results obtained (Fig. 2.14) were very similar to those obtained when panning with 8E4, with an enrichment of T cells in the non-adherent population from 67% to 82% and a depletion of B cells by half. However, there were still 14% B cells in this population which is quite a high level of contamination. The flow cytometric data on adherent cells are difficult to interpret due to the poor staining of this population with either 8E4 or ALC mAbs. It appears likely, however, that the vast majority of adherent cells (Ig light chain^{+ve}) are indeed B cells with low fluorescence intensity of 8E4 and ALC staining.

2.3.3.2 Panning for T cells

Panning experiments were initially performed with 2B1 which is thought to recognise the CD5 antigen on *Xenopus* T cells (Jurgens *et al*, 1995). Figure 2.15 demonstrates that panning has enriched the non-adherent population for 8E4⁺ cells (B cells) from 27% to 48%, but is unable to remove all the 2B1⁺ cells (T cells), since there are still 20% 2B1⁺ cells present (about half of which are AM22⁺ cells - results not shown).

Adherent splenocytes were stained with 2B1/D8 (rather than 8E4/2B1) so that all of the 2B1 was on the FITC channel, leaving the PE channel able to clearly detect any B cell contamination. This is important because 2B1 is a fairly 'sticky' antibody, and so if there is any left on the cells after panning, the 2^o FITC-conjugated anti-mouse Ig Ab will bind to it and give a positive reading. Therefore, if 8E4-FITC/2B1-PE is used, any 2B1⁺ cells will have a double fluorescence :- red from the 2B1-PE binding directly to the CD5, and green from the 2^o FITC-conjugated Ab binding to the Fc region of the 2B1 used for coating the cells. (This would lead to 'false +ve B cells'). Using 2B1-FITC/D8-PE avoids this because the only marker that can show on the red channel is D8-PE which binds to sIg⁺ cells. Thus, staining with 2B1/D8 shows that the adherent cells have been enriched for 2B1⁺ cells (T cells) from 62% to 86% and that B cell contamination is minimised.

In a further 2B1-panning experiment, non-panned or panned splenocyte populations were each treated with both Con A and LPS (Fig. 2.16). As expected, the non-panned splenocytes were able to respond well to both mitogens, with stimulation indices of 30.74 and 10.43 for Con A and LPS respectively (Table 2.7). The non-adherent cells (T cell depleted) also proliferate to both mitogens, although the LPS response is greater since this population is now enriched for B cells. (The Con A response is due to the contaminating T cells). Surprisingly, the adherent cells

(ie. the T cell-rich population) responded to Con A with an SI of only half that of the non-panned population, and were still able to show proliferation induced by LPS.

Subsequent panning of T cells was attempted with AM22 (or AM22 and F17 simultaneously) to enrich for CD8⁺ T cells. Figure 2.17 illustrates the results from a separation using AM22 alone, although AM22 and F17 together yielded similar findings. The non-panned splenocytes contain ca. 82% T cells (which is unusually high), of which just under a third (33% of all cells) are cytotoxic. The panning procedure was quite successful at producing a purified CD8⁺ adherent population (approximately 95% pure), but it was not efficient at removing all CD8⁺ cells from the non-adherents, since this population was contaminated with 14% CD8⁺ cells. Also, the actual number of recoverable adherents was very poor (only 7.4×10^5 adherents were recovered from 1×10^7 total cells). A ³HTdR uptake assay after *in vitro* treatment with Con A demonstrates that both the non-panned and non-AM22-adherent populations are able to respond well to the Con A (SI's = 46.2 and 38.6 for the non-panned, and 30.5 and 28.7 for the non-adherent cells, for Con A 1µg/ml and Con A 2.5µg/ml respectively). In contrast, the CD8⁺ adherent cells showed minimal response (SI = 3.03 for Con A 2.5µg/ml). (Fig. 2.18, Table 2.8).

2.3.4 Enrichment of Cell Populations by Magnetic Bead Separation

Antibody-coated magnetic beads (Dynal[®] M-450 magnetic beads) were employed in an attempt to improve upon the efficiency of cell separation achieved using 'panning'. Resulting populations were cultured overnight (to allow adherent cells to detach from the beads), then dual-labelled with mAbs and analysed by flow cytometry to assess the success of the separation.

2.3.4.1 B cell depletion / enrichment of T cells

Attempts to remove B cells employed the mAb 8E4, and required several modifications to determine optimum conditions. Three controls were initially set up to ensure that the cells were not affected by incubation with the mAb or by the beads themselves. Figure 2.19 demonstrates that, indeed, the cells are not affected since all these controls have ca. 21% 8E4⁺ cells and ca. 70% 2B1⁺ cells, and thus any separation observed experimentally is genuine.

To determine the optimum number of beads required, fresh splenocytes from a control animal were incubated with 8E4 at a 1 in 4 dilution, and separated using Dynabeads® M-450 at bead : lymphocyte ratios of 5:1, 10:1 and 20:1. Figure 2.20 shows both the non-adherent and adherent populations from each separation after overnight culture. Increasing the bead : cell ratio did not increase the extent to which the non-adherents were enriched for 2B1⁺ cells since all three ratios resulted in an enrichment of 2B1⁺ cells from 65% in the non-beaded population to 81/82% in the non-adherents. However, there were differences in the percentage contamination with B cells : ratios of 10:1 and 20:1 were both able to deplete the non-adherents of B cells from 19% to 3%, but a ratio of 5:1 was only able to deplete to 5%. The adherent population (which should have been pure 8E4⁺ cells) were odd in all three cases with a significant contamination of 2B1⁺ cells (ca. 27%) and maximally only 37% 8E4⁺ cells were recorded. These latter percentages are probably misleading due to the low level of sIgM on B cells that may have been activated overnight by the anti-IgM attached to the beads.

During these initial experiments, it was observed that the number of recoverable adherent cells was very poor, and it was hypothesised that this was because many of the splenocytes were still bound to the Dynabeads® even after overnight culture, and were hence being removed with the beads when placed in the Magnetic Particle Concentrator. Consequently, an 8E4-separation was set up to

investigate the effects of a longer culture period following Dynabead® separation. The separation was performed in duplicate so that one batch of adherent cells could be cultured over one night (17 hours), and the other batch could be cultured over two nights (41 hours). As Figure 2.21 demonstrates, the longer culture period actually produces worse results since the adherent population contains only a very low percentage of 8E4⁺ cells (ca. 16%), and those that were present showed a high incidence of dead cells (data not shown). In comparison, the separation with only one night culture enriched the non-adherent population for 2B1⁺ cells from 75% to 87% (with only 8% 8E4⁺ contamination). Furthermore, the adherent cells contained a distinctly elevated (47%) population of 8E4⁺ cells (albeit a dull population), although was still contaminated with a significant percentage of 2B1⁺ cells (17%).

In an attempt to reduce this 2B1⁺ contamination of the adherent population (which may be 2B1⁺ cells trapped by the adherent cells moving to the side), a post-separation wash was added. Thus, after removal of the initial non-adherent cells, the eppendorf was removed from the MPC and the adherents thoroughly resuspended in 1ml APBS/FCS. These re-washed splenocytes were then returned to the MPC and the non-adherent supernatant added to the 'non-adherent' sample. As Figure 2.22 demonstrates, this extra wash had great effect, with virtually no 2B1⁺ contaminants (2% in the 'washed' sample compared with 18% contamination in the 'unwashed' adherent sample). This was a significant improvement (which was thus employed in all further experiments), and demonstrated that the technique was successful in binding only to 8E4⁺ cells. The percentage of 8E4⁺ cells in the 'washed' adherent population was still recorded as only 4.5% by positive analysis, but the shift in the FL1 brightness peak indicates that perhaps all these cells are sIgM^{low}.

The final experiment employed 8E4 at 1 in 4 (better separation than using 8E4 at 1 in 8 - results not shown), utilised extra washing of the initial adherent cells, and examined cells by flow cytometry after overnight culture. The only variation used here was to determine if the initial volume of 8E4 used to coat IgM⁺ cells was of

any importance. Thus, fresh splenocytes (1×10^6) were incubated in either 1ml or 0.5ml of 8E4 (1 in 4) before being rotated with the beads. As Figure 2.23 demonstrates, both 1ml and 0.5ml of 8E4 produced extremely similar results, reducing the number of 8E4⁺ cells from 29% in the non-beaded population to 7% and 6% in the non-adherent population for 1ml and 0.5ml respectively. Similarly, 2B1⁺ cells were enriched from 63% in the non-beaded population to 80% and 81% in the non-adherent populations. The adherent populations in each separation were also very similar; both consisting of dull 8E4⁺ cells and only 3% CD5⁺ T cell contamination. The mAb incubation volume was thus kept at 0.5ml for all further experiments. Furthermore, the conditions used in this experiment produced the cleanest enrichment utilising the 8E4 mAb, and were thus utilised in subsequent experiments.

2.3.4.2 T cell depletion / enrichment of B cells

Following the optimum conditions determined for removing adherent B cells and enriching for T cells (ie. incubation in 0.5ml, 10:1 beads, overnight culture, washing of adherents), T-cell removal was attempted using the mAb 2B1 (which binds to the *Xenopus* CD5 antigen).

Two 2B1 concentrations were used : 1 in 5 and 1 in 10, and the results are shown in Figure 2.24. The technique was successful, reducing the number of 2B1⁺ cells from 62% in the non-beaded population to only 5% and 6% (for 1 in 5 and 1 in 10 respectively) in the non-adherent cell populations. Concurrently, 8E4⁺ cells are enriched from 29% in non-beaded splenocytes to 61% and 64% (for 1 in 5 and 1 in 10 respectively) in the non-adherent populations. Although the percentages of 2B1⁺ cells in the adherent populations are recorded by the flow cytometer as fairly low (36% and 59% for 1 in 5 and 1 in 10 respectively), these values may be misleading because the 2B1⁺ staining is dull. Also, the 2B1⁺ adherents are duller following initial incubation with the more concentrated mAb (ie. 1 in 5 : mean fluorescence =

1.56, compared with 1.93 at 1 in 10 - results not shown), suggesting that the 2B1 used for coating the cells is down-regulating CD5 expression. (Consequently, further experiments were set up using 2B1 at 1 in 15 and 1 in 20. Although the resulting 2B1⁺ adherents were brighter, the separation was not as clean and therefore the populations not as useful - results not shown). Cells found in quadrant 3, where the PE-fluorescence intensity has somewhat increased compared with control Ab-stained cells, may actually be CD5^{low} T cells. The adherents are probably, therefore, predominantly CD5⁺ T cells, as the cells present are a single, discreet population, and there is only a negligible contamination with 8E4⁺ B cells (1% in the 1 in 10 sample).

Separations were also attempted using AM22 (a μ isotype antibody) to try and remove CD8⁺ cells. Figure 2.25 shows the results of the separation using AM22 at 1 in 125. Unfortunately there were not enough adherent cells to analyse. Non-beaded splenocytes (Figure 2.25a) contained ca. 60% 2B1⁺ cells, of which just over a third were F17⁺ cells (24% of the total lymphocyte population). Beading depletes the non-adherent population of 2B1⁺ cells to 46% and AM22⁺ cells to 10%, ie. removing about half of the CD8⁺ population. The CT3/Ig stain of the beaded population shows a 9% CT3-FITC⁺ population - this is likely to represent AM22 - coated cells still in this population.

2.3.5 Assessment of the Killing Activity of Cell Populations Enriched by Beading.

In an attempt to identify the particular cell population responsible for spontaneous cytotoxicity, splenocytes from a control *Xenopus* were subjected to magnetic bead separation using either 2B1 (at 1 in 10) or 8E4 (at 1 in 4), and the resulting populations used as effectors in ⁵¹Chromium release assays. (Unfortunately, there were usually insufficient numbers of adherent cells available for their killing ability to be assayed). All other conditions for separation were those determined as optimum in this study (see sections 2.3.4.1 and 2.3.4.2). The animal had been

injected three days previously with 1×10^6 B₃B₇ tumour cells, via the dorsal lymph sac, in order to boost the level of cells exhibiting natural killer activity.

Figure 2.26 illustrates the flow cytometric data from these separations, and demonstrates that both separations have been successful in enriching each population for the appropriate cell type. (Beading with 2B1 depletes the non-adherent population of 2B1⁺ cells from 76% [in the non-beaded population] to 24%, while enriching the adherent population to 93% 2B1⁺ cells. Beading with 8E4 depletes the non-adherent population of sIg⁺ cells from 21% [in the non-beaded population] to 6%, while enriching the adherent population to 86% D8⁺ cells). Figure 2.27 illustrates the killing activity towards B₃B₇ cells observed in each of these populations and compares them with the killing observed in a corresponding 'unseparated' splenocyte sample. The most noticeable difference in killing activity is that displayed by the '2B1-adherents' which are almost pure 2B1⁺ cells (93%). These cells exhibit the lowest amount of killing out of all the populations, reaching only 15% lysis at an E:T ratio of 13:1. (At a similar E:T ratio, the other populations are displaying between 40% and 50% lysis). These 2B1⁺ (adherent) cells are viable since they can respond well (by ³HTdR uptake) to Con A (data from lab, unpublished).

The other populations - 'unseparated', '2B1-non-adherents' and '8E4-non-adherents' - show very similar killing patterns, although there are slight differences in the actual percentage lysis observed. Generally, for each E:T ratio, the greatest killing is seen in the '8E4-non-adherents' (T cell enriched), followed by the '2B1-non-adherents' (B cell enriched), and then least is seen in the unseparated population. For example, at an E:T ratio of 10:1, percentage lysis is 47%, 43% and 38% for '8E4-non-ads', '2B1-non-ads', and 'unseparated' respectively.

Figure 2.3 : Assessment of optimal Con A dose for *Xenopus* splenocyte stimulation in L15 culture medium

Splenocytes prepared directly *ex vivo* from a 6 month old control *Xenopus laevis* were dispensed into each well of a 96-well, flat-bottomed plate in 100 μ l aliquots (1×10^6 lymphocytes/ml) in 1% FCS-supplemented L15 medium. Cultures were set up in triplicate, each well was pulsed with 1 μ Ci 3 HTdR at 48 hours and harvested at 66 hours. Con A dilutions refer to 'in-well' concentrations. DPM = degenarations per minute 3 H thymidine.

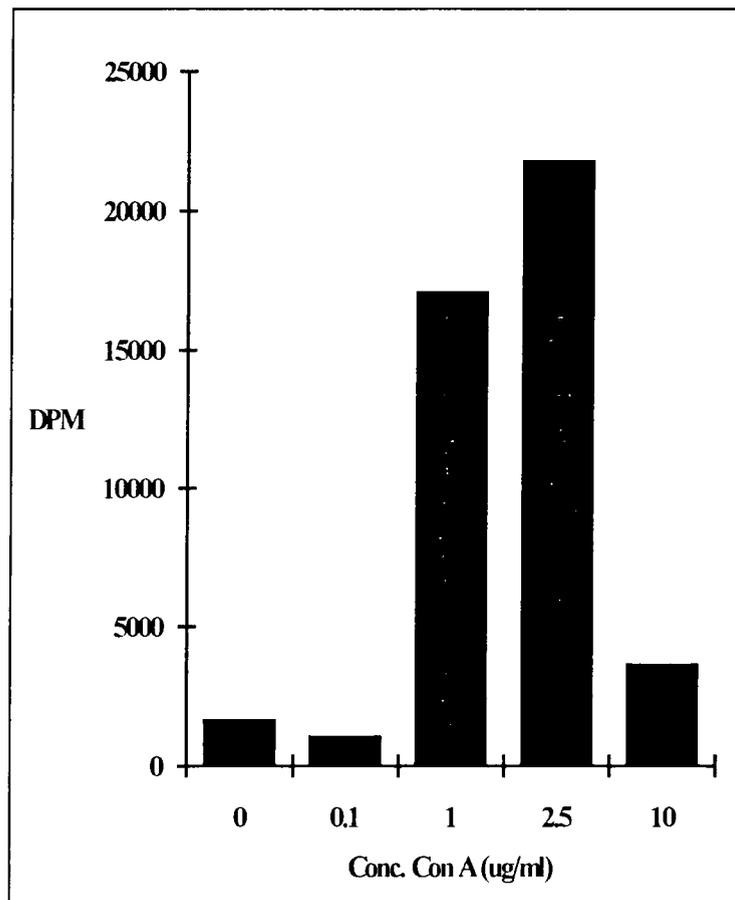


Figure 2.4 : $^3\text{HTdR}$ uptake of control and both early (day 7) - and late (day 35) - thymectomised (Tx) *Xenopus* when stimulated with Con A at $1\mu\text{g/ml}$. All animals were 23 months old. (Some cells were set up with Con A + 0.1M $\alpha\text{-mm}$ to test the ability of $\alpha\text{-mm}$ to abrogate Con A stimulation).

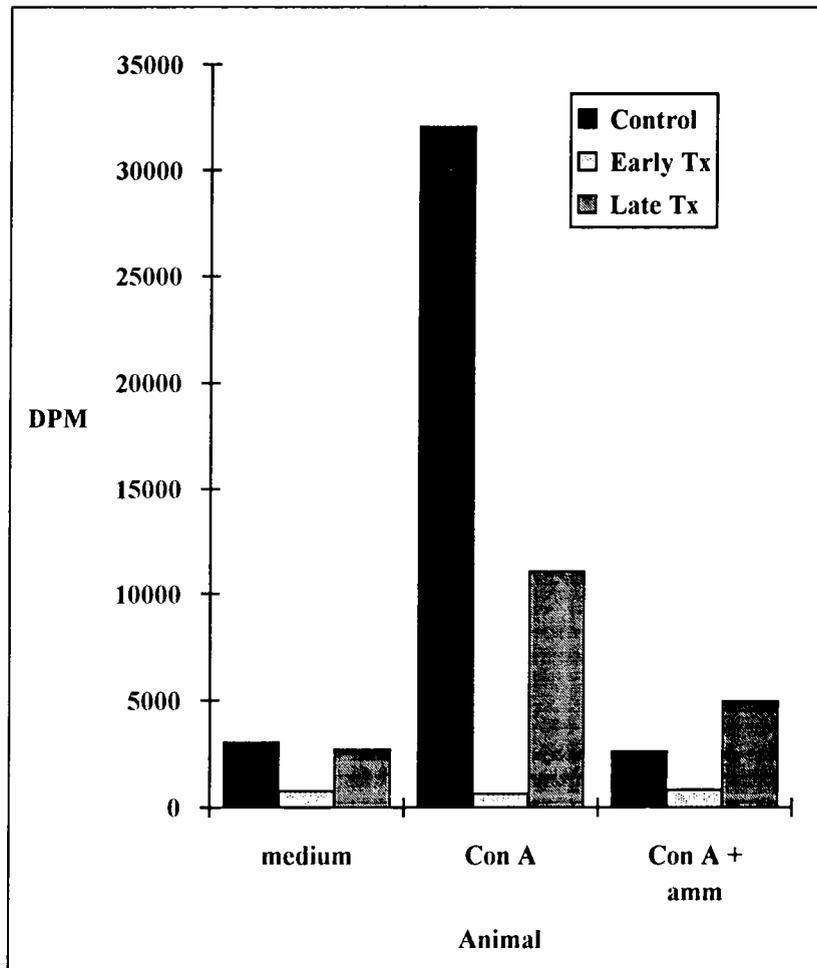


Figure 2.5 : $^3\text{HTdR}$ uptake of control *Xenopus* splenocytes (pre-cultured with either L15 for 96 hours or Con A [either $1\mu\text{g}/\text{ml}$ or $2.5\mu\text{g}/\text{ml}$] for 48 hours then L15 for 48 hours) when treated for 48 hours with either CSN, ASN 1 or ASN 2.5. *Xenopus* were 23 months old.

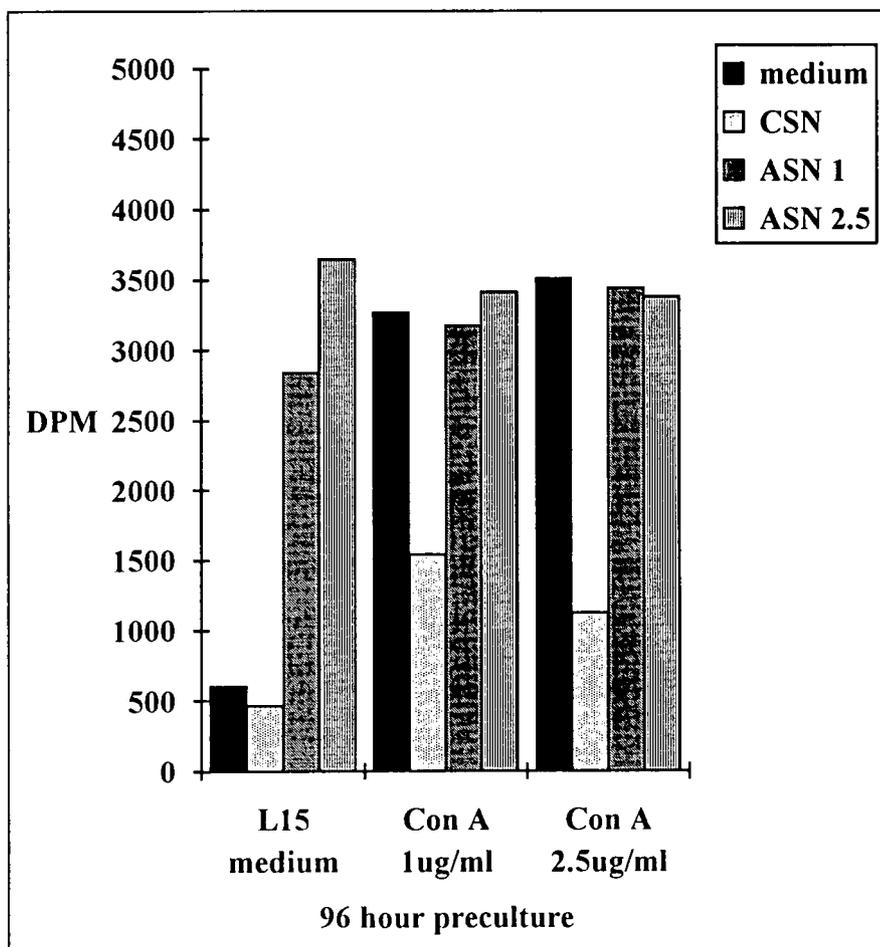


Table 2.1 : Stimulation indices produced by 48 hour CSN- and ASN- treatment of control *Xenopus* splenocytes (that had been precultured with either L15 for 96 hours or Con A [either $1\mu\text{g}/\text{ml}$ or $2.5\mu\text{g}/\text{ml}$] for 48 hours then L15 for 48 hours). *Xenopus* were 23 months old.

96 hour preculture	Stimulation indices when splenocytes treated for 48 hours with:		
	CSN	ASN 1	ASN 2.5
L15 medium	0.77	4.72	6.07
Con A $1\mu\text{g}/\text{ml}$	0.47	0.97	1.04
Con A $2.5\mu\text{g}/\text{ml}$	0.32	0.98	0.96

Figure 2.6 : $^3\text{HTdR}$ uptake of control *Xenopus* splenocytes (pre-cultured with either L15 for 9 days or with either $1\mu\text{g/ml}$ or $2.5\mu\text{g/ml}$ Con A for 48 hours then L15 for 7 days) when treated for a further 48 hours with either CSN or ASN 2.5. *Xenopus* were 26 months old.

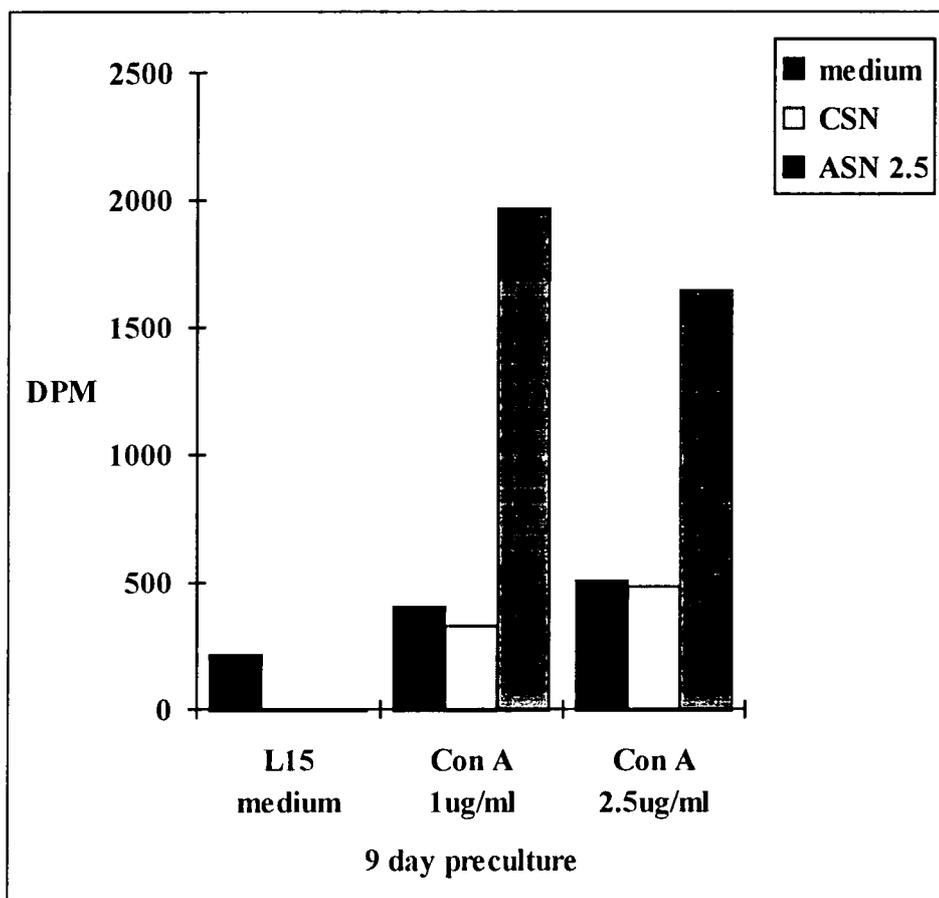


Table 2.2 : Stimulation indices produced by CSN- and ASN- treatment for 48 hours of control *Xenopus* splenocytes (pre-cultured with either L15 for 9 days or Con A for 48 hours then L15 for 7 days). *Xenopus* were 26 months old.

9 day preculture	Stimulation indices when splenocytes treated with:	
	CSN	ASN 2.5
L15 medium	-	-
Con A $1\mu\text{g/ml}$	0.81	4.86
Con A $2.5\mu\text{g/ml}$	0.96	3.25

Figure 2.7: $^3\text{HTdR}$ uptake of control and early-thymectomised *Xenopus* splenocytes when treated for 48 hours with various CSNs and ASNs produced from either control (C) or late (stage 56/7) - thymectomised (Tx) animals. *Xenopus* were 6 months old.

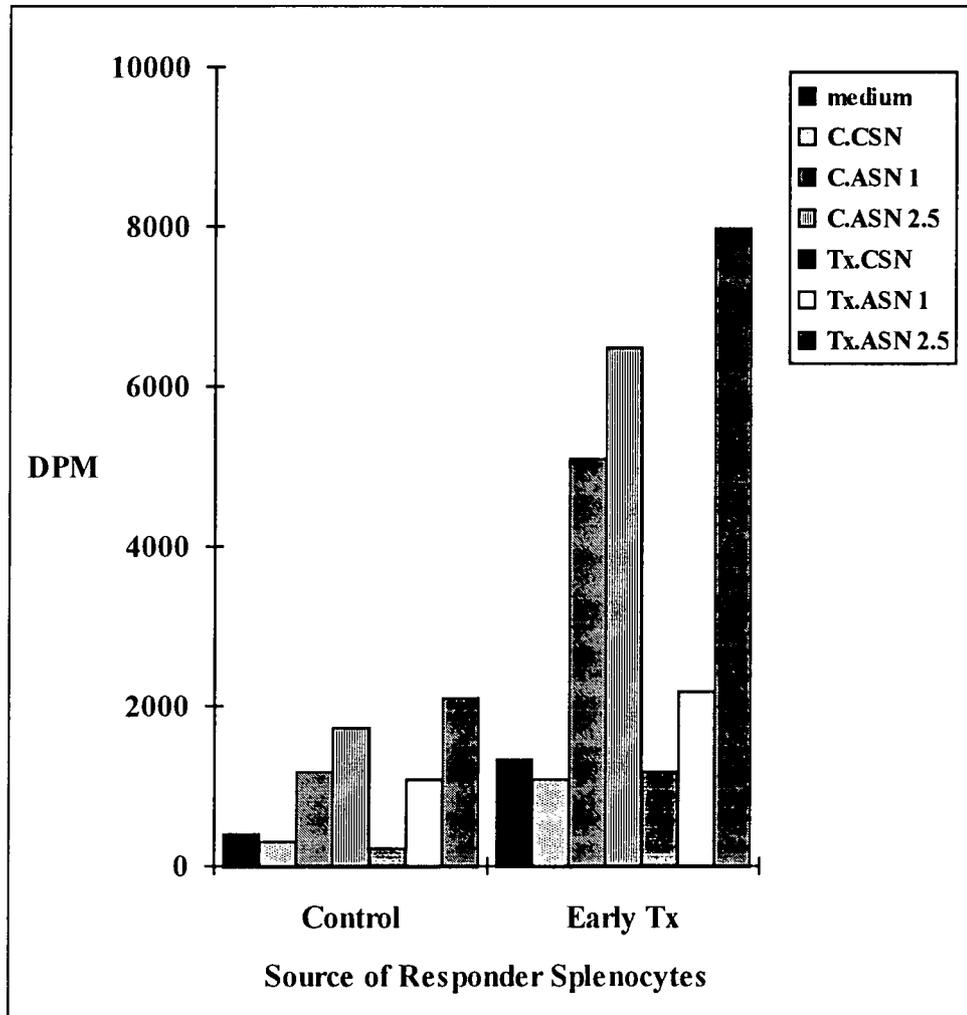


Table 2.3 : Stimulation indices produced by CSN- and ASN- treatment of control and early-thymectomised (Tx) *Xenopus* splenocytes. *Xenopus* were six months old.

Source of Responder Splenocytes	Stimulation indices when splenocytes treated with:		
	C.CSN	C.ASN 1	C.ASN 2.5
Control	0.76	2.94	4.33
Early Tx	0.81	3.84	4.88

Source of Responder Splenocytes	Stimulation indices when splenocytes treated with:		
	Tx. CSN	Tx. ASN 1	Tx. ASN 2.5
Control	0.54	2.70	5.26
Early Tx	0.89	1.64	6.01

Table 2.4 : Percentage ^{51}Cr release from B_3B_7 tumour target cells exhibited by control splenocytes, control thymocytes, and Tx splenocytes in a 6 hour assay. *Xenopus* were 11 months old.

Effector cells were assayed fresh from the animal, at effector : target ratios of 29:1 → 100:1

Effector cell	E:T ratio	% lysis
Control splenocytes	50:1	-2
	100:1	6
Control thymocytes	50:1	-3
	100:1	-7
Tx splenocytes	29:1	17
	58:1	16

Figure 2.8 : Percentage 51 chromium release from B₃B₇ tumour target cells induced by control and Tx splenocytes after their treatment with either medium or ASN. Both *Xenopus* were 20 months old.

Splenocytes were cultured at 5×10^6 for 48 hours in either B₃B₇ tumour medium or ASN, then washed and recounted, and used as effectors at E:T ratios of 3:1 → 45:1.

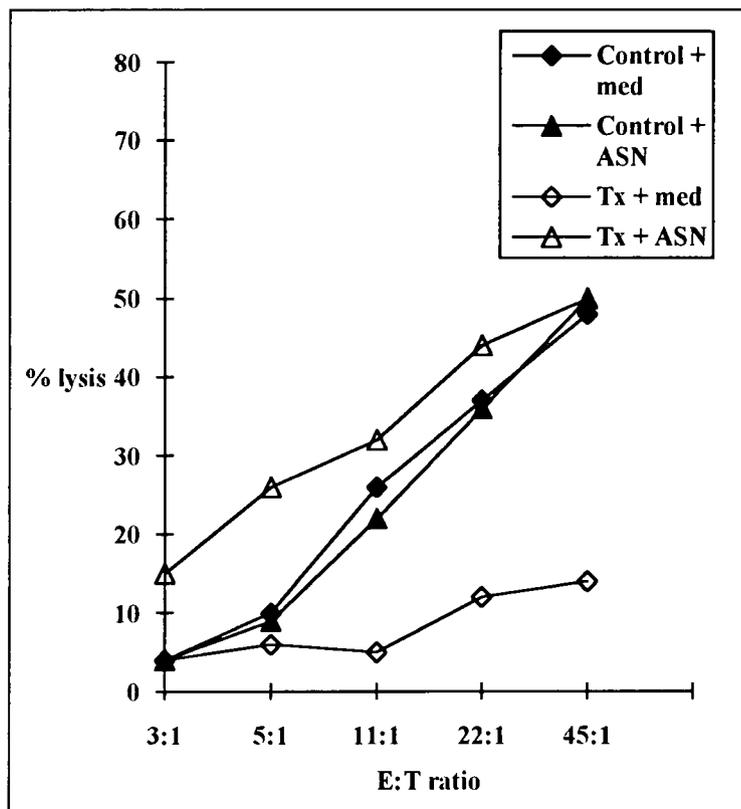


Table 2.5 : Percentage ⁵¹chromium release from B₃B₇ tumour target cells exhibited (in a 6 hour assay) by control and Tx splenocytes after treatment with either medium or ASN. Both *Xenopus* were 20 months old.

Splenocytes were cultured at 5×10^6 for 48 hours in either B₃B₇ tumour medium, with or without ASN, then washed and recounted, and used as effectors at E:T ratios of 3:1 → 45:1.

Effector Cells (Splenocytes)	Percentage lysis at the following E:T ratios :				
	3:1	5:1	11:1	22:1	45:1
Control + Medium	4	10	26	37	48
Control + ASN	4	9	22	36	50
Tx + Medium	4	6	5	12	14
Tx + ASN	15	26	32	44	50

Figure 2.9: Flow cytometric analysis of splenocytes from both a control and a thymectomised *Xenopus laevis* after 48 hour culture in B₃B₇ tumour medium. Both *Xenopus* were 20 months old.

Cells were dual labelled for flow cytometry with the pan T cell mAb 2B1 (1:80) and the anti-CD8 mAb F17 (1:200).

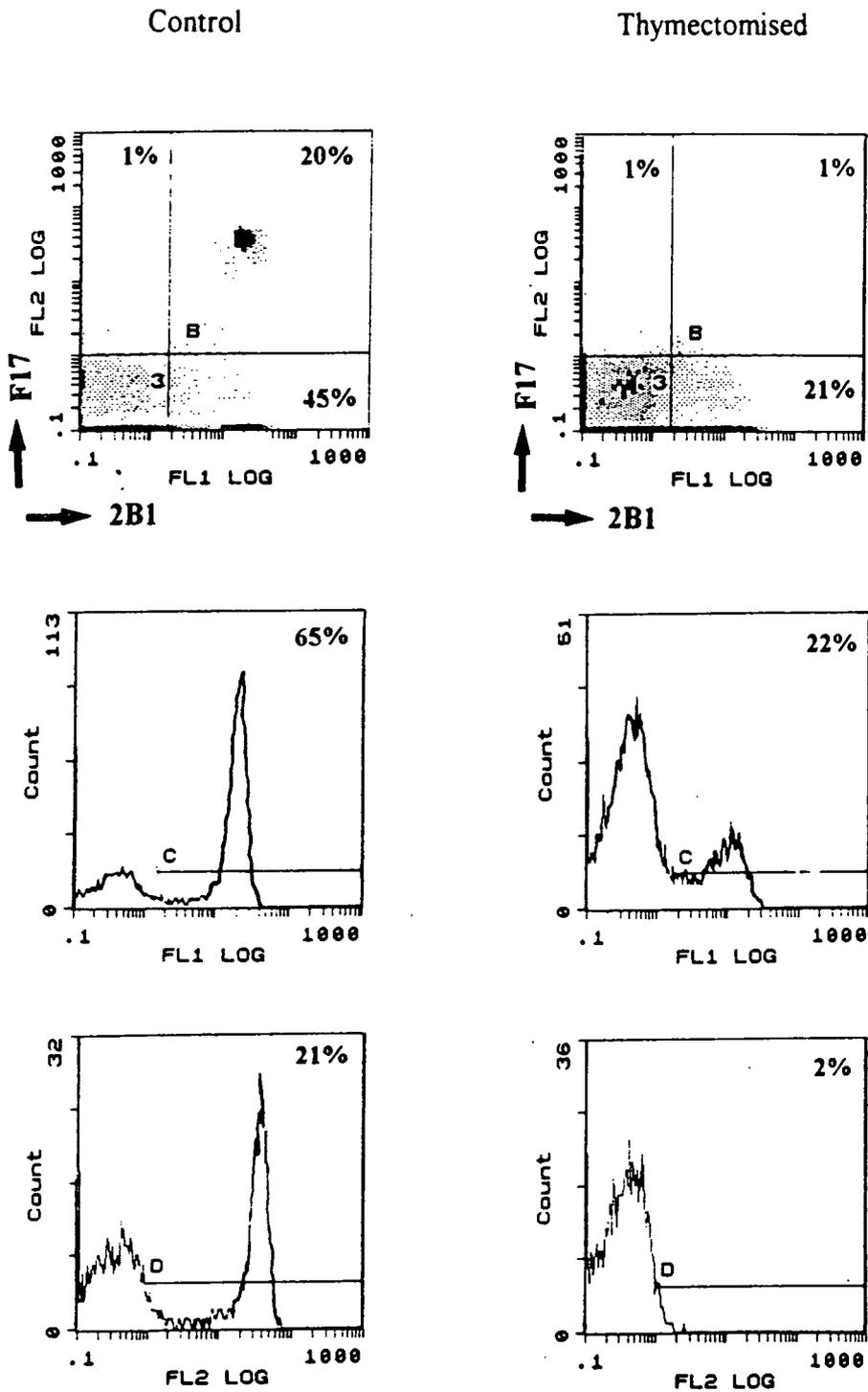


Figure 2.10 : Percentage $^{51}\text{Chromium}$ release from B_3B_7 tumour target cells induced by either fresh control splenocytes or those cultured in medium or ASN. The *Xenopus* was 13 months old.

Splenocytes were cultured at 5×10^6 for 48 hours in either B_3B_7 tumour medium or ASN, then washed and recounted, and used as effectors at E:T ratios of 3:1 \rightarrow 40:1.

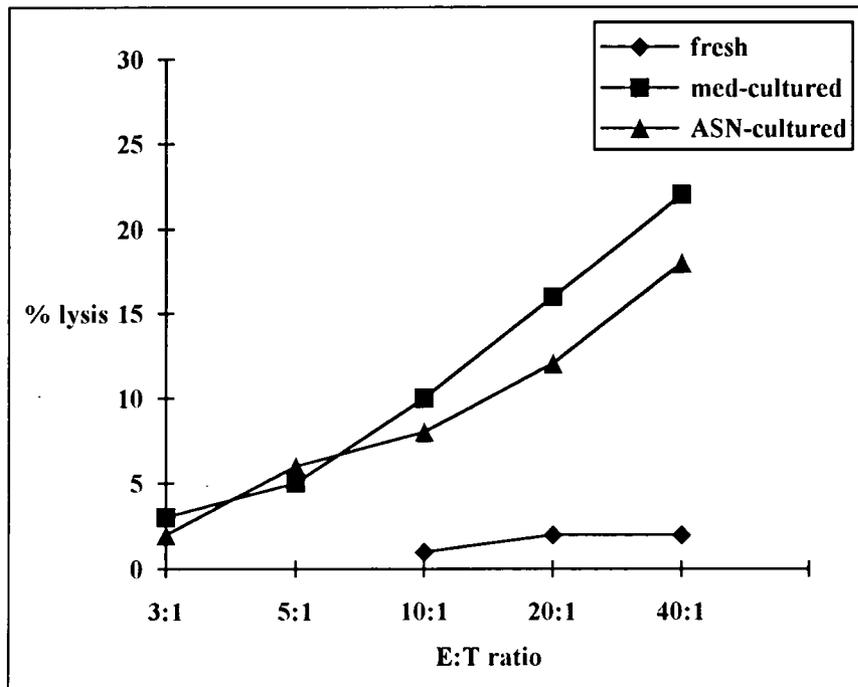


Figure 2.11 : Flow cytometric analysis of splenocytes from an 8 month old control *Xenopus laevis*.

Cells were dual labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5], or AM22 (1:400) [anti-CD8] and 2B1 (1:80).

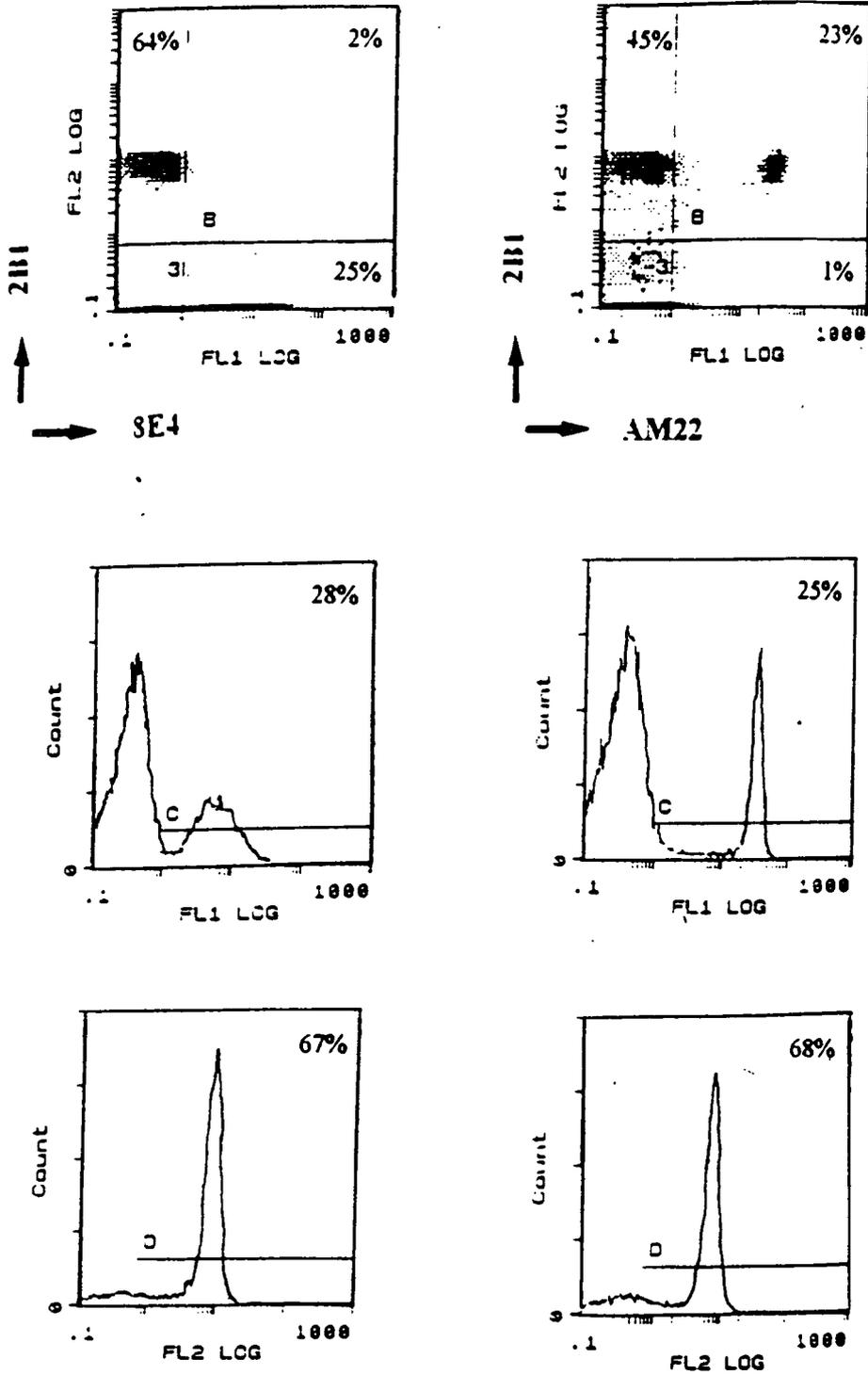


Figure 2.12: Flow cytometric analysis of the non-panned, non-adherent and adherent splenocytes from a 7 month old control *Xenopus laevis*, panned with 8E4.

Panning was performed with 8E4 (anti-IgM) at a 1 in 60 dilution. Cells were dual labelled for flow cytometry with the mAbs 10A9 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

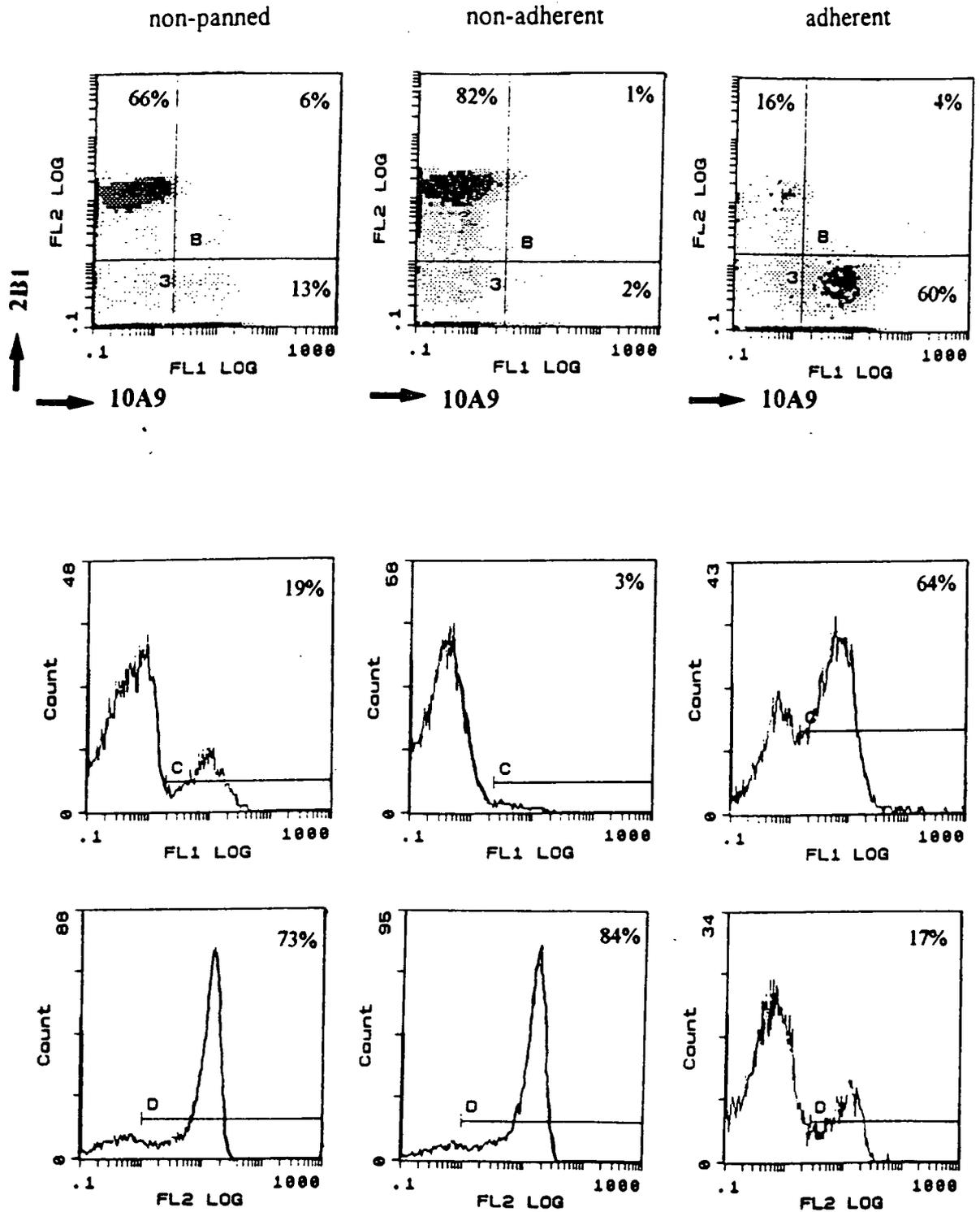


Figure 2.13 : $^3\text{HTdR}$ uptake (DPM) of the non-panned, non-adherent and adherent splenocytes from a 10 month old control *Xenopus laevis* (panned with 8E4), when treated with Con A or LPS.

Panning was performed with 8E4 at a 1 in 60 dilution. Con A was used at $2.5\mu\text{g/ml}$, and LPS was used at 0.5mg/ml .

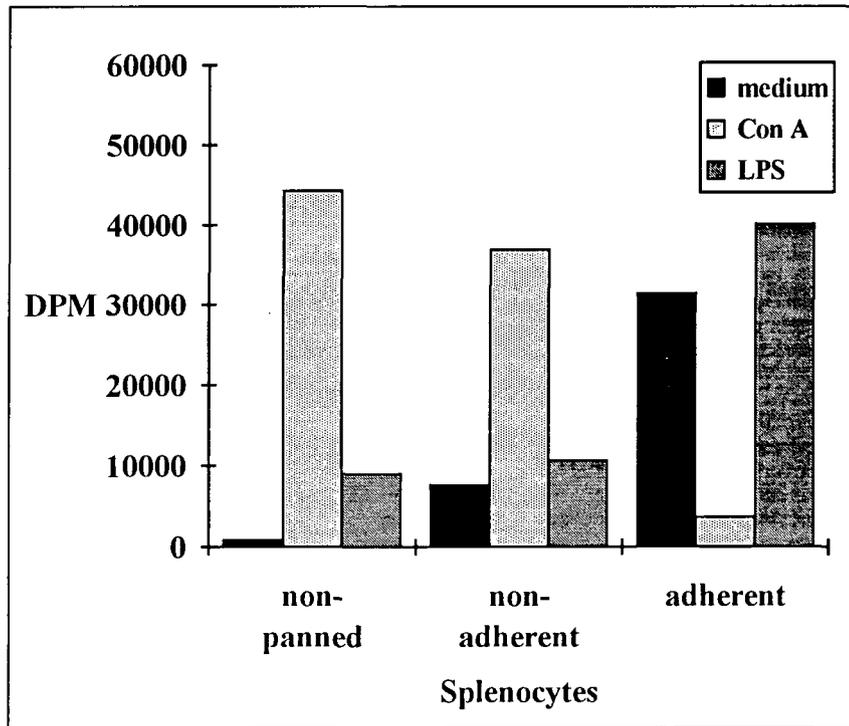


Table 2.6 : Stimulation indices produced by Con A and LPS treatment of the non-panned, non-adherent and adherent splenocytes from a 10 month old control *Xenopus laevis* panned with 8E4.

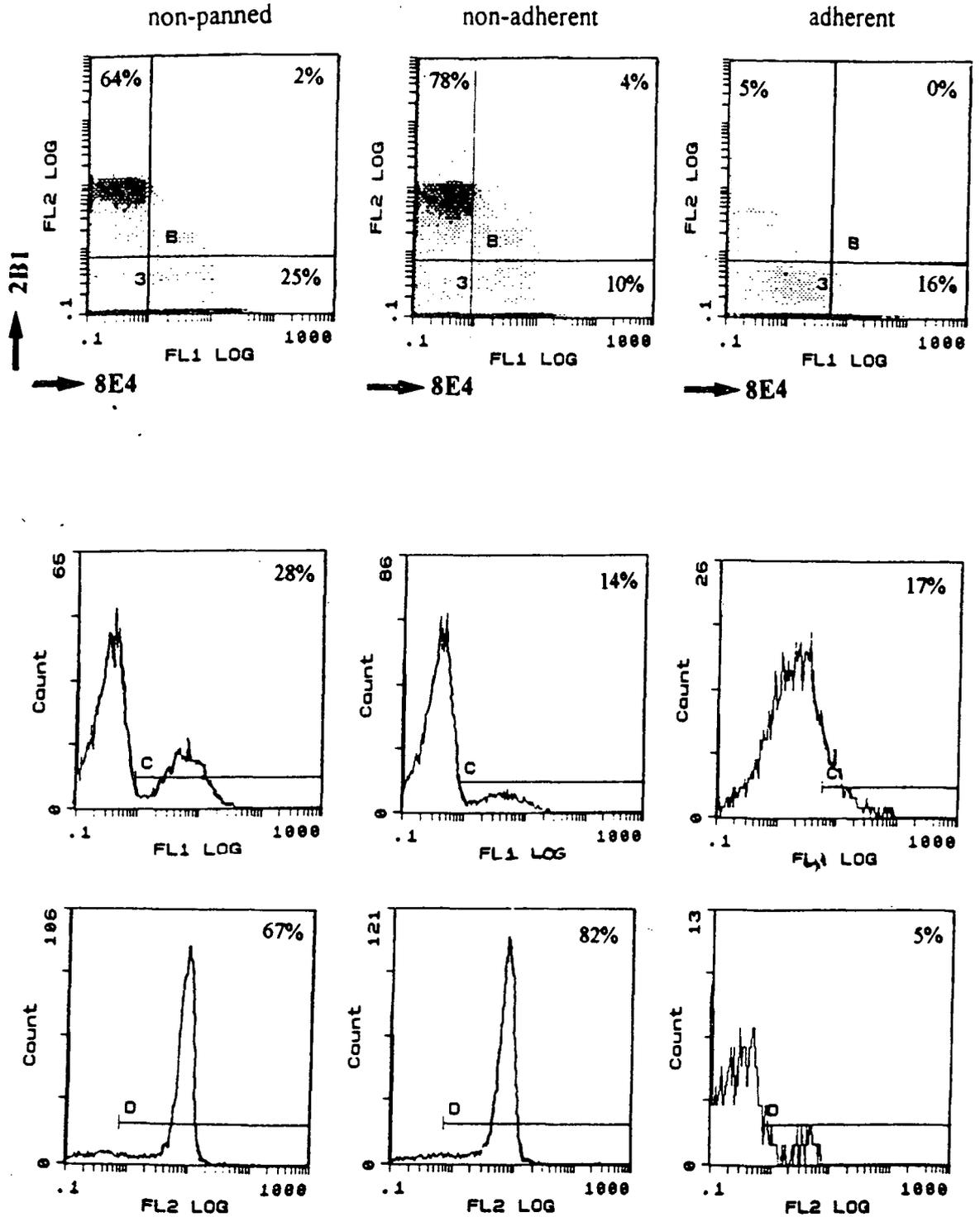
Panning was performed with 8E4 at a 1 in 60 dilution. Con A was used at $2.5\mu\text{g/ml}$, and LPS was used at 0.5mg/ml .

Splenocyte population	Stimulation indices when splenocytes treated with:	
	Con A	LPS
non-panned	48.6	9.80
non-adherent	4.87	1.40
adherent	0.11	1.28

Figure 2.14: Flow cytometric analysis of the non-panned, non-adherent and adherent splenocytes from an 8 month old control *Xenopus laevis* panned with anti-light chain mAbs (ALC mix).

Panning was performed with ALC mix at 1 in 50 dilution. Cells were dual labelled for flow cytometry with the mAbs a) 8E4 (1:60) [anti-IgM] and 2B1 (1:100) [anti-CD5] or b) ALC (1:50) [anti-sIg] and 2B1 (1:100).

a)



b)

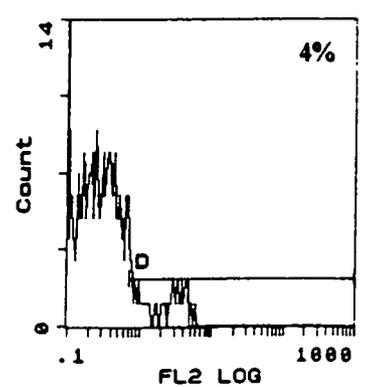
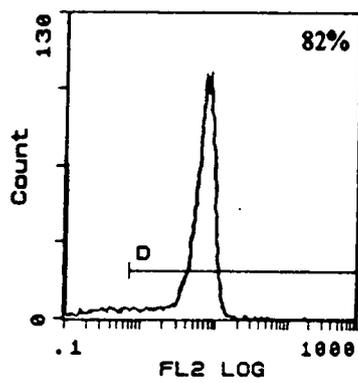
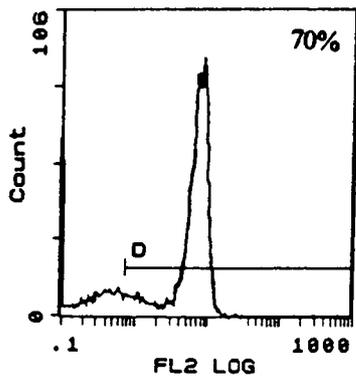
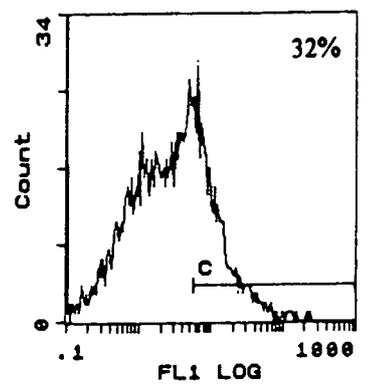
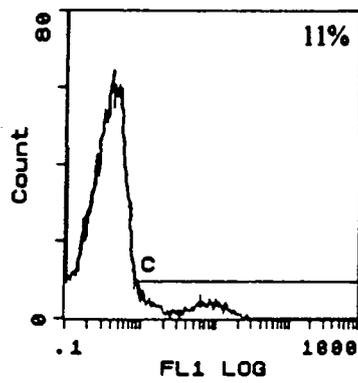
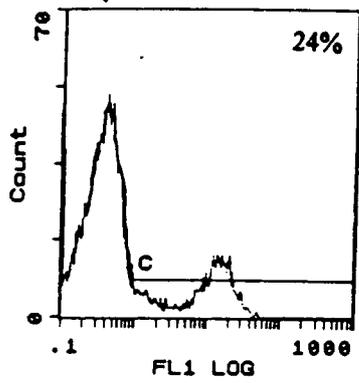
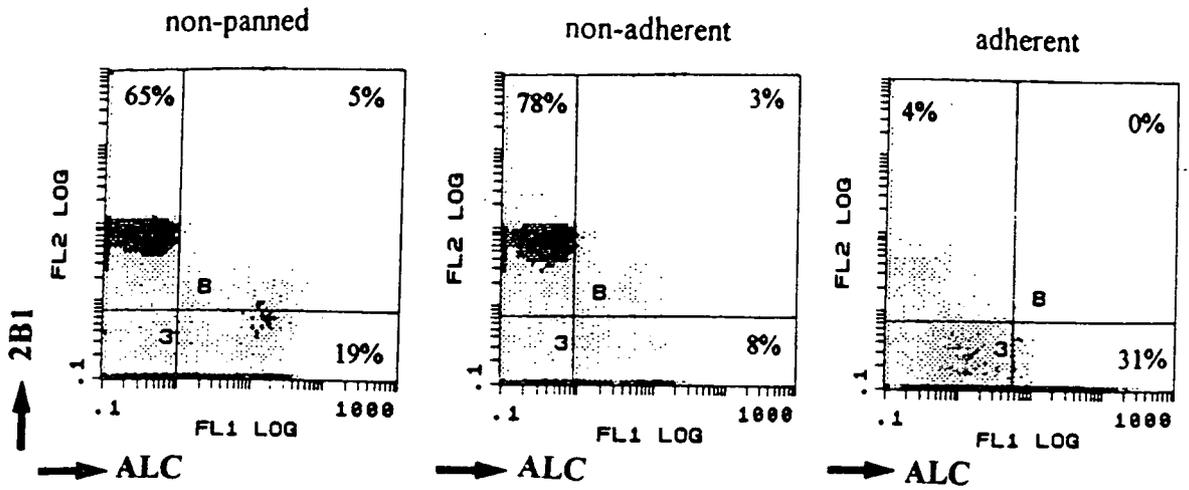


Figure 2.15: Flow cytometric analysis of the non-panned, non-adherent and adherent splenocytes from an 8 month old control *Xenopus laevis*, panned with 2B1.

Panning was performed with 2B1 SN at 1 in 5 dilution. Cells were dual labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5], or 2B1(1:200) and D8 (1:300) [anti-IgM].

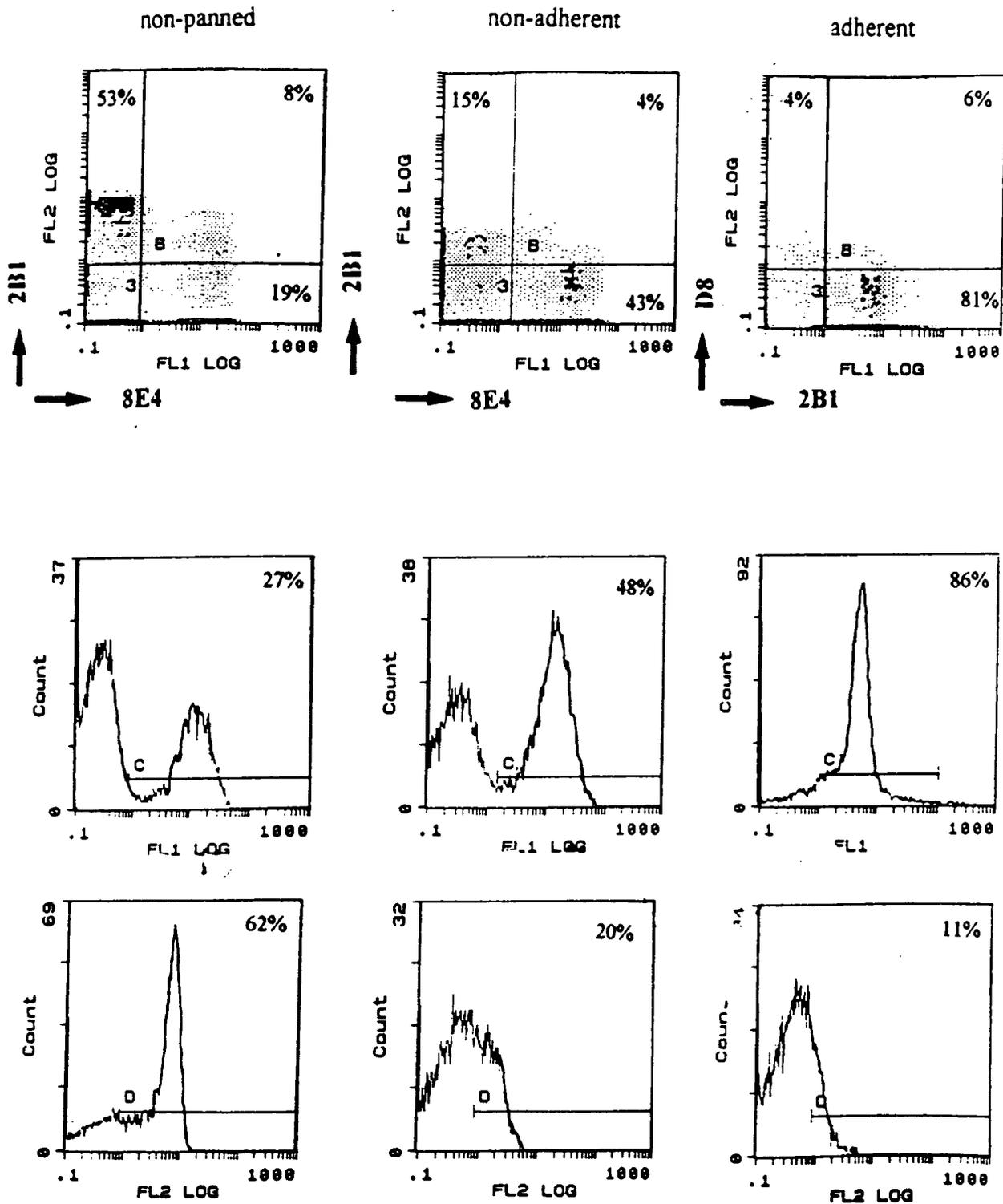


Figure 2.16 : $^3\text{HTdR}$ uptake of the non-panned, non-adherent and adherent splenocytes from a 10 month old control *Xenopus laevis* (panned with 2B1), when treated with Con A or LPS.

Panning was performed with 2B1 at a 1 in 5 dilution. Con A was used at $2.5\mu\text{g/ml}$, and LPS was used at 0.5mg/ml .

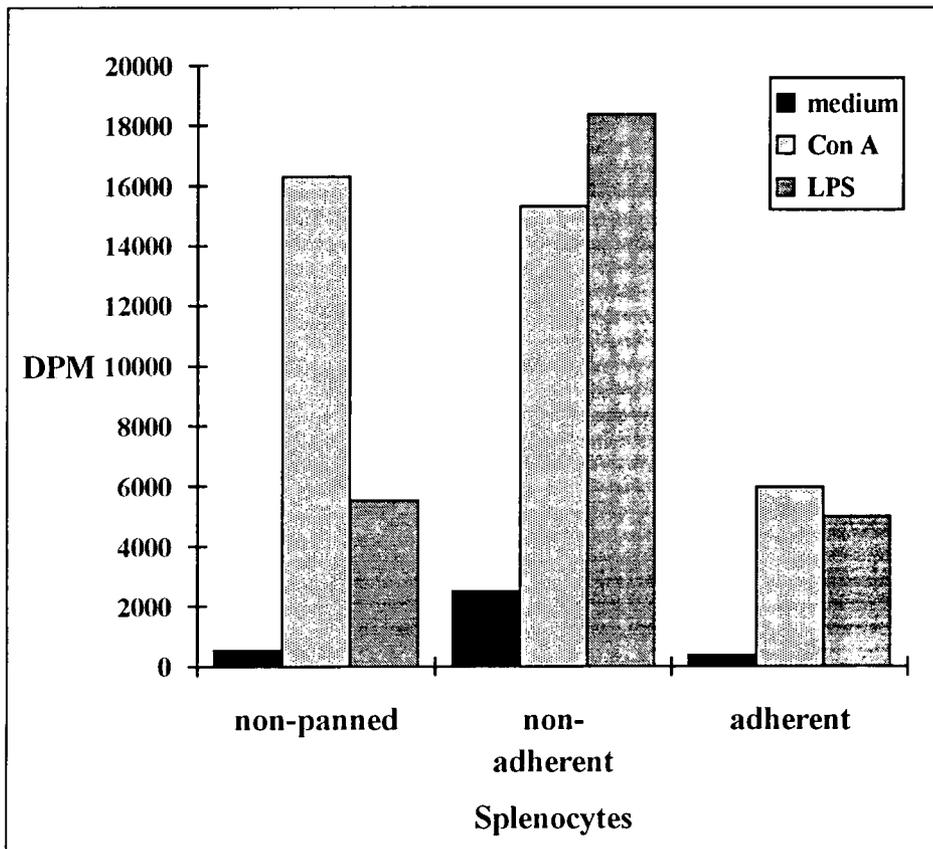


Table 2.7 : Stimulation indices produced by Con A- and LPS- treatment of the non-panned, non-adherent and adherent splenocytes from a 10 month old control *Xenopus laevis*, panned with 2B1.

Panning was performed with 2B1 at a 1 in 5 dilution. Con A was used at $2.5\mu\text{g/ml}$, and LPS was used at 0.5mg/ml .

Splenocyte population	Stimulation indices when splenocytes treated with:	
	Con A	LPS
non-panned	30.74	10.43
non-adherent	6.13	7.36
adherent	16.15	13.52

Figure 2.17: Flow cytometric analysis of the non-panned, non-adherent and adherent splenocytes from an 8 month old control *Xenopus laevis*, panned with AM22.

Panning was performed with AM22 at a 1 in 400 dilution. Cells were dual-labelled for flow cytometry with the mAbs AM22 (1:400) [anti-CD8] and 2B1 (1:80) [anti-CD5].

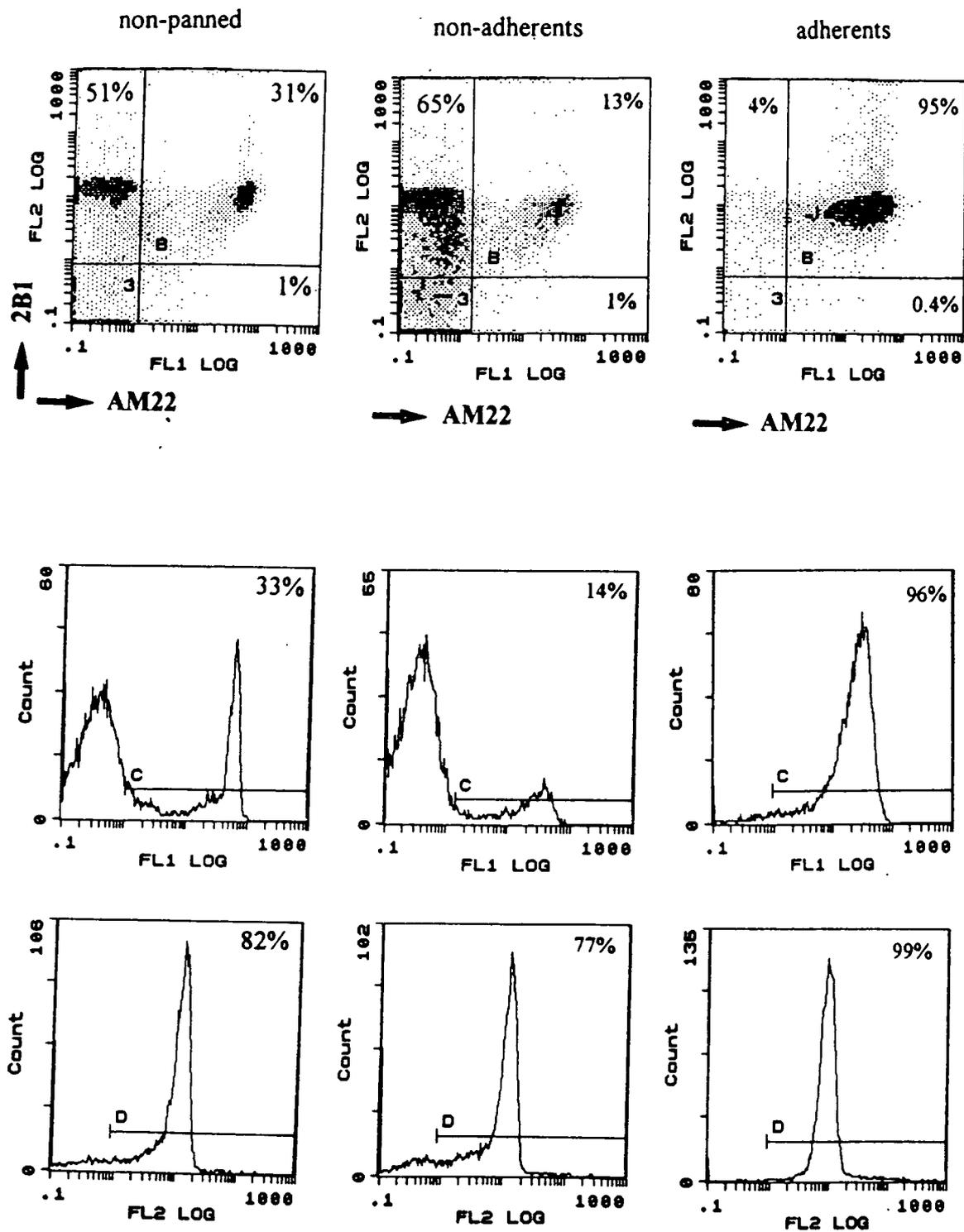


Figure 2.18 : $^3\text{HTdR}$ uptake of the non-panned, non-adherent and adherent splenocytes from an 8 month old control *Xenopus laevis* (panned with AM22), when treated with Con A for 48 hours.

Panning was performed with AM22 at a 1 in 400 dilution. Con A was used at both $1\mu\text{g/ml}$ and $2.5\mu\text{g/ml}$.

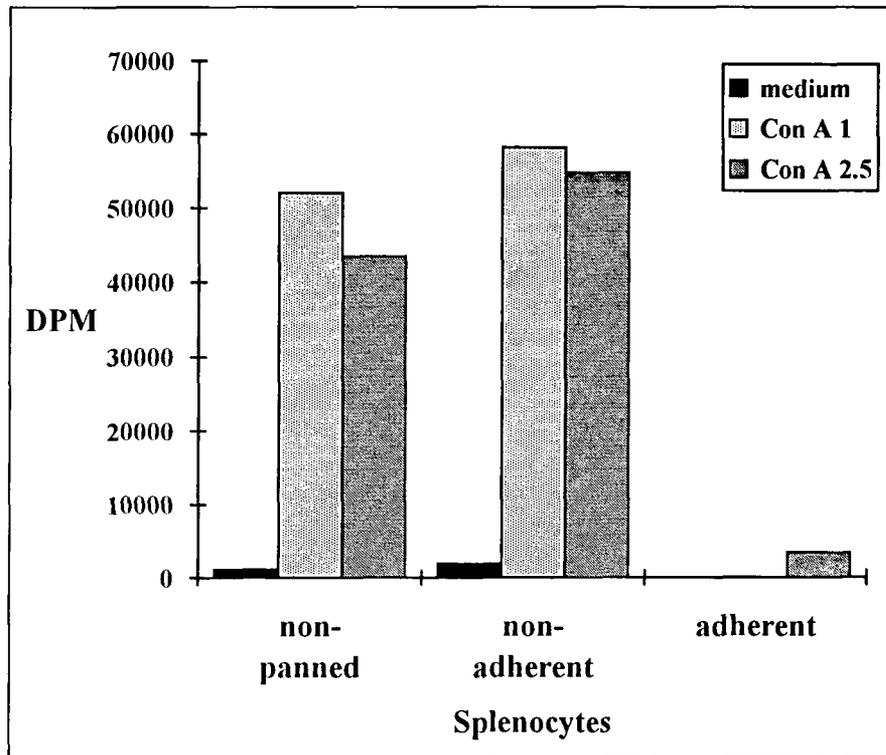


Table 2.8 : Stimulation indices produced by Con A treatment of the non-panned, non-adherent and adherent splenocytes from an 8 month old control *Xenopus laevis*, panned with AM22.

Panning was performed with AM22 at a 1 in 400 dilution. Con A was used at both $1\mu\text{g/ml}$ and $2.5\mu\text{g/ml}$.

Splenocyte population	Stimulation indices when splenocytes treated with:	
	Con A 1	Con A 2.5
non-panned	46.2	38.6
non-adherent	30.5	28.7
adherent	-	3.03

Figure 2.19 : Flow cytometric analysis of the non-adherent splenocytes from three different control Dynabead® separations.

Freshly prepared splenocytes from a 15 month old control *Xenopus laevis* were incubated in either APBS/FCS or 8E4 (diluted at 1 in 4 in APBS/FCS). After 45 minutes one set of control cells (no 8E4) were mixed with Dynabeads® M-450 at 10:1 beads:cells, the other two cell samples were rotated without beads. After overnight culture (to achieve detachment of adherent cells from beads), non-adherents from each sample were dual-labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1 :80) [anti-CD5].

(Figure 2.19 is shown on the following page).

Control cells (no 8E4)
no beads

Control cells (no 8E4)
1:10 beads

8E4-treated cells
no beads

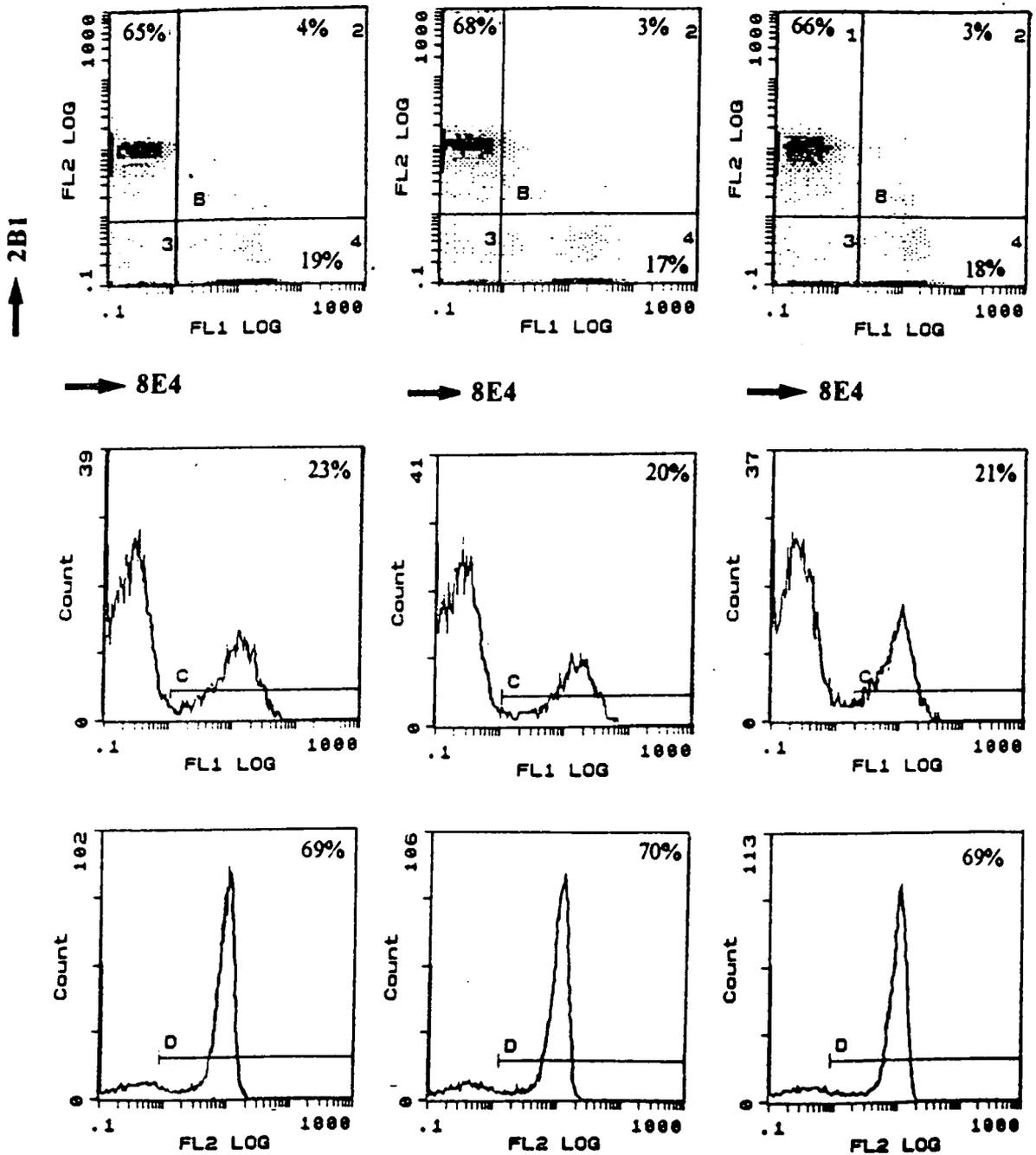


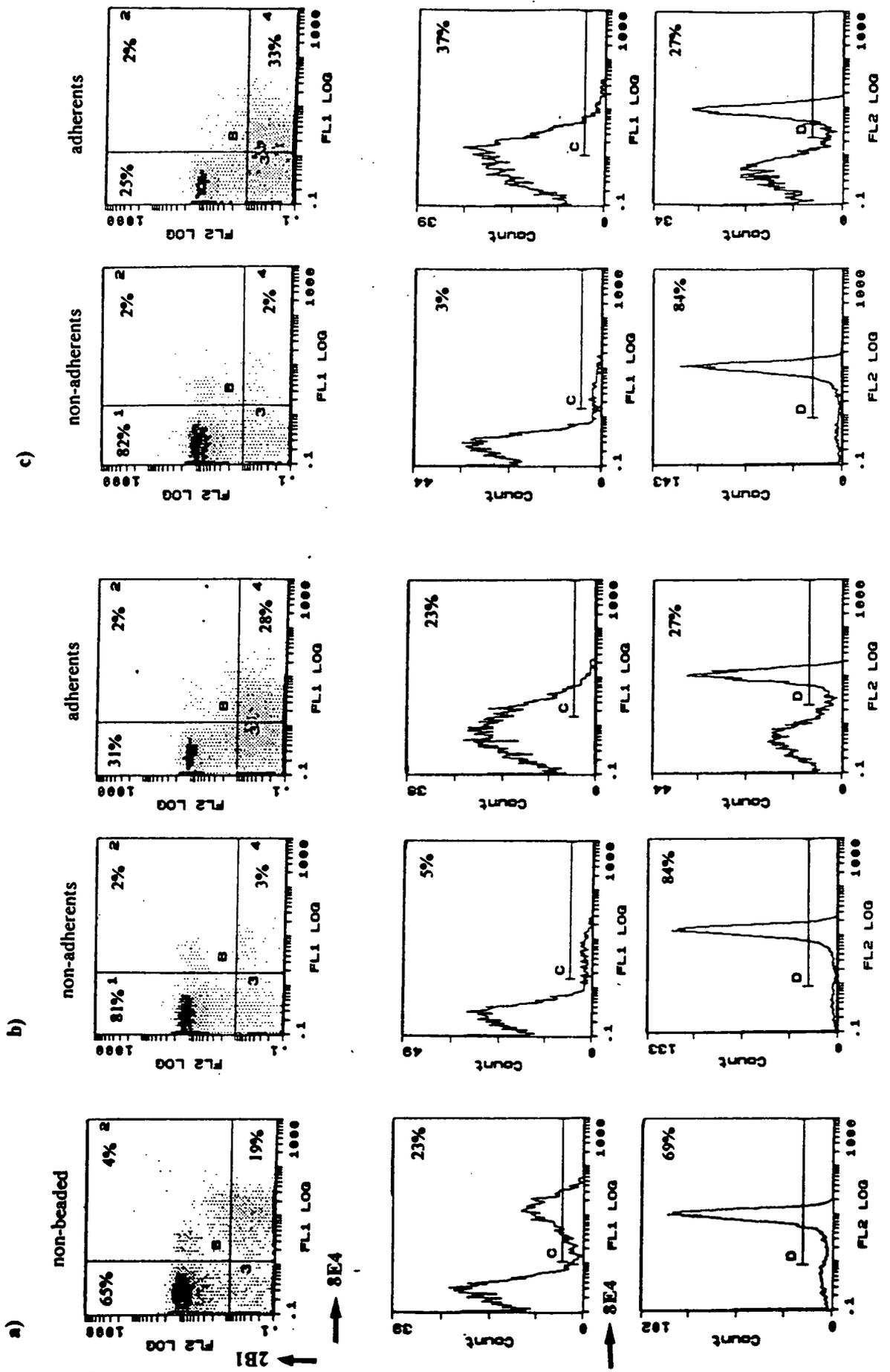
Figure 2.20 : Flow cytometric analysis of the non-adherent and adherent splenocytes from Dynabead® separation using 8E4 at bead : lymphocyte ratios of 5:1, 10:1 and 20:1.

Freshly prepared splenocytes from a 15 month old control *Xenopus laevis* were subjected to Dynabead® separations under the following conditions:

- a) no 1° Ab, no beads
- b) cells incubated with 8E4 (1 in 4), beads : lymphocytes 5:1
- c) cells incubated with 8E4 (1 in 4), beads : lymphocytes 10:1
- d) cells incubated with 8E4 (1 in 4), beads : lymphocytes 20:1

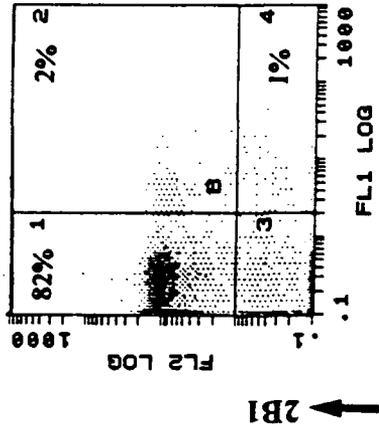
After overnight culture, all populations were dual-labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

(Figure 2.20 shown on the following two pages)

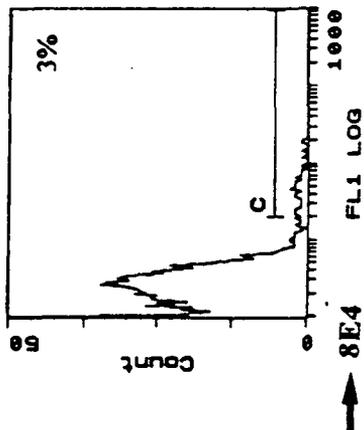


d)

non-adherents

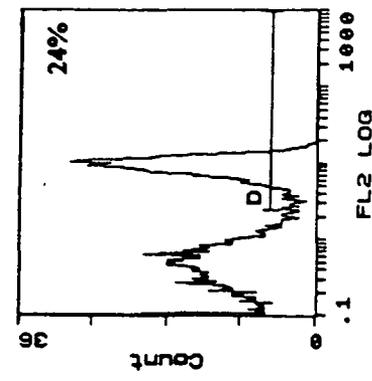
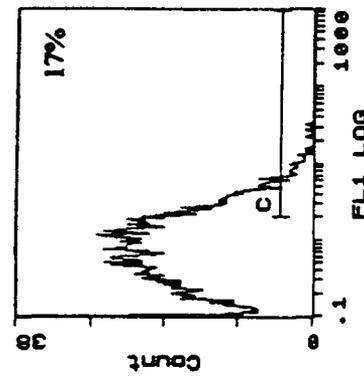
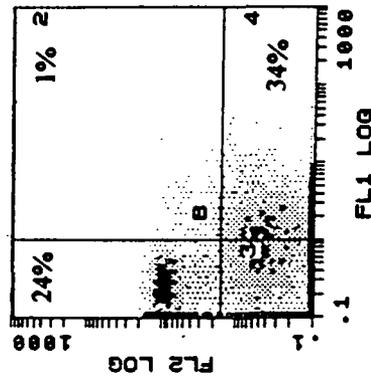


8E4



8E4

adherents



2B1

Figure 2.21 : Flow cytometric analysis of the non-beaded, non-adherent and adherent splenocytes from a Dynabead® separation using 8E4, after 17 or 41 hours culture *in vitro*.

All splenocytes were from a 15 month old control *Xenopus laevis*. Experimental cells were incubated in 8E4 (1 in 4), and mixed with beads : cells 10:1. Separations were performed in duplicate and cultured for either:

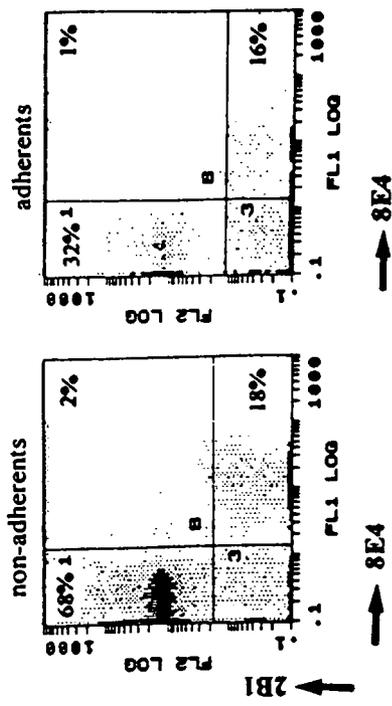
a) 17 hours or

b) 41 hours,

before being analysed by flow cytometry. All populations were dual-labelled with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

(Figure 2.21 is shown on the following page)

b)



a)

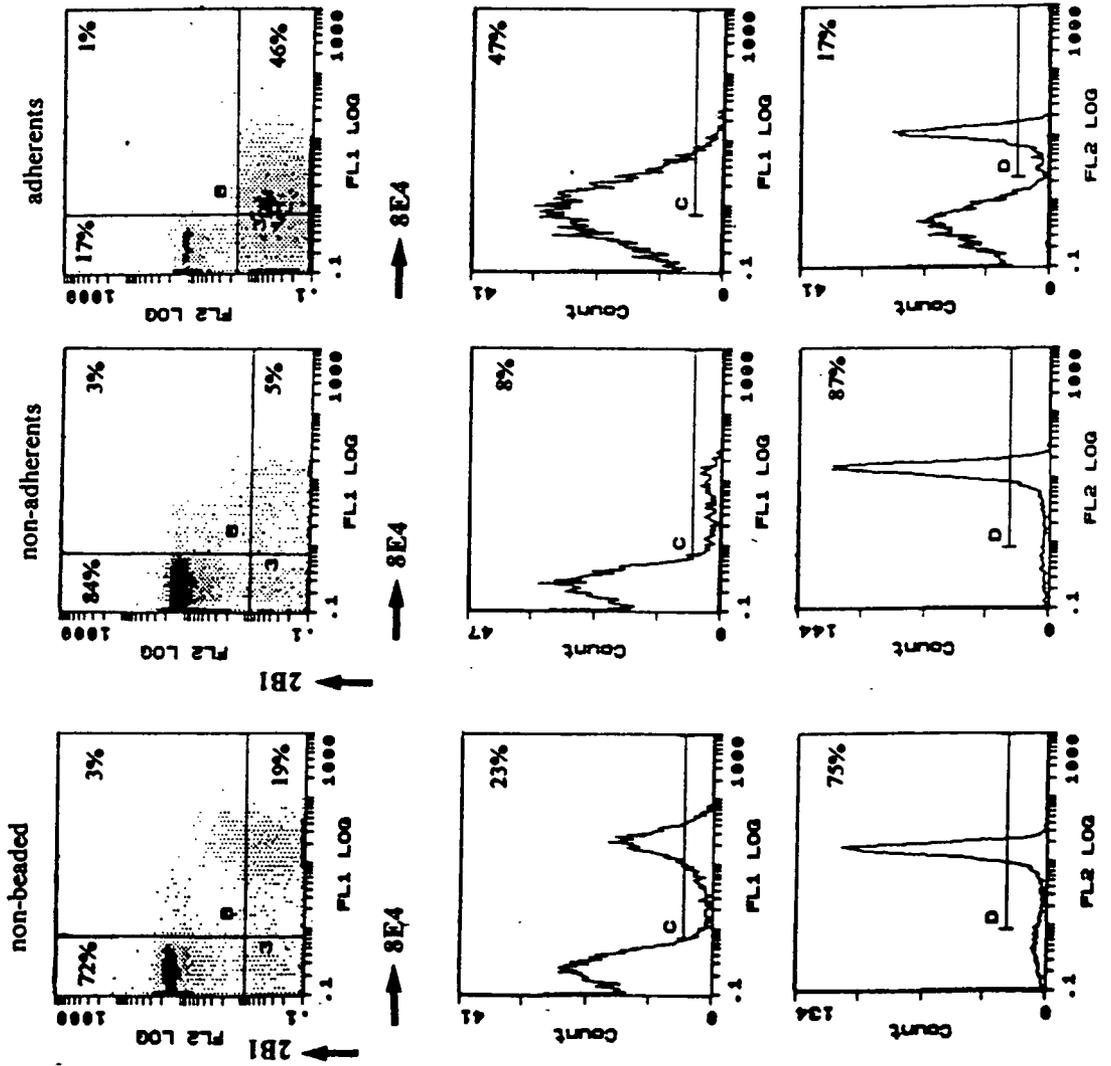


Figure 2.22 : Flow cytometric analysis of the non-beaded and either 'washed' or 'unwashed' adherent splenocytes from a Dynabead® separation using 8E4.

Freshly prepared splenocytes from a 15 month old control *Xenopus laevis* were treated with 8E4 at a 1 in 4 dilution. Duplicate separations were set up (Beads : cells 10:1), and the resulting adherent populations either removed directly in the MPC (unwashed) or removed, washed with 1ml APBS/FCS and then removed again in the MPC (washed). All populations were cultured overnight then dual-labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

(Figure 2.22 is shown on the following page).

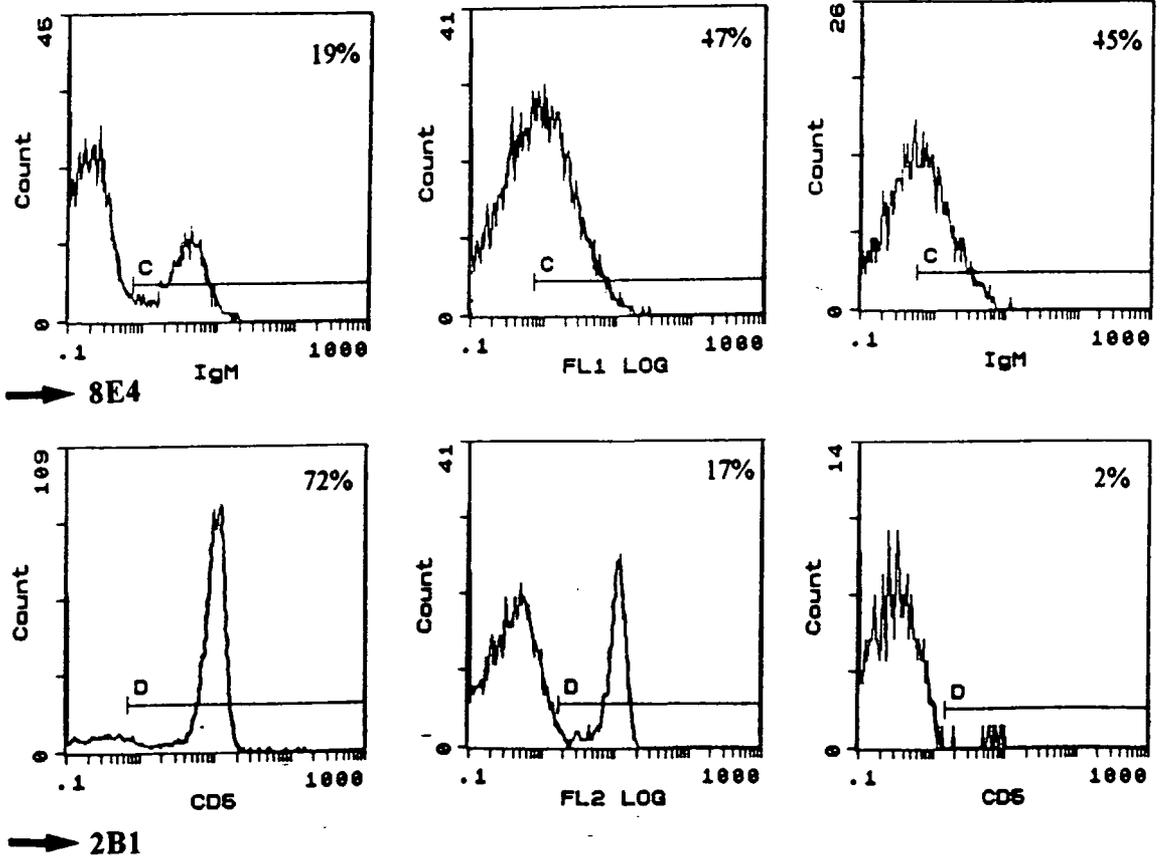
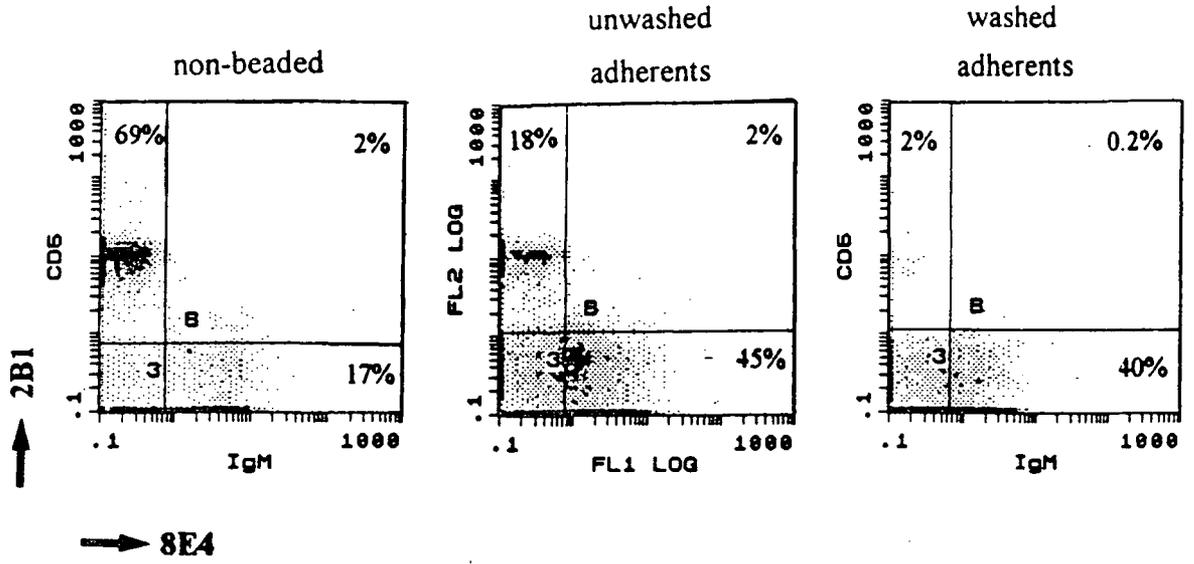


Figure 2.23 : Flow cytometric analysis of overnight-cultured non-beaded, non-adherent and adherent splenocytes from Dynabead® separations utilising different initial mAb incubation volumes.

All splenocytes were from a 15 month old control *Xenopus laevis*. Experimental cells were incubated in either

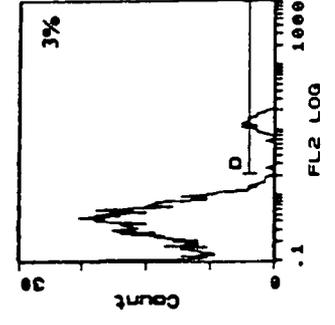
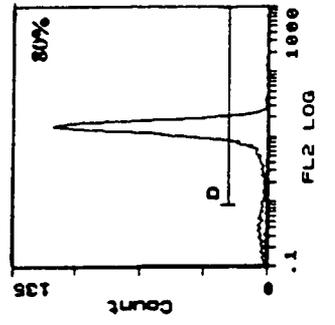
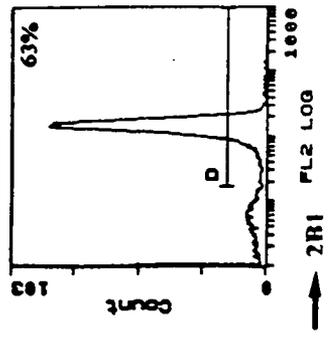
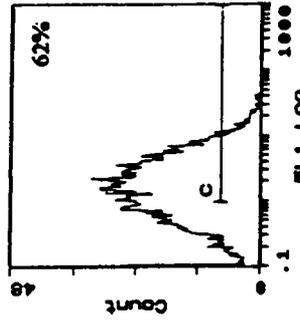
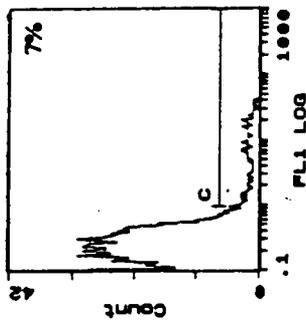
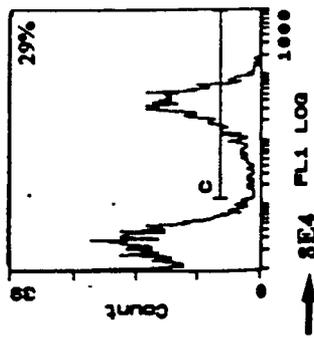
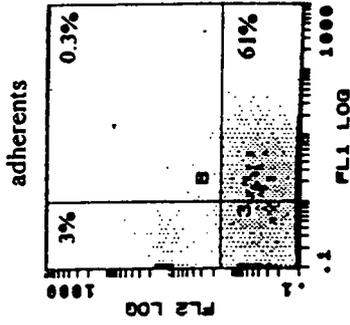
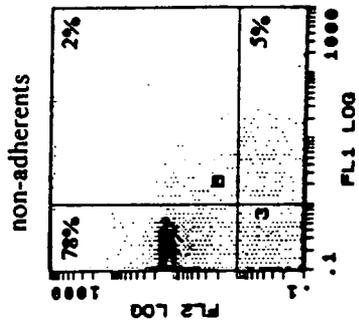
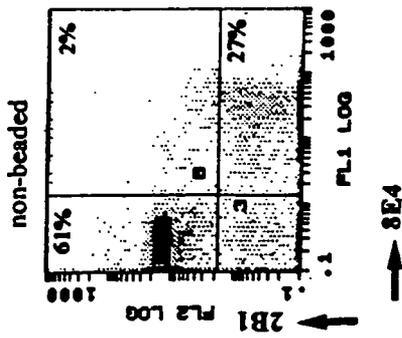
a) 1ml or

b) 0.5ml

of 8E4 (1 in 4), then mixed with beads at 10:1 beads : cells. Washing of cells during magnetic bead separation was carried out. All populations were dual-labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

(Figure 2.23 is shown on the following page)

a) 1ml



b) 0.5ml

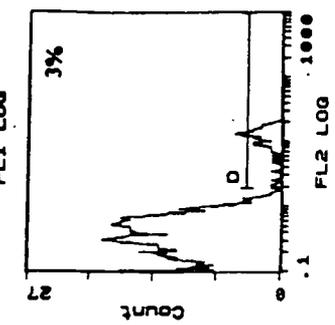
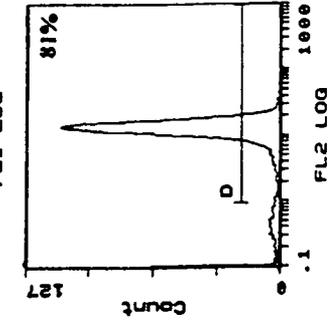
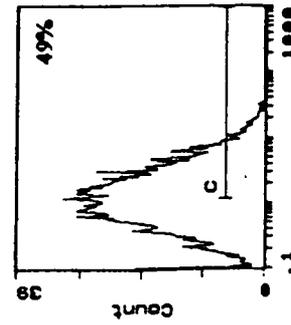
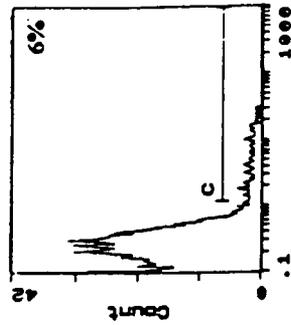
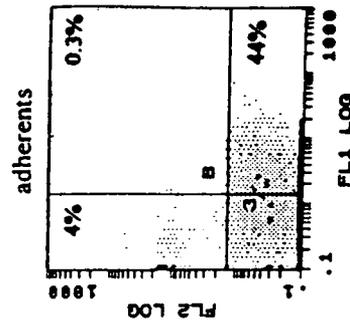
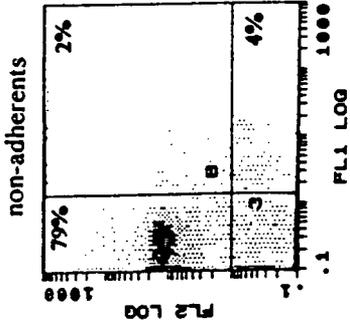


Figure 2.24 : Flow cytometric analysis of the non-beaded, non-adherent and adherent splenocytes from a Dynabead® separation using 2B1, assessing two 2B1 concentrations, under optimal conditions described in 2.3.4.1.

All splenocytes were from a 15 month old control *Xenopus laevis*. Experimental cells were incubated in 2B1 at either

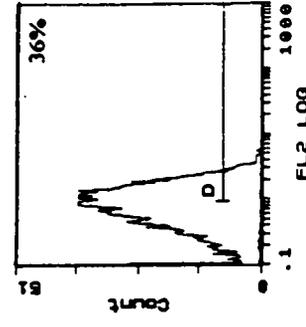
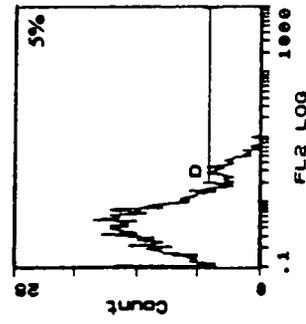
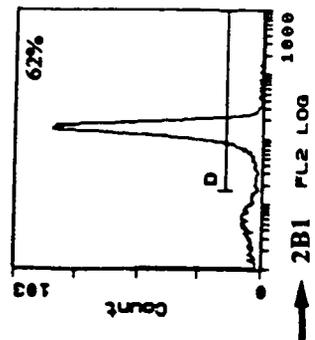
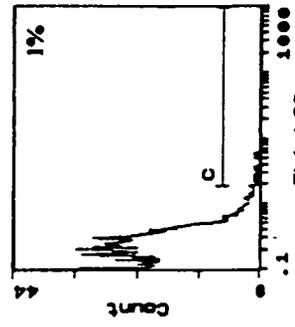
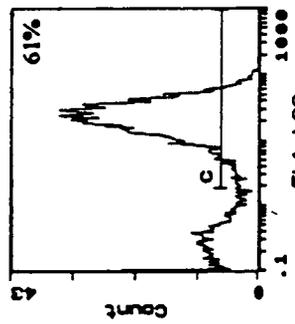
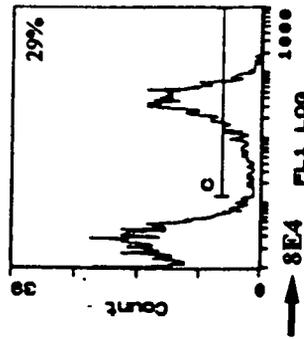
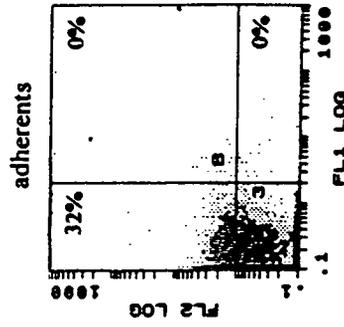
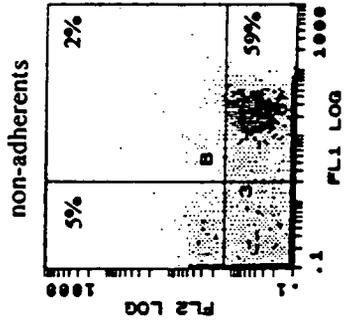
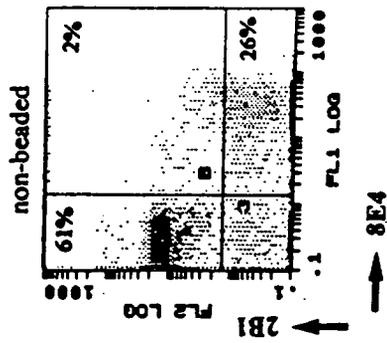
a) 1 in 5 or

b) 1 in 10,

then mixed with beads at 10:1 beads : cells. All populations were cultured overnight and then dual-labelled for flow cytometry with the mAbs 8E4 (1:60) [anti-IgM] and 2B1 (1:80) [anti-CD5].

(Figure 2.24 is shown on the following page)

a) 2B1(1 in 5)



b) 2B1(1 in 10)

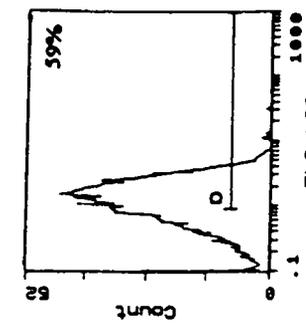
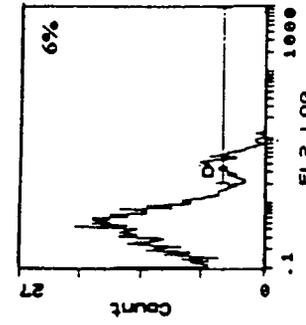
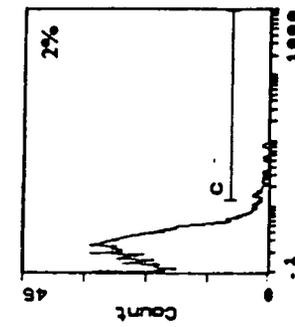
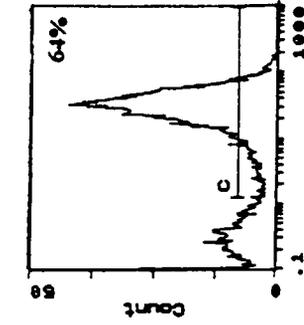
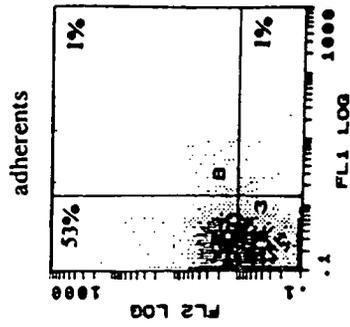
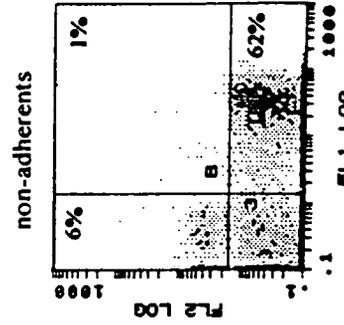


Figure 2.25 : Flow cytometric analysis of the non-adherent splenocytes from a Dynabead® separation using AM22.

Freshly prepared splenocytes from a 16 month old control *Xenopus laevis* were subjected to Dynabead® separation:

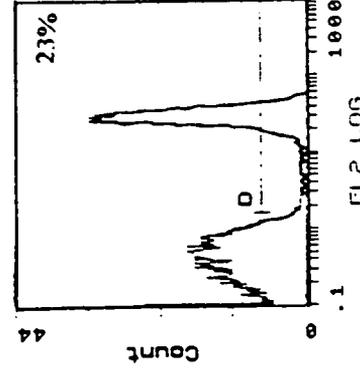
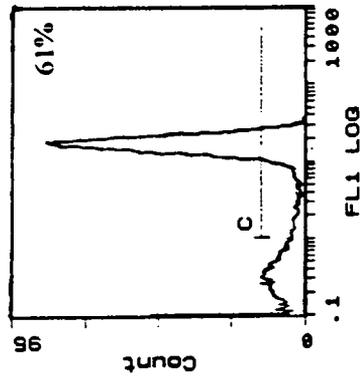
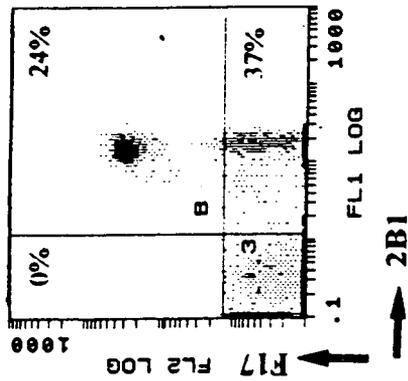
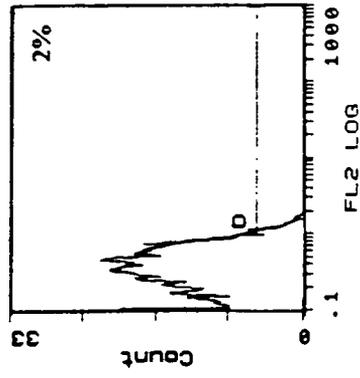
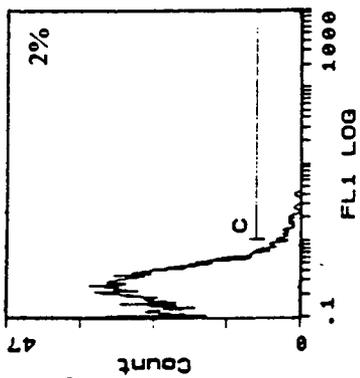
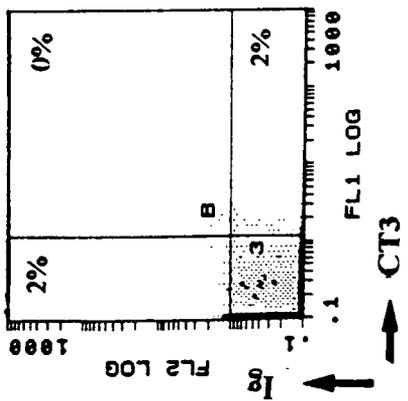
a) no 1° Ab, no beads

b) cells incubated with AM22 (1 in 125), beads : cells 10:1

After overnight culture, populations were dual-labelled for flow cytometry with the mAbs CT3 (1:20) [anti-chicken CD3] and Ig (1:40) [mouse IgG₁], or 2B1 (1:15) [anti-CD5] and F17 (1:200) [anti-CD8].

(Figure 2.25 is shown on the following page)

a) no 1° Ab, no beads



b) AM22(1 in 125), 1:10 beads non-adherents

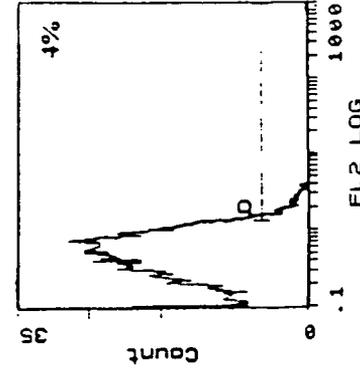
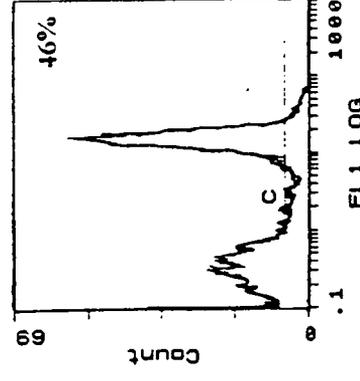
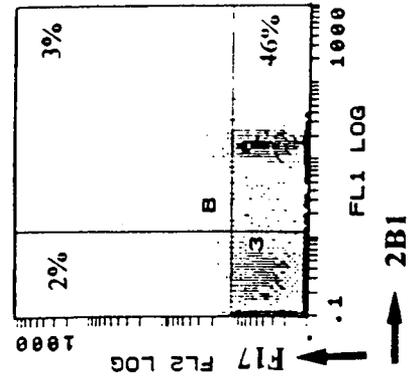
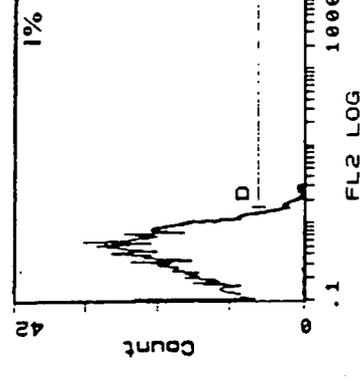
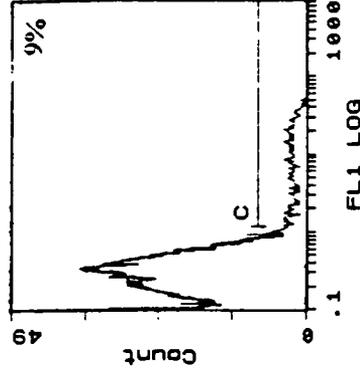
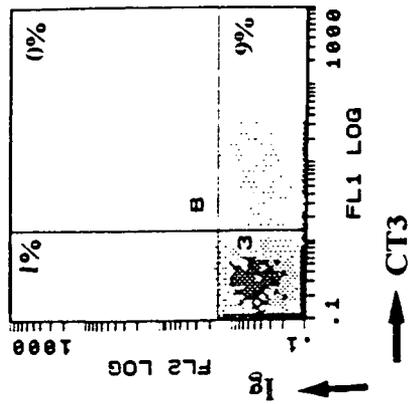


Figure 2.26 : Flow cytometric analysis of the splenocyte populations (from Dynabead® separations using either 8E4 or 2B1) tested for their killing ability in a separate ⁵¹Cr-release assay.

Freshly prepared splenocytes from a 17 month old control *Xenopus laevis* were incubated with either

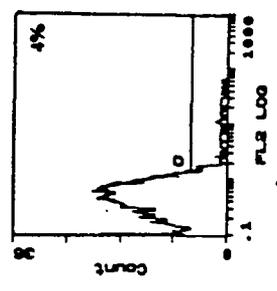
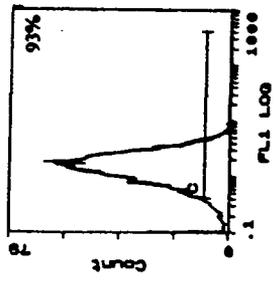
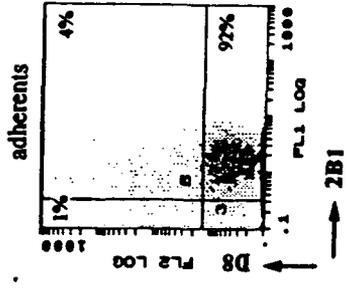
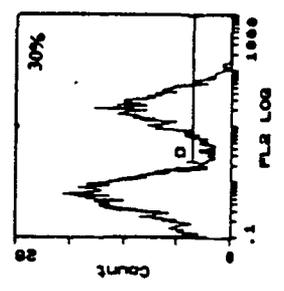
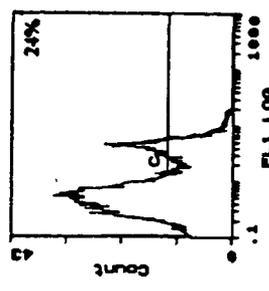
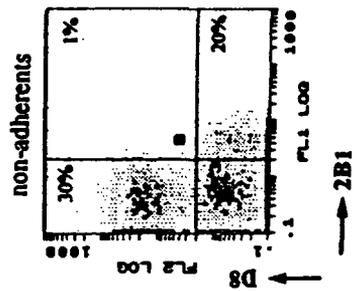
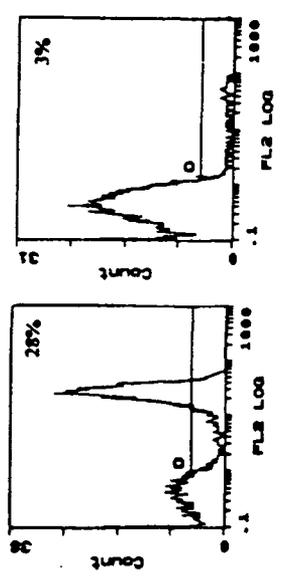
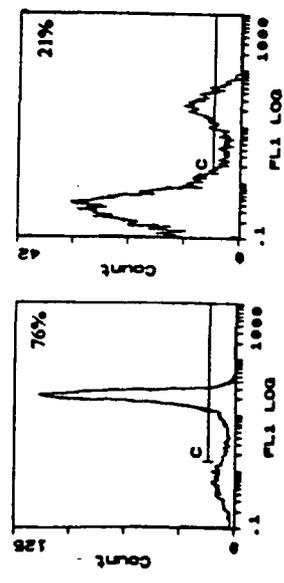
a) 2B1 (1 in 10) or

b) 8E4 (1 in 4),

then mixed with beads at 10:1 beads : cells. After separation and overnight culture, an aliquot of each population was removed and dual-labelled for flow cytometry with the mAbs 2B1 (1:15) [anti-CD5] and D8 (1:100) [anti-IgM]. (Non-beaded cells were labelled with either 2B1 (1:80) and F17 (1:200) [anti-CD8], or 8E4 (1:60) [anti-IgM] and D12.2 (1:200)).

(Figure 2.26 is shown on the following page)

a) 2B1(1 in 10)



b) 8E4(1 in 4)

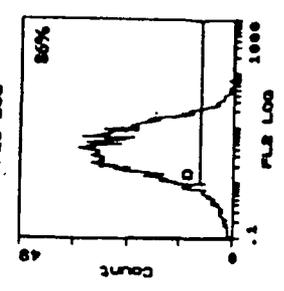
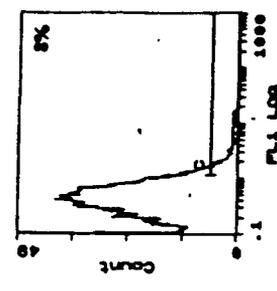
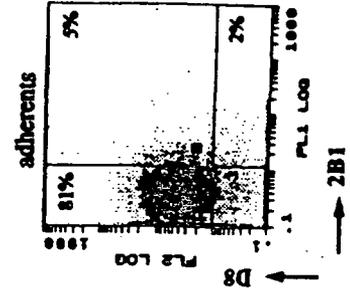
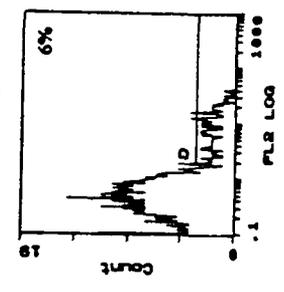
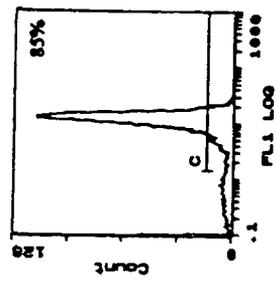
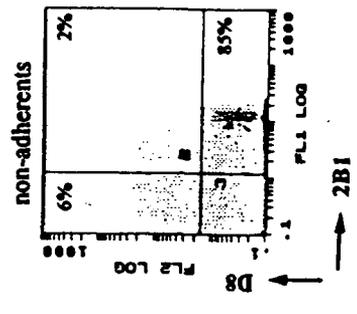


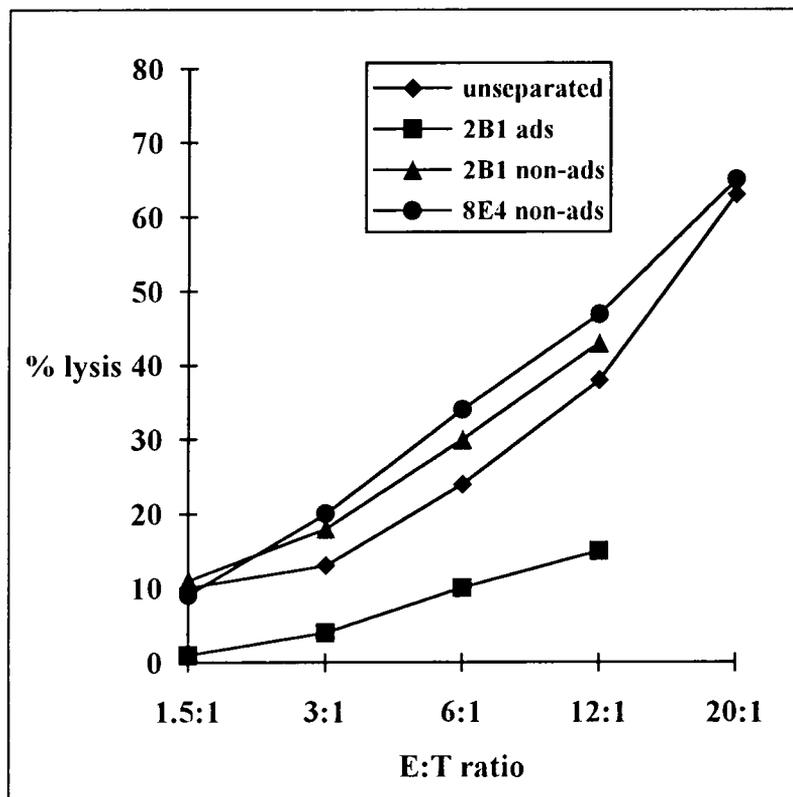
Figure 2.27 : Percentage lysis of $^{51}\text{Chromium}$ -labelled B_3B_7 tumour target cells exhibited by the splenocyte populations from Dynabead[®] separations using either 8E4 or 2B1. Unseparated splenocytes were also assayed.

Freshly prepared splenocytes from a 17 month old control *Xenopus laevis* were incubated with either

a) 2B1 (1 in 10) or

b) 8E4 (1 in 4),

then mixed with anti-Ig magnetic beads at 10:1 beads : cells. After separation and overnight culture, the cells were washed and recounted, then used as effectors in $^{51}\text{Chromium}$ -release assays at E:T ratios of 1.5:1 \rightarrow 20:1. FACS traces shown in previous figure.



2.4 Discussion

2.4.1 Generation of Active Supernatants by Con A Stimulation of Splenocytes.

The T-cell mitogen Concanavalin A was used in a series of *in vitro* stimulation experiments to probe optimal conditions for generating active supernatants (ASNs) from *Xenopus* splenocytes. The extent to which the splenocytes were stimulated by Con A was assessed in tritiated thymidine ($^3\text{HTdR}$) assays, where stimulated cells display high $^3\text{HTdR}$ incorporation (due to their active proliferation) compared to medium-cultured cells. In this preliminary study, Con A concentrations of both $1\mu\text{g/ml}$ and $2.5\mu\text{g/ml}$ were capable of stimulating splenocytes. Although the range of stimulation indices varied from animal to animal, the optimum Con A concentration was always found to be $2.5\mu\text{g/ml}$, which is in agreement with Ho (1992), and therefore this concentration was used throughout the subsequent experiments. Since Con A is a T cell mitogen, it would be expected that there would be no response to this mitogen in early-thymectomised *Xenopus*, as these should have no T cells (see Gravenor *et al*, 1995). This proved to be the case, since no stimulation was observed in the early- (stage 48, days 7 old) thymectomised animals. In contrast, there was clearly some stimulation in the late- (stage 56/57, 35 days old) thymectomised frogs, presumably due to T cells that had escaped to the periphery before late thymectomy was performed.

These preliminary experiments pointed to the likelihood that Con A would be capable of generating active culture supernatants from *Xenopus* T cells, for use in promoting NK-like cell activity. Further studies revealed that the sugar α -methylmannoside (at 0.1M) was effective at removing all the Con A activity (in agreement with Ho, 1992). Consequently, any stimulation seen in later assays of

these ASNs is likely to be assigned to secreted cytokine, rather than residual Con A in the culture. ASNs were then tested for their stimulatory capabilities against different lymphocyte populations.

Initial experiments were carried out on pre-cultured cells. Splenic lymphoblasts (ie. cells that had already been pre-cultured for 48 hours with Con A) were not induced to incorporate more $^3\text{HTdR}$ than cultures lacking ASN. Presumably such cells, which were already stimulated by the Con A during the pre-culture period, are unresponsive to further stimulation, possibly due to their own lymphokine production. However, when these Con A-derived lymphoblasts were left for a further 5 days in L15 culture medium, they became susceptible to addition of growth factors in the ASN. This phenomenon could relate to their own cytokine production now being insufficient at this longer time point after Con A stimulation.

Further batches of Con A-derived ASNs were generated from both control and late-thymectomised animals. Both were tested against fresh splenocytes (ie. *ex vivo*), rather than pre-cultured cells, and were able to stimulate $^3\text{HTdR}$ incorporation in such targets. ASNs from the control were able to stimulate fresh splenocytes from both control and early-thymectomised animals. This is in agreement with Turner *et al.* (1991) who demonstrated that cells from early-thymectomised *Xenopus* (especially the non-Ig⁺ cell population) could proliferate well in response to both PHA- (phytohaemagglutinin) and MLC-(mixed leukocyte culture) induced supernatants. Since early-thymectomised animals should contain no T cells, this suggests that there are non-T / non-B [NK-like ?] cells present in these animals that are able to proliferate to cytokines (such as T cell growth factors [IL-2-like] that are found in ASNs of T mitogen-stimulated cells. (Watkins & Cohen, 1987; Haynes & Cohen, 1993). Interestingly, the early-Tx cells exhibit a slightly better proliferative response than the control cells, which could conceivably be due to either a high proportion of TCGF-reactive cells, or to an enhanced responsiveness of individual splenocytes in Tx frogs to respond to the ASNs.

In conclusion, it was likely that the Con A-derived ASNs generated in this study contained cytokines, particularly IL-2 or IL-2-like factors. Such ASNs are therefore potentially useful in promoting activation of natural killers (which are known to constitutively express IL-2 receptors, Kos & Engelman, 1996). The ability of such ASNs to potentiate spontaneous cytotoxicity of *Xenopus* lymphocytes towards tumour target cells was then addressed.

2.4.2 Tumour Cytotoxicity Mediated by *Xenopus* Splenocytes : Effects of ASN Treatment

Before the effects of the ASNs on the ability of splenocytes to lyse tumour targets could be determined, it was first necessary to assess the level of spontaneous cytotoxicity effected by *Xenopus* lymphocytes without added cytokine. Consequently, several experiments were performed assessing the ability of *Xenopus* splenocytes to lyse ⁵¹Cr-labelled B₃B₇ tumour cell targets (a *Xenopus* thymus tumour cell line obtained from Louis Du Pasquier and established in our laboratory - see Du Pasquier *et al*, 1992; Robert *et al*, 1994). These B₃B₇ cells fail to express MHC I and II proteins and, given that the presence of MHC proteins on target cells renders them resistant to NK cell cytolytic activity (Kärre *et al*, 1986; Ljunggren and Kärre, 1990; Storkus *et al*, 1989), are therefore good candidates as NK targets.

Fresh, untreated splenocytes from control animals exhibited only minimal spontaneous lysis of tumour targets, whereas those from early-thymectomised animals exhibited significant spontaneous cytotoxicity. These results are interesting since they suggest that the lymphocytes in thymectomised animals exhibit more 'natural-killer-like' cell activity than do those from control animals. This implies that *Xenopus* NK-like cells develop extrathymically. Furthermore, if natural killer cells

represent a primitive form of immune defence, active before T cells are fully developed (Janeway, 1989), then it might be expected that they play a greater role in a thymectomised animal (who has no T cells) than in a control (whose defence strategy is dominated by T cells). Natural killer cells may, therefore, be the means by which thymectomised animals can survive.

Several investigations have researched the effect of interleukin-2 on mammalian lymphocytes, in particular on natural killer cells. For example, Trinchieri, *et al* (1984) found that recombinant interleukin-2 (purified to homogeneity) induced a rapid and potent enhancement of the spontaneous cytotoxicity displayed by human peripheral lymphocytes. Furthermore, when the cells mediating the cytotoxicity were examined, they were found to have surface markers characteristic of natural killer cells, and were generated from the same lymphocyte subset that mediated NK activity. To the best of our knowledge, such cytokine activation of natural killer cell activity has not previously been investigated in *Xenopus*. Several experiments were performed here whereby splenocytes from both control and thymectomised animals were cultured with putative cytokine-rich Con A-derived ASNs, and then assessed for their cytolytic activity (against B₃B₇ tumour cells).

When control splenocytes were cultured for 48 hours with medium alone, cytotoxicity towards tumour cells was now readily apparent, suggesting that there is some factor in the medium which is stimulating the cells into becoming active killers. Quite possibly this phenomenon is actually due to a component of the medium stimulating the T cells present in the control animal - these stimulated T cells then produce cytokines, and it is these cytokines which could then promote NK-like cell activity. Consequently, any further addition of cytokines, i.e. from the ASN, has no effect because the cells are already highly stimulated.

When splenocytes from a thymectomised *Xenopus* were cultured with medium alone, the level of anti-tumour lysis was approximately the same as that

observed in Tx splenocytes assayed directly *ex vivo*, indicating that for these (T-deficient) lymphocytes, 48 hour culture in medium has no effect. However, when these Tx lymphocytes were cultured for 48 hours in ASN, there was a significant increase in cytolytic activity. The fact that Tx splenocytes cultured in ASN became especially effective killers of the tumour cells is interesting. This finding is indicative of lymphokine-activated killing in *Xenopus*, and lends support to the concept that the tumour cell lysis is mediated by NK-like cells.

2.4.3 Enrichment / Depletion of Spleen Cell Populations By 'Panning'.

One of the goals of this study was to enrich *Xenopus* splenocyte populations for natural killer cells, in order to obtain significant numbers of such purified cells for raising anti-NK cell antibodies, and for probing NK cell functioning. This was initially attempted through 'panning' with appropriate monoclonal antibodies.

Removal of B cells :

At the outset of these experiments, it was considered that if B cells could be removed from the spleen, this would offer an excellent way to eventually probe NK-like cells in Tx animals, which are already devoid of T cells. However, in order to avoid wasting valuable Tx frogs, initial experiments were, in fact, carried out on control animals, using the mAb 8E4 which binds to surface immunoglobulin (sIg) on B cells (Langeberg *et al*, 1987). Removal of B cells by panning was reasonably successful, although the purity of the panned populations was sometimes a little disappointing. ³HTdR assays of each of the three populations (non-panned, non-adherents and adherents), following stimulation with either Con A or LPS, confirmed that the separated lymphocyte subsets are still functional after panning. However, the very high counts for the adherent cells treated with medium alone (ie. no mitogen),

suggests that the panning procedure itself causes massive stimulation of the cells, which is likely to be a disadvantage of the technique. (It has been shown, in fact, that lymphocytes may be stimulated by treatment with anti-Ig - Schwager & Hadji-Azini, 1985. This is due to cross-linking of the receptors such that the cell thinks that it has contacted antigen).

In order to improve B cell removal through panning, an experiment was set up to examine the ability of a mix of anti-light chain mAbs (ALC - Hsu, Lefkovits *et al*, 1991) to remove B cells. Unfortunately, the mAb mix was not as successful as 8E4, since it failed to remove a significant proportion of B cells from the non-adherent population.

Removal of T Cells :

Panning experiments were also performed with the mAb 2B1 (against the CD5 antigen - Jurgens *et al*, 1995), as a means of depleting a cell population of T cells, since if this were successful, it might represent a useful means of obtaining a 'non-T' population without using up precious thymectomised animals. Panning with 2B1 was reasonably successful at producing a 'pure' adherent population of T cells. Unfortunately, it was not so successful at producing a fully T-cell-depleted non-adherent population since there were still 20% 2B1⁺ cells present. (One of the inherent problems with panning for T cells in control animals is that the large percentage of T cells present makes it extremely difficult to remove them all. Potentially, T-cell-panning would be a better technique for removing any residual T cells remaining in a thymectomised animal, to ensure a total lack of T cells). ³HTdR assays showed that all populations were functional after panning.

Panning experiments were also performed with AM22 (or AM22 - Flajnik *et al*, 1990 - and F17 - Ibrahim *et al*, 1991 - simultaneously) in an attempt to deplete the splenocyte population of CD8⁺ T cells. If these could be successfully removed, any

cytolytic activity observed in the remaining population could be assigned to a lymphoid subset other than the CD8⁺, putative cytotoxic cell subset (Flajnik *et al*, 1990). The procedure was quite successful at producing a purified CD8⁺ adherent population, but was inefficient at depleting all CD8⁺ cells from the non-adherents since there was still a high level of CD8⁺ contamination in this population. A ³HTdR assay following *in vitro* treatment with Con A demonstrated that both the non-panned and non-adherent populations were able to respond well, and are therefore still functional after panning, but the CD8⁺ adherents were not. This is extremely interesting, since it is known that in mammals, the main producers of IL-2 are CD4⁺ helper T cells (Roitt et al, 1989), with only a small amount being produced by CD8⁺ cells. Therefore, it is possible that the lack of stimulation in the adherent (CD8⁺) population is because these cells do not normally produce IL-2, and are therefore refractory to Con A stimulation.

This study was attempting to obtain an enriched NK cell population through the selective depletion of other lymphocytes (negative selection). Although the panning technique generally produced a reasonably pure adherent population, it was never possible to totally deplete the non-adherent population of any particular type of lymphocyte, regardless of which monoclonal antibody was used. It was therefore concluded that panning was not the ideal cell separation technique with regards to this study, and so magnetic bead separation was attempted instead.

2.4.4 Enrichment / Depletion of Cell Populations by Magnetic Bead Separation.

Immunomagnetic separation is becoming increasingly popular as a means of cell separation or cell enrichment, due to the variable ways it can be employed (direct or indirect, positive or negative selection - Funderud *et al.* 1987), and because of the excellent results it achieves. For example, Hansel *et al.* (1989) found that negative immunoselection on human blood produced eosinophils of greater purity and activity than those purified by other techniques they had attempted. Furthermore, such negative selection had no effect on the functionality of the resulting lymphocyte populations (Funderud *et al.*, 1987). Consequently, negative immunoselection of *Xenopus* lymphocytes was attempted via the indirect method, where cells are first sensitised with monoclonal antibody, and then magnetically separated with Dynabeads® coated with anti-Ig.

As with the panning experiments, initial trials attempted to remove B cells with the mAb 8E4, and required several modifications to determine optimum conditions. This series of magnetic cell separation experiments successfully determined the optimum conditions required for B cell depletion / enrichment of T cells, and these were as follows:- a) incubation of cells in 0.5ml of 8E4 at a 1 in 4 dilution, b) separation with a bead : cell ratio of 10:1, c) washing of adherents before removing them to culture, and d) overnight (17 hour) culture of populations before utilisation.

Following these optimum conditions, T cell removal was attempted using the mAb 2B1. Initial experiments using 2B1 at concentrations of 1 in 5 and 1 in 10 were extremely successful, reducing 2B1⁺ contamination in the non-adherent population to only minimal levels. Considering the large proportion of T cells that are present in

control animals, this is a highly effective technique for efficient T cell removal, and one that achieves far superior results than panning. This is in agreement with a review by Funderud *et al.* (1987) which cites very efficient depletion of T cells using both direct and indirect methods. Such good T cell depletion is beneficial because it allows a 'non-T cell' population to be generated (on which further separations can be made) without using up precious thymectomised animals.

Subsequent removal of T cells was attempted using AM22, a μ isotype antibody that recognises the CD8 antigen (Flajnik *et al.*, 1990). It was expected that separation might be very poor since the anti-Ig attached to the M-450 Dynabeads[®] recognises chiefly immunoglobulin of the γ isotype (and loosely the μ isotype - Dynal[®] product information guide), and therefore binding might not be strong enough to pull out the cells. However, beading managed to successfully remove about half of the CD8⁺ population.

On comparison of the two cell separation techniques investigated in this study, it was found that both panning and magnetic bead separation were able to deplete a splenocyte culture of B cells to the same extent, but that magnetic sorting produced far superior results for depletion of T cells. Furthermore, immunomagnetic separation had proved to be a much simpler and less time consuming technique than panning. Although it was not tested here, other workers have found that the functionality of the non-adherents is unaffected by the beading procedure (Funderud *et al.*, 1987). (Perhaps an additional experiment could be performed to confirm this with *Xenopus* lymphocytes by treating the resultant populations with Con A and LPS and assessing the stimulation in ³HTdR assays, as was performed for panning). Consequently, immunomagnetic separation utilising Dynabeads[®] M-450 was selected as the technique for generating enriched populations for further analyses.

2.4.5 Assessment of the Killing Activity of Cell Populations Enriched by Magnetic Bead Separation.

Although spontaneous cytolytic activity of allogeneic tumour cells had already been observed in *Xenopus* (see section 2.3.2), the specific cell population responsible for the activity had not been identified. Thus, in an attempt to locate this activity, control cells (from a *Xenopus* that had been primed with B₃B₇ tumour cells to boost the level of NK cells) were subjected to immunomagnetic separation under optimum conditions using either 2B1 or 8E4, and then assessed for their cytolytic activity against B₃B₇ cells in ⁵¹Cr-release assays.

Interestingly, the least spontaneous cytotoxicity was exhibited by the 2B1 adherents which were almost pure T cells (2B1⁺), suggesting that T cells are not responsible for such 'natural' killing. In comparison, the other 'less pure' populations ('unseparated', 2B1 non-adherents' and '8E4 non-adherents') all exhibited a much higher cytolytic ability. Interestingly, removal of either 2B1⁺ or 8E4⁺ cells from the splenocyte culture results in an elevated level of cytotoxicity, with depletion of 8E4⁺ cells resulting in the highest. This increase in killing in the non-adherent populations compared to the unseparated populations may be due to the removal of cells which do not contribute to natural killing, and thus the relative enrichment of 'killing' cells in the remaining sample (at the same E : T ratio). Had time allowed, it would have been interesting to assess the cytolytic activity of magnetic bead separated populations from thymectomised animals.

From these preliminary studies, it can be therefore be postulated that the lymphocytes responsible for natural killer activity in *Xenopus* are likely to be a 'non-T, non-B' lymphoid subset, which agrees with numerous studies on mammalian natural killer cells (see review by O'Shea and Ortaldo, 1992). In order to locate more specifically the natural killer cell population, monoclonal antibodies specific for



Xenopus natural killer cells are required. Since there is no mAb to *Xenopus* NK cells as yet, it might have been interesting to attempt isolation using the 5C6 mAb, a mouse mAb raised against catfish NK-like cells by Evans *et al.* (1988). This mAb is believed to identify receptors on human NK cells, but its suitability to probe *Xenopus* NK-like cells has recently been questioned (Horton *et al.*, 1996), especially as it binds a high proportion of *Xenopus* B lymphocytes.

The preparation of antibodies against *Xenopus* NK cell surface antigens is now clearly required. One method being considered in this laboratory is to use phage display technology for this purpose. In Chapter 3, preliminary molecular biology experiments, necessary to establish such technology, are outlined.

Chapter 3

Preliminary investigations into the development of 'Phage Display' technology for the generation of scFv fragments

3.1 Introduction

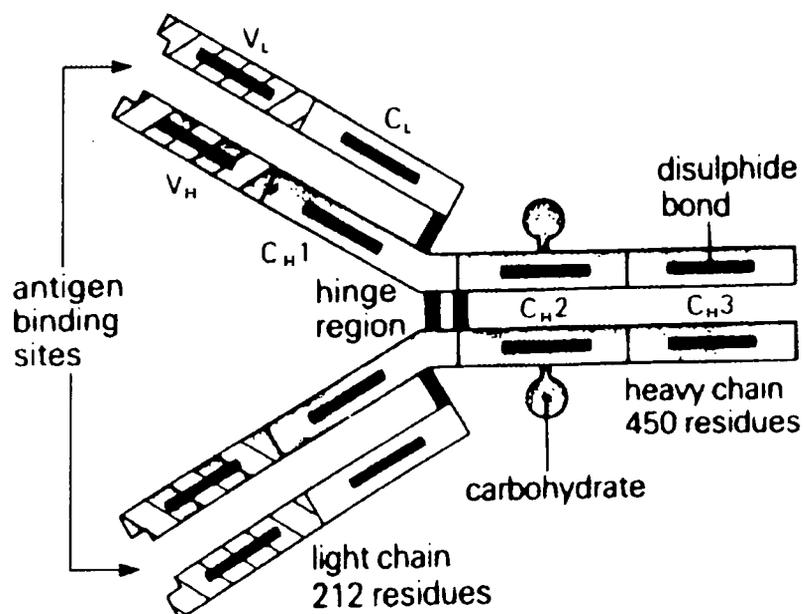
One of the long-term goals of natural killer cell study is to identify and characterize the cell surface markers that are involved in the recognition, binding and activation of the natural killer cells. Such a characterization would then allow a greater understanding of the mechanisms by which natural killer cells operate, and hence could potentially be exploited for therapeutic benefit. However, identifying cell surface markers, especially those which are involved in the killing activity of the cell, can prove difficult. One method of approaching this problem is to generate antibodies against the surface markers, and then to characterize these antibodies, identify what they bind to, and use them to modulate the activity of the surface markers. Such generation of antibodies is currently being attempted using the technique of 'phage display' (discussed in greater detail later), which mimics antibody production in the natural immune system.

In nature, organisms generate sufficient antibodies to recognize and combat any antigens that they might encounter during their lifetime, a number which far exceeds the number of genes available to code for them. This puzzling situation has

been solved by the discovery of several mechanisms that generate extra diversity from the genetic material available (reviewed by Hay (1989); Stryer (1988); Alberts *et. al.* (1989) and Mathews & van Holde (1990)):-

Antibodies (or immunoglobulins) are basically comprised of two identical heavy chains and two identical light chains linked together by disulphide bonds, and each chain contains both a variable domain and one or more constant domains (Figure 3.1). The first mechanism of generating diversity is that these variable and constant domains are encoded by separate segments of DNA with one or a few genes coding for the constant domain, and a very large number (several hundred) of genes coding for the variable domain. These different segments of DNA (which are often quite far apart in the germ line) are brought together during the development of a fully differentiated B cell, such that any one 'V' gene becomes joined to one 'C' gene.

Figure 3.1 : The basic structure of an immunoglobulin (IgG). (Taken from Roitt et al, 1989)



Diversity is further increased by the fact that the variable domain is not encoded completely by the 'variable' (V) genes. Instead, the last few amino acid residues are encoded by one of five 'joining' (or 'J') genes in the light chain, and by one of five 'J' genes plus one of fifteen 'diversity' (or 'D') genes in the heavy chain. These last 'J' and 'DJ' segments make an important contribution to diversity because they encode part of the last hypervariable region, which is involved in antigen binding. Mechanisms exist to ensure that like segments do not join, and that different segments join in the correct order, i.e. V genes are joined to J genes which are joined to C genes in light chains (and V to J to D to C in heavy chains). However, the accuracy with which joining occurs is not particularly precise, meaning that the same V and J genes could create three different amino acid sequences. This is known as recombinational inaccuracy. Finally, heavy chain genes are on different chromosomes to light chain genes, such that any light chain can combine with any heavy chain (therefore 10^4 different light chains capable of binding with 10^4 heavy chains would generate 10^8 different binding specificities). Added on to all these mechanisms, the fact that cells undergo somatic point mutation and change their amino acid sequence, and it is easy to see that nature has developed a complex and sophisticated process for generating vast antibody diversity.

Following their production, these antibodies circulate in the body until they contact antigen and are stimulated into proliferation. During this stimulation, further somatic mutation occurs which potentially generates antibodies of a higher affinity. Due to Darwinian selection over repeated cycles of antigen selection and somatic hypermutation, these higher affinity antibodies will be selected to survive in a process known as affinity maturation. Thus, from a limited pool of genetic material, nature can generate highly specific antibodies to what may be even rare antigens. Such a capacity would be extremely useful in scientific investigations and thus researchers are currently attempting to imitate these natural processes using 'phage display' technology. As an insight into how scientists have attempted to achieve this, a brief

history of the development of the technology is now given. (Reviewed by Nissim *et. al.* (1995), Winter *et. al.* (1994), Irving *et. al.* (1996) and Winter & Milstein (1991).

Original techniques for generating monoclonal antibodies involved 'hybridoma technology', in which antibody-producing cells were fused to myeloma cells, making them immortalized hybridoma cells, each one secreting homogenous antibody of a desired specificity. (Discovered by Milstein and Köhler in 1975 - see Stryer, 1988). As in the natural immune system, somatic cell mutation allowed some diversity to be generated, but the antibody repertoire was somewhat restricted, especially against self-antigens. The advent of gene technology began to change this because it meant that antibody genes could be manipulated and altered *in vitro* (Winter and Milstein, (1991) and references therein).

Initially, rearranged antibody genes were amplified from the hybridomas (using the polymerase chain reaction), cloned into plasmid vectors and expressed first as complete antibodies in mammalian cells (Winter and Milstein review (1991)), and then as antibody fragments in bacteria. For example, Skerra and Plückthun (1988) demonstrated that the Fv fragment of McPC603 (a monoclonal antibody against phosphorylcholine) could be produced in *Escherichia coli*. This Fv fragment was functional and had an identical affinity constant to the native McPC603. Their technique mimicked the assembly pathway of antibodies in eukaryotic cells, in that transport of the two variable domains (V_H and V_L) into the periplasmic space of *E. coli* (and their subsequent association) is functionally equivalent to the transport of the immunoglobulin chains into the lumen of the endoplasmic reticulum in eukaryotic cells (and their subsequent assembly into a whole antibody).

Although these soluble antibody fragments secreted from the bacteria could then be screened for binding activities, it was hypothesised that screening, especially for antibodies against rare antigens, would be greatly facilitated if the Fv fragments could be expressed on the surface of bacteriophage (phage) i.e. 'phage display'. In 1990, McCafferty *et. al.* constructed a single chain Fv fragment (scFv) of the anti-

lysozyme antibody D1.3 by joining the V_H and V_L domains with a flexible linker ($Gly_4 - Ser$)₃. This construct was cloned into an Fd phage vector at the amino terminal end of the gene III minor coat protein (3 or 4 copies of which are usually expressed at the tip of the phage), such that an antibody - gene III fusion protein was produced, anchoring the scFv protein to the outside of the phage coat. They then demonstrated that this 'phage antibody' bound specifically to lysozyme (with an affinity similar to that of the parent D1.3 antibody), and that affinity chromatography using lysozyme-Sepharose was capable of isolating rare phage (one in a million) through binding between the lysozyme and the displayed scFv D1.3 fragment.

Developing this technique further, the use of hybridomas as a starting point was superseded by the cloning of antibody genes directly from the lymphocytes of an immunized animal (Huse *et. al.*, 1989). The advantages of using such a heterogeneous cell population are that the repertoire of potential genes is much greater than in hybridomas and it mimics the natural immune system much more closely. Kang, Burton and Lerner (1991) used PCR to obtain the Fd ($V_H + C_{HI}$) and light chain genes from mouse spleen RNA, cloned them into separate λ expression vectors, (generating separate heavy and light chain libraries, just as the natural immune system has separate 'pools' of heavy and light genes), and then combined them randomly to generate single λ vectors containing one heavy and one light gene. This process (which produces functional antibodies that are found in the lysed bacteria of the phage plaque) mimics exactly the random shuffling of heavy and light chains that is used to generate diversity in nature. However, only 0.1 - 1% of the combinations could be accessed by screening.

Thus, Kang, Barbas *et. al.* (1991) went on to develop a more powerful technique for accessing these very large combinatorial libraries. Instead of using phage λ , they used the filamentous M13 bacteriophage which is related to Fd phage. In this system, Fd ($V_H + C_{HI}$) is fused to gene VIII (the major coat protein of M13 phage, with approximately 3000 copies per phage particle), such that several copies

of Fd become anchored along the entire length of the phage surface. The concomitant secretion of κ light chains into the periplasm allows functional Fab molecules to assemble and through binding to antigen, relevant phage can be selected. Each phage displays a single antibody species (Winter *et. al.*, 1994), and contains all the genetic material needed to code for that antibody, just as B-cells do in nature. Since M13 display multivalent Fabs, a wider range of affinities is able to be captured. Furthermore, since all the genetic material is present, the pool of selected antibodies can be mutated and further selected, or exposed to limiting amounts of antigen, such that higher-affinity antibodies (and the genes for them) are generated. This process is akin to affinity maturation in the natural immune system.

Finally, the systems used have been engineered to mimic the plasma cell in producing soluble antibody fragments (Irving *et. al.*, 1996; Winter *et. al.*, 1994). To do this, amber stop codons have been incorporated at the site between the antibody fragment and the coat protein (pIII or pVIII). When the phage is grown in suppressor bacteria, the amber codon is suppressed, the fragment becomes fused to the coat protein and is displayed on the surface. When the phage is grown in wild type bacteria, the amber codon is functional and the antibody fragment is secreted. Thus, technology has developed to imitate almost every stage of antibody production. (Figure 3.2). Furthermore, phage selection appears to be at least as powerful as immune selection.

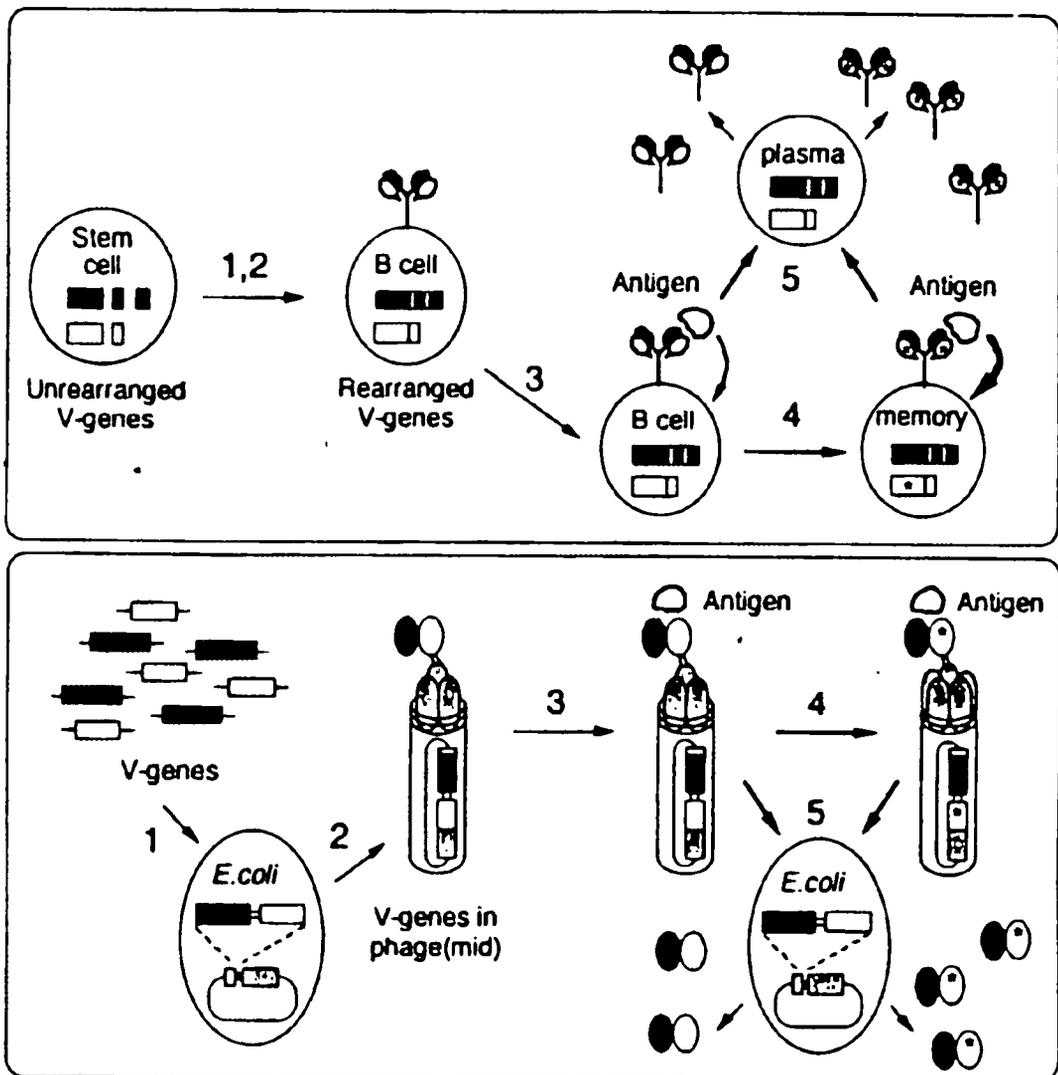
In this study, generation of recombinant antibodies (scFv fragments) was attempted using a modified M13 system (also developed in Kang's laboratory - personal communication). The details of the procedure are discussed in greater depth later (see section 3.4), but in brief, the technique involves immunization of mice with the relevant antigen (to boost levels of the appropriate V-gene repertoire), amplification of the heavy and light chain domains (generating 'pools' or libraries of different V genes), random combination of heavy and light chain genes (mimicking

natural random association) and selection of high affinity fragments through repeated rounds of antigen-selection and mutation (mimicking natural affinity maturation).

In order to learn the technique, and to simplify the results, initial experiments were performed using a single antigen - Carboxypeptidase Y. The aim was, that having perfected this technique on the CPY, the study would move on to generating antibodies against a cell population, and for this, the B₃B₇ *Xenopus* thymic tumour cell line was chosen. However, ultimately the aim was to immunize mice using pure natural killer cell populations (or at least the NK - enriched populations generated through the beading experiments detailed in Chapter 2), such that monoclonal antibodies could be generated against the natural killer cell surface markers. Unfortunately, neither time nor the success of the initial experiments allowed this goal to be realized, but it is still feasible for further study.

Figure 3.2 : Generation of antibodies by the immune system and phage technology. Steps : (1) rearrangement or assembly of germline V genes; (2) surface display of antibody (fragment); (3) antigen-driven or affinity selection; (4) affinity maturation; (5) production of soluble antibody (fragment).

(Taken directly from Winter, Griffiths *et. al.*, 1994)



3.2 Materials and Methods

3.2.1 Preparation of Oligonucleotides

Oligonucleotide primer pellets (prepared by John Gilroy, Department of Biological Sciences, University of Durham on ABI) were resuspended in 1ml sterile water, and their concentration determined by measuring their optical density at 260nm (OD₂₆₀). (Samples were measured as a 1 in 20 dilution in distilled water, and blanked against distilled water).

Since OD₂₆₀ 1.0 = 33 µg/ml for an oligo,
concentration of oligo = OD₂₆₀ x 33 x 20 µg/ml.

3.2.2 Immunisation of Mice

Two Balb c mice were used in each immunisation. 300µl PBS containing either 50µg Carboxypeptidase Y or 10⁷ B₃B₇ cells was injected intraperitoneally at a single site. Each animal was boosted with the same antigen (in 300µl PBS) 14 days later, and then killed a further 14 days later.

3.2.3 Removal of Spleen and Blood from Mice

All dissection equipment and eppendorfs had been pre-treated with either RNase-away or DEPC-water to remove any contaminating RNAses. Mice were anaesthetised by P. Hunter using ether. The spleen was then dissected out ventrally by M. D. Watson, transferred to a sterile 1.5ml eppendorf, and placed immediately on ice while the blood was being obtained. Blood was obtained by cardiac puncture

using a 2ml syringe and a 19-gauge needle (performed by P. Hunter). This was also transferred to a 1.5ml eppendorf and placed on ice.

If RNA extraction from the spleen was to be delayed for any reason, the spleen was then frozen and stored at -80°C until ready for use, otherwise, extraction was performed immediately. RNA extraction was performed using either the Scotlab Nucleon, Pharmacia, or Stratagene extraction kits according to the manufacturers instructions (see sections 3.2.5, 3.2.6 and 3.2.7 respectively).

3.2.4 Preparation of anti-serum from the Blood

The blood was placed in a 37°C heat block (Grant Instruments BT3) for approximately 3 hours to help it to clot. Then it was centrifuged for 20 minutes at full speed in a bench-top microcentrifuge (Hettich Mikroliter), and the supernatant (anti-serum) was removed to a sterile 1.5ml eppendorf. Sodium azide was added as a preservative to a final concentration of 0.1% v/v., and the samples were stored at -20°C until use.

3.2.5 Scotlab NUCLEON_{RNA} Extraction Kit

All equipment was pre-treated with either RNase-away or DEPC-water to remove any contaminating RNAses. The spleen was first chopped up into small pieces using a sterile scalpel, before being transferred to an homogeniser and homogenised in lysis solution. The rest of the protocol was performed according to the manufacturers instructions, except in section C1.2 of the Quicktrack RNA method. (Here, the alteration was to place the RNA-containing supernatant at -20°C for 40 minutes, then to centrifuge at $10,000g$ for 20 minutes at 4°C). Finally, the RNA pellet was resuspended in $100\mu\text{l}$ RNase - free water and stored at -20°C .

3.2.6 Pharmacia RNA Extraction Kit

All equipment was pre-treated with 'RNase-Away' or DEPC-treated water to remove any contaminating RNAses. After weighing, the spleen was chopped into small pieces with a sterile scalpel, transferred to an homogeniser and homogenised in extraction buffer (18ml/g tissue). The homogenate was then filtered through sterile glass wool (instead of centrifuging at 5000g for 20 minutes at 15°C, as this had been attempted and found to be ineffective), and then passed through a 19 gauge needle 20 times to shear the DNA. Ultracentrifugation of the sample was performed in a Beckman SW28 rotor for 16 hours, and then the RNA was recovered as described in the manufacturer's instructions, including the ethanol precipitation step. (RNA was stored at -20°C).

3.2.7 Stratagene RNA Extraction Kit

All equipment was pre-treated with 'RNase-away' or DEPC-treated water to remove any contaminating RNAses. After weighing, the spleen was either chopped up with a sterile scalpel and then homogenised, or pierced several times with a sterile needle and syringe and flushed with PBS until all the cells were out, and the spleen appeared 'white'. The rest of the protocol, which included a phenol : chloroform extraction and an ethanol precipitation, was followed exactly as per the manufacturers instructions, with no alterations. The RNA pellet was resuspended in DEPC-treated water and stored at -20°C.

3.2.8 Assay of RNA

RNA quality was assayed by spectrophotometry. A small aliquot (usually 5µl) of the RNA preparation was diluted 20 fold with sterile, DEPC-treated water, and its absorbance at 260nm measured on a spectrophotometer which had been

blanked against distilled water. Each sample was measured for its absorbance at 260nm (A_{260}) and its ratio of absorbance at 260nm to absorbance at 280nm (A_{260}/A_{280}). Each sample was also measured three times so that an average recording could be calculated.

3.2.9 1st Strand cDNA Synthesis

A reaction mix of 5 μ l total RNA, 3 μ l primer (either MVH-link *or* DB3 BLAB) and 6 μ l DEPC-treated water was incubated at 70°C for 10 minutes, then on ice for at least 1 minute. To this was added (from the BRL SuperScript Pre-amplification Kit) 2 μ l 10 x PCR buffer, 2 μ l 25mM MgCl₂, 1 μ l 10mM dNTP, 2 μ l 0.1M DTT, and 1 μ l SuperScript II Reverse Transcriptase. On a Techne PHC-3 thermal cycler, this mixture was then incubated at 26°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes, then 4°C for at least 1 minute before the addition of 1 μ l RNase H (from kit) and a further incubation of 20 minutes at 37°C. This was stored at -20°C until use.

3.2.10 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a thermal cycler (PHC-3, Techne). Typically, each reaction contained ca.60 pmoles of each primer (detailed in Appendix A), 20 nmol of each dNTP, 1.5mM MgCl₂ PCR buffer (provided with the enzyme) and 1 unit of *Taq* DNA Polymerase (Gibco-BRL) in a final volume of 100 μ l in a 0.5ml microcentrifuge tube / eppendorf. The quantity of template in each reaction and the cycling conditions varied according to what was to be amplified, and are therefore described where individual PCR reactions appear in the results chapter. *Taq* polymerase was the last component to be added to the reaction before mixing by brief centrifugation. The reaction mixture was then overlaid with 100 μ l sterile mineral oil

to prevent evaporation during the heat cycles. After completion of the PCR, samples were stored at 4°C until ready for analysis by agarose gel electrophoresis.

3.2.11 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in either a Biorad (50ml) or Appligene (80ml) mini-gel system. Agarose powder (Gibco BRL) was added to the appropriate volume of 1 x TAE buffer (usually to a concentration of 2% w/v, but this was varied according to the size of DNA to be separated) and dissolved by microwaving. (50x TAE = 242g TRIS, 57.1ml glacial acetic acid, 100ml 500mM EDTA pH 8.0). After allowing this to cool, ethidium bromide was added to a final concentration of 0.5µg/ml, and the mixture was poured into a gel mould containing a well-former. This was left to set before being transferred to an electrophoresis tank and covered with 1 x TAE buffer containing 0.5µg/ml ethidium bromide. Molecular weight markers and DNA samples in loading buffer were loaded into separate wells, and electrophoresis was performed until the dyes had migrated an appropriate distance (For a 2% gel this was usually 90V for 1¼ hours). The gel was then photographed on a UV illumination apparatus, using a red filter and a Polaroid camera.

3.3 Results

3.3.1 Amplification of Kappa Chains

Initial experiments were performed on spleens from mice that had been injected with 300µl of PBS containing 50µg Carboxypeptidase Y (CPY), so that optimum conditions could be determined before using precious *Xenopus* lymphocytes.

Thus, two Balb c mice were injected with CPY, boosted 14 days later under the same conditions, and then had their spleens and some blood removed a further 14 days later as described in section 3.2.3. Both spleens were frozen at -80°C. Two days later, one of the spleens was removed from storage, weighed (140mg), and an RNA extraction was performed on it using the Nucleon_{RNA} Test Kit (as described in section 3.2.5.), storing the pellet at -20°C overnight.

1st strand cDNA synthesis was performed on this RNA using the DB3-BLAB primer to obtain the Kappa chain cDNA. Six reaction mixtures were then prepared for PCR, each one containing 2µl of the DB3-BLAB cDNA and 3µl of one of the light chain primers (0.1µg/ml), before being treated to 91°C for 1 min 15 sec and 72°C for 1 min 30 sec, ending at 4°C until ready for use. 10µl of each PCR product was then analysed by agarose gel electrophoresis (2% agarose, Biorad), and the results are shown in Figure 3.3. As Figure 3.3 demonstrates, nothing could be seen on this gel, not even any of the primers, which suggested that the primer working solutions were too dilute for amplification to work. Consequently, different dilutions of the light chain primers were analysed by agarose gel electrophoresis, and it was

found that only the 'stock' primers were visible on the gel (results not shown), and therefore only these primers were suitable for amplification purposes.

Thus, a repeat PCR was set up, following the same conditions as just described except that the stock primers (variable concentration) were used instead of the 0.1 µg/ml dilution. However, although there was a row of primers just visible at <100bp, there were no amplification bands present at all (expected at ca. 660bp) - results not shown. Given that all the light chain primers were visible, it was thought that the problem could have been with either the DB3-BLAB or the RNA, and so both were analysed by agarose gel electrophoresis (2% agarose, Biorad, 10 µl total RNA, and 5 µl stock DB3-BLAB). Figure 3.4 quite clearly shows a 'streak' of sample in the RNA lane (lane 3), which is characteristic of RNA, while nothing is visible in the DB3-BLAB lane (lane 7), suggesting that it is the DB3-BLAB primer that is the problem.

Fortunately, a second sample of DB3-BLAB primer was available (used successfully by M. D. Watson in The Scripps Institute, San Diego), and so another repeat 1st strand cDNA synthesis and Kappa PCR amplification was performed using this second sample (all other conditions the same). Unfortunately the experiment was again a failure, with no bands visible other than the primer bands at <100bp (results not shown). It was thus suspected that this DB3-BLAB was no good either, and had deteriorated over time. However, when it was analysed by agarose gel electrophoresis, it was quite visible with the other light chain primers (Figure 3.5, all stock solutions), and hence should be working fine. Given that all the primers were not a problem, and that RNA was present, it was hypothesised that it must, in fact, be the *quality* of the RNA which was poor. For instance, the RNA present may be in small fragments, such that the complete template region necessary for amplification is not intact, and therefore it would be impossible to amplify up the light chain genes successfully. Consequently, it was decided to extract the RNA from the second 'CPY' spleen using a different technique.

RNA extraction from the spleen (which weighed 120mg) of the second mouse injected with Carboxypeptidase Y was thus attempted using the Pharmacia RNA extraction kit (see section 3.2.6 for details), and the resulting sample assayed by agarose gel electrophoresis. Figure 3.6 demonstrates that the technique was clearly successful at producing a good RNA preparation, since the RNA sample is now readily visible on the gel, and the smear is very long, covering a wide range of molecular weights (unlike the Nucleon-prepared RNA which only appears at the lower molecular weights - less than 200 or 300 bp long - see Figure 3.4).

Thus, 1st strand cDNA synthesis was performed on this new RNA (using 2µl of the stock San Diego DB3 BLAB primer), and then the light chains were amplified up using the same PCR conditions as last described (using 2µl of the stock DB3 BLAB and Lc primers). 10µl of each PCR product was analysed by agarose gel electrophoresis, and the results are shown in Figure 3.7 . Faint bands were visible for Lc3 and Lc7 (tracks 5 and 13 respectively) at approximately 660bp, while even fainter bands were just visible at the same molecular weight for Lc1, Lc4 and Lc6 (tracks 3,7 and 11 respectively). Consequently, it was decided to optimise the conditions from these promising results by adjusting the annealing temperature during the PCR cycle.

For convenience, only two samples were chosen for the adjusted amplification - Lc1 (which had previously appeared as a faint band) and Lc3 (which had previously appeared as a faint clear band). The reaction mixture was prepared exactly as last described, but the cycling conditions were altered to either +3°C or -3°C on the annealing temperature.

i.e.	+3°C:	91°C	1 min		
		91°C	1 min	15 sec	} 35X
		55°C	1 min	15 sec	
		72°C	1 min	30 sec	
		4°C			

-3°C	91°C	1 min		
	91°C	1 min	15 sec	} 35X
	49°C	1 min	15 sec	
	72°C	1 min	30 sec	
	4°C			

10µl of each of the four PCR products was then analysed by agarose gel electrophoresis, and the results are shown in Figure 3.8 . There are no bands present for Lc1 at either of the adjusted temperatures, but there are bands clearly visible for Lc3 (at ca. 650/700bp) at both temperatures. The strongest Lc3 band is at +3°C, i.e. when the annealing temperature was increased by 3°C to 55°C. Consequently, a PCR was performed for all the Kappa light chains at +3°C annealing temperature, (all other conditions were the same as last described) and the products analysed by agarose gel electrophoresis. When the gel was viewed under the ultra-violet light, definite bands at ca. 660bp could be seen for Lc3, 4, 5 and 7 (tracks 5, 7, 9 and 13 respectively), with Lc3 being by far the strongest. However, these bands are not quite so clear on the polaroid photograph of the gel (see Figure 3.9). No bands were visible for Lc1 or Lc6 (tracks 3 and 11 respectively), although it is possible they were there, just extremely faint.

Given that bands were visible in most samples, an attempt was made to increase the amount of amplified product present by increasing the amount of total RNA used in the first strand cDNA synthesis. (i.e. increasing the amount of template available). Thus, 8µl total RNA (instead of 5µl) was mixed with 1µl DB3 BLAB primer, (the DEPC-treated water being used to adjust the volume), and the rest of the protocol was performed as described in section 3.2.9. As a trial PCR amplification of this cDNA was first only attempted for Lc1, 3 and 4. PCR was performed at +3°C annealing temperature, all other conditions being as previously described. 10µl of each of these PCR products was analysed by agarose gel electrophoresis (2% agarose, Biorad), and the results (shown in Figure 3.10) were found to be the best produced so far, with very clear, distinct bands visible for both Lc3 and Lc4 (tracks 5

and 7 respectively) at ca. 700bp. Consequently, the first strand cDNA synthesis and PCR was repeated for the remaining Kappa light chains (Lc5, 6 and 7), except that the amount of total RNA used in the cDNA synthesis was again increased, to 10 μ l. As Figure 3.11 demonstrates, bands were visible in all three samples at ca. 700bp, such that Lc3, 4, 5, 6 and 7 had all now been successfully amplified.

However, the strength of the visible bands was still fairly weak, with not nearly the quantity of product present as had been obtained previously by M. D. Watson. It was considered still, that the problem lay in the quality of the RNA template, in that there was not enough good condition RNA of sufficient length to allow successful amplification. Consequently, RNA extraction was finally attempted using the Stratagene RNA extraction kit (used successfully by M. D. Watson and others in San Diego). Furthermore, the extraction was performed on fresh spleens, ie. directly they were taken from the animal, (rather than being stored at -80°C first. This was because other researchers had found that freezing the spleens to -80°C before extracting the RNA, significantly decreased both the quality and quantity of RNA produced (Chris Secombes - personal communication).

As a trial, and simply to check that the extraction was successful, initial experiments were carried out on spleens (both weighing 120mg) taken from unimmunised mice. Also, two different methods of cell extraction were tested - the first spleen was chopped up into small pieces and homogenised in the same fashion as had been performed with both the Nucleon and Pharmacia extraction kits, this preparation was termed the 'minced' sample. The second spleen was pierced several times with a needle, and then repeatedly flushed out with PBS, until the spleen appeared 'white'; this preparation was termed the 'pierced' spleen. RNA extraction was then performed on both of these samples using the Stratagene kit (see section 3.2.7 for details). Each sample was then assayed for its RNA quality/quantity, and it was found that the 'minced' sample had an average A_{260}/A_{280} of 1.03, whereas the 'pierced' sample had an average A_{260}/A_{280} of 2.03 (Table 3.1). Given that the

Stratagene protocol states that the A_{260}/A_{280} should be at least 1.7, this would suggest that the 'pierced' RNA sample alone is of a sufficiently high quality to be suitable for further use. The low A_{260}/A_{280} ratio of the 'minced' RNA sample (which falls below the recommended level) may explain why the previous experiments have had only limited success - the rough chopping and homogenisation of the spleen may destroy some of the RNA, or cause it to fragment into small pieces.

Although the results of the RNA assay suggested that only the 'pierced' RNA preparation would produce any useful results, both samples were used in separate first strand cDNA synthesis and PCR amplifications of the Kappa chains. 10 μ l of each total RNA preparation was used with 1 μ l DB3 BLAB in the cDNA synthesis; the rest of the protocol being as previously described (section 3.2.9). PCR amplification was only performed for Lc1 and Lc3 in each case, and the annealing temperature was 55°C (i.e. +3°C from the original conditions). All other conditions were as previously described. 10 μ l of each PCR product was analysed by agarose gel electrophoresis (2% agarose, Biorad), and the results are shown in Figure 3.12. Bands were visible in all four samples - at ca 550bp for Lc1 (both 'minced' and 'pierced' - tracks 3 and 7 respectively), and at ca. 660bp for Lc3 (both 'minced' and 'pierced' - tracks 5 and 9 respectively) - although the bands present in the 'pierced' sample are significantly more intense (hence more product) than those in the 'minced' sample (particularly noticeable when comparing 'pierced' Lc3 - track 9 - with 'minced' Lc3 - track 5). This is perhaps not surprising given that the 'pierced' RNA was of a much higher quality, and therefore there was 'more' template available for the PCR to work successfully.

Consequently, this PCR was repeated under exactly the same conditions, except that only the 'pierced' RNA was used, for all the Kappa light chains. Figure 3.13 illustrates the results of this experiment, and shows that bands are visible at ca. 700bp for Lc3, 4, 5 and 7 (tracks 5, 7, 9, and 13 respectively), with particularly strong bands for Lc4 and 5. There is a band present for Lc1 (track 3), but at ca.

590bp it is perhaps a bit smaller than would be expected. No bands are visible for Lc6 (track 11), although it is possible that product is there - it just may be very faint. It is further likely that all of the bands could be made more intense (i.e. increasing the amount of PCR product present) by adjusting various conditions and concentrations. However, at this point it was decided to leave the conditions as they were, and to attempt amplification on the experimental spleens, which may contain a higher titre of antibodies anyway.

Finally, amplification was attempted on spleens from two mice that had been injected with B₃B₇ *Xenopus* thymus tumour cells. Splenocyte preparations were produced by piercing the spleens, and RNA was extracted using the Stratagene RNA extraction kit. Each sample was resuspended in 50µl of DEPC-treated water, and then the two samples were pooled. A spectrophotometric RNA assay of the pooled samples showed an average A_{260}/A_{280} of 1.9826 (Table 3.2), which is above the recommended 1.7 for suitable quality RNA. Thus, first strand cDNA was synthesised from this RNA, using 10µl total RNA and 1µl DB3 BLAB - all other conditions being as previously described. PCR amplification was then performed for all the kappa light chains (at an annealing temperature of 55°C, i.e. +3°C, all other conditions as previously described), and the products were analysed by agarose gel electrophoresis. As Figure 3.14 quite clearly demonstrates, the experiment was extremely successful, with very intense bands present at ca. 700bp for Lc 3, 4, 5, 6 and 7 (tracks 5, 7, 9, 11 and 13 respectively). Unfortunately, there were still no bands present in Lc1, which suggested that maybe there were no antibodies of this particular type generated during the immunisation procedure. Thus it was established that the conditions used in this last experiment were the optimum for amplification of kappa chain genes, and hence these products were at a stage where they were ready for pooling and cloning to continue with the protocol.

3.3.2 Amplification of V_H Chains

Most of the initial experiments attempting to amplify the V_H chains were performed alongside those for the kappa chains, and hence the details of and reasons for doing many of the V_H experiments will not be given here - instead refer to section 3.3.1. (Sometimes, for convenience, experiments were only performed for the kappa chains, and are therefore not described here for V_H chains). Briefly, the results were as follows :

RNA extracted (using the Scotlab Nucleon_{RNA} Test Kit) from the spleen of a mouse that had been injected with CPY, was subjected to first strand cDNA synthesis using the MVH-link primer (0.1µg/ml) to obtain the V_H chain cDNA. Nine reaction mixtures were then prepared for PCR, each one containing 2µl of the MVH-link cDNA and 3µl of one of the V_H primers (0.1µg/ml), before being treated to 91°C for 1 min, and then cycled 35 times through 91°C for 1 min 15 sec, 48°C for 1 min 15 sec, and 72°C for 1 min 30 sec, ending at 4°C until ready for use. Agarose gel electrophoresis of the PCR products revealed that the amplification had not worked since there were no bands visible (Figure 3.15). When the *stock* primers were analysed by agarose gel electrophoresis, it was found that all the primers were present and visible, and therefore should not be the cause of the problem. Consequently, RNA from the second 'CPY' spleen was extracted using the Pharmacia RNA extraction kit, which generated lots of RNA (refer to Figure 3.6). First strand cDNA was synthesised as first described, with 5µl total RNA, and PCR amplification was performed for all nine V_H primers (using 2µl of each stock primer, all other conditions being as previously described). Unfortunately, no bands of the expected length - ca. 440bp - were visible (see Figure 3.16). Instead, there was a mixture of bands present :- at ca. 290bp for Hc 1, 3 and 7 (tracks 3, 5 and 9 respectively), and at ca. 190bp for Hc 2 and 4 (tracks 4 and 6 respectively).

Although these bands were in the wrong position, it was more promising than none at all, and so an attempt was made to improve these results by adjusting the annealing temperature of the PCR by +3°C and +6°C (all other conditions as before).

i.e. +3°C : 91°C 1 min
 91°C 1 min 15 sec
 51°C 1 min 15 sec
 72°C 1 min 30 sec
 4°C

 +6°C : 91°C 1 min
 91°C 1 min 15 sec
 54°C 1 min 15 sec
 72°C 1 min 30 sec
 4°C

For convenience, PCR was only performed on Hc1 and Hc2 but, as Figure 3.17 demonstrates, increasing the annealing temperature has no positive effects - in fact, the results are worse, with only vague bands for Hc2 at ca. 190bp, and nothing for Hc1. PCR was thus attempted with an annealing temperature of 46°C, i.e. +2°C, but again the results were not as good as the original 48°C (a band is visible at ca. 290bp in Hc1, but it is slightly less intense at 46°C than 48°C - see Figure 3.18), and thus the rest of the experiments were annealed at 48°C.

Given that the concentration of MgCl₂ in the reaction buffer can be critical to the success of a PCR amplification, an experiment was performed to assess the effect of varying the MgCl₂ concentration. Four reaction mixtures of Hc1 were prepared, with either 1.5mM, 3.0mM, 6.0mM or 9.0mM MgCl₂. (All previous PCR amplifications have used 1.5mM MgCl₂, which is the concentration in the supplied PCR buffer). The volume of water was adjusted in each reaction so that the final volume was still 100µl, and all other conditions were as previously described. However, the experiment was again a failure, with no bands present whatsoever (not even at the wrong base pair length), results not shown.

At this point, relative success had been achieved with the kappa chains by increasing the amount of total RNA used in the first strand cDNA synthesis, and thus, this was attempted for the heavy chains too, increasing the amount of total RNA used to 10µl. PCR was performed on Hc1, 2 and 3, using 1.5mM MgCl₂ and 1µl stock primers, and the products were analysed by agarose gel electrophoresis. Disappointingly, increasing the amount of total RNA used did not improve the results (see Figure 3.19) - there are bands visible at ca. 290bp for Hc1 and Hc3 (tracks 3 and 9 respectively) which are not the bands required, and there are no bands present at all for Hc2 (track 5).

Work then continued on spleens from unimmunised mice, with the RNA being extracted using the Stratagene kit, immediately after the spleen was removed from the animal. PCR amplification for all the Hc primers produced a mixture of different sized bands, most of which were the wrong size (Figure 3.20). However, if studied very carefully, a row of faint bands could be seen at ca. 440bp (i.e. the expected length), particularly in Hc1, 3, 6 and 7 (tracks 3, 5, 7 and 8 respectively). Given that these were the most promising results produced so far, and given that even better results had been achieved for the kappa chains on the experimental spleens, it was decided to attempt V_H amplification on the RNA from the B₃B₇-injected mice. (See section 3.2.2. for details of RNA production).

First strand cDNA was synthesised using 10µl total 'B₃B₇' RNA and 60 picomoles of new MVH-link (synthesised by Perkin Elmer, 30 picomoles/µl), all other conditions being as previously described. PCR amplification was performed for all Hc primers under the original conditions described, except for the use of the new MVH-link (used 60 picomoles per reaction). As Figure 3.21 demonstrates, there is a clear band at approximately the correct position (400bp instead of 440bp) for Hc2 (track 4), but unfortunately not for all the others. (Most of the others do have bands in them, but not at the correct length). Attempts were made to improve upon these results by both adjusting the annealing temperature and increasing the amount of Hc

primer used in each reaction. (It was postulated that the concentration of even the stock Hc primers was not sufficiently high to allow successful amplification).

Consequently, PCR was attempted for all Hc primers, using 4 μ l of the appropriate primer (adjusting the volume with the water), and annealed at either 50°C, i.e. +2°C (Figure 3.22) or 56°C, i.e. +8°C (Figure 3.23). Agarose gel electrophoresis of all the PCR products revealed that neither of the experiments had been successful. Although there were several bands present, with particularly strong ones at ca. 290bp for Hc 3, 7 and 9 at both temperatures, there were no bands of the correct position (440bp) in any of the samples. These results were extremely disappointing, since at this point, all the kappa chains were successfully amplified and ready to pool.

Figure 3.3 : Agarose gel electrophoresis of the Kappa chain PCR products obtained from the spleen of a mouse immunised with Carboxypeptidase Y. PCR was performed for all the Lc primers, at 0.1µg/ml.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc1;
- Lane 4 = Lc3;
- Lane 5 = Lc4;
- Lane 6 = Lc5;
- Lane 7 = Lc6;
- Lane 8 = Lc7.

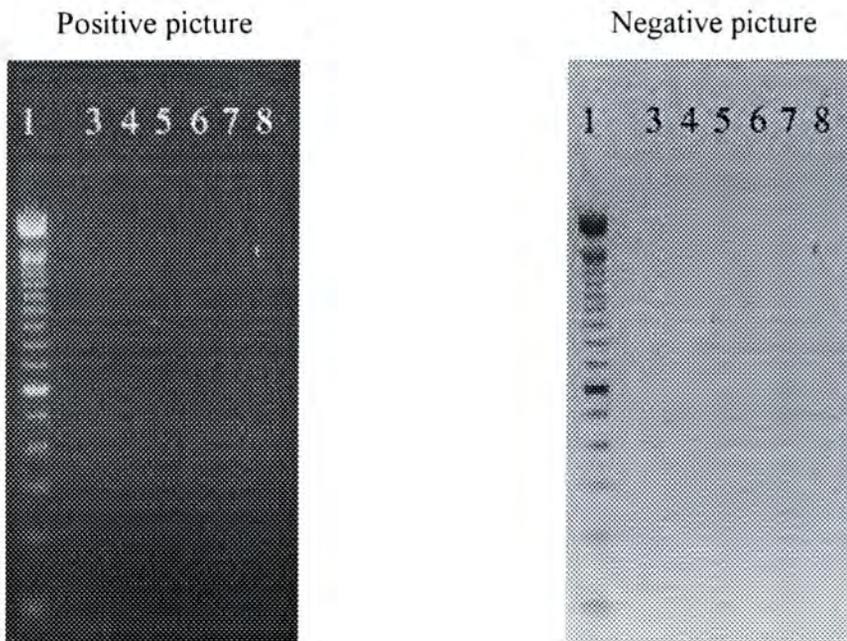


Figure 3.4 : Agarose gel electrophoresis of the RNA extracted using the Scott Nucleon_{RNA} Test Kit from the spleen of a mouse immunised with Carboxypeptidase Y; and of the DB3-BLAB primer.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = RNA;
- Lane 7 = DB3 - BLAB (stock strength).

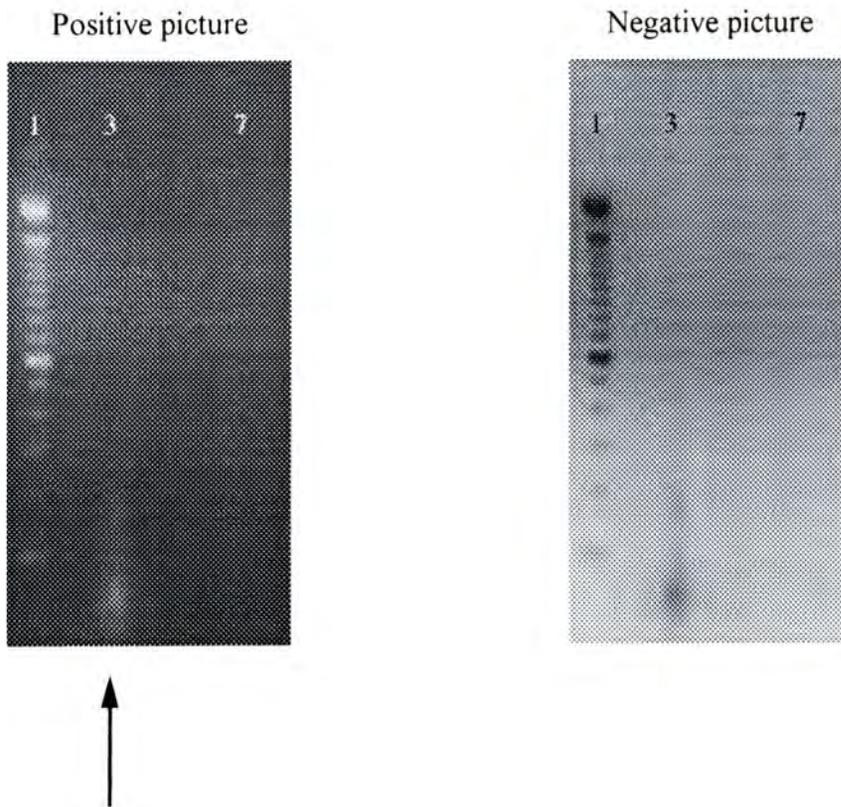


Figure 3.5 : Agarose gel electrophoresis of all the stock Lc primers and stock DB3-BLAB.

- Lane 1 = 100 bp molecular weight markers;
- Lane 3 = DB3 BLAB;
- Lane 5 = Lc1;
- Lane 7 = Lc3;
- Lane 9 = Lc4;
- Lane 11 = Lc5;
- Lane 13 = Lc6;
- Lane 15 = Lc7.

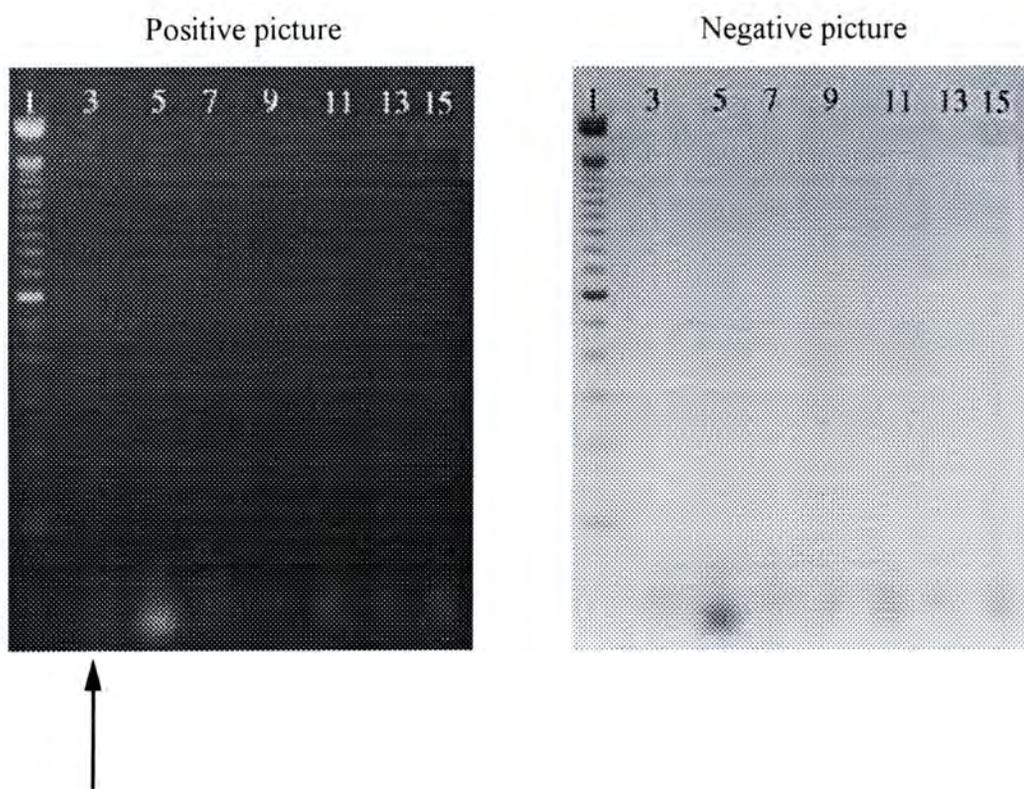
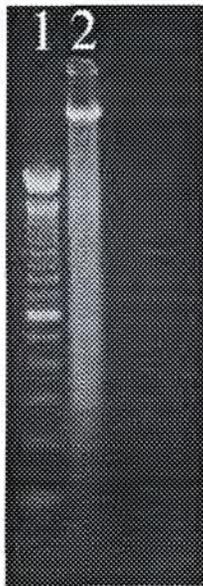


Figure 3.6: Agarose gel electrophoresis of the RNA extracted using the Pharmacia Kit from the spleen of a mouse immunised with Carboxypeptidase Y.

Lane 1 = 100 bp molecular weight markers;
Lane 2 = RNA.

Positive picture



Negative picture

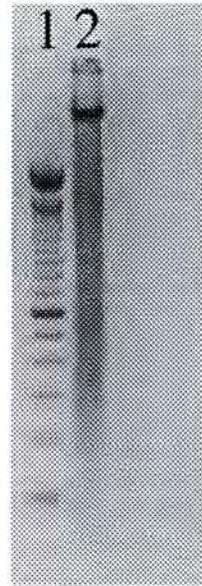


Figure 3.7: Agarose gel electrophoresis of the Kappa chain PCR products obtained from the Pharmacia-extracted RNA of the CPY-injected mouse.

PCR was performed with all the stock Lc primers and the 'San Diego' DB3 BLAB primer.

- Lane 1 = 100 bp molecular weight markers;
- Lane 3 = Lc1;
- Lane 5 = Lc3;
- Lane 7 = Lc4;
- Lane 9 = Lc5;
- Lane 11 = Lc6;
- Lane 13 = Lc7.

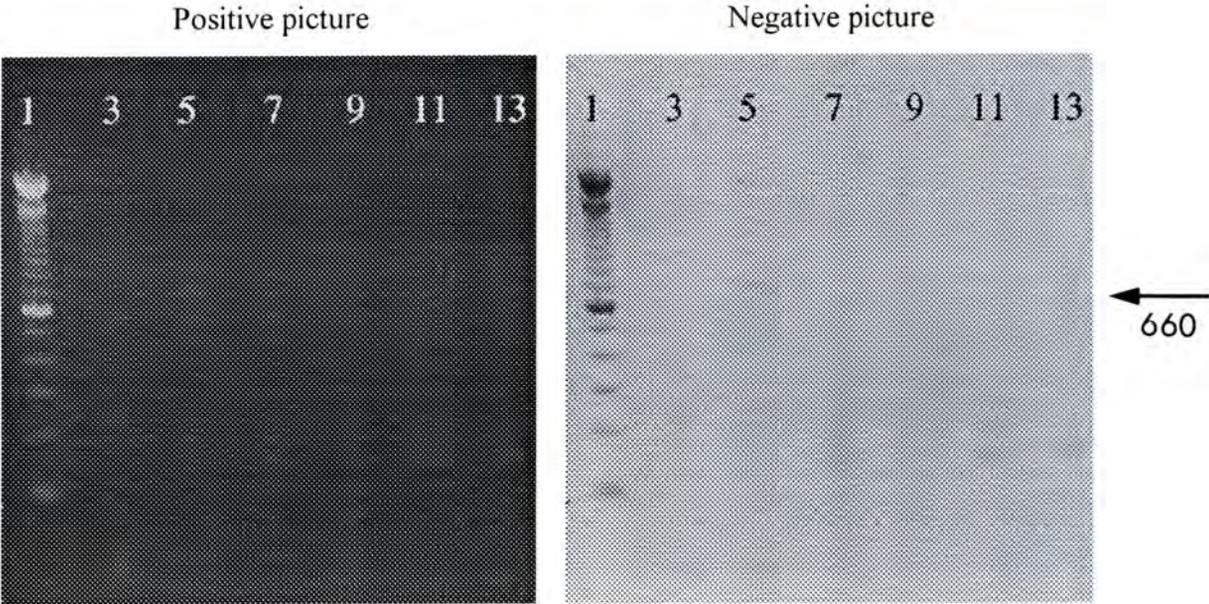


Figure 3.8 : Agarose gel electrophoresis of the Lc1 and Lc3 Kappa chain PCR products obtained with adjusted PCR annealing temperatures of +3°C and -3°C from the original 57°C.

RNA was extracted using the Pharmacia Kit from the CPY-injected mouse.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc1 at +3°C;
- Lane 5 = Lc3 at +3°C;
- Lane 7 = Lc1 at -3°C;
- Lane 9 = Lc3 at -3°C.

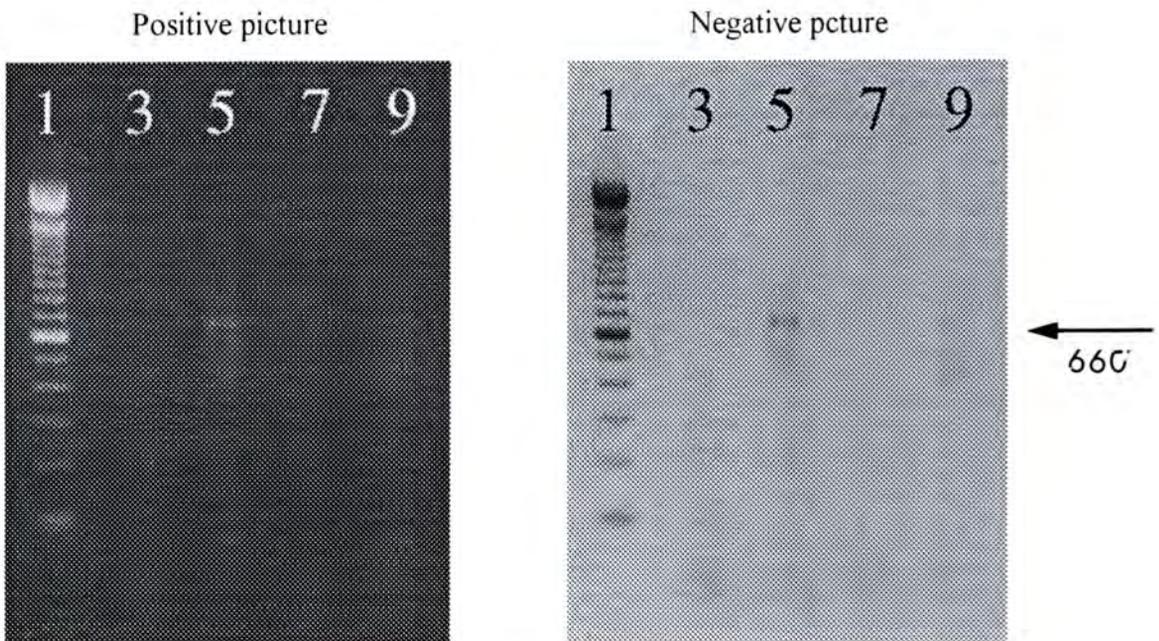


Figure 3.10 Agarose gel electrophoresis of the Lc1, Lc3 and Lc4 Kappa chain PCR products obtained using increased amounts of RNA in the initial cDNA synthesis.

8 μ l total RNA, Pharmacia-extracted from the CPY-injected mouse, was used instead of the 5 μ l used previously.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc3;
- Lane 5 = Lc3;
- Lane 7 = Lc4.

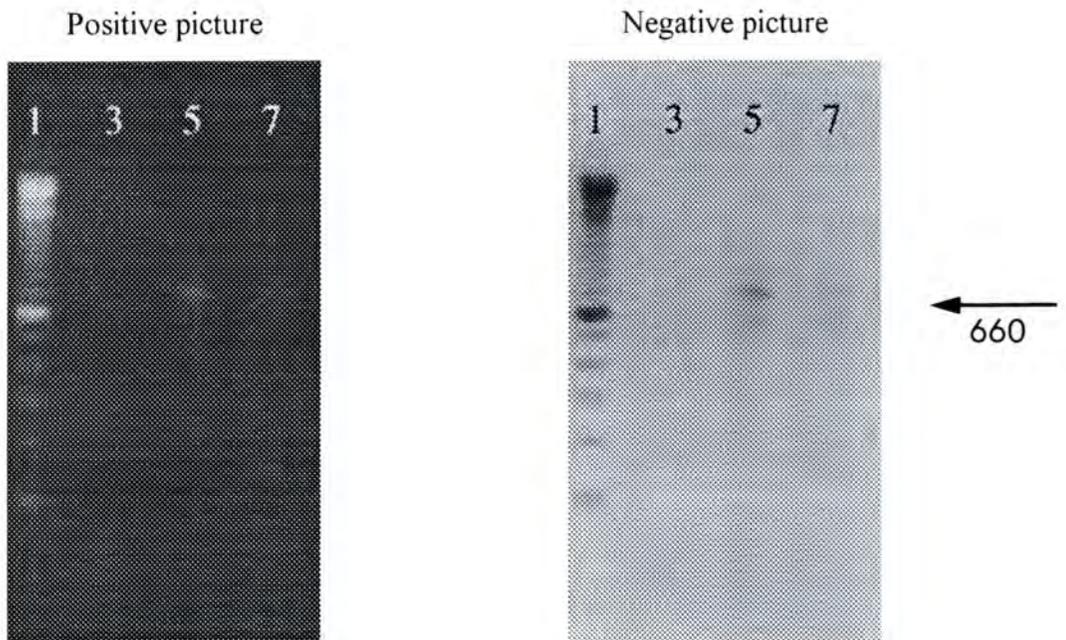


Figure 3.11 : Agarose gel electrophoresis of the Lc5, Lc6 and Lc7 Kappa chain PCR products obtained using increased amounts of RNA in the initial cDNA synthesis.

10µl total RNA, Pharmacia-extracted from the CPY-injected mouse, was used.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc5;
- Lane 5 = Lc6;
- Lane 7 = Lc7.

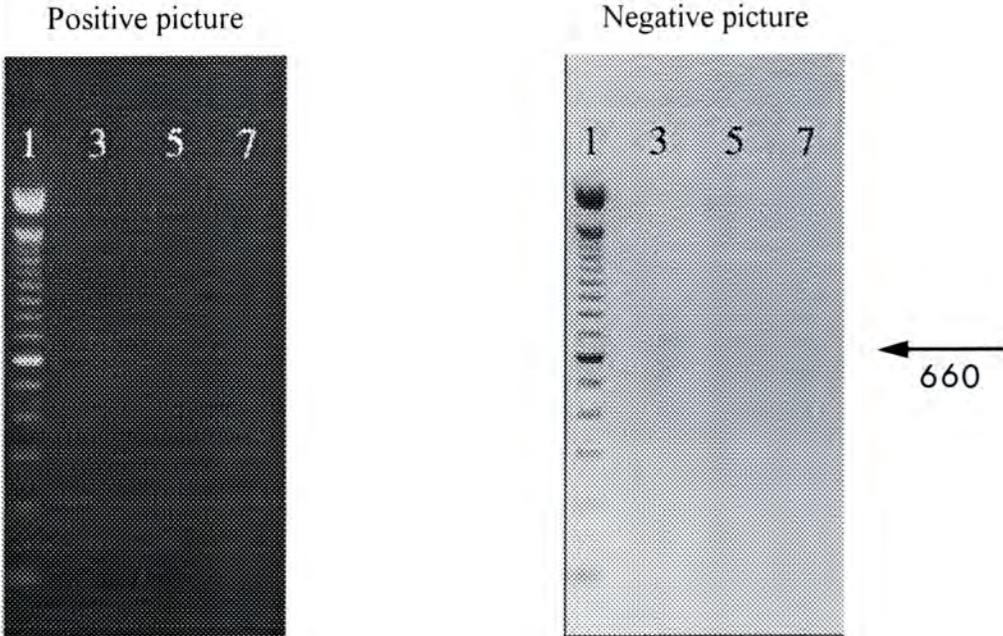


Table 3.1 : Spectrophotometric assay of the RNA extracted using the Stratagene Kit from both 'minced' and 'pierced' *fresh* spleens of unimmunised mice.

Samples were blanked against distilled water, and their absorbance was measured three times, both at 260nm and 280nm.

Source of RNA sample	A ₂₆₀	A ₂₆₀ / A ₂₈₀	Average A ₂₆₀ / A ₂₈₀
Minced spleen	2.3697	0.9765	} 1.0347
	2.3005	1.0211	
	2.8780	1.1066	
Pierced spleen	1.1811	2.0258	} 2.0288
	1.1822	2.0371	
	1.1850	2.0235	

Figure 3.12 : Agarose gel electrophoresis of the Lc1 and Lc3 Kappa chain PCR products obtained from RNA that had been extracted using the Stratagene Kit from either minced or pierced *fresh* spleens of unimmunised mice.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc1 from a minced spleen;
- Lane 5 = Lc3 from a minced spleen;
- Lane 7 = Lc1 from a pierced spleen;
- Lane 9 = Lc3 from a pierced spleen.

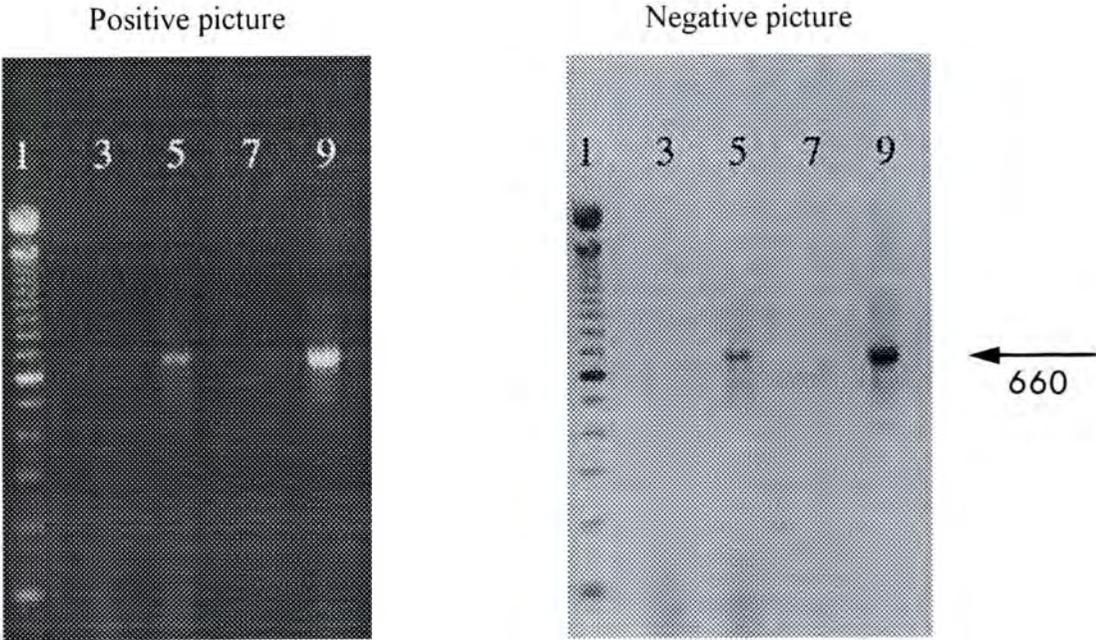


Figure 3.13 : Agarose gel electrophoresis of all Kappa chain PCR products obtained from Stratagene-extracted RNA from a fresh 'pierced' spleen of an unimmunised mouse.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc1;
- Lane 5 = Lc3;
- Lane 7 = Lc4;
- Lane 9 = Lc5;
- Lane 11 = Lc6;
- Lane 13 = Lc7;
- Lane 15 = 100bp molecular markers.

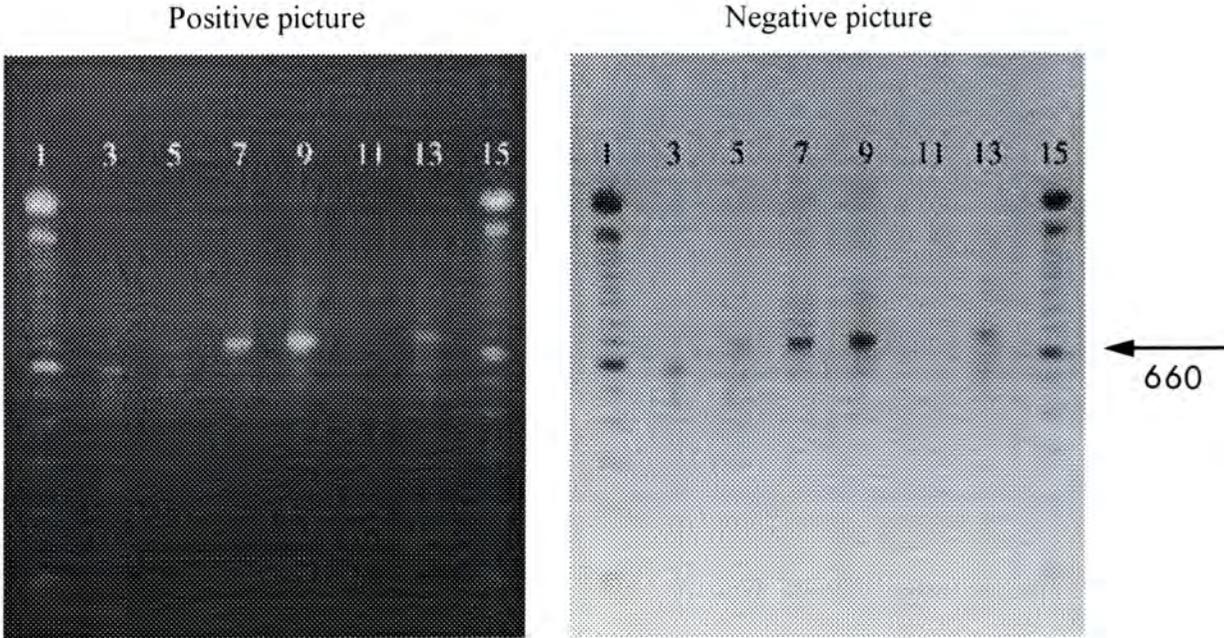


Table 3.2 : Spectrophotometric assay of the RNA extracted using the Stratagene Kit from the ‘pierced’, fresh spleens of mice immunised with *Xenopus* B₃B₇ tumour cells.

Sample was blanked against distilled water, and it’s absorbance measured four times both at 260nm and 280nm.

Source of RNA sample	A ₂₆₀	A ₂₈₀	A _{260 / 280}
Pierced spleens of B ₃ B ₇ - injected mice	0.5670	1.9914	} 1.9826
	0.5578	1.9739	
	0.5514	1.9683	
	0.5578	1.9667	

Figure 3.14 Agarose gel electrophoresis of all Kappa chain PCR products obtained from Stratagene-extracted RNA from the fresh spleen of a mouse immunised with *Xenopus* B₃B₇ tumour cells.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Lc1;
- Lane 5 = Lc3;
- Lane 7 = Lc4;
- Lane 9 = Lc5;
- Lane 11 = Lc6;
- Lane 13 = Lc7;
- Lane 15 = 100bp molecular weight markers.

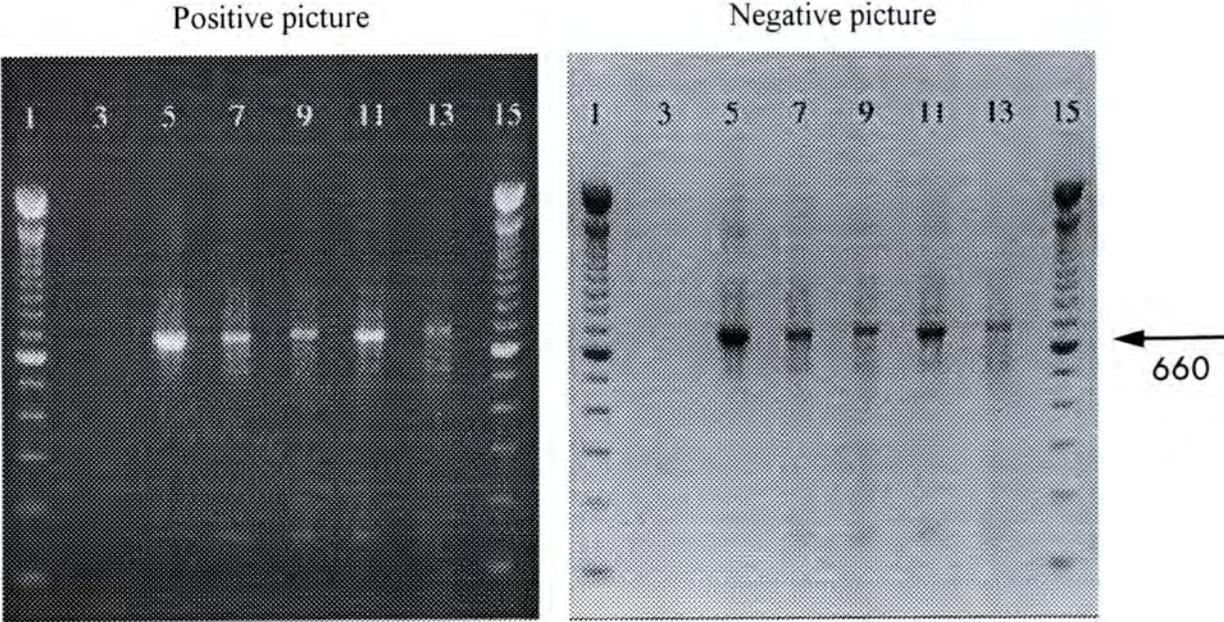


Figure 3.15 : Agarose gel electrophoresis of all V_H PCR products obtained from the spleen of a mouse immunised with Carboxypeptidase Y.

RNA was extracted using the Scott Nucleon_{RNA} Test Kit, and PCR was performed with 0.1µg/ml primers.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9.

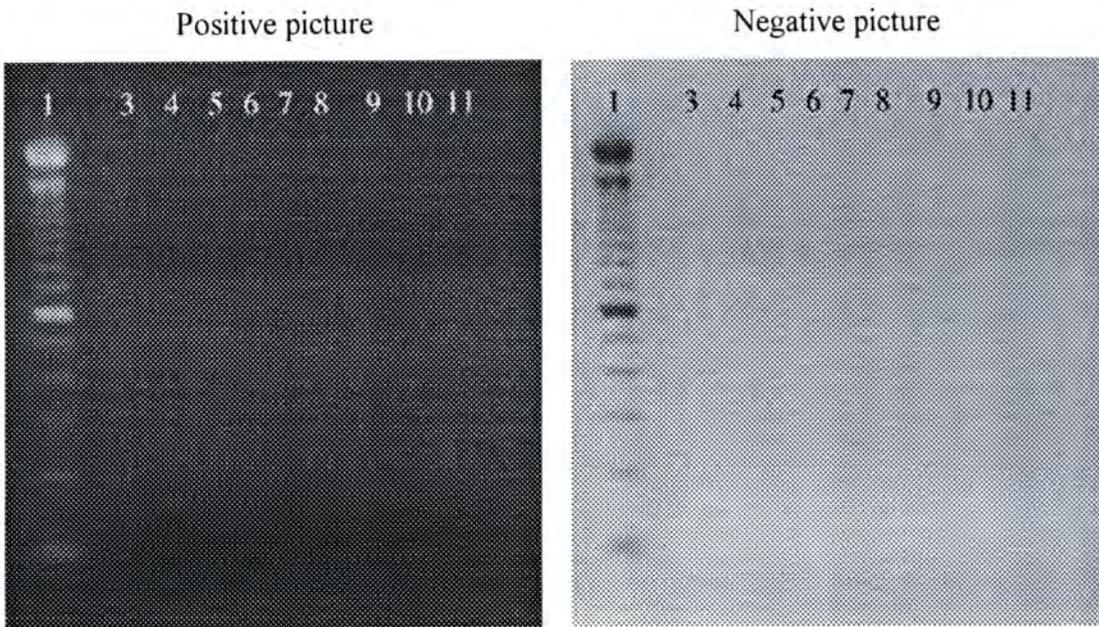


Figure 3.16 : Agarose gel electrophoresis of all V_H PCR products obtained using stock primers on Pharmacia-extracted RNA from the spleen of a mouse immunised with Carboxypeptidase Y.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9.

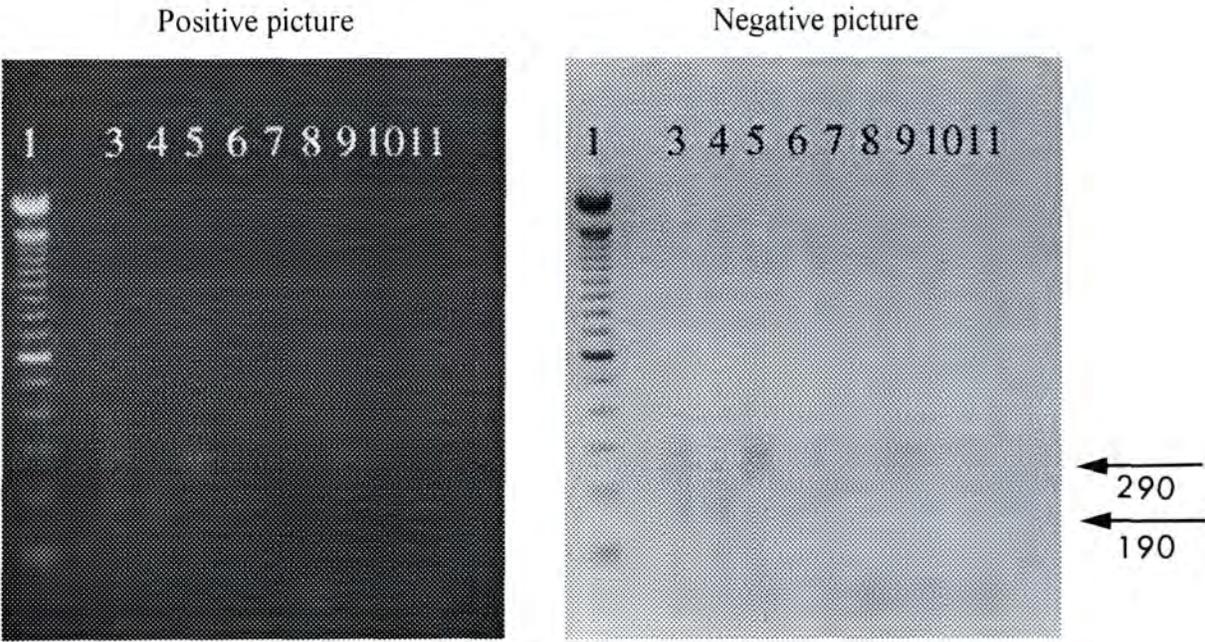


Figure 3.17 : Agarose gel electrophoresis of the Hc1 and Hc2 PCR products obtained with adjusted annealing temperatures of +3°C and +6°C from the original 48°C.

RNA was Pharmacia-extracted from the CPY-injected mouse.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1 at +3°C;
- Lane 5 = Hc1 at +6°C;
- Lane 7 = Hc2 at +3°C;
- Lane 9 = Hc2 at +6°C.

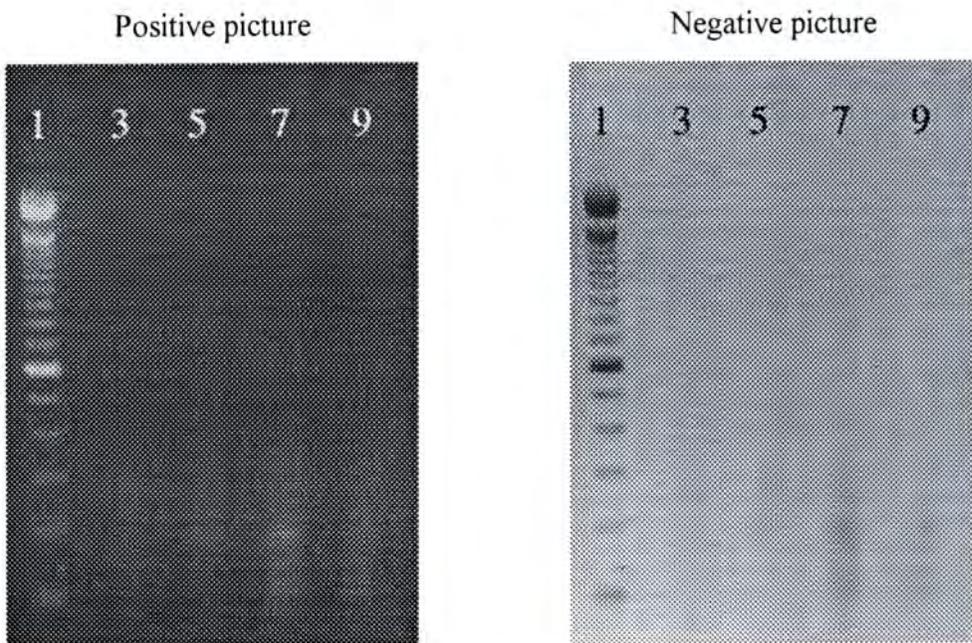


Figure 3.18 : Agarose gel electrophoresis of the Hc1 and Hc2 PCR products obtained at both the recommended annealing temperature of 48°C, and at 46°C (ie. - 2°C).

RNA was Pharmacia-extracted from the CPY-injected mouse.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1 at 48°C;
- Lane 5 = Hc2 at 48°C;
- Lane 7 = Hc1 at 46°C;
- Lane 9 = Hc2 at 46°C.

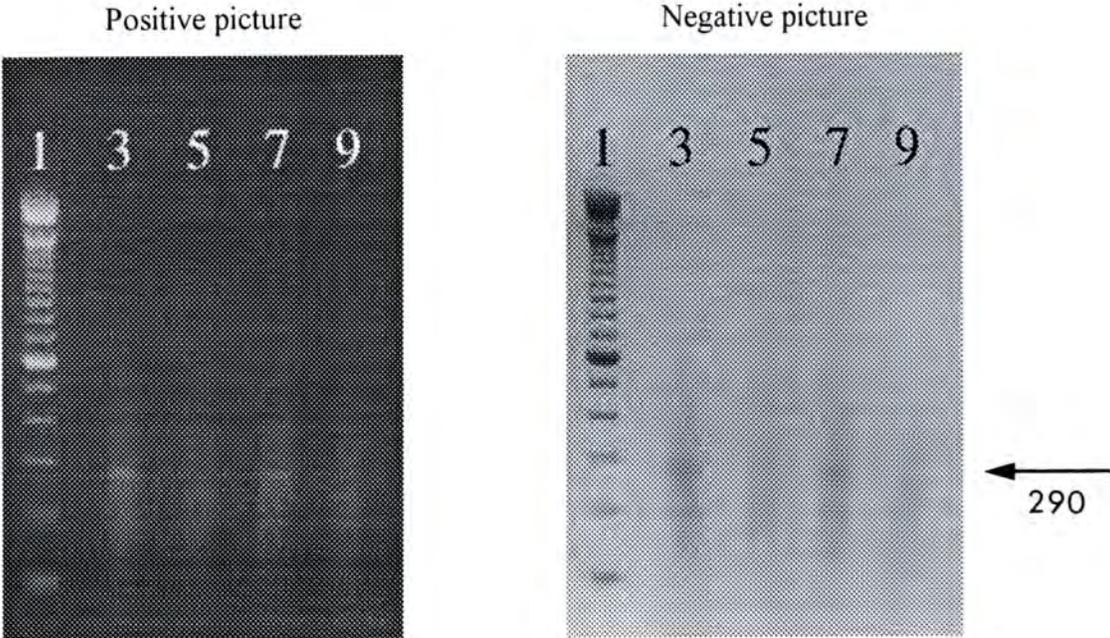


Figure 3.19 : Agarose gel electrophoresis of the Hc1, Hc2 and Hc3 PCR products obtained using increased amounts of RNA in the initial cDNA synthesis.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 5 = Hc2;
- Lane 7 = Hc3.

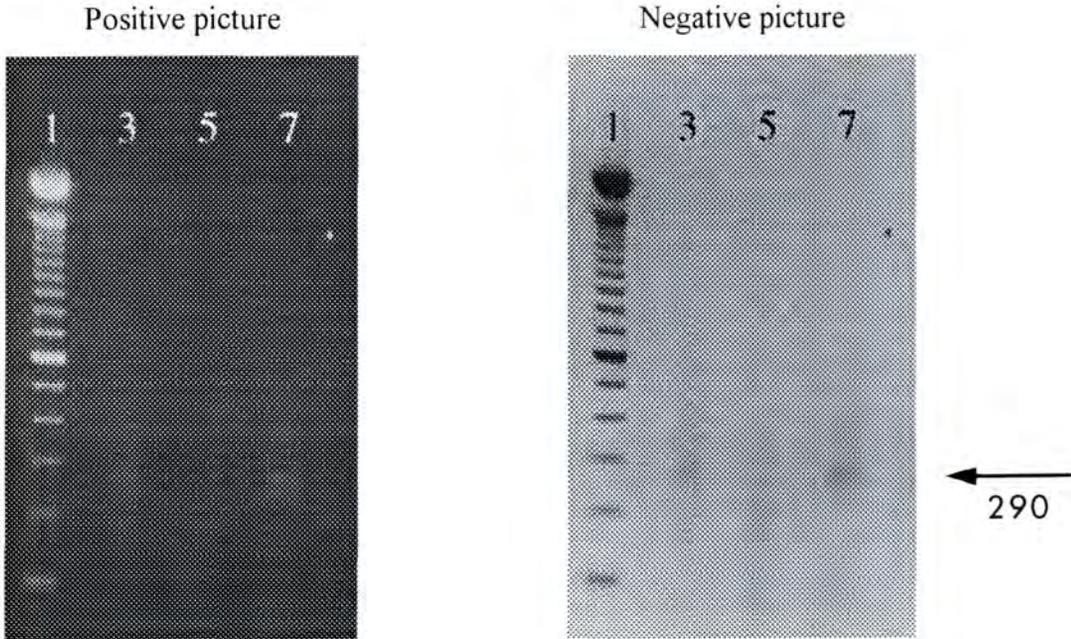


Figure 3.20 : Agarose gel electrophoresis of all V_H PCR products obtained from Stratagene-extracted RNA from a fresh 'pierced' spleen of an unimmunised mouse.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9;
- Lane 13 = 100bp molecular weight markers.

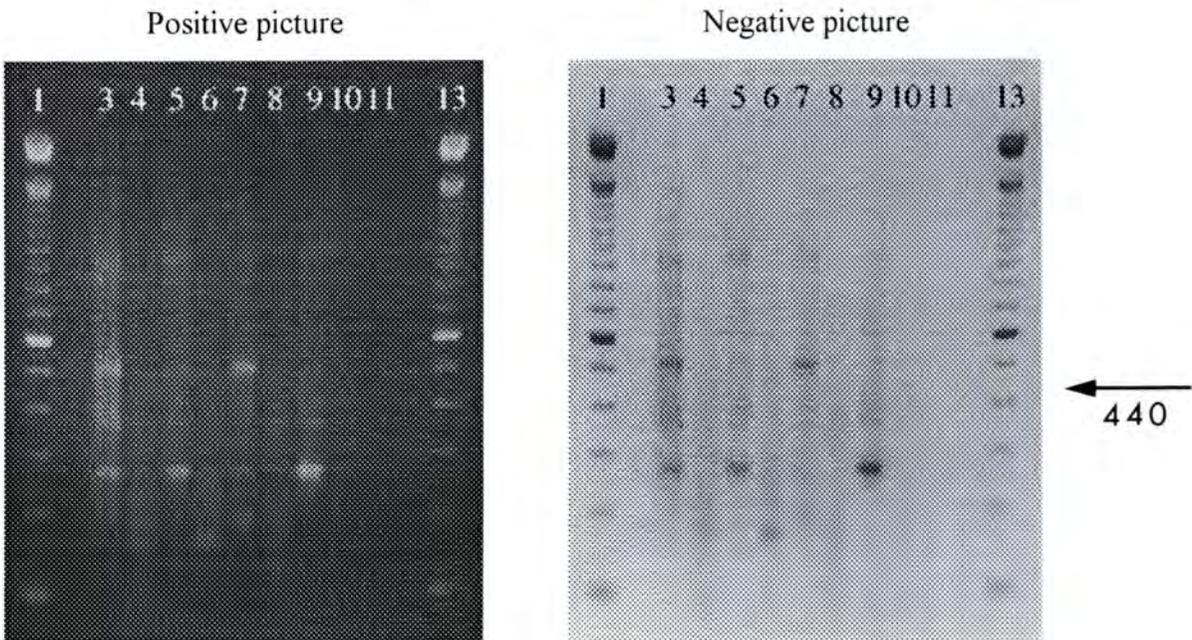


Figure 3.21 : Agarose gel electrophoresis of all V_H PCR products obtained from Stratagene-extracted RNA from the fresh spleen of a mouse immunised with *Xenopus* B₃B₇ tumour cells.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9;
- Lane 13 = 100bp molecular weight markers.

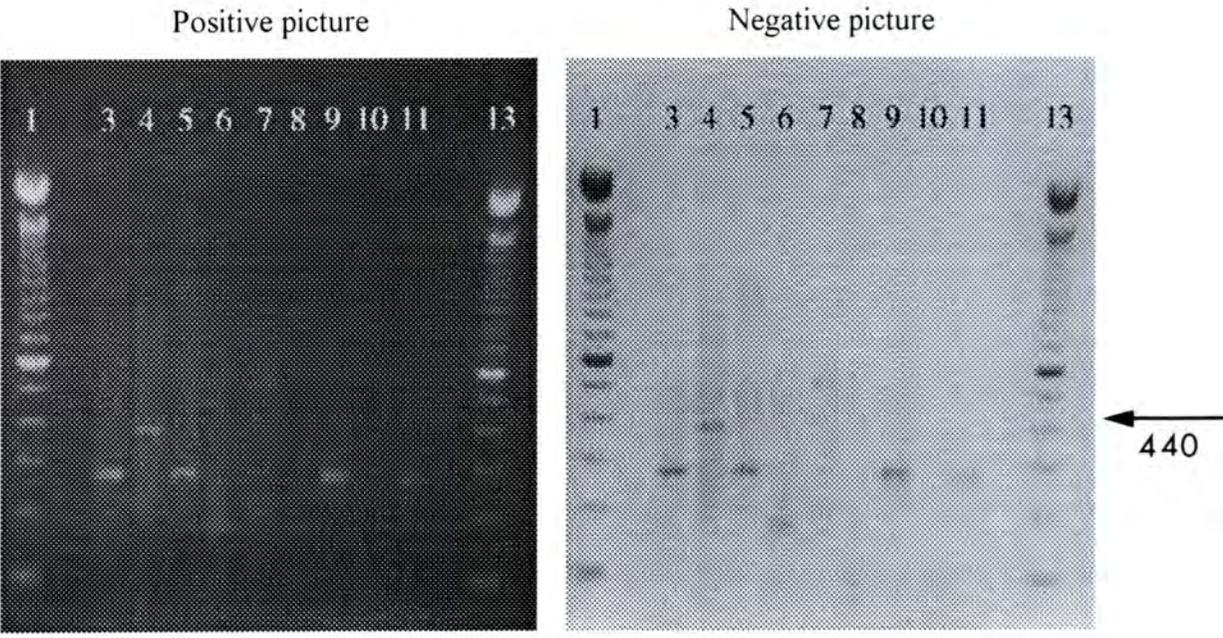


Figure 3.22 : Agarose gel electrophoresis of all V_H PCR products obtained using an increased amount of each Hc primer and an adjusted PCR annealing temperature of 50°C (ie. +2°C). RNA was Stratagene-extracted from the *fresh* spleen of a B₃B₇-injected mouse.

4µl of each stock Hc primer was used, rather than 1µl

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9;
- Lane 13 = 100bp molecular weight markers.

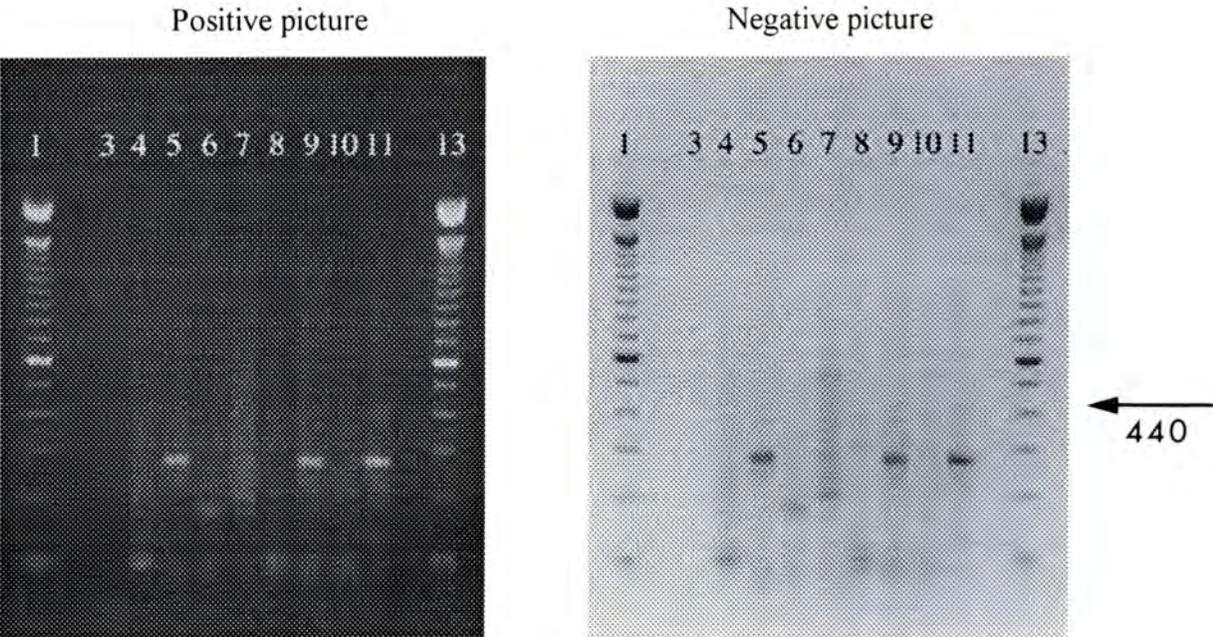
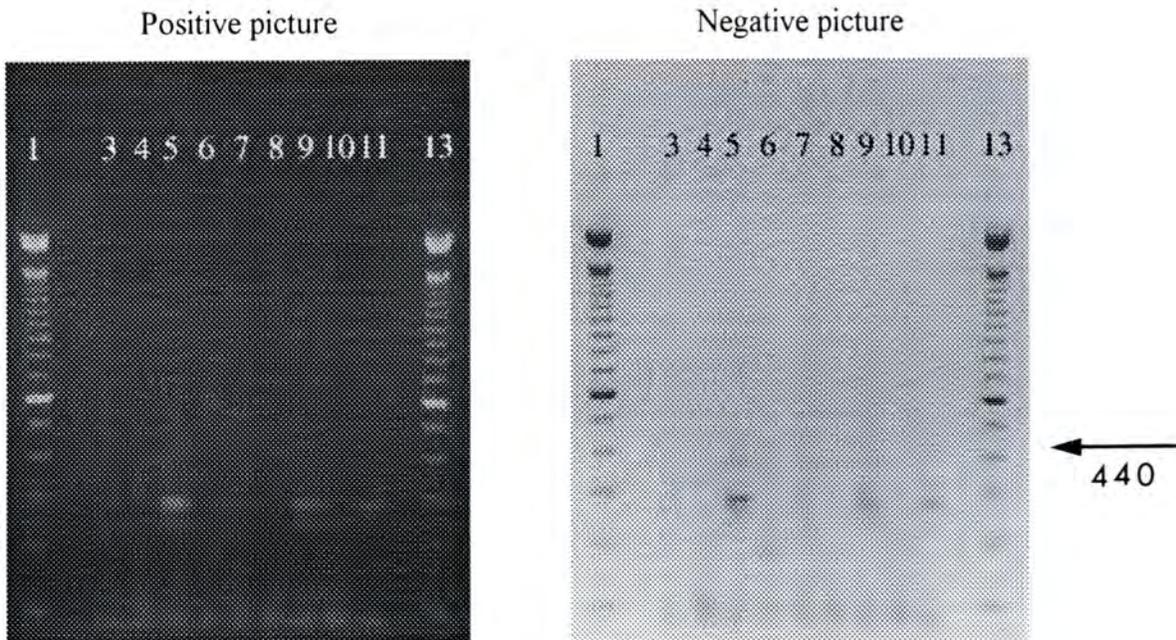


Figure 3.23 : Agarose gel electrophoresis of all V_H PCR products obtained at an adjusted annealing temperature of 56°C (ie. +8°C), from the Stratagene-extracted RNA from the *fresh* spleen of a B₃B₇-injected mouse.

4µl of each Hc primer was used.

- Lane 1 = 100bp molecular weight markers;
- Lane 3 = Hc1;
- Lane 4 = Hc2;
- Lane 5 = Hc3;
- Lane 6 = Hc4;
- Lane 7 = Hc5;
- Lane 8 = Hc6;
- Lane 9 = Hc7;
- Lane 10 = Hc8;
- Lane 11 = Hc9;
- Lane 13 = 100bp molecular weight markers.



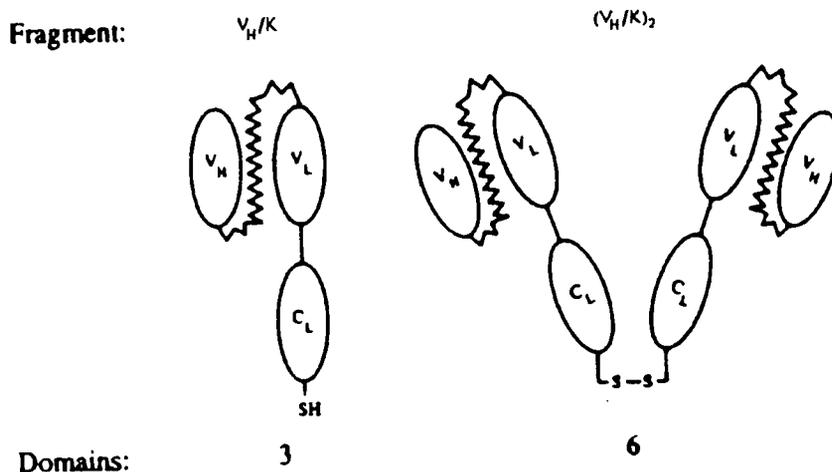
3.4 Discussion

In this study, the generation of recombinant monoclonal antibodies against natural killer cell surface markers was to be attempted through the use of 'phage display' technology. However, since this was a technique not attempted in our laboratory before, it was decided to trial the technology against a single antigen, Carboxypeptidase Y (CPY) first. Although antibody variable domain genes can be amplified from naive (i.e. unimmunised) sources, immunisation increases the number of cells producing an immune response, and therefore also increases the levels of mRNA encoding the specific immunoglobulins. (Winter *et. al.*, 1994; Clackson *et. al.*, 1991). Thus, in each of our trials, mice were immunised with the relevant antigen (CPY or B₃B₇ cells) in an attempt to increase the chances of successfully amplifying the relevant variable domains. Four weeks following the initial immunisation, the spleens were removed from the mice and the RNA was extracted from them. A series of different experiments confirmed that the best quality RNA was obtained if extraction was performed on fresh spleens (i.e. immediately after removal from mice, and *not* frozen) using the Stratagene RNA extraction kit. Furthermore, better quality RNA was obtained if the spleen was pierced and flushed out with PBS rather than minced and homogenised. (This latter technique was clearly too vigorous, resulting in fragmented RNA that was unsuitable for amplification).

Once the RNA was obtained and reverse transcribed into cDNA, PCR was performed to obtain the variable heavy (V_H) and kappa light chain genes. (Kappa light chains were chosen because less than 5% of murine antibodies possess lambda light chains - Roitt *et. al.*, 1989. Therefore, by amplifying kappa chains, most antibodies should be represented). Fortunately, the constant regions flanking the murine variable domains have been well characterised (Irving *et. al.*, 1996), allowing primers to these coding sequences to be designed. The primers used in this study were those detailed by Kang, Burton and Lerner (1991) (see Appendix A), with nine

5' primers for the heavy chain variable domains and six 5' primers for the kappa chain variable domains. In comparison, only one 3' primer is needed for the heavy chains (MVH - link) and one for the kappa chains (DB3-BLAB) because these primers are based on the sequence of the first part of the constant regions of the heavy and light chain genes. The MVH - link also includes the V_H - C_{H1} , elbow region which will act as a short linker sequence to join V_H and kappa together to create a single chain Fv fragment (scFv). A similar construct was used by He *et. al.* (1995) to successfully generate an scFv fragment of the anti-progesterone monoclonal antibody DB3. See Figure 3.24.

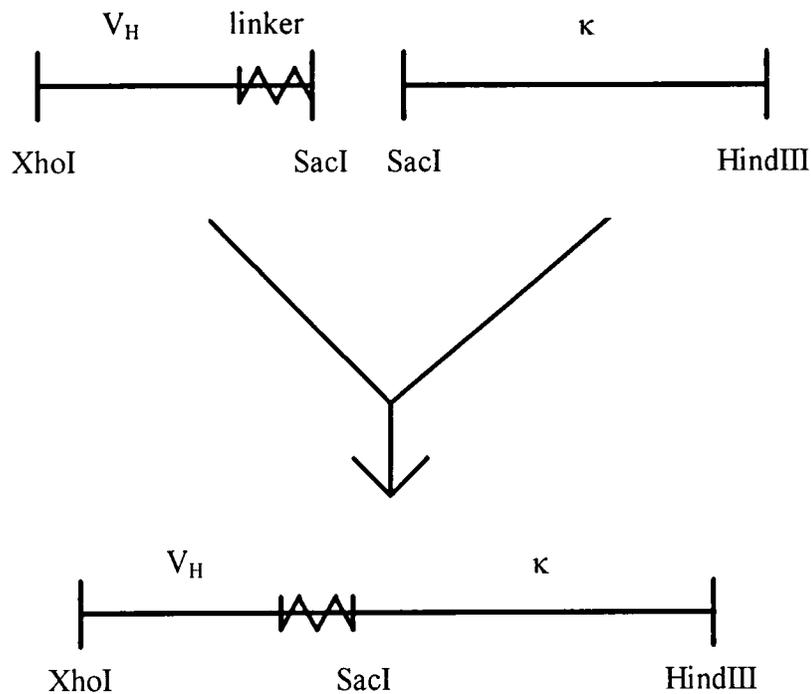
Figure 3.24 : Diagrammatic structures of monomer and dimer forms of scFv DB3 fragments expressed in *E. coli* (taken from He *et al.*, 1995)



(ScFvs have been shown to have similar binding specificity and affinity to the parent antibody, and are also produced at higher levels than Fvs because they are produced as a single, fused fragment, rather than having to associate in the bacterial periplasm - Irving *et. al.*, 1996). Furthermore, each primer also contains a restriction site to allow cloning of the V_H and kappa domains into the pSelect DB3 plasmid. These sites are as follows: V_H domain: 5' primer contains a Xho I site; 3' primer

contains a Sac I site. κ chain: 5' primer contains a Sac I site; 3' primer contains a Hind III site. These sites further allow the two domains to be cloned together, as follows:-

Figure 3.25 : Diagrammatic representation of the cloning together of V_H and kappa chains using various restriction sites.



Using the primers just described, PCR amplification of the V_H and kappa chains was attempted on RNA extracted from CPY-injected mice, B_3B_7 -injected mice, and unimmunised mice.

As described in section 3.3, numerous parameters were adjusted to try and optimise conditions for amplification. For example, $MgCl_2$ was used at different concentrations (of which none worked any better than the original 1.5mM), different RNA extraction kits were employed (of which the Stratagene kit appeared to give the best results, for the kappa chains at least), and different annealing temperatures were tried. (All of the kappa chains amplified better at $+3^\circ C$, i.e. at $55^\circ C$, rather than the

recommended 52°C annealing temperature, but it was difficult to assess the effect of annealing temperature on the heavy chains because PCR products of the correct size were rarely seen for any of the heavy chains). Problems were also encountered initially with the primers used. 60 picomoles of each primer was needed for each reaction, but when the appropriate primer dilution was analysed by agarose gel electrophoresis, it appeared that there was insufficient primer present, and hence no PCR products. Consequently, the ‘stock’ primers were used instead, and although it produced improved results, it meant that the absolute amount of primer added was inaccurate. However, the most significant improvement in results was observed when the spleen was pierced, rather than minced, to remove all the cells prior to extraction. Presumably, this was because the RNA was in better condition following the more gentle technique.

Interestingly, it appears that these problems with amplification of immunoglobulin genes are not unique. Stinson *et. al.* (1995), who were also attempting to generate scFv fragments from spleen cells, found that certain primer combinations resulted in no PCR products, or that some amplifications generated products that were quite varied in size. (This is particularly interesting because this was a feature noted several times in this study, especially for the heavy chain amplifications, where products were present but not at the correct size). Unfortunately, Stinson *et. al.* are also unclear about the factors that are involved in these odd situations. They do suggest, however, that like many PCR reactions, optimisation of the various parameters is what is needed, so maybe with the heavy chains in this study, it is simply the case that we hadn’t identified the optimum conditions for successful amplification.

Irving *et. al.* (1996) noted in their review that poorer amplification results were sometimes obtained when restriction sites (for subsequent cloning) were incorporated into the primers. They suggest that this is possibly due to an adverse effect of the extra sequence encoding the restriction site being non-complementary to the immunoglobulin cDNA. However, although the removal of the restriction sites

from the primers used in this study might increase the success of the PCR amplifications, the fact that the resulting products could not be cloned into vectors, nor subcloned into phage for display, renders this suggestion not an option for improvement. Another suggestion proposed by Irving *et. al.* is to 'hot start' the PCR, since this reduces the chance of V_H primer-dimers forming, a problem which decreases the efficiency of product amplification. 'Hot-starting' involves heating the reaction mixture (e.g. to 94°C) before adding the *taq* polymerase enzyme, and is therefore a simple modification which could easily be trialed for this study.

Unfortunately, the V_H domains were not successfully amplified here, and hence work with the kappa chains was also halted. However, had all amplifications been achieved, the technique would have continued as follows.

All the PCR products from the kappa chain amplifications would have been pooled together into one 'kappa pool', and all the PCR products from the V_H amplifications would have been pooled together into a separate 'V_H pool'. Using the restriction sites described previously, each PCR product pool would have been cloned into the appropriately -cut plasmid pSelect DB3. Thus two libraries of fragments in pSelect would be generated - one library for all the different kappa chains, and another library for all the different V_H domains. The plasmid pSelect DB3 contains an ampicillin - resistance gene (directly after the inserted V_H or kappa gene) that is only expressed if a functional V_H or kappa gene has been inserted. Thus, by culturing in ampicillin - containing media, it is possible to select for only those plasmids which are expressing a functional gene.

Random recombination of the two genes is then performed by subcloning the two libraries together. In other words, using the same restriction sites as described earlier, the kappa genes from the kappa chain library are excised and subcloned into the V_H library (pSelect + V_H), and the V_H genes from the V_H library are excised and subcloned into the kappa library (pSelect + kappa), both methods generating plasmids containing both V_H and kappa genes (pSelect + V_H + kappa). Again, the

ampicillin resistance gene is only expressed if both genes are present and functional, and therefore only successful recombinations are selected. Most pairings in this combinatorial repertoire will be artificial (Winter *et. al.*, 1994), although if the libraries were large enough initially, then original pairings are likely to be present.

At this stage, the recombined gene pairs are excised using *XhoI* and *HindIII* and subcloned into a modified strain of the filamentous M13 phage; JCM1388. This JCM1388 strain has been modified to contain two copies of the gene VIII (major coat) protein, the two having identical amino acid sequences, but different nucleotide sequences. One copy of the gene is used to create a fusion product with the V_H/κ construct, such that it may become displayed on the phage surface, while the other, wild type gene VIII, is necessary to be displayed normally for the phage to be viable. (Winter *et. al.*, 1994). The advantages of using gene VIII over gene III are that several scFv fragments are incorporated over the phage surface, i.e. it is multivalent, allowing a wider range of affinities to be captured, and secondly, that the phage is still infective (Kang, Barbas *et. al.*, 1991).

Each phage contains only one V_H and one kappa chain, and therefore displays only one species of antibody, just like a B-cell. Furthermore, as with unstimulated natural antibodies, most of these scFv fragments are likely to be of low affinity. However, just as in the natural immune system, they may undergo 'affinity maturation' through various rounds of selection against antigen, and mutation. Once a particular scFv fragment (and its gene) has been selected by phage display, it is generally straightforward to transfer the gene to a plasmid system to facilitate both mutation and high level expression (Irving *et. al.*, 1996). Since gene technology combined with phage display immortalises the genes encoding the desired antibody specificities, the potential possibilities for their use, both in scientific research and medical treatments is vast.

Clearly, phage display is an extremely powerful technique, capable of overcoming many of the problems faced by hybridoma technology in generating

monoclonal antibodies against a wide variety of different antigens. It is a shame that only the first step was reached in this study, but it is certain that research into this area will continue, and hopefully the technique will be mastered, such that work can go on into generating monoclonal antibodies against natural killer cell surface markers. From this, the ultimate goal of identifying what surface markers are present, and involved in natural killing, is possible.

Chapter 4

Concluding Remarks and Suggestions for Further Work

This study investigated the presence of natural killer cells in the amphibian *Xenopus laevis*, to determine whether this proposed 'primitive' immune system exists at lower levels of evolution (and potentially provide an insight into the development of this system).

Initial studies (detailed in Chapter 2) probed the ability of *Xenopus laevis* lymphocytes to spontaneously lyse allogeneic B₃B₇ thymic tumour cell targets (which is indicative of natural killer cell activity), through the use of ⁵¹chromium release microcytotoxicity assays. Fresh, untreated splenocytes from control animals exhibited only minimal cytotoxic activity, whereas those from early-thymectomised animals displayed significant cytotoxicity. This suggested that 'NK - like' activity is greater in thymectomised animals (that have no T cells), which is in agreement with Janeway (1989) who proposes that NK cells are a primitive form of immune defence, active before T cells are fully developed.

Furthermore, the cytolytic activity observed in splenocytes from a thymectomised animal could be significantly enhanced by Con A-derived supernatants. (These ASNs, which were generated under optimal conditions were likely to be rich in IL-2 or IL-2-like factors (Haynes & Cohen, 1993), and thus would be expected to activate natural killer cells). Such enhancement was typical of the 'lymphokine activated killing' observed by other researchers (Mason *et al.*, 1990; Trinchieri *et al.*, 1984), supporting the theory that tumour cell lysis recorded here was mediated by NK-like cells, and hence substantiating the presence of such cells in *Xenopus laevis*.

Following this observation, attempts were made to locate the specific cell population responsible for the cytolytic activity, by enriching the splenocytes for natural killer cells through the selective depletion of other lymphocyte subsets, in particular T cells, B cells and T_c cells. Two techniques were employed in this study - separation by 'panning', and magnetic bead separation, both of which are detailed in Chapter 2. On comparison of the two techniques, it was found that both were able to deplete a splenocyte culture of B cells to the same extent, but that magnetic sorting produced far superior results for depletion of T cells. Further, separation trials could have been carried out to try and achieve absolute purity of populations, but at this point it was deemed unnecessary since sufficient purity had been achieved to allow further cytolytic and molecular work to be undertaken. Thus, under conditions determined in this study as optimum for magnetic sorting, various 'purified' populations were generated, which were then assessed for their cytotoxic activity in ⁵¹chromium release microcytotoxicity assays. These preliminary analyses suggested that natural killer - like activity in *Xenopus* is likely to be mediated by a 'non-T / non-B' lymphoid subset. However, more detailed analysis is required to confirm this. In particular, a mAb against NK cell surface antigens would be an extremely useful tool for enriching NK populations through magnetic sorting.

Work detailed in Chapter 3 outlines preliminary investigations designed to address the problem of generating such mAbs against NK cell surface antigens. These initial experiments attempted to develop the technique of 'phage display' for the generation of single chain F_v antibody fragments against chosen antigens. In order to learn the technique, trials were made against the single antigen Carboxypeptidase Y, and a population of B₃B₇ *Xenopus* thymic tumour cells, although ultimately mAbs would have been generated against enriched natural killer cell populations. Following RNA extraction under conditions determined as optimum, Kappa light chains were successfully amplified from the experimental

spleens, but unfortunately the V_H chains were not, which meant that work could not continue.

However, in the period since the research for this thesis was completed, work has continued in the laboratory, and now both Kappa and V_H chains have been successfully amplified (Martin Watson - personal communication). V_H chains were finally amplified using Reverse Transcriptase PCR (RT - PCR), which incorporates the transcription of RNA into cDNA with the PCR amplification into one reaction mix. Furthermore, trials have been made using rat spleen RNA rather than mouse spleen RNA, and have produced surprisingly good results - in fact, better bands were achieved than with the murine system for both Kappa and V_H chains!! Consequently, it seems likely that rats will be used for future immunization and antibody-generation purposes.

Given that both V_H and Kappa have now been successfully amplified, the rest of the technology could now be continued as detailed in Chapter 3 (section 3.4). Providing that this progressed well, and an antibody-displaying phage population was successfully generated, a further goal of any future work would be to select all phage that are displaying antibodies against NK cell surface antigens. According to de Kruif *et al.* (1996), there are four steps in the selection of phage-Abs from libraries: “(1) binding of phages to the target antigen; (2) removal of nonbound phages by washing; (3) elution of bound phages; and (4) infection and propagation of eluted phages in *Escherichia coli*.” Various techniques have been employed for such selection, including binding of the phage-Abs to purified antigens coated on to plastic surfaces, capture on to a solid phase through monoclonal antibodies, selection using intact eukaryotic cells (in suspension or as monolayers) as targets, or even using flow cytometric sorting with fluorochrome-labeled mAbs against the required subpopulation of cells (de Kruif *et al.*, 1996).

For this study, where phage-Abs are to be generated against whole natural killer cells, the technique of choice would be to mix phage-Abs with the enriched NK cell population, and then to centrifuge the mix. Phage which are displaying antibodies against NK cell surface antigens will bind to the cells and be centrifuged down to the bottom of the tube as a pellet. The other phage (displaying non-NK cell antibodies) will remain in solution and can be removed in the supernatant. Once these selected phage-Abs have been purified, a further, long-term goal of the project could be to characterize the antibodies and identify the surface antigens that they bind to (involving such techniques as Western blotting and SDS-PAGE [sodium dodecyl sulphate polyacrylamide gel electrophoresis]).

Although beyond the scope of this study, these suggestions are exciting and realistic possibilities for long-term investigation. The work detailed here provides a sound and thorough basis from which such possibilities can be developed, and will provide useful information regarding the evolution of natural killer cells within the immune system.

Appendix A

Murine Immunoglobulin PCR Primers

The primers used in this study for the amplification of murine immunoglobulin genes were those detailed by Kang, Burton and Lerner (1991), and were named Lc1,3,4,5,6,7, and Hc1,2,3,4,5,6,7,8,9 as follows :

Murine light-chain variable 5' primers

Lc1 : 5'-CCA GTT CCG AGC TCG TTG TGA CTC AGG AAT CT-3'

Lc3 : 5'-CCA GTT CCG AGC TCG TGC TCA CCC AGT CTC CA-3'

Lc4 : 5'-CCA GTT CCG AGC TCC AGA TGA CCC AGT CTC CA-3'

Lc5 : 5'-CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA-3'

Lc6 : 5'-CCA GAT GTG AGC TCG TCA TGA CCC AGT CTC CA-3'

Lc7 : 5'-CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA-3'

Heavy-chain variable 5' primers

Hc1 : 5'-AGG TCC AGC TGC TCG AGT CTG G-3'

Hc2 : 5'-AGG TCC AGC TGC TCG AGT CAG G-3'

Hc3 : 5'-AGG TCC AGC TTC TCG AGT CTG G-3'

Hc4 : 5'-AGG TCC AGC TTC TCG AGT CAG G-3'

Hc5 : 5'-AGG TCC AAC TGC TCG AGT CTG G-3'

Hc6 : 5'-AGG TCC AAC TGC TCG AGT CAG G-3'

Hc7 : 5'-AGG TCC AAC TTC TCG AGT CTG G-3'

Hc8 : 5'-AGG TCC AAC TTC TCG AGT CAG G-3'

Hc9 : 5'-AGG TII AIC TIC TCG AGT C(TA)G G-3'

I = Inosine

DB3-BLAB (Murine kappa chain 3' primer)

5'-TCT AGA AGC TTG CCC ACC CTC ATT CCT GTT GAA GCT-3'

MVH-LINK (Murine heavy chain 3' primer)

5'-CTG GGT CAT CTG GAG CTC GGC CAG TGG ATA GAC AGA TGG GGG
TGT CGT TTT GGC-3'

Chapter 5

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