MANIPULATION OF THE IMMUNOSTIMULATORY CAPACITY OF A HUMAN MYELOID LEUKAEMIA CELL LINE HL-60.

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ABSTRACT

The aim of this project was to determine the reason for the lack of ability of many myeloid leukaemic cell populations to stimulate allogeneic lymphocytes in mixed leucocyte culture (MLC), with a view to manipulating the immunogenicity of these cells for therapeutic purposes. The myelogenous leukaemic cell line, HL-60, was chosen as a model system since it has been reported that cells of this line can be induced, by a variety of agents, to differentiate along the granulocytic or monocytic pathways, with or without the acquisition of class II major histocompatability (MHC) antigens. It was predicted that at least some of these treatments would result in the acquisition of allostimulatory activity, and would thus allow analysis of the requirements for this activity.

HL-60 cells cultured with a physiological concentration (10 nM) of all-*trans* retinoic acid (RA) for 7 days (HL-60-R7) possessed an enhanced capacity to stimulate the proliferation of $CD2^{+}$-enriched lymphocytes derived from the peripheral blood of healthy human donors in MLC. The lymphocyte populations responding to HL-60R7 possessed NK activity (i.e. the ability to lyse K562 cells) and an enhanced capacity to lyse HL-60 and HL-60-R7 cells. Examination of the HL-60 cells cultured for 7 and 12 days with RA, carried out by means of enzyme histochemistry, cell surface marker analysis and morphology, revealed a somewhat heterogeneous population of cells with monocyte-like, neutrophil-like and other less readily definable cell types being present. The presence of monocyte-like cells was indicated by the cell surface expression of CD36 by a proportion of the cells and, although no cells expressed detectable levels of MHC class II molecules on day 7, by day 12 a small percentage (15%) were positive for low-medium levels of class II antigen. However morphological and cytochemical studies failed to indicate the emergence of a clearly identifiable population of mature cells.

Additional cell surface marker analysis revealed an up-regulation of CD11b and ICAM-1, along with a down-regulation of MHC class I molecules and transferrin receptor (CD71). Analysis of the levels of expression of mRNA for four immunologically relevant cytokines by HL-60 and HL-60-R7 cells was performed by the RNase protection assay, which revealed that both populations failed to express detectable levels of IL-6 mRNA and barely detectable levels of IL-1α and β mRNA, with the most prominent mRNA species being that coding for TNFα. The levels of expression of TNFα mRNA appeared to remain unchanged after R.A.-treatment.

The stimulatory activity of HL-60-R7 cells in MLC despite their failure to express detectable MHC class II antigens prompted examination of the nature of the responding lymphocytes. Two-colour immunofluorescence analysis using CD25 expression as an indicator of cell activation revealed that the predominant phenotype of the responding cells was $CD16^+$ and/or $CD8^+$, with $CD4^+$ lymphocytes being stimulated to a lesser extent. However, removal of the $CD4^+$ population significantly impaired the proliferative response to HL-60-R7 cells as did the addition of monoclonal antibodies specific for MHC class II antigen. In contrast, removal of MHC-class II^+ cells from the lymphocyte populations had no effect on the proliferative response. The addition of anti-TNFα almost completely abrogated the response to HL-60, HL-60-R7 and RC-2a cells, whereas anti-IL-1 and anti-IL-12 appeared to have no effect.

A wide variety of mostly myeloid cell lines were also tested for their cell surface marker expression as well as their immunostimulatory nature in MLC, where it was found that the majority of these cell lines were only weakly stimulatory.

STATEMENT

This thesis contains no material which has been accepted as full or part requirement for the award of any other degree or diploma in any other university. To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

Sean Michael Geary

November, 1993

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ABBREVIATIONS

3D3.3-Fl	Fluorescein-labelled 3D3.3
3H-thymidine	Tritiated thymidine
Abs	Absorbance
amp	Ampicillin
APC	Antigen presenting cells
ATP	Adenosine triphosphate
ATTC	American Type Culture Collection
Az	Sodium azide
Biot	Biotinylated
bp	base pairs
BSA	Bovine serum albumin
CAE	Chloroacetate esterase
cpm	Counts per minute
CTP	Cytidine-5'-triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	Ethidium monoazide
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate

FL	Fluorescence intensity (logarithmic)
FLUOS	Fluorescein-isothiocyanate, 5(6)-carboxyfluorescein-N-succinamide ester
FSC	Forward scatter
GαM	Goat anti-mouse antibodies
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
H.E.L.	Human embryonic lung cells
HAc	Acetic acid
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine N'-2-ethane sulphonic acid
HL-60-R12	HL-60 cells cultured with 10 nM RA for 12 days
HL-60-R7	HL-60 cells cultured with 10 nM RA for 7 days
HLA	Human leucocyte antigen complex
ICAM-1,-2,-3 or -R	Intercellular adhesion molecule-1, 2 or 3
IFN or IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIF	Indirect immunofluorescence
IL-1	Interleukin-1
IL-12	Interleukin-12
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
IL-6	Interleukin-6
IMDM	Iscove's modification of Dulbecco's medium
IMVS	Institute of Medical and Vetinary Science

IU	International units
L-B	Luria-broth
LEP	$CD2^+$-lymphocyte-enriched lymphocyte populations
LFA-1 ,2 or 3	Leucocyte function associated molecule-1,2 or 3
LPS	Lipopolysaccharide
mAb	Monoclonal antibodies
MHC	Major histocompatibility complex
MLC	Mixed leucocyte cultures
MNC	Mononuclear cells
mRNA	Messenger RNA
NK	Natural killer
NRS	Normal rabbit serum
NSE	Non-specific esterase
O. D.	Optical density
OPD	o-Phenylenediamine.2HCl
PBS	Phosphate-buffered saline
PE	Phyco-erythrin
PHA	Phytohaemagglutanin-P
PIPES	Piperazine-N',N'-bis[2-ethanesulfonic acid]
PMA	4β-Phorbol 12β-myristate 13α-acetate
RA	All-*trans* retinoic acid
RAR-α	Retinoic acid receptor-α
RMF	Relative mean fluorescence intensity
RT	Room temperature
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SPE	Streptavidin-phyco-erythrin

SSC	Side scatter
TBE	Tris-borate-EDTA
TC	Tissue culture
TCR	T cell receptor
TE	Tris-EDTA
Temed	NNN'N'-tetramethylethylene-diamine
TNF	Tumour necrosis factor
TPA	N,N,N'N'-Tetramethyl-p-phenylenediamine
Tris	Tris(hydroxymethyl)amino-methane
tRNA	transfer RNA
UTP	Uridine-5'-triphosphate
Vit D_3	1, 25 Dihydroxyvitamin D_3

* See amendment 3 at the back of this thesis for list of alternative names used for some of the cell surface molecules

Contents

Chapter 2: Materials and methods

Presentations

1989 and 1990: abstracts and posters were presented, relating to the work with retinoic acid-treated HL-60 cells and their ability to activate peripheral blood lymphocytes, at the annual meetings of the Australian Society of Immunology.

.1991: a seminar was presented, relating to Ph.D. thesis, at the annual meeting of the Australasian Society of Pathology.

.1993: a seminar was presented, relating to Ph.D. thesis, at the Department of Haematology.

Publications in preparation

1. Geary, S.M. and Ashman, L.K. Activation of Cytotoxic Lymphocytes by a Myeloid Leukaemic Cell Line Treated with all-*trans* Retinoic Acid. *Br. J. Haematol.*, in submission.

2. Geary, S.M. and Ashman, L.K. Requirements for the Activation of Lymphocytes by HL-60 cells Treated with *all-trans* Retinoic Acid, *in preparation.*

CHAPTER I

INTRODUCTION

INTRODUCTION

1.1 Leukaemia

Leukaemia is a rare disease of the bone marrow, which appears to result from the production of an abnormal immature haemopoietic cell clone that is in some way resistant to the strict regulatory control of the bone marrow micro-environment that governs the haemopoietic process. As a result, these cells tend to overpopulate the marrow, as well as the blood and tissues, leading to a disruption of normal haemopoiesis. This disruption may arise from overcrowding, competition between leukaemic and normal cells for essential factors and/or haemopoietic niches, or the production of inhibitory factors by the leukaemic cells. Due to the lack of production of effective levels of functionally normal white and red blood cells, the patient usually succumbs to infection, haemorrhage or anaemia and will eventually die unless some form of treatment is administered.

The earliest recorded case of this disease was described by Velpeau in 1827 (Velpeau, 1827) and the term "leukaemia" which, literally translated, means "white blood" was first used by Virchow in 1847 (Virchow, 1847). It was subsequently recognized that there were 2 major types of leukaemia; acute and chronic. These leukaemias have been sub-categorized into acute and chronic lymphocytic leukaemia (ALL and CLL) and acute and chronic myeloid leukaemia (AML and CML). Acute leukaemias have been further delineated, according to morphological and histochemical criteria, on the basis of the stage of differentiation of the majority of the leukaemic cell population, and these categories were defined by the French-American-British (FAB) system (Bennett *et al*, 1976).

Chronic leukaemia is generally less severe than the acute form, initially at least, presumably because the aberrant cells are still capable of differentiating *in vivo*. In fact the early phase of chronic myeloid leukaemia (CML) is often referred to as

"preleukaemic" (reviewed by Sawyers *et al*, 1991). Nevertheless, the lineage(s) affected is expanded as a result of a lack of proliferative control, eventually leading to a disruption of the normal processes of haemopoiesis. In the case of acute leukaemia there is not only a lack of proliferative control but also an inability of the aberrant cells to respond to the local differentiation inducing factors, generally resulting in a more rapid over-production of immature cells. Hence the onset of life threatening symptoms generally occurs more quickly with a median survival of less than 2 months for untreated patients (Tivey *et al*, 1955).

With most human leukaemias the nature of the transforming events is unknown. However the majority of CML cells possess what is known as the "Philadelphia chromosome", which results from a translocation event between chromosomes 9 and 22 that can lead to the expression of a fusion protein (p210) encoded partially by c-*abl*, a proto-oncogene coding for a tyrosine kinase (reviewed by Morgan and Wiedemann, 1989), and partially by another gene fragment known as *bcr* (breakpoint cluster region) (reviewed by Kurzrock *et al*, 1988). The Philadelphia chromosome is present in all the haemopoietic lineages of patients with CML, suggesting that the translocation event occurs in a pluripotent stem cell. The bcr fragment can trigger the tyrosine kinase activity of the juxtaposed c-abl fragment (Konopka *et al*, 1984) and result in the transformation of haemopoietic cells (McLaughlin *et al*, 1987). The *bcr-abl* gene product may confer an enhanced proliferative capacity on cells of the myeloid compartment through an ability to confer independence of growth factors such as IL-3 or GM-CSF, as suggested by experiments performed on haemopoietic myeloid cell lines that became independent of such cytokines upon the introduction of the *bcr-abl* gene (Daley and Baltimore, 1988). A murine study involving *in vitro* colony assays revealed that multipotent haemopoietic stem cells, infected with a retrovirus carrying *bcr-abl,* were initially dependent on growth factors, but eventually became growth factor-independent (Gishizky and Witte,

1992). These researchers also found that the growth factor-independent cells were nonmalignant, supporting previous studies that suggested that multiple genetic alterations are required for the generation of a leukaemic phenotype (Fialkow *et al*, 1981)

With CML, as already mentioned, there is eventually a block in differentiation of the leukaemic cell. Indirect evidence that some genetic alteration could be responsible for this block in differentiation comes from the discovery that a substantial percentage of patients with myelodysplastic syndromes (MDS) have genetic abnormalities in the N-*ras* or Ki-*ras* genes (Bos, 1989), the products of which have been proposed to possess GTPase activity (McCormick, 1989). MDS is often referred to as a "preleukaemic" disorder where there is a block in differentiation yet there is no substantial over-production of the haemopoietic precursors (Sawyers *et al*, 1991). This disease is often associated with the loss of the long arms of chromosomes 5 and 7, generally progresses to AML and has a tendency to occur in elderly people (Fialkow *et al*, 1987). Interestingly, the 5q chromosomal region encodes many haemopoietic growth factors, including IL-3, IL-5, M-CSF and GM-CSF, as well as growth factor receptors, such as the M-CSF receptor and the PDGF receptor, implicating a direct or indirect role for one or more of these molecules in the generation of MDS (Groopman *et al*, 1989).

Myeloproliferative disorders are also considered to be "preleukaemic" and may result from the overexpression of growth factors such as IL-3 or GM-CSF (Chang *et al*, 1989; Johnson *et al*, 1988). Experiments performed with mice transgenic for either of these cytokines would suggest that growth factor overexpression is probably not a sufficient stimulus on its own to induce an acute form of leukaemia (Chang *et al*, 1989; Johnson *et al*, 1988). Thus, the generation of acute leukaemia would appear to depend on at least two alterations to the candidate haemopoietic cell; 1) the acquisition of an enhanced capacity to proliferate over normal haemopoietic cells and 2) the loss of

the ability to respond to the normal differentiation inducers of the bone marrow. The former alteration occurs in chronic leukaemias and myeloproliferative disorders whilst the latter alteration is found in MDS.

Experimental models have supported the idea that the generation of acute leukaemia requires genes effecting both growth and differentiation (reviewed by Hunter, 1991). For instance, it has been shown that while the overproduction of IL-3 was not sufficient to transform murine haemopoietic cells, the introduction of both IL-3 overexpression and a homeobox gene did result in transformation (Perkins *et al*, 1990). Finally, the fact that only a small percentage of cells became tumours in mice transgenic for both the *ras* and *myc* oncogenes, suggests that more than two alterations are sometimes necessary and that the requirements for transformation may depend on the cell type and/or its stage of differentiation (Sinn *et al*, 1987).

In the case of acute promyelocytic leukaemias (APML), which constitute approximately 10% of AML in adults (Stone and Mayer, 1990), there appears to be an invariant association with a translocation between chromosomes 15 and 17 (Brazell *et al*, 1983; Longo *et al*, 1990). The translocation event often leads to the production of fusion mRNA derived from the transcription of a portion of the novel zinc finger encoding gene, PML (Pandolfi *et al*, 1992), from chromosome 15 in combination with a portion of the RA receptor (RAR)-α gene from chromosome 17 (de The *et al*, 1990). As a result, the RAR-α/PML fusion protein is suspected of playing a role in leukaemogenesis of APML (Solomon *et al*, 1991), although this is yet to be directly proven.

1.2 Growth requirements for leukaemic cells

The growth characteristics of myeloid leukaemias are possibly better understood than lymphoid leukaemias as a result of the comparative ease with which the former can be cultured *in vitro*. ALL cells, in the past, have proven to be inefficient at forming colonies under similar conditions to those used for AML colony formation and thus defining growth factors responsible has not been easy (Touw *et al*, 1985; Uckun *et al*, 1986). The ability of ALL cells to form colonies in methylcellulose supplemented with IL-1, IL-3 and a pool of B cell growth factors has met with some measure of success (Uckun *et al*, 1987). On the other hand, primary AML cells have sometimes been reported to proliferate in culture in the absence of exogenously supplied growth factors (Young and Griffen, 1986). One possible explanation for this phenomenon is that the leukaemia cells were capable of autocrine stimulation of proliferation. The likelihood of such an event was given credence by Young and Griffen (1986), who showed that primary AML blasts were capable of producing, and proliferating in response to, their own GM-CSF. Similar studies with primary AML blasts have also indicated a possible role for M-CSF and G-CSF in an autocrine system (Murahashi *et al*, 1988). There have even been rare reports of primary AML cells which could proliferate in the absence of both endogenous and exogenous sources of growth factors (Delwel *et al*, 1990), suggesting that a point mutation or chromosomal translocation had led to the constitutive expression of an activated regulatory protein, such as a growth factor receptor. A possible example of this is the M-CSF receptor, coded for by the c-*fms* proto-oncogene. A mutant form of this gene, possessing a point mutation at codon 301, has been found to be expressed by some primary AML (Sherr, 1990; Ridge *et al*, 1990). The majority of primary AML cells, however, cannot form colonies in semisolid media (reviewed by Griffen and Lowenberg, 1986) and require the presence of at least one exogenously supplied growth factor if they are to proliferate *in vitro*, the most common of which are IL-3 (Delwel *et al*, 1987), GM-CSF and G-CSF (Vellenga *et al*, 1987; Miyauchi *et al*, 1987).

1.3 Leukaemic cell lines

1.3.1 General characteristics of leukaemic cell lines

Whether or not primary AML cells require endogenous or exogenous growth factors to survive and grow in suspension culture, they nevertheless possess a limited proliferative capacity and life span. Leukaemic cell lines, on the other hand, have attained immortality and can usually also grow in liquid suspension cultures in the absence of exogenously added growth factors. Such cell lines are generally derived from primary cultures of leukaemic cells which have been sustained with rich growth media, but have eventually become independent of exogenous growth factors for their maintenance and can survive in culture indefinitely. Attaining immortality though does not imply absolutely that the cell has become growth factor-independent as growth factor-dependent cell lines do exist (Hendrie *et al*, 1991; Kitamura *et al*, 1989).

Leukaemic cell lines are believed to be derived from single leukaemic cells which have attained immortality, thus making them attractive to work with as they are therefore considered to be an inexhaustible supply of homogeneous cells. All myeloid cell lines have been generated from leukaemic patients and are each believed to be representative of a particular stage of differentiation in haemopoiesis. However, despite their apparent clonal derivation, cell lines are still somewhat heterogeneous. For instance cells of the same population can differ with respect to their capacity to form colonies in semi-solid media. Some cells have been shown to only be capable of self-renewal whilst others of the same cell line can spontaneously differentiate (Fibach *et al*, 1990).

1.3.2 HL-60

The HL-60 cell line was derived from a 36 year old female patient originally diagnosed as having acute promyelocytic leukaemia (Collins *et al*, 1977) (M3 according to the FAB classification) but the disease was posthumously rediagnosed as being "acute myeloblastic leukaemia with maturation" (FAB-M2) (Dalton *et al*, 1988).

Neither the HL-60 cells nor the original leukaemic cells possessed the t(15:17) translocation, which is characteristic of APML (Dalton *et al*, 1988). The primary leukaemic cells derived from the peripheral blood of this patient were cultured in conditioned medium for approximately three weeks, by which time cells were found to grow in RPMI + 15% FCS independently of exogenous growth factors. These cultures were comprised predominantly of promyelocytes (55 - 65%) and myeloblasts (20 - 30%) along with a small percentage of myelocytes and metamyelocytes (Collins *et al*, 1977).

A substantial amount of characterization of HL-60 cells has been performed since. They have a doubling time of 30 - 40 hr and grow exponentially up to a density of 3 x 10^6 cells/ml (Foa *et al*, 1982). A large proportion of them express chloro-acetate esterase (Gallagher *et al*, 1979), an enzyme characteristic of neutrophils, whilst approximately 1 - 5% of HL-60 cells express the monocyte-specific enzyme non-specific esterase (Dayton *et al*, 1985), emphasising the non-homogeneous nature of this cell line. They express little or no functional traits characteristic of monocytes or granulocytes, such as superoxide anion production, antibody dependent cellular cytotoxicity (ADCC) or phagocytosis (Collins *et al*, 1979). With respect to antigen expression, the majority of HL-60 cells lack detectable levels of many immunologically relevant antigens on their cell surface, such as MHC class II molecules (Dayton *et al*, 1985), the C3bi receptor (CD11b) (Hickstein *et al*, 1989) and the intercellular adhesion molecule (ICAM-1 or CD54) (Most *et al*, 1992) but do express MHC class I (Trinchieri *et al*, 1987), and LFA-1 (Back *et al*, 1992). Cytogenetic studies have shown that 75% of cells from the original bone marrow sample from the patient were 44, XX, -5, -8, -17, +mar 3; 20% were 45, XX, -5, -8, +mar 3; 5% were 46 XX, -5, -8, -17, +18, +mar 2, +mar 3 (Dalton *et al*, 1988). Not long after HL-60 cells had been established, chromosomal analysis revealed that the majority of cells analysed (27/40) had 44 chromosomes with the other cells possessing numbers of 43 to 47 (Collins *et al*, 1977). Subsequently, another group performed cytogenetic studies on HL-60 cells and

found that the predominant karyotype was 45, X, -5, -8, -X, + an A group marker, + an acrocentric D group marker, + a submetacentric E group marker (Koeffler *et al*, 1980). These differences may well reflect the genetic instability of HL-60 cells and may explain the contradictory data obtained by independent research groups, with respect to certain experiments performed with HL-60 cells. Further studies have revealed that HL-60 cells have altered N-*ras* and c-*neu* genes (Murray *et al*, 1983), a stably amplified c-*myc* oncogene (16 - 32 fold) (Dalla Favera *et al*, 1982), a deletion of a large part of the gene normally encoding the p53 protein (Wolf and Rotter, 1985), and a truncated GM-CSF gene (Huebner *et al*, 1985).

Chromosomal abnormalities are not peculiar to HL-60 cells, as most other cell lines are also cytogenetically aberrant in some way, with the monocytic cell line, THP-1, having been reported as one of the few exceptions, possessing an apparently normal complement of chromosomes (Tsuchiya *et al*, 1980). Despite the high frequency of chromosomal abnormalities amongst cell lines, no common chromosomal aberration is apparent.

1.3.3 Differentiation of HL-60 cells

Many of the myeloid cell lines studied can be induced to differentiate *in vitro* with a variety of factors despite their inability to differentiate *in vivo*. One of the main reasons that the differentiation of cell lines has been of such great interest to researchers is that it provides an insight into the normal processes of haemopoiesis. This apparent isolation of the separate stages of haemopoiesis has made it possible to study and characterize the maturation process. Another motivation for inducing leukaemic cell lines to differentiate is that some factors, especially physiological ones, may be useful for treatment of patients with particular types of leukaemia, as acquisition of maturity generally results in a loss of proliferative capacity of the malignant cells (Greaves, 1982).

HL-60 has been the most widely studied of the leukaemic cell lines, mainly due to the readiness with which it differentiates in response to a host of different factors (reviewed by Collins *et al*, 1987). One of the earliest discoveries was that HL-60 cells were readily induced to differentiate with polar compounds such as dimethyl sulphoxide (DMSO) and the phobol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Collins *et al*, 1978; Rovera *et al*, 1979). Since then a myriad of other factors, and combinations of factors, have been shown capable of inducing HL-60 cells to differentiate along a variety of lineage pathways. To illustrate, HL-60 cells, previously passaged in alkaline medium, can be induced to differentiate into basophilic cells in the presence of sodium butyrate and an, as yet, undefined factor present in monocyte-conditioned medium (Hutt-Taylor *et al*, 1988). Factors secreted by the hairy T cell leukaemic cell line were shown to induce eosinophil differentiation of HL-60 cells (Tanno *et al*, 1987). 1,25-Dihydroxyvitamin D_3 (Vit D_3), which is the most biologically active form of vitamin D, was shown to induce HL-60 cells along the monocyte pathway (Rigby *et al*, 1985). Monocyte differentiation of HL-60 cells can also be induced by IFN-γ (Ball *et al*, 1984), TNF (Trinchieri *et al*, 1986) as well as combinations of factors which act synergistically, such as all-*trans* retinoic acid (RA) with IFN-γ (Hemmi and Breitman, 1987), RA with IFN-α (Grant *et al*, 1985; Hemmi and Breitman, 1987) and TNF-α with IFN-γ (Trinchieri *et al*, 1986). That HL-60 cells were becoming monocyte-like was determined by researchers using one or more criteria, which included morphology, expression of NSE, adherence to plastic, expression of monocyte-specific antigens including the expression of MHC class II molecules.

1.3.4 The role of RA in the differentiation of HL-60 cells

As early as the 1920s, researchers had discovered that vitamin A depleted animals developed, amongst other maladies, marrow hypoplasia (Findlay and McKenzie, 1922; Wolbach and Howe, 1925) suggesting that this vitamin or its

derivatives played an important role in haemopoiesis. Since then all-*trans* retinoic acid (RA), a derivative of vitamin A, along with other retinoids, has been shown to possess numerous activities which include the ability to inhibit carcinogenesis in a variety of *in vivo* models (Chatterjee and Banerjee, 1982), the capacity to inhibit the growth of several tumour cell lines (Lotan, 1980), the ability to regulate foetal development during vertebrate embryogenesis (Balling *et al*, 1989; Thaller and Eichele, 1990) and the capacity to modulate the immune system (Eccles, 1985), including T lymphocyte and NK cell activity (Prabhala, 1990).

All-*trans* retinoic acid (RA) is believed to mediate its multiple effects through specific nuclear retinoic acid receptors (RAR)-α, β, and γ, which have been shown to be related to the steroid/thyroid hormone receptor superfamily of nuclear transcription factors (Evans, 1988). RA is capable of diffusing through the cellular and nuclear membranes and binding directly to RAR (reviewed by Warrell *et al*, 1993). These RAR all share a substantial structural similarity with respect to their DNA-binding and ligand-binding domains, but vary at their NH_2 domains (Mangelsdorf *et al*, 1990). It was discovered that the RA-induced differentiation of HL-60 cells can be mediated by RAR-α, β or γ (Collins *et al*, 1990; Robertson *et al*, 1992). More broadly though, it has been shown that different receptor isoforms exhibit distinct tissue-specific patterns of expression (de The *et al*, 1989; Kastner *et al*, 1990). When bound to RA, the RAR are capable of acting as transcription factors for a number of genes by binding to RA-responsive elements (RARE) (Mangelsdorf *et al*, 1990; Evans *et al*, 1988; Mader *et al*, 1993). The wide variety of RA-responsive genes include those that encode transcription factors, such as HOX genes (Reid, 1990).

RA is found in the plasma of healthy individuals at concentrations approximating 5 nM (Tang and Russel, 1988) and such small concentrations have been shown to induce HL-60 cells to differentiate. However, the majority of researchers have used higher concentrations of RA to induce the differentiation of HL-60 cells into what were defined as granulocytes (Breitman *et al*, 1980), based on their nuclear

morphology and their ability to reduce NBT. The differentiation of HL-60 cells by RA was shown to be potentiated by other cytokines such as IFN-α, -β and -γ, TNF-α, IL-1-α and IL-4 (Bollag, 1991). However the combination of RA and TNF-α or RA and IFN-γ has been reported to induce HL-60 cells to differentiate along the monocyte pathway rather than the granulocyte pathway (Hemmi and Breitman, 1987; Trinchieri *et al*, 1987)

1.4 T lymphocyte activation

1.4.1 T lymphocytes

Before delving into the numerous studies investigating the requirements for T lymphocyte activation, it is important to first define T lymphocytes. T lymphocytes originate in the bone marrow and undergo a process of maturation and selection in the thymus, after which they enter the circulatory system as naive, or virgin, T lymphocytes (von Boehmer *et al*, 1989; reviewed by Rothenberg, 1992). All T lymphocytes express a variable T cell receptor (TCR) which usually comprises two disulphide linked glycoprotein chains, α and β, capable of recognizing allogeneic MHC class II or MHC class I molecules, or the combination of syngeneic MHC products in association with antigen (Trinchieri *et al*, 1973, Zinkernagel and Doherty, 1974; Doyle and Strominger, 1987; Davis and Bjorkman, 1988).

A small percentage of T lymphocytes from the peripheral blood express a different type of TCR comprising a γ and a δ chain. These cells have also been shown to be capable of recognizing MHC products (reviewed by Haas *et al*, 1993)). The TCR (αβ or γδ) is associated non-covalently with the invariant CD3-ζ-ζ or CD3-ζ-η complex (reviewed by Ashwell and Klausner, 1990). Expression of this CD3 complex is necessary for the expression of a functional TCR, and vice versa (Weiss and Stobo, 1984). The majority of T lymphocytes also express CD2 and either CD4 or CD8, but unlike the CD3 complex and the TCR, none of these antigens are exclusive to the T cell lineage.

Many experiments relating to T lymphocyte activation have been performed on resting T lymphocytes derived from the peripheral blood of healthy individuals. Resting T lymphocytes can simply be defined as those T lymphocytes which do not produce IL-2 or express the high affinity IL-2 receptor (IL-2R), and may include "naive" and "memory" T cells. Activated lymphocytes, which express the high affinity IL-2R and MHC class II antigen, can be removed from the population, along with other potential accessory cells such as B lymphocytes and monocytes, prior to the performance of activation assays, thus eliminating any contributors to the activation process other than the resting T lymphocytes themselves and the exogenously added factors. Some researchers choose to leave the natural killer (NK) cells in the resting population whilst others remove them. Their role as accessory cells is uncertain, however NK cells are capable of expressing MHC class II and IL-1β (London *et al*, 1985).

Resting peripheral blood T lymphocytes can be grouped into a number of sub-categories. For instance, the vast majority express either CD4 or CD8 molecules on their cell surface and expression of one generally excludes expression of the other. $CD4^+$ lymphocytes are often described as "helper" cells and are considered the initiators of immune responses, but it is probably more correct to consider them as those cells which can interact with syngenic MHC class II molecules plus antigen or allogeneic MHC class II molecules (Doyle and Strominger, 1987). $CD8^+$ lymphocytes can interact with MHC class I molecule and are often referred to as cytotoxic lymphocytes (Norment *et al*, 1988). The specificity of these two sub-populations is probably the result of the ability of CD4 and CD8 to recognize non-polymorphic regions of MHC class II and MHC class I molecules respectively during T cell development in the thymus (Emmrich, 1988). A model for the classical interaction between MHC antigen and the TCR was put forward (Bjorkman *et al*, 1987b) based on the results from crystallographic studies of the product of the MHC class I allele, HLA-A2 (Bjorkman *et al*, 1987a). This model proposed that certain variable regions of the

TCR could interact with the α helices of the MHC, whilst the highly polymorphic VDJ junctional regions of the TCR could interact with antigenic peptides bound in the MHC cleft. Crystallographic studies of MHC class II molecules have not yet been performed however it is possible that their structure is similar to MHC class I molecules. It has been suggested that perturbation of the TCR by MHC class I antigen generates a signal within the lymphocyte that induces CD8 to bind to MHC class I (O'Rourke *et al*, 1990), resulting in stabilization of the bond between the TCR and MHC class I. An analogous role for CD4 and MHC class II is also possible but is yet to be described.

Resting T lymphocytes can also be delineated into memory and naive lymphocytes based on cell surface marker expression. These two subsets were first distinguished with the monoclonal antibodies (mAb) 2H4 and UCHL1, which recognize different isoforms of CD45, CD45RA and CD45RO, on naive and memory T lymphocytes respectively (Dalchau and Fabre, 1981). That CD45RO is a marker for memory cells is a contentious issue since it has been discovered that recently activated T lymphocytes expressed this marker (Taga *et al*, 1991) and that transition to the CD45RO phenotype was not irreversible (Bell and Sparshott, 1990).

Further characterization of the CD45RA and CD45RO sub-populations has been performed, and it was found that naive lymphocytes expressed low levels of CD29, LFA-3, CD-2, LFA-1 and CD45RO and high levels of CD45RA, whilst memory cells expressed high levels of CD29, LFA-3, CD-2, LFA-1 and CD45RO and low levels of CD45RA (Sanders *et al*, 1988). The same study found that CD3 expression remained constant. Thus memory cells would appear to express higher levels of adhesion molecules than naive cells, possibly enhancing the capacity of these lymphocytes to form clusters with themselves and/or antigen presenting cells (APC) and, as a result, lessening the stringency of activation requirements. The majority of activation studies however have not been performed with lymphocyte populations depleted of either of these two populations, although much work has been done with T

cell clones, which may be considered analogous to memory T cells in that they have been previously activated. Nevertheless, the few studies that have been performed on separated naive and memory populations showed that memory lymphocytes were more readily activated with mAb to CD3 or CD2 than naive cells (Tedder *et al*, 1985), however both populations seemed to respond similarly to agents such as TPA plus an ionophore (Budd *et al*, 1987) or allogeneic cells (Morimoto *et al*, 1985a; Morimoto *et al*, 1985b).

1.4.2 Requirements for T lymphocyte activation

From early studies performed with rodents, it was discovered that the role of activating lymphocytes seemed to be a property of specialised cells that are now referred to as antigen-presenting cells (APC) (Greineder and Rosenthal, 1975; Pulvertaft and Pulvertaft, 1967; Hardy and Ling, 1969). A proposal was put forward by Lafferty *et al* (1978) that the activation of lymphocytes required two signals, with the first signal being the interaction of the TCR with its ligand. This ligand was later defined as membrane-bound syngeneic MHC class I plus antigen for the TCR on $CD8^+$ lymphocytes, or membrane-bound syngeneic MHC class II plus antigen for the TCR on $CD4^+$ lymphocytes (Norment *et al*, 1988; Doyle and Strominger, 1987). The second signal was also proposed to be supplied by the stimulator cell, possibly in the form of a cytokine. This second signal, or costimulator, was originally described as a constituent of the supernatant of Concanavalin A-treated spleen cells or human leukocytes and was required at the initiation of the MLC and throughout the culture period (Lafferty *et al*, 1978).

The conventional views on how immune responses are initiated are based primarily on *in vitro* and *in vivo* murine studies as well as *in vitro* assays performed with human leukocytes. The mixed leukocyte culture (MLC) has been one of the primary *in vitro* activation assays used for understanding immune responses in the human system, and generally involves the culturing of lymphocytes with allogeneic, or

antigen-pulsed, stimulator cells for approximately 5 days, after which the degree of activation can be measured by the level of cellular proliferation. It was through the MLC that evidence for the existence of polymorphism in MHC class II molecules was first reported. Evidence from these assays also suggested that lymphocyte responses to allogeneic MHC class II were due to lymphocytes recognizing the MHC product in a similar fashion to syngeneic MHC class II plus non-self antigen (Malissen *et al*, 1988).

The importance of the interaction of the TCR and MHC class II molecules was shown indirectly through autologous and allogeneic MLC where purified resting T lymphocytes depleted of HLA-DR$^+$ cells were capable of proliferation and these activated lymphocytes were capable of absorbing exogenously added IL-2. When mAb specific for HLA-DR were added to the MLC this would reduce by 60 - 70% the capacity of the lymphocytes to absorb exogenously added IL-2, tentatively suggesting that the TCR-MHC class II interaction led to IL-2 receptor expression (Palacios, 1981; Palacios and Moller, 1981) and implying that CD4$^+$ lymphocytes were likely to be of importance in the development of the proliferative response. Additionally, it was shown that phytohaemagglutinin (PHA), Conconavalin A (Con A) or OKT3, a mAb possessing specificity for CD3, were each capable of triggering the expression of the IL-2 receptor, and that this phenomenon was not inhibitable by mAb to HLA-DR (Palacios, 1982). Another group found that triggering the TCR led to an up-regulation of the receptors for IL-1 and IL-2 on T lymphocytes, but these cells failed to proliferate, suggesting another signal(s) was necessary (Meuer and Meyer zum Buschenfelde, 1986).

In order to discover the minimum requirements for the activation of resting T lymphocytes many experiments have been performed using PHA, Con A or anti-CD3 mAb. In terms of defining such requirements, anti-CD3 mAb would appear to be of much greater value than either PHA or Con A since the mAb are more likely to mimic the effect of allogeneic MHC class II antigen, as they are unlikely to bind to any cell

surface structures on the lymphocyte other than the TCR complex. The same cannot be said for Con A and PHA which may bind to other molecules on the lymphocyte surface that in turn may have some input into the lymphocyte activation process.

The task of obtaining a comprehensible overview of the results from such experiments has proven to be difficult because of the apparently contradictory nature of the various reports regarding T lymphocyte activation requirements. Many of these contradictions probably stem from the use of different lymphocyte populations or different implements of activation. For instance, it has been reported on many occassions, by independent researchers, that the addition of a cross-linking form of an anti-CD3 mAb to purified human peripheral blood resting T cells was not sufficient to stimulate lymphocyte proliferation (Meuer and Meyer zum Buschenfelde, 1986; Manger *et al*, 1985; Scheurich *et al*, 1985; Palacios *et al*, 1985; Williams *et al*, 1985; Ledbetter *et al*, 1986a; Kawakami *et al*, 1989; Vine *et al*, 1989). However, there are also a number of reports that have found that the addition of anti-CD3 mAb was the only requirement necessary to promote lymphocyte proliferation, so long as the mAb was bound to a substrate, such as sepharose, or the plastic walls of the culture well, such that crosslinking of the TCR complex was achieved, therefore mimicking the effect of cell surface MHC molecules (Geppert and Lipsky, 1987; Van Lier *et al*, 1989; Bank and Chess, 1985; Verwilghen *et al*, 1991).

The seemingly paradoxical nature of these reports may, in some cases, be the result of differing degrees of T lymphocyte purification leading to varying levels of contaminating cells with potential for accessory function. Such cells include activated B or T cells, monocytes, dendritic cells and possibly activated NK cells, most of which have been found capable of functioning as APC (Krieger *et al*, 1986; Chestnut and Grey, 1981; Glimcher *et al*, 1982). On the other hand, resting T and B lymphocytes have been shown to be weak APC (Krieger *et al*, 1986). In an attempt to exclude the possibility of the contribution of APC to the proliferation assays, many researchers purified the T lymphocytes such that > 99% were $CD3^+$ and < 1% were MHC class

II^+. For instance, Verwilghen *et al,* (1991) found that some immobilized mAb to CD3, such as UCHT1, WT32, 64.1, anti-Leu-4 and anti-T3, were capable of stimulating T lymphocyte proliferation on their own and that anti-IL-1β did not inhibit this activation process. In contrast, however, are results obtained by Williams *et al,* (1985) which showed that the mAb, 64.1, required the presence of IL-1 to stimulate lymphocyte proliferation. Additionally, it was shown by Meuer and Meyer zum Buschenfelde, (1986) that immobilized anti-T3B was incapable of activating lymphocytes unless IL-1 was supplied.

A feasible explanation for these anomalies is that there was variation in the concentration of cross-linked anti-CD3 available to the lymphocytes and that this concentration may have partially depended upon the mode of presentation of the anti-CD3 mAb. To illustrate, both Dixon *et al,* (1989) and Verwilghen *et al,* (1991) used wells coated with 10 - 20 μg/ml of goat anti-mouse antibodies (GαM) to immobilize anti-CD3 mouse mAb which were added to the wells at 0.01 - 1 μg/ml, whereas Williams *et al,* (1985) and Meuer and Meyer zum Buschenfelde, (1985) used sepharose beads to immobilize the anti-CD3 mAb. Furthermore Verwilghen *et al,* (1991) found that varying the amount of anti-CD3 added to the wells coated with GαM resulted in variations in levels of lymphocyte proliferation. It may be that using wells coated with anti-CD3 mAb is a more effective way of activating T lymphocytes than using coated sepharose beads. Thus it would appear that, under certain conditions, purified resting T lymphocytes can be activated by merely cross-linking the TCR complex. However, these experiments may represent an extreme that is, physiologically speaking, unrealistic. Additionally, there is an overwhelming amount of data, supporting the two signal hypothesis, that has been derived from experiments performed with mAb to CD3 as well as from mixed leukocyte reactions, the latter perhaps bearing greater biological relevance.

1.4.3 The biochemistry of lymphocyte activation

Biochemically, T lymphocyte activation can also be defined in terms of two required signals; 1) an increase in intracellular concentrations of calcium ions ($[Ca^{++}]_i$) and 2) the activation of protein kinase C (PKC) (Weiss and Imboden, 1987; Altman *et al*, 1990). It would appear that an increase in $[Ca^{++}]_i$ and the activation of PKC results in the mobilization of a number of key DNA-binding proteins (e.g. AP-1, Oct-2 and NF-κB) involved in the induction of IL-2 and IL-2R-α response genes (Ullman *et al*, 1990) which then leads to T lymphocyte proliferation (reviewed by Rothenberg *et al*, 1992).

It has been shown that, when anti-CD3 or anti-TCR mAb interact with CD3 or TCR respectively, there is an increase in $[Ca^{++}]_i$ (Hesketh *et al*, 1977; Tsien *et al*, 1982) that is due to the generation of inositol-1,4,5-trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) and consequent triggering of the release of Ca^{++} from the endoplasmic reticulum (Streb et al, 1983; Abdel.latif, 1986) within one min of ligand-receptor association and can be maintained for greater than 30 min (Imboden and Stobo, 1985). When the TCR complex interacts with a ligand there is generally a resultant activation of a phospholipase C (PLC), perhaps via a G-protein (Desai *et al*, 1990) or a protein tyrosine kinase (PTK) (Weiss *et al*, 1991), which in turn hydrolyses PIP_2 to create diacyl glycerol (DAG) and IP_3. Increases in $[Ca^{++}]_i$ alone can lead to the expression of IL-2R-α (Isakov and Altman, 1985; Isakov *et al*, 1986; Leonard *et al*, 1985) reinforcing the proposal by Palacios, (1982), that the interaction of MHC antigen with the TCR leads to the expression of IL-2R on the cell surface. Recent studies suggest that one function of an increase in $[Ca^{++}]_i$ is the activation of the protein phosphatase, calcineurin, which is capable of enhancing IL-2 gene inducibility (O'Keefe *et al*, 1992; Clipstone and Crabtree, 1992). Ca^{++} alone is incapable of activating PKC (Isakov and Altman, 1987), however the addition of both a Ca^{++} ionophore and TPA, an analog of DAG and an activator of PKC, resulted in the

stimulation of resting T lymphocyte proliferation, so long as extracellular Ca^{++} was present (Niedel *et al*, 1983; Kikkawa *et al*, 1983).

Interestingly, DAG, a product of the hydrolysis of PIP_2 is involved in the activation of PKC (Kaibuchi *et al*, 1981). DAG has been shown to increase the affinity of PKC for Ca^{++} and phospholipid (Kaibuchi *et al*, 1981) which can directly activate PKC (Takai *et al*, 1979). Why then is the interaction between the TCR complex and its ligand, i.e. the first signal, usually not sufficient for activation of T lymphocytes since this interaction leads to the production of both DAG and $[Ca^{++}]_i$? One reason could be that DAG is not produced at sufficient levels to efficiently activate PKC. This could possibly explain the aforementioned observations that cross-linking of the TCR complex alone is often incapable of inducing substantial proliferation of resting T lymphocytes. If the first signal is strong enough however perhaps enough DAG is produced to significantly activate PKC, thereby circumventing the need for an exogenously supplied second signal.

One of the earliest biochemical responses to the perturbation of the TCR complex is believed to be the activation of protein tyrosine kinases (PTK) and at least three have been shown to be important in the initiation of lymphocyte activation. Two of these PTK are associated with the TCR complex:; pp59*fyn* (Samelson *et al*, 1990) and ZAP-70 have both been shown to be associated with the ζ subunit of the CD3 complex (Samelson *et al*, 1990; Chan *et al*, 1991). The third is associated with CD4 and CD8 molecules and is known as pp56*lck* (Rudd *et al*, 1988) and is expressed specifically by T lymphocytes (Koga *et al*, 1986). There is still much controversy over the roles played by PTK versus G-proteins in the regulation of the initial intracellular signalling events of lymphocyte activation. G-proteins are a structurally heterogeneous group of proteins which are capable of binding to and hydrolysing GTP and, as a result, play an intrinsic part in many cellular functions, such as transmembrane signalling, transcription, protein trafficking, proliferation and differentiation (Harnett and Klaus, 1988; Simon *et al*, 1991). Although G-proteins have been shown to be involved in T

lymphocyte activation via pathways that involve PIP_2 hydrolysis (Harnett and Klaus, 1988; Graves and Cantrell, 1991; Phillips *et al*, 1991) there is little direct evidence to suggest that they are responsible for PLC activation via the TCR. However the TCR has been shown to be associated with pp59*fyn* (Samelson *et al*, 1990) and perturbation of the TCR on certain T cell lines can result in tyrosine phosphorylation and activation of PLC-γ1 (Park *et al*, 1991; Dasgupta *et al*, 1992). Further indirect evidence to support the idea that PTK are important in TCR mediated intracellular signalling was provided with the discovery that CD45, a protein tyrosine phosphatase, was crucial for signalling via the TCR to occur (Mustelin *et al*, 1989; Koretzky *et al*, 1990).

1.4.4 Cytokines as second signals

Whilst the two signal model may still hold sway amongst the cellular immunologists, the nature of the second signal has indeed been the subject of much controversy. IL-1 was initially proposed as the cytokine responsible and has been shown to act as an activation signal for T lymphocytes stimulated with immobilized anti-CD3 mAb (Williams *et al*, 1985; Manger *et al*, 1985). The outcome of IL-1 binding to its receptor on an antigen-stimulated T cell is the production of IL-2 and CD25 (Durum *et al*, 1985). It has been proposed that IL-1 can act by increasing PKC activity and increasing cytosolic calcium concentrations (Dinarello, 1988) and that the binding of IL-1 to its receptor leads to internalization of the receptor-ligand complex, which is not degraded and may be transported to the nucleus where it may have a role in regulating intranuclear processes (Lowenthal and MacDonald, 1985; Mizel *et al*, 1987). However other studies reported that when IL-1 stimulated lymphocyte proliferation in combination with immobilized anti-CD3, a different species of DAG, to that resulting from PIP_2 hydrolysis, was produced (Altman *et al*, 1990). Additionally, evidence that the signalling pathway of IL-1 does not involve the activation of PKC came from experiments where PKC inhibitors had no effect on IL-1-induced biological responses such as CD25 expression (Abraham *et al*, 1987). Instead, IL-1 appears to be

capable of signalling via a G-protein which activates adenylate cyclase to produce cAMP (Chedid *et al*, 1989), which in turn activates protein kinase A (PKA). PKA has the ability to convert the DNA-binding protein, NF-κB, from an inactive to an active form (Shirakawa and Mizel, 1989) which then translocates to the nucleus, binds to the promoter regions of the IL-2 gene (Hoyos *et al*, 1989) and the gene encoding the light chain of the IL-2R (CD25) (Cross *et al*, 1989) to enhance their transcription. As a result of these findings, a model was proposed by Mizel *et al* for one hypothesised pathway of signal transduction utilized by IL-1 (Mizel *et al*, 1990). Such findings however appear to contradict the discovery that an increase in intracellular cAMP levels is capable of inhibiting T cell signalling, partly through the uncoupling of the TCR-complex from PLC (Kim *et al*, 1988; Mary *et al*, 1987; Gajewski *et al*, 1990).

It has been suggested that TNF-α may trigger IL-2R-α expression via a similar pathway to that described above for IL-1 (Lee *et al*, 1987). TNF-α, which can be produced by monocytes in MLC (Lee *et al*, 1993) and by activated T lymphocytes (Anegon *et al*, 1988), is also capable of triggering IL-2 production by T lymphocytes (Lowenthal *et al*, 1989) and enhancing the proliferation of T lymphocytes in response to mitogen, antigen or alloantigen (Yokota *et al*, 1988; Shalaby *et al*, 1988). Another cytokine that has been shown to play a role in lymphocyte activation is IL-6, which was recognized as a costimulator for human T lymphocytes and thymocytes (Lotz *et al*, 1988) but was often inadequate unless IL-1 was also added, resulting in a synergistic effect at inducing T lymphocyte proliferation (Houssiau *et al*, 1988). It has been suggested that IL-6 may act by enhancing the expression of the high affinity IL-2R (Houssiau *et al*, 1989). IL-6 has also been shown to act in synergy with TNF-α to induce proliferation of PHA-stimulated T cells, where the effect of TNF-α did not result from the secondary induction of IL-1 (Kuhweide *et al*, 1990).

Thus the classical immune response is believed to be initiated by an APC which presents allogeneic MHC class II antigen to $CD4^+$ lymphocytes, which upon activation produce a number of lymphokines including IL-2 which in turn induces their own

proliferation as well as the proliferation of effector $CD8^+$ lymphocytes, NK cells or B lymphocytes. Although this scenario is generally true for B lymphocyte responses (Doherty *et al*, 1992), it has been discovered through murine studies with skin allografts that $CD4^+$ and $CD8^+$ lymphocyte populations can possess both helper and effector (cytotoxic) function (Golding and Singer, 1985; Golding *et al*, 1987). Thus it would appear that a proportion $CD8^+$ lymphocytes can respond to APC without addition of exogenous IL-2 or the involvement of $CD4^+$ lymphocytes. In the mouse, purified resting $CD8^+$ lymphocytes have been induced to proliferate in response to MHC class II^- peritoneal macrophages (Inaba *et al*, 1987). Additionally, P815, a MHC class II^- mastocytoma cell line has been reported to directly stimulate the proliferation of $CD8^+$ lymphocytes as well as generating primary CTL responses (Sprent and Schaefer, 1986). Whilst allogeneic T lymphocytes are not generally allostimulatory, after treatment with a neuraminidase, an enzyme that cleaves terminal sialic acid residues, the T lymphocytes demonstrated reasonably strong APC function for murine $CD8^+$ lymphocytes (Sprent and Schaefer, 1989). It has also been reported that human $CD8^+$ T cells can be activated by allogeneic cells, as determined by their capacity to produce IL-2, in the absence of $CD4^+$ T cells (Jooss *et al*, 1989). However, based on analysis by limiting dilution and cell cloning, it would seem that most $CD4^+$ lymphocytes are biased toward being "helpers" since approximately 90% of human peripheral blood $CD4^+$ lymphocytes are reported to be capable of secreting IL-2 as compared with 10 - 15% of $CD8^+$ lymphocytes (Moretta, 1985).

1.4.5 The role of accessory molecules in T lymphocyte activation

1.4.5(a) CD4 and CD8

The main focus thus far has been on the role of the TCR complex-ligand interaction in T lymphocyte activation. However it should be noted that a vast array of molecules on both the surface of the T cell and the APC can play a role in at least increasing the efficiency of the activation process. These molecules are often referred

to as accessory molecules as they are capable of enhancing the ability of lymphocytes to respond to triggering of the TCR complex, but alone are generally not stimulatory for lymphocytes. Molecules such as CD4 and CD8 molecules have been shown to enhance the binding of the TCR to MHC class II and MHC class I molecules respectively, probably due to their ability to bind to non-polymorphic regions of the MHC molecules, resulting in stabilization of the MHC-TCR interaction (Emmrich, 1988; Saizawa *et al*, 1987; Gay *et al*, 1987). CD4 and CD8 also appear to be capable of cell signalling through their cytoplasmic association with pp56^{lck} (Veilette *et al*, 1988). CD4 and CD8 themselves possess no PTK domain (Maddon *et al*, 1985), however cross-linking of either of these molecules has been shown to result in an up-regulation of pp56^{lck} activity leading to phosphorylation of the ε chain of CD3 (Veilette *et al* 1989). Phosphorylation of the Tyr505 in pp56^{lck} leads to down-regulation in the activity of this enzyme and mutant forms, which cannot be phosphorylated at this residue, have been shown to be capable of transforming transfected fibroblasts (Marth *et al*, 1988). Rudd *et al*, (1989) showed that the pp56^{lck} associated with CD4 and CD8 is probably identical, but there is evidence to suggest that CD4 and CD8 could nevertheless signal differently. To illustrate, Hurley *et al*, (1989) showed that, subsequent to treatment with TPA, pp56^{lck} dissociated from CD4 but not from CD8.

1.4.5(b) LFA-1

Other receptor-ligand interactions shown to sometimes play a role in lymphocyte activation involve the adhesion molecules, such as LFA-1 and one or more of its ligands, ICAM-1, 2 or 3; or LFA-2 (CD2) and its ligand LFA-3. LFA-1, which is expressed constitutively by lymphocytes, as it is by most leukocytes (Marlin and Springer, 1987), has been shown to have an important part to play in both T cell-mediated cytotoxicity (Makgoba *et al*, 1988) as well as homotypic and heterotypic antigen-independent conjugate formation (Rothlein *et al*, 1986). Such information was gained from *in vitro* experiments utilizing mAb to LFA-1 or ICAM-1, which is a ligand

for LFA-1 (Makgoba *et al*, 1988). Unlike LFA-1, ICAM-1 expression has not been detectable on resting lymphocytes (Dougherty *et al*, 1988), however it can be induced after activation (Boyd *et al*, 1989; Schulz *et al*, 1988). LFA-1 expression is also up-regulated after activation with PHA (Rothlein and Springer, 1986) and cross-linking the TCR can result in a transient increase in the avidity of LFA-1 for ICAM-1 (Dustin and Springer, 1989). Once activated, PKC has been shown to be capable of phosphorylating the β chain of LFA-1, an event proposed to result in the enhancement of the affinity of LFA-1 for its ligand (Dustin and Springer, 1989). That the LFA-1-ICAM-1 interaction is important in antigen presentation, leading to lymphocyte proliferation, was shown through transfection studies where L-cell transfectants, expressing HLA-DR at levels similar to those of monocytes, were only capable of stimulating lymphocytes if the L-cells were supertransfected with ICAM-1. However, L-cell transfectants expressing higher levels of HLA-DR were capable of stimulating lymphocyte proliferation in the absence of ICAM-1 (Altman *et al*, 1989). Both LFA-1 and ICAM-1 have been implicated in cell signalling (Van Noesel *et al*, 1988; Geissler *et al*, 1990; Van Seventer *et al*, 1990; Vermot Desroches *et al*, 1991). For instance, mAb to LFA-1α were shown to enhance the proliferation of T lymphocytes using a T cell activation model that was independent of cellular adhesion (Van Noesel *et al*, 1988). Interestingly, mAb to LFA-1β were found to be inhibitory in the same system. Other recently discovered ligands for LFA-1 include ICAM-2, ICAM-3 and ICAM-R (de Fougerolles *et al*, 1991; de Fougerolles *et al*, 1993; Vazeux *et al*, 1992). De Fougerolles and Springer, (1992) have proposed that ICAM-3 may play an important role in the initiation of immune responses based on, 1) the discovery that resting T lymphocytes adhered to purified LFA-1 primarily via ICAM-3 and 2) the finding that monocytes and resting T cells expressed ICAM-3 at much higher levels than ICAM-1 or ICAM-2.

1.4.5(c) CD2

CD2, a molecule expressed by all T lymphocytes, but not exclusively, is currently defined as having one physiological ligand, known as LFA-3, or CD58 (Selvaraj *et al*, 1987; Dustin, *et al*, 1987). Like the LFA-1-ICAM-1 interaction, the CD2-LFA-3 interaction has been shown to be functionally involved in cell-mediated lysis (Sanchez-Madrid *et al*, 1982; Shaw and Luce, 1987), and ligand binding to CD2 on T cells has been shown to mimic the effect of IL-1 or accessory cells in providing the second signal (Hara *et al*, 1985). As is the case for the activation of lymphocytes via CD3/TCR, perturbation of CD2 results in the activation of PLC, which in turn leads to an increase in $[Ca^{++}]_i$ (Sanders *et al*, 1988; Baynasco *et al*, 1989).

Additionally, the induction of T lymphocyte proliferation can be made to occur in an antigen-independent fashion, using a combination of mAb directed against discrete epitopes of the CD2 molecule. Meuer *et al* showed that mAb to the epitopes $T11_2$ and $T11_3$ resulted in the activation of T lymphocytes in the absence of accessory cells (Meuer *et al*, 1984). $T11_3$ is initially unavailable for binding, but during activation this epitope is exposed as a result of a conformational change in the CD2 molecule. This mode of lymphocyte activation is often referred to as "the alternative pathway" and has been reported to be less dependent on accessory signals such as IL-1, than activation via CD3 (Meuer *et al*, 1983; Meuer and Meyer zum Buschenfelde, 1986). However, there are contrary reports which demonstrate that IL-1 and IL-6 can act synergistically to aid in the CD2 driven proliferation of lymphocytes, where IL-6 was shown to enhance the expression of the high affinity IL-2 receptor (Lorre *et al*, 1990). The binding of mAb to CD2 can bring about lymphocyte proliferation, however the interaction of CD2 with its natural ligand, LFA-3, is not a sufficient stimulus and actually requires the addition of mAb to $T11_2$ and $T11_3$ as LFA-3 only binds to CD2 via $T11_{1a}$ and $T11_{1b}$ (Meuer *et al*, 1989). Although considered to be an antigen-independent mode of lymphocyte activation, in some circumstances triggering of proliferation via CD2 appears to be dependent on the presence of CD3, as it was shown

that modulation of CD3 molecules with non-mitogenic concentrations of an IgM anti-CD3 mAb resulted in non-responsiveness of lymphocytes to the subsequent addition of a normally mitogenic combination of anti-$T11_1$ mAb (Brottier *et al*, 1985).

1.4.5(d) CD28

The combination of TPA and a mAb to CD28 induces resting T cells to express IL-2, high affinity IL-2R and to proliferate, and this means of activation was shown to be independent of the CD3/TCR complex (Hara *et al*, 1985). Additionally, the signal transduction pathway of CD28 seems to be distinct from that of CD3 as there was found to be no increase in intracellular Ca^{++} concentrations or PKC activation upon perturbation of CD28 (Ledbetter *et al*, 1986a; Ledbetter *et al*, 1987). Also, stimulation of T lymphocyte proliferation and IL-2 synthesis via CD28 does not appear to involve an increase in phospholipid hydrolysis (June *et al*, 1990). Instead, increased levels of cGMP have been observed (Ledbetter *et al*, 1986a). However, like mAb to CD3, mAb to CD28 were capable of triggering TNF-α mRNA production in peripheral blood T lymphocytes (Thompson *et al*, 1989). There is evidence which suggests that CD28 can deliver the second signal. For instance, the cross-linking of CD3 was found by some researchers to be insufficient for the activation of T lymphocytes and it was found that CD28, present on T cells, needed to interact with anti-CD28 mAb or with the ligand for CD28, known as B7, on the surface of transfected cells, before lymphocyte proliferation could occur (Jenkins *et al*, 1991; Linsley *et al*, 1991).

* See amendment 1 at the back of this thesis for further elaboration on CD28

1.4.5(e) CD45

As mentioned previously, two different isoforms of CD45, known as CD45RA and CD45RO, are used to distinguish naive cells from memory cells respectively. However 8 isoforms of CD45, which are generated through differential usage of 3 of the exons (A, B and C) of the CD45 gene (Streuli *et al*, 1987), have actually been

discovered. CD45RA is partly encoded by the A exon whilst exons A, B and C do not contribute to CD45RO (Akbar *et al*, 1988). $CD45RA^+$ lymphocytes convert to a $CD45RO^+$ phenotype upon activation, with a concomitant loss of CD45 isoforms containing the A exon (Akbar *et al*, 1988). There is sufficient evidence to suggest that CD45 plays an intrinsic role in lymphocyte activation but just what that role(s) is, at this stage, is uncertain. The physiological ligand for CD45 is yet to be defined, however it is known that CD45 has a cytoplasmic domain possessing phosphotyrosine phosphatase (PTPase) activity. CD45 PTPase has been shown to dephosphorylate both $pp56^{lck}$ and $pp59^{fyn}$ *in vitro* (Mustelin *et al*, 1989; Mustelin *et al*, 1992), although there is no direct evidence which demonstrates that CD45 regulates the activity of $pp56^{lck}$ or $pp59^{fyn}$ *in vivo*. It has been observed however that $CD45^-$ human acute lymphocytic leukaemic cells possessed much lower levels of $pp59^{fyn}$ kinase activity than those transfected with CD45 (Shiroo *et al*, 1992).

The concomitant cross-linking of CD3 and CD45 with mAb has been reported to result in the inhibition of lymphocyte activation as detected by the repression of calcium signals (Ledbetter *et al*, 1988), inositol phosphate production (Ledbetter *et al*, 1991) and proliferation (Ledbetter *et al*, 1988). In contrast, it was discovered that in $CD45^-$ mutant lymphocytes of T leukaemia cell lines, the TCR was uncoupled from mitogenic intracellular pathways, suggesting a positive role in the process of lymphocyte activation (Koretzky *et al*, 1990). A possible solution to the apparently paradoxical nature of these results has been suggested by Shivnan *et al*, (1992) who found that when the ratio of cross-linked CD3 to cross-linked CD45 was sustained at 1:1 or 1:2 then inhibition of lymphocyte activation did not occur. It was assumed that, in previous experiments where manipulations were not performed using controlled levels of cross-linking mAb, the ratio of cross-linked CD3 to CD45 was 1:10 since CD45 is expressed 10 times more abundantly on T cells than CD3 (Shivnan *et al*, 1992), and thus this ratio may have resulted in negative signalling.

$CD45RO^+$ T lymphocytes proliferate to a greater extent than $CD45RA^+$ cells in response to recall antigen and mitogenic mAb (Merkenschlager *et al*, 1988). Also PKC activation appears higher in $CD45RO^+$ lymphocytes (Merkenschlager *et al*, 1988). Such phenomena are probably, at least partially due to the higher basal levels of secondary signalling molecules, such as DAG, in the $CD45RO^+$ population (Robinson *et al*, 1993) rather than reflecting a functional disparity between the two molecules, however this is yet to be clearly ascertained.

1.5 Natural killer cells

1.5.1 Definition of natural killer cells

Natural Killer (NK) cells are a heterogeneous population of cells, functionally and phenotypically distinct from both T and B lymphocytes, and comprising approximately 10 - 15% of human peripheral blood lymphocytes (reviewed by Trinchieri, 1989). They were originally identified through (and defined by) their capacity to spontaneously lyse allogeneic tumour cell lines in a non-MHC restricted fashion without prior sensitization and without the addition of exogenous cytokines (Heberman *et al*, 1975a; Heberman *et al*, 1975b; West *et al*, 1977). However, NK cells have since been more precisely defined through their cell surface antigen expression ($CD3^-$, $CD2^+$, $CD56^+$ and usually $CD16^+$), and a number of phenotypically distinct subsets have been described, with some possessing different activation requirements (see later). NK or NK-like activity is believed to have been developed very early in the evolution of the immune system since virtually all vertebrates, including fish, as well as some invertebrates, such as earthworms, possess this activity (Decker *et al*, 1981). Human NK cells are capable of lysing a broad spectrum of cell-types including autologous, allogeneic or xenogeneic tumours (Hansson *et al*, 1979), virally infected cells (Herberman and Ortaldo, 1981) and proliferating normal cells such as haemopoietic cells (Kiessling *et al*, 1977).

Studies of NK cells have been aided by the development of the limiting dilution technique which many researchers have used to generate NK cell clones (Hercend *et al*, 1982; Kornbluth *et al*, 1982). Such clones can continue to grow for several years with the retention of phenotypic and functional traits. However, NK cell clones are dependent on the presence of allogeneic or autologous feeder cells in order to constantly proliferate. These feeder cells are usually irradiated peripheral blood mononuclear cells plus Epstein-Barr virus-transformed cells (Hercend *et al*, 1983). A wide variety of phenotypically distinct NK cell clones have been generated and all NK cell clones appear capable of lysing K562 cells, but differ with respect to their ability to lyse other targets (Ritz *et al*, 1988). Along with T cells, NK cells have been shown to be precursors of lymphokine-activated killer cells (LAK) which are capable of lysing NK-resistant targets (Ortaldo *et al*, 1986). Such a conversion can be induced by culturing NK cells with high levels of IL-2.

The majority of researchers in the past have tended to detect the presence of NK cells, or NK cell activity, through their ability to lyse cells of the MHC class I^-, class II^- leukaemic cell line, K562 without the requirement for prior sensitization (Jondal and Pross, 1975). However, such a characteristic could also be assigned to T lymphocytes that are no longer MHC-restricted (Phillips and Lanier, 1987; Phillips *et al*, 1987) and does not tend to delineate subpopulations of human peripheral blood NK cells. Recently, a number of mAb, specific for what are considered to be NK cell markers, have become available. Two of the most useful antigens detected thus far are CD16 and CD56. CD16, or FcγRIII, is expressed by 80 - 90% of NK cells and only 1% of T lymphocytes and is not expressed by B lymphocytes (Lanier *et al*, 1985). This molecule is expressed by NK cells as a transmembrane protein with a cytoplasmic domain and is considered to be a marker of NK cell differentiation (Nagler *et al*, 1989) but has been reported to not be involved in the differentiation process itself as indicated by the inability of a solid-phase-bound mAb to CD16 to either promote or inhibit the generation of NK cells (Warren and Skipsey, 1991). Subpopulations can be delineated

on the basis of lack of expression of CD16, $CD16^-$, low level expression, $CD16^{dim}$, or expression of higher levels, $CD16^{bright}$, the latter of which make up the majority of the NK population in the peripheral blood. CD56, also known as NKH1, is expressed by virtually all (> 95%) NK cells and < 5% of T cells (Ritz *et al*, 1988). Using a combination of mAb simultaneously in an immunofluorescence assay, one can define a number of phenotypically distinct NK cell subpopulations, the most abundant being $CD56^{dim}$, $CD16^{bright}$, $CD3^-$ (80 - 90%) (Perussia *et al*, 1982). Another reasonably prominent population are the $CD56^{bright}$, $CD16^-$, $CD3^-$ cells, which express CD56 at higher levels than the $CD16^{bright}$ group (Nagler *et al*, 1989) but are found not to comprise the high density NK cell population (Warren and Skipsey, 1991). Instead, the high density NK cell population has been reported to possess a greater proportion of $CD16^{dim}$ cells than the total peripheral blood NK cell population. Other molecules found expressed by NK cells include the pan T lymphocyte marker CD2, which is detected on 80 - 90% of NK cells (Meuer *et al*, 1985), CD57, also known as HNK1, which is found on 50 - 60% of NK cells as well as a large proportion of T lymphocytes (Abo *et al*, 1981), CD8, which is present on approximately 30% of NK cells, is generally expressed at lower levels than on T cells (Schmidt *et al*, 1986) and is usually present as a homodimer (α/α) rather than the heterodimer (α/β) found associated with T lymphocytes (Norment and Littman, 1988; Baume *et al*, 1991).

NK cells are often referred to as large granular lymphocytes (LGL) (Timonen *et al*, 1981; Fitzgerald-Bocarsly *et al*, 1988) and the majority of these circulating cells are considered to be in the resting state as defined by their lack of expression of activation antigens such as CD25 and MHC class II antigen (Phillips *et al*, 1984; Perussia *et al*, 1987; Trinchieri, 1989). However not all NK cells are considered to be LGL, as non-activated NK precursors in the peripheral blood have the same buoyant density as resting T cells (Warren, 1984). The ontogeny of NK cells is not well defined but it is generally conceded that they form a lineage distinct from T and B lymphocytes, since most reports have indicated that they possess the germ-line configuration of both

immunoglobulin (Ig) and TCR genes (Lanier *et al*, 1986; Ritz *et al*, 1985; Reynolds *et al*, 1985). However, some NK cell clones have been found to possess a rearranged TCR gene (Schmidt *et al*, 1986). Such contradictory data probably stems from confusion as to the definition of a NK cell where some researchers have chosen to refer to $CD3^+$ lymphocytes possessing NK activity and NK markers such as CD56 and CD16 as being NK cells (Schmidt *et al*, 1986) whilst most researchers refer to such cells as non-MHC-restricted T lymphocytes (Lanier *et al*, 1986; Trinchieri, 1989). In fact, the fifth international workshop on natural killer cells defined NK cells as being $CD3^-$ and TCR^- (Fitzgerald-Bocarsly *et al*, 1988).

To this point, no progenitor cell which can give rise to T, B and NK cells exclusively has been discovered (Lanier *et al*, 1992). NK cells apparently develop normally in *scid* mice which lack the capacity to produce T or B cells with rearranged Ig or TCR genes (Hackett *et al*, 1986). NK cells also appear not to require the thymus for maturation since they develop normally in athymic *nude* mice (Herberman *et al*, 1975). However, unlike T lymphocytes they have been shown, in mice, to be dependent on the bone marrow microenvironment for their terminal differentiation (Hackett *et al*, 1985; Bennett *et al*, 1976)

1.5.2 Mechanism of NK cell activation

Far less research has been performed regarding the minimal requirements for NK cell activation than for T cell activation, partly due to the difficulties in obtaining pure populations. The results obtained have probably been dependent on the level of purity of the NK cells and also on the techniques for purifying them (see review by Trinchieri, 1989). For instance, some groups have purified NK cells via positive selection and, in doing so, may have inadvertently caused cell signalling, potentially leading to an influence upon the outcome of the experiments. For example, London *et al* (1986) purified NK cells using a mAb to CD16, an antigen which has subsequently been shown to play a role in cell signalling (Ting *et al*, 1992). Additionally, many

activation experiments have been performed using the total peripheral blood NK population (Trinchieri *et al*, 1984; Lanier *et al*, 1985), however, different sub-populations of NK cells appear to have different activation requirements. For instance $CD56^{bright}$, $CD16^{-}$ NK cells have been reported to be capable of proliferating in response to low concentrations (e.g. < 10 IU/ml) of IL-2 alone (Nagler *et al*, 1990; Caligiuri *et al*, 1990), whilst $CD56^{dim}$, $CD16^{bright}$ NK cells could only respond to high concentrations (> 100 IU/ml) of IL-2 (Nagler *et al*, 1989; Nagler *et al*, 1990). It has also been reported that the peripheral blood NK population, as a whole could proliferate in response to high levels of IL-2 (500 U/ml), however a subpopulation of NK cells (with the same buoyant density as resting T cells), were not responsive to IL-2 unless additional signals were supplied, despite the constitutive expression of p75 (IL-2 β chain) by the NK cells. These additional signals, whilst currently undefined, can be partially provided by the human malignant melanoma cell line MM-170 or mitomycin C-treated activated autologous T cells (Warren and Skipsey, 1991; Warren, 1984). In further studies, IL-2-containing conditioned medium was shown to be far more effective than rIL-2 at triggering the proliferation and cytotoxic activity of resting NK cells (cultured with γ-irradiated MM-170 cells), implicating the importance of another cytokine in this activation process (Warren *et al*, 1993). Additionally, research performed by London *et al*, (1985) with LGL revealed that the combination of IL-2-containing cultured medium and irradiated allogeneic lymphoblastoid cells (Daudi cells) was capable of inducing the proliferation and cytotoxic activity of resting NK cells. Here too it was discovered that other undefined factors in the culture medium other than IL-2 were involved. In both of the examples described above IFN-γ did not appear to be the factor responsible.

One recently discovered cytokine shown capable of enhancing the cytotoxic activity of resting NK cells is IL-12. IL-12, or NK stimulatory factor, was originally isolated from the culture supernatants of phorbol diester-stimulated B lymphoblastoid cell lines (Stern et al, 1990). This cytokine was shown to be capable of enhancing the

proliferation and cytotoxicity of activated NK cells (Gately et al, 1991). A more recent study has revealed that, although IL-12 is capable of enhancing the cytolytic activity of resting and activated NK cells, it had little effect upon the proliferation of purified resting NK cells and was actually shown to inhibit the IL-2-induced proliferation of NK cells (Robertson *et al*, 1992). IL-12 had similar effects to IL-2 in that it could directly up-regulate the expression of adhesion molecules important in the cytolytic process, such as CD2, CD11b and ICAM-1, nevertheless IL-12 appeared to be capable of activating NK cells via a pathway distinct from that used by IL-2 since anti-IL-2 was not capable of inhibiting the effects of IL-12 (Robertson *et al*, 1990; Robertson *et al*, 1992). IL-12 seemed to preferentially stimulate cytotoxic activity in $CD16^+$ NK cells over $CD16^-$ NK cells (Chehimi *et al*, 1992) and was active at concentrations as low as 0.1 - 1 pM (Kobayashi *et al*, 1989). Although IL-12 inhibited the proliferation of IL-2 activated NK cells this cytokine has been found to be capable of stimulating the proliferation of PHA-activated T lymphoblasts as well as synergizing with IL-2 in the generation of LAK cells (Robertson *et al*, 1992).

NK cells (LGL) derived from the peripheral blood have been shown to have enhanced cytotoxic potential when cultured with IL-2 and this enhancement was not inhibitable with anti-Tac antibody, which binds to CD25 (Trinchieri *et al*, 1984), suggesting that IL-2 can act directly through IL-2R-β (p75) which NK cells constitutively express (Kehrl *et al*, 1988). Subsequent to treatment with IL-2, NK cells were shown to express activation antigens such as HLA-DR, CD71 and the high affinity IL-2 receptor (London *et al*, 1985), leading to cell proliferation (London *et al*, 1986) and often resulting in the generation of lymphokine-activated killer (LAK) cells which have the capacity to kill tumour cells that are usually resistant to standard NK cell killing (Grimm *et al*, 1983). It has been suggested that p75, after interacting with IL-2, is capable of triggering the transcription of p55 mRNA (Kehrl *et al*, 1988). However, IL-2 alone is not normally sufficient for optimal NK activation and therefore other factors such as the presence of a tumour cell line are considered necessary. For

instance, K562 cells, or MM-170 cells (discussed earlier), have been shown to enhance the proliferation and cytoxic activity induced by IL-2 (Harris *et al*, 1989; Warren and Skipsey, 1991). Also, CD16, when cross-linked, was reported to be capable of synergistically enhancing the effects of IL-2 (Harris *et al*, 1989). As discussed above, IL-2 was found capable of inducing CD25 expression on NK cells, an event which was also found to be triggered by other factors such as IL-1 or IFN-γ (Shirakawa *et al*, 1986). IFN-γ has also been shown to increase the cytolytic activity of NK cells against such targets as fresh tumour cells (Vanky *et al*, 1980) and furthermore, IFN-γ can synergize with IL-2 to enhance NK cell cytotoxicity (Brunda *et al*, 1986).

CD16 has been shown on many occasions to be important in triggering NK cell cytotoxic activity. Cross-linking of this receptor on NK cells has been shown to lead to an increase in Ca^{++}, IP_3, IP_4 (Cassatella *et al*, 1989) and DAG, or more specifically *sn*-1,2-DAG (Berridge and Irvine, 1989), as well as an increase in transcription of mRNA for IFN-γ and TNF-α (Anegon *et al*, 1988), eventually resulting in exocytosis of preformed cytolytic granules (Henkart and Yue, 1988). NK cells have been shown to express at least two isoforms of PLC, PLC-γ1 and PLC-γ2, both of which become phosphorylated, probably by a protein tyrosine kinase, within 1 minute of cross-linking of CD16 (Ting *et al*, 1992). CD16 has also been implicated in the activation of NK cells via CD2 through experiments performed with a TCR^- variant of the Jurkat T cell line which was nonresponsive to CD2 triggering, but became responsive after these cells were transfected with CD16 (Reinherz *et al*, 1992). These studies went further to show that CD2 signal transduction in NK cells was dependent on the presence of the ζ protein (normally associated with CD3) which is expressed by NK cells and has been shown to be associated with CD16 in NK cells (Anderson *et al*, 1990), and to be crucial for its expression (Vivier *et al*, 1991). Perturbation of CD16 can result in the phosphorylation of a tyrosine residue on the ζ protein (Vivier *et al*, 1991), suggesting that this subunit may play a role in the cytolytic activity of NK cells.

CD16 has also been shown, indirectly, to be responsible for antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells (Perussia *et al*, 1979; Lanier *et al*, 1988).

The mechanism of activation of NK cell antibody-independent cytotoxicity is still a mystery in many respects, due in part to the difficulty in distinguishing between ligand-receptor interactions important in target cell binding and/or triggering of cytotoxic activity. Those molecules on the NK cell shown to be involved in target cell-NK cell conjugation include LFA-1, CD2 and ICAM-1 (Schmidt *et al*, 1985; Springer *et al*, 1987). These molecules bind to their respective ligands on the target cell, ICAM-1, LFA-3 and LFA-1. When mouse L-cells were transfected with human LFA-3 they became susceptible to both conjugation with, and cytolysis by, human NK cells (Anasetti *et al*, 1989). Conjugation between NK cells and K562, CEM or MOLT-4 cells could be partially (50 - 80%) inhibited by mAb to LFA-1, but these mAb had no effect on the binding of NK cells to another tumour cell line HSB (Schmidt *et al*, 1985). MAb to ICAM-1 were capable of inhibiting NK cell-target cell binding, but only at the effector cell level (Wawryk *et al*, 1989; Stade *et al*, 1989). Another molecule implicated in triggering cytolytic activity in NK cells is CD45, since a mAb specific for this antigen could inhibit the ability of NK cells to lyse K562 cells (Newman *et al*, 1983).

It is likely that different killing mechanisms are involved in NK cell-mediated cytotoxicity. These include cytolysis via the release of perforin, a complement-like protein that can form holes in target cells (Young and Liu, 1988). Other possibilities include the triggering of programmed cell death, or apoptosis, within the target cell or the secretion of lethal cytotoxins (Duke *et al*, 1986; Chow and Jondal, 1990; Gromkowski, 1986). These mechanisms have proven to be dependent upon the presence of Ca^{++} (Clark *et al*, 1988) and it has been subsequently shown that phosphoinositide hydrolysis is an important early step in the activation of the lytic process in NK cells with PKC also playing a crucial role (Chow and Jondal, 1990).

NK cells are capable of producing TNF-α and the binding of TNF-α to the TNF receptor can lead to lysis of certain targets (Tsujimoto *et al*, 1985). It has been suggested that internalization of the receptor-ligand complex is required before TNF-α can exert its biological effects. (Kull and Cuatrecasus, 1981; Liddil *et al*, 1989). The lytic effects of TNF have been shown to be O_2-dependent (Matthews *et al*, 1987) possibly implicating a role for oxygen radicals as mediators of TNF-induced cell killing. It has been reported that TNF-α on the surface of NK cells is capable of killing targets such as K562 cells that are normally resistant to the cytotoxic effects of soluble TNF-α (Peck *et al*, 1989).

1.6 Tumour immunology

1.6.1 The role of T cells in controlling cancer

The role of the immune system in the protection against cancer is a topic bristling with controversy. The idea of tumour-specific immunity had its origins in the 1950's when it was shown that methylcholanthrene-induced murine tumours had immunogenic properties in inbred mice of the same strain (Foley, 1953; Prehn and Main, 1957). It was discovered that the primary host, after resection of a chemically-induced sarcoma, was capable of rejecting subsequent challenges of the same malignant cells (Klein *et al*, 1960).

The idea that there were such things as tumour-specific antigens gained impetus with the discovery that each individual chemically-induced tumour was apparently capable of inducing a specific immune response (Old *et al*, 1962; Prehn 1962; Baldwin 1955; Globerson and Feldman, 1964). Such a phenomenon was also found to occur with U.V.-induced (Kripke, 1974) and virally-induced tumours (Sjogren *et al* 1961; Klein, 1966) which suggested that the immune system had a critical role in defending the host against malignant cells. Amidst the euphoria of these findings, it was proposed that one of the major roles of T lymphocytes was to eliminate spontaneously arisen malignancies (see Burnet, 1970). However, the promise of such a

role appeared unlikely with the discovery that *nude* (athymic) mice, which lack mature T lymphocytes, did not develop spontaneous tumours more readily than normal mice (Rygaard and Povlsen, 1976). Possibly the most optimistic interpretation of these results was that *nude* mice were not completely devoid of mature T cells as a result of the existence of extrathymic microenvironments suitable for T lymphocyte maturation. Another interpretation includes the idea that NK cells, rather than T lymphocytes, may play a role in the surveillance of tumours, a notion that is discussed in more detail later.

The most probable of conclusions, however, is that the majority of spontaneous tumours are not capable of being eliminated by the host's immune system. Why not? A number of feasible explanations have been proposed, and include; 1) that many tumours may not be immunogenic, either because they fail to express any antigens which can be recognized by the immune system as non-self, or alternatively, the tumour cells may possess tumour rejection antigens but there may be a failure, directly or indirectly, to provide the additional signals required for T lymphocyte activation, which may include cell surface antigens and/or cytokines. This inability to provide an appropriate second signal, whilst providing the first, could possibly lead to T cell anergy (Mueller *et al*, 1989) or merely unresponsiveness on the part of the T lymphocyte. 2) The tumour may be immunogenic but by the time it is recognized by the immune system the tumour mass is too large to be effectively eliminated. 3) Some tumours are capable of releasing immunosuppressive factors, such as TGF-β or prostaglandins (Broxmeyer *et al*, 1978; Farram, 1982), thus reducing the chances of lymphocyte-mediated tumour control.

It would appear, in general, that T cell surveillance of naturally occurring tumours can be successful in those instances where the tumour is virally induced (Moss *et al*, 1977; Thorley-Lawson, 1980), but is not successful where non-virally-induced tumours are concerned. Rare exceptions include the observation that immunodeficient renal transplant patients develop UV-induced squamous cell cancers at higher frequencies than normal individuals (Hardie *et al*, 1980).

The scepticism as to the role of immunology in cancer biology was further fuelled during the 1970's by the inability of researchers to directly identify tumour-specific antigens. However, since then the molecular mechanics of antigen processing and presentation have been defined (reviews by Brodsky and Guagliardi, 1991; Schwarz, 1990 and Harding and Unanue, 1990). In brief, internally manufactured proteins can be processed and small denatured segments (≈ 10 amino acids) of these proteins can be presented in association with MHC class I antigen, and it is this combination of the MHC product and antigen which is recognized by the TCR of $CD8^+$ lymphocytes (Rothbard and Gefter, 1991; Townsend *et al*, 1986; Bjorkman *et al*, 1987b; Rotzschke *et al*, 1990). Thus the native protein need not be secreted or expressed on the cell surface to be potentially immunogenic. Additionally, and concomitant to these findings, were the many reported examples of T cell clones specific for tumour cells of cancer patients from which the clones were derived (Vose and Bonnard, 1982; Slovin *et al* 1986; Barnd *et al*, 1989). Furthermore, tumour-infiltrating T lymphocytes (TIL) can be isolated and enriched and subsequently found to have unique specificity for the tumour-type from which they were derived (Topalian *et al*, 1987; Topalian *et al*, 1989; Belldegrun *et al*, 1989). It has been shown in humans that TIL have little activity against large tumour masses as opposed to LAK cells plus IL-2, which are capable of effectively attacking such lesions (Rosenberg *et al*, 1986; Rosenberg and Lotze, 1986). However, if the tumour burden is diminished by treatment with cyclophosphamide for example, TIL are much more effective (Rosenberg *et al*, 1986; Rosenberg *et al*, 1988a).

It was discovered that P815, a murine mastocytoma, was capable of presenting two tumour rejection antigens (TRA) derived from the same peptide and coded for by a gene with an identical sequence to a gene found in normal DBA/2 mice from which P185 cells were derived (Van den Eynde *et al*, 1991). This gene, P1A, is expressed by P185 cells but there is little or no expression by normal adult mouse

tissues (Lethe *et al*, 1992), possibly explaining the antigenicity sometimes displayed in syngeneic mice.

The use of human melanoma cells and lymphocytes derived from the same patient in mixed lymphocyte-tumour cell cultures often results in the generation of specific CTL (Mukherji *et al*, 1983; Knuth *et al*, 1984; Herin *et al*, 1987; Topalian *et al*, 1989). The gene responsible for one of the antigens that was recognized by autologous CTL was isolated and sequenced (Traversari *et al*, 1992; Brasseur *et al*, 1992). The gene was identical to that found in normal human tissues, however unlike > 40% of melanoma samples this gene was not found to be expressed in normal tissues.

Thus it would appear that the immune system can recognize, or be triggered to respond to, the host's own normal antigens which are inappropriately expressed. Additionally, it has been found that some human cancers express mutant proteins that can be recognized as tumour-specific antigens (reviewed by Urban and Schreiber, 1992). These mutant proteins have been found to derive from genetically aberrant proto-oncogenes or tumour suppressor genes whose abnormalities are the result of point mutations, chromosomal translocations, deletions or insertions. For instance, the intracellular protein encoded by the *ras* proto-oncogene, p21 *ras*, is involved in signal transduction and appears to become potentially oncogenic after an amino acid substitution, resulting from a point mutation, at residue 12, 13 or 61 (Taparowski *et al*, 1982; Seeburg *et al*, 1984). More recently, in a murine system, it has been found that peptides encoded by mutant *ras* proto-oncogenes were capable of associating with MHC-class II antigen and eliciting a host T lymphocyte response (Peace *et al*, 1991)

An example of a chromosomal abnormality which has the potential to result in the expression of a tumour-specific antigen, is the 9;22 translocation (Philadelphia chromosome) which is found in patients with CML and was discussed in some detail in section 1.1. The fact that this translocation event leads to the expression of an abnormal fusion protein makes those cells expressing this protein, possible targets for the immune system if the appropriate association with MHC products can occur. As

discussed previously, APML is also characterized by a chromosomal translocation, t(15;17), which results in the production of a fusion protein. If induced to travel along the monocyte pathway such cells may be capable of supplying all the signals required for T lymphocyte activation.

1.6.2 The role of NK cells in controlling cancer

The majority of primary and metastatic tumours are resistant to NK cells or NK-like activity (reviewed by Ortaldo and Herberman, 1984). Nevertheless NK cells have been shown, indirectly, to be important in controlling some forms of cancer. For instance, it was discovered that mice homozygous for the beige gene (*bg/bg*) and therefore deficient in NK activity, possessed a higher frequency of spontaneous lymphomas and were more susceptible to pulmonary metastasis formation from a B16 melanoma than their normal counterparts (Haliotis *et al*, 1985; Ritchie, 1984). Such a discovery supports the idea that NK cells may be very important in the surveillance of spontaneous tumours in *nude* mice, thus at least partially explaining why, in the previously mentioned experiments of Rygaard and Povlson (1976), there was little difference between *nude* and normal mice with respect to the frequency of spontaneous tumours. Contrary to such a notion, however, is the work of Fodstad *et al* (1984) which suggests that NK cells play little part in tumour surveillance since the growth of a number of murine and human tumours was not increased in *beige-nude* mice compared with *nude* mice. The same researchers also found that *beige-nude* mice had a similar lifespan to *nude* mice.

Susceptibility of tumour cells to NK activity has often been shown to be inversely proportional to the level of MHC class I antigen expression, as was found with human colorectal cell lines where MHC class I deficient cells were more readily lysed by NK cells. Such a correlation has been noted by many other researchers (Harel-Bellam *et al* 1986; Ohlen *et al*, 1989; Karre *et al* 1986; Ljunggren *et al*, 1988; Storkus *et al*, 1987; Tanaka *et al*, 1988). It has been suggested that the presence of MHC class I antigen

may inhibit cytolysis or target cell killing, and that this interference could occur at the binding step (Carbone *et al*, 1991). However, others have reported the interference to occur at a post-binding step where it was shown that murine NK cells could bind equally well to a MHC class I^+ murine lymphoma cells and their MHC class I^- variants, but only the MHC class I^- cells could be lysed (Ljunggren *et al*, 1988).

Studies with a variant of the murine lymphoma cell line, EL-4, which did not express β-2 microglobulin, revealed that these cells had a reduced tumourigenicity, compared to their normal counterparts, when introduced into syngeneic mice, and that the tumourigenicity was restored when the cells were transfected with β-2 microglobulin. Interestingly, the β-2 microglobulin -ve cells also had reduced tumourigenicity in nude mice, but not in mice depleted of NK cells (Glas *et al*, 1992). Experiments performed with the "Lewis lung" carcinoma (3LL) in mice revealed that the cells expressing predominantly H-2D MHC class I molecules as opposed to H-2K molecules were less immunogenic and metastasized far more readily than those cells expressing predominantly H-2K molecules (Eisenbach *et al*, 1986). The idea that only certain MHC class I molecules are involved in protecting the target from NK cell-mediated cell lysis was further supported by the finding that P815 cells transfected with human HLA-Cw3, but not other HLA alleles, were protected from lysis by allospecific human NK cell clones (Ciccone *et al*, 1992b). Although originally defined through their capacity to lyse tumour cells non-specifically, NK cells, or at least those with the $CD16^+$, $CD3^-$ phenotype, have recently been demonstrated to specifically recognize allogeneic cells (Ciccone *et al*, 1988) and susceptibility of the target cells to lysis was shown to be inherited in an autosomic recessive fashion (Ciccone *et al*, 1990a; Ciccone *et al*, 1990b). To date 5 distinct allospecificities have been defined using NK cell clones (Ciccone *et al*, 1992a). It has been proposed that NK cells are capable of recognizing self-epitopes in association with MHC class I products and that this recognition leads to an inhibitory signal to the NK cell resulting in inhibition of cytolysis (Ljunggren and Karre, 1990). Such a proposal would explain many of the above

findings of Ciccone *et al* as well as the observation that susceptibility of tumour cells is often inversely proportional to MHC class I expression.

When cells of the leukaemia cell line, K562, were pretreated with IFN-γ, they became less susceptible to cytolysis by NK cells and NK cytotoxic factors, although target-effector binding was not affected. It was shown that IFN-γ was capable of inducing MHC class I antigen expression in some of the K562 population and that the class I^+ cells were less susceptible to cytotoxic factors released by NK cells as well as lysis by NK cell themselves. Such a finding may further implicate MHC class I molecules in the role of resistance to NK cell mediated killing. However the same researchers found that other IFN-γ-pretreated cell lines also attained an enhanced capacity to resist NK cytolysis without there being a recognizable change in the levels of MHC class I expressed on their cell surface (Ramirez *et al*, 1992). Such a result suggests that IFN-γ can induce resistance to NK cell cytolysis by altering the target cell in some other way apart from increasing MHC class I expression, at least for some cell lines.

Human melanoma cells are generally sensitive to the lytic activity of NK and LAK cells *in vitro*. However after these cells were induced to differentiate with TPA they were more resistant to NK activity (Correale *et al*, 1992). Once again there was no observable change in MHC class I expression, but there was down-regulation of ICAM-1, collagen IV and the integrin vibronectin receptor (Correale *et al*, 1992). However, the role of ICAM-1, expressed on target cells, in inducing cytolytic activity in NK cells would appear to be minimal, as indicated by the findings that mAb to ICAM-1 could only inhibit conjugate formation of target-NK cells at the effector level (Wawryk *et al*, 1989; Stade *et al*, 1989). Furthermore, it has been noticed that many metastatic tumours express ICAM-1 (Ruoslahti *et al*, 1989) whilst non-metastatic solid tumours do not (Stade *et al*, 1989). LFA-1, on the other hand, has proven to be important in target-NK conjugation and it is possible that ICAM-2 and/or ICAM-3 expression by the target are the crucial ligands with which LFA-1 interacts.

1.6.3 Therapeutic strategies for acute myeloid leukaemias

For the past twenty years the standard treatment for patients with AML has been the application of the combination of two chemotherapeutic agents, cytosine arabinoside (CA) and, usually, the anthracycline, daunorubicin (D). Such a regime results in complete remission rates of approximately 65% of patients under 60 years of age (Gale and Foon, 1987; Sauter *et al*, 1984; Mayer *et al*, 1985; Rees *et al*, 1986). The median age of patients with AML is approximately 60 years (Brincker, 1982) and the percentage of patients who enter complete remission has been reported to decrease with increasing age (Preisler *et al*, 1987).

A number of new agents have also been tried alone and in combination with others, sometimes resulting in even greater CR rates for particular age groups. For instance, the treatment of newly diagnosed AML patients, below the age of 61 years, with continuous intravenous infusion of CA plus idarubicin (I), which is another anthracycline, resulted in a higher CR rate compared to those patients treated with CA plus D (Berman *et al*, 1991). However, another study has shown there to be little difference between the two treatments (Petti and Mandelli, 1989). Further data involving various combinations of agents have been briefly summarized in a review by Vogler (Vogler, 1992).

Despite the reasonably high CR rate achieved using chemotherapeutic agents the majority of patients eventually relapse (Preisler *et al*, 1989). Thus, once CR has been achieved it would appear that consolidation and/or maintenance therapy should be embarked upon as such treatment lengthens the duration of the remission, on average, as well as extending the overall survival rate of patients (Cassileth *et al*, 1988). However, even with the best scenario involving therapy with chemotherapeutic agents only, greater than 50% of AML patients who achieve CR will relapse within 5 years (Vogler, 1992).

An alternative to chemotherapeutic consolidation therapy (CCT) are HLA-matched allogeneic bone marrow transplants (BMT). Such treatment has sometimes proven to decrease the rate of relapse of AML patients when compared to CCT but often made little difference to survival as a result of BMT complications such as graft vs host disease (Champlin and Gale, 1987; Zander *et al*, 1988). Since many patients cannot receive allogeneic BMT, often as a result of the unavailability of a suitable donor, autologous BMT have recently been implemented as consolidation therapy. It would appear that ridding the patient's marrow of any residual leukaemic cells, by treatment *in vitro* with cyclophosphamide derivatives prior to transplantation, may significantly lower relapse rate compared to transplants with non-treated marrow (Gorin *et al*, 1990).

At present, 33% of adult patients under the age of 45 years and 23% of patients between the age of 45 and 60 are expected to survive, disease-free, several years after the diagnosis of AML (reviewed by Stone and Mayer, 1993). Thus, the discovery that some leukaemic cell lines and fresh leukaemic cells could be induced to differentiate *in vitro* with non-physiological or physiological factors inspired clinical investigations with some of these agents as an alternative to chemotherapy. The most encouraging results of differentation therapy have been the effects of all-*trans* retinoic acid (RA), mentioned previously, where most APML patients (newly diagnosed or resistant to chemotherapy) treated with the vitamin A derivative achieved CR (Huang *et al*, 1988; Chen *et al*, 1991; Warrel *et al*, 1991). However, there proved to be a high incidence of relapse and subsequent resistance to RA treatment, with only 1/5 of the relapsing patients achieving a second CR (Castaigne *et al*, 1990). RA-resistance *in vitro* for some leukaemic cell lines has been reported and possible mechanisms include mutations in nuclear retinoid receptors (Pratt *et al*, 1990; Collins *et al*, 1990) or alterations in the binding affinity of cellular RA binding proteins (CRABP) (Boylan and Gudus, 1991). Whether such mechanisms could apply in the case of clinical RA resistance is controversial since it has been found that leukaemic cells of RA-resistant

patients were still susceptible to the differentiating activity of RA (Muindi *et al*, 1992). A preliminary study reported that treatment of newly diagnosed APML patients with RA followed by intensive chemotherapy led to 25/26 patients achieving CR and apparently prolonged remissions (Fenaux *et al*, 1993). The idea that RA plus intensive chemotherapy is more successful at reducing the incidence of relapses than intensive chemotherapy alone is currently being tested by different groups, where early are apparently supporting this notion (Fenaux *et al*, unpublished; Warrell and Ohno, unpublished - refer to Fenaux *et al*, 1993)

Immunotherapy with respect to AML is currently at the "embryonic" stage. IL-2 has recently been used as maintenance therapy in the hope that the hosts own immune system can be stimulated to attack any residual leukaemic cells (Wiernik *et al*, 1991). IL-2 has also been given to patients, after autologous BMT, sometimes in combination with LAK cells (Higuchi *et al*, 1989; Benyenes *et al*, 1992). From an immunological standpoint such treatments appear crude but are hopefully a step towards a more tumour-specific approach involving the implementation of the knowledge and tools of both molecular biology and immunology.

1.6.4 Aim

At the initiation of this body of research very little focus had been directed towards the role of the immune system in attacking leukaemia. Limited *in vitro* studies using primary leukaemic cells and leukaemic cell lines in allogeneic MLC have revealed that few are particularly immunostimulatory, as has also proven to be the case for many other forms of cancer. Thus despite the expression of allogeneic MHC class I or MHC class II antigen, which can be considered analogous to syngeneic MHC product plus non-self antigen, the majority of leukaemic cell lines are not capable of triggering lymphocyte proliferation.

The primary aim of these studies was to induce a weakly immunostimulatory myeloid leukaemic cell line, HL-60, to become significantly more immunopotent, as

measured by the allogeneic MLC, and to then compare the differences between the stimulatory and non-stimulatory HL-60 cells as well as characterizing the lymphocyte response and its requirements. Such studies, it was hoped, would reveal the factors important in triggering a lymphocyte response. HL-60 cells were chosen as they are readily induced along a number of maturation pathways, including the monocyte pathway, with a variety agents.

In order to gain a broader perspective of leukaemia immunology it was thought important to also analyse a wide range of other leukaemic cell lines with respect to their cell surface marker expression and their capacity to stimulate lymphocyte proliferation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Tissue culture

2.1.1 Media

To make RPMI 1640 medium, one sachet of RPMI 1640 powder (Gibco, cat. no. g-140-1800 EB) was added, along with 2 g $NaHCO_3$ (BDH, USA, cat. no. 10247), 2.5 ml 1 N HCl, 3.57 mg N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) (Boehringer-Mannheim, Australia, cat. no. 737151), 100 mg streptomycin sulphate (Sigma, USA, cat. no. S 9137), 1 x 10^5 international units (IU) penicillin (Sigma, USA, cat. no. P. 3032) and made up to 1 litre with tissue culture grade Milli Q-purified water, which had been deionized using a Milli Q RO60 system (Millipore corp., USA) and then further purified by passing through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli Q system (Millipore, USA). The pH of the medium was 7.4. This medium was filter sterilized using a sterivex GS 0.22 μm filter unit with filling bell (Millipore, USA, cat. no. SVGSB1010). Medium was supplemented with glutamine (BDH, cat. no. 37107) to a final concentration of 2 mM, and 10% v/v foetal calf serum (FCS) (Flow Laboratories, Australia, cat. no. 29-101-54), the latter of which had been previously heat inactivated by incubating at 56°C for 30 min. After 7 days of storage, medium was resupplemented with glutamine (2 mM).

Iscove's modified Dulbecco's medium (IMDM) was made by adding one sachet of IMDM powder with HEPES (Gibco, USA), along with 2 g $NaHCO_3$ and streptomycin sulphate and penicillin as described previously for RPMI 1640. Then made up to 1 litre with Milli Q-purified H_2O. The pH of the medium was 7.4.

Hank's balanced salt solution (HBSS) was made by adding 8 g NaCl (Ajax Chemicals, Australia, cat. no. 465), 0.4 g KCl (Ajax Chemicals, Australia, cat. no. 383), 0.12 g $Na_2HPO_4.12H_2O$ (BDH, Australia, cat. no. 10248), 0.06 g KH_2PO_4 (BDH, Australia, cat. no. 10203), 1 g glucose (BDH, Australia cat. no. 10117), 0.35 g $NaHCO_3$ and 1.7 ml 1% Phenol Red (M & B, England, cat. no. P152/18/61) (in 0.1 M NaOH) and making up to 1 litre with Milli Q-purified H_2O. The pH of the medium was 7.4.

Cell line	Description	Reference
HL-60	Promyelocytic	Collins *et al*, 1977
RC-2a	Myelomonocytic	Bradley *et al*, 1982
HEL	Erythroblastic	Martin and Papayannopoulou, 1982
HEL-DR	Erythroblastic (subline of HEL)	Dr. B. Torok-Storb, (unpublished)
BALM-1	B lymphoblastic	Minowada *et al*, 1977
MOLT-4	T lymphoblastic	Minowada *et al*, 1973
K562	Early myeloblastic/ erythroblast	Lozzio & Lozzio, 1975
U937	Monocytoid	Sundstrom & Nilsson, 1976
KG-1	Myeloblastic	Koeffler & Golde, 1978
MCF-7	Epithelial-like	Soule *et al*, 1973

Table 2.1.2(a): Human leukaemic cell lines. A list of the human leukaemia cell lines used in these studies.

2.1.2 Maintenance of cell lines

The cell lines U937, K562, HL-60, BALM-1, HEL-DR, HEL, MOLT-4 and RC-2a were maintained at log phase in RPMI 1640 (+ 10% FCS), whilst KG-1 was grown in IMDM (+ 10% FCS), at cell densities ranging from 1 x 10^5/ml to 1.5 x 10^6/ml in tissue culture flasks of 25 cm^2 (Nunc, Denmark, cat. no. 1 63371) or 75 cm^2 (Corning, USA, cat. no. 25110-75) growth area in a 37°C incubator (Forma Scientific, USA) with a fully humidified atmosphere and containing 5% $C0_2$ in air.

HL-60 and KG-1 cells were obtained from the American type culture collection (ATCC); RC-2a and K562 cells were provided by Glenn Pilkington (Cancer Institute, Melbourne); U937 cells were a gift from Bronwyn Cambareri (I.M.V.S., Adelaide); BALM-1 and MOLT-4 cells were obtained from Dr. Heddy Zola (Flinders Medical Centre, Adelaide); HEL cells were obtained from the ATCC; HEL-DR cells were obtained from Dr. Beverly Torok-Storb (Fred Hutchinson Cancer Centre, Seattle, USA); MCF-7 were provided by Dr. Rob Sutherland (Garvin Institute of Medical Research, New South Wales). A list of all the human cell lines used in this project, and the stage of haemopoiesis they are considered to represent, is given in table 2.1.2(a). The viabilities of the cell cultures were ascertained via a Trypan blue exclusion assay where 50 µl of Trypan blue (0.8% in saline) were mixed with 50 µl of the cell culture and then a sample was placed on a haemocytometer and, using a microscope, at least 100 cells were counted with those cells taking up the blue stain being classified as dead.

2.1.3 Induction of differentiation of cell lines

HL-60 cells were cultured in the presence of 10 nM all-*trans* retinoic acid (Sigma, USA, cat. no. R-2625) for 7 days. All-*trans* retinoic acid (RA) was stored in a light-free environment at 2 x 10^{-3} M in absolute ethanol (Ajax Chemicals, Australia, cat. no. 214) at -20°C in a glass container. To attain a final concentration of 10 nM RA a dilution of 1/1000 was made of the stock solution in RPMI 1640 followed by a 1/200

dilution into the culture containing HL-60 cells at a density of 4 x 10^5/ml. When the cell density was equal to, or exceeded, 1 x 10^6/ml the culture was replenished with fresh RPMI 1640 containing 10 nM RA to give a 1/2 dilution, unless otherwise stated.

HL-60 cells were cultured with 1000 U/ml interferon-γ (IFN-γ) (a gift from Jenny Gamble, Hanson Centre, Adelaide), a combination of 1000 U/ml IFN-γ and 10 nM RA, or with 5 x 10^{-8} M 1,25-dihydroxyvitamin D_3 (a gift from Hoffman-La Roche, Switzerland).

U937 cells were cultured in the presence of 5 x 10^{-9} M N,N,N'N'-Tetramethyl-p-phenylenediamine (TPA) (Sigma, USA, cat. no. P-8139) for 6 days. A stock solution of TPA (1 mg/ml in analytical grade dimethylsulphoxide (DMSO) (BDH, Australia, cat. no. 10323), which was stored at -20°C, was diluted to 5 x 10^{-7} M in RPMI 1640 prior to addition to the cell cultures.

2.1.4 Cryopreservation of cells

Cells were resuspended at 1 - 4 x 10^7/ml in RPMI 1640 (+ 10% FCS) and an equal volume of cryoprotectant (30% heat-inactivated FCS, 20% analytical grade DMSO and 50% RPMI 1640) was added. 1 ml aliquots of this cell suspension were then added to 1 ml cryotubes (Nunc, Denmark, cat. no. 3-66656) and cooled to approximately -100°C via a controlled-rate freezer (Paton Industries, South Australia). The rate of freezing was 5°C/min down to 0°C, 1°C/min to -25°C and 5°C/min to -100 °C. The cryotubes were then stored in liquid nitrogen-filled canisters (Thermolyne, USA, cat. no. LT509X9).

2.1.5 Thawing cells

Cryotubes containing frozen cells were rapidly thawed in a 37°C water-bath. The cell suspension was then transferred to 10 ml polystyrene tubes (Disposable Plastics, S. Australia, cat. no. 21829) and diluted drop-wise with RPMI 1640 (+ 10% FCS), mixed and then left to stand for 8 min. An equal volume of medium was again

gently mixed into the cell suspension and left to stand for a further 8 min. The tube was then topped up with medium and centrifuged (200g) for 5 min. The supernatant was aspirated and the cells were washed 2 times with medium (RPMI 1640 + 10% FCS).

2.2 Histochemical staining techniques

2.2.1 Making cell smears

Cells were harvested from culture flasks, washed once in their respective growth media (+ 10% FCS) and resuspended at 8 x 10^7/ml in FCS. 10 µl of this cell suspension was then placed onto a ethanol-cleaned microscope slide (Sail Brand, China, cat. no. 7105) and immediately flick-dried. These smears were stored in an air tight container at -20°C in the presence of self-indicating 2 - 4 mm silica gel (Ajax Chemicals, Australia, cat. no. 3681) until ready for use.

2.2.2 Staining for non-specific esterase (NSE) and chloroacetate esterase (CAE)

This technique was based on the protocol described by Yam *et al*, 1971. Cell smears were fixed in esterase fixative (see appendix A2.1.1 for preparation) for 30 sec at 4°C, then washed briefly 3 times with distilled water. 30 ml of 0.066 M phosphate buffer (pH 6.3) (see appendix A2.1.4 for preparation) was added to a Coplin jar. To the buffer was added 1 ml of 10 mg/ml α-naphthyl acetate (Sigma, USA, N-8505) in acetone (Ajax Chemicals, Australia, cat. no. 6). Then 0.3 ml of para-rosanalin solution (see appendix A2.1.2 for preparation) was mixed with 0.3 ml of 4% sodium nitrite (M & B, England, cat. no. 64248), left to stand for 1 min and then 0.5 ml of this mixture was mixed with the buffer solution in the Coplin jar. The pH of this solution was then adjusted to 6.1 using 1 - 2 drops of 0.5 M NaOH. The smears were then added to this mixture and left to stand at RT for 45 min, after which they were washed 3 times with distilled water. Those cells expressing NSE would now be detectable through the presence of a red-brown stain.

To a clean and dry Coplin jar was added 38 ml of 0.066 M phosphate buffer (pH 7.4) (see appendix A2.1.3 for preparation) with 20 mg fast blue BB (Sigma, USA, cat. no. F0125). Then 5 mg of AS-D chloroacetate (Sigma, USA, cat. no. N-0758) was mixed with 2.5 ml of N-N dimethyl formamide (BDH, Australia, cat. no. 10322) and then 2 ml of this solution was stirred into the buffer solution. The stained smears were placed into this solution and left to stand for 1 hr at RT. Those cells expressing CAE would now be detectable through the presence of a blue stain. The smears were then washed and counter-stained for 10 min with 2% methyl-green (see appendix A2.1.5 for preparation). After air-drying, the cells were mounted in Apothy's mounting fluid which was kindly provided by Llewelyn Spargo (Dept. of Microbiology and Immunology, Adelaide University).

2.2.3 Toluidine blue staining

Added a few drops of 1% Toluidine blue O (Chroma-Gesellshaft, Germany, cat. no. (chroma) 11645) (in methanol) to unfixed cell smears and left them to stand at RT for 5 min, after which they were rinsed with distilled H_2O. The cell smears were then dried and mounted in D. P. X. mounting fluid (Ajax Chemicals, Australia, cat. no. P3197).

2.2.4 Luxol fast blue staining

This technique was based on a protocol described by Dacie and Lewis, 1984. Cell smears were fixed in methanol for 2 min and then allowed to dry. The fixed smears were added to a Coplin jar containing 0.15% Luxol fast blue MBS (BDH, England, cat. no. 34044) in a 70% ethanol solution containing saturating amounts of urea and let stand for 2 hr and then washed with running tap water for 5 min. The stained smears were then counterstained with 0.25% Safranin-O (BDH, England, cat. no. 34067) for 2 min and subsequently washed with tap water. After air-drying, the cells were mounted in D.P.X. mounting fluid.

2.2.5 Nuclear staining: the Diff-Quick method

This technique was described by the manufacturer of the staining chemicals (Lab-Aids, Australia, cat. no. LP-6485). The cell smears were dipped in fixative (Lab-Aids, Australia, cat. no. LP-64851A) for three seconds, followed by immersion of smears into Solution 1 (Lab-Aids, Australia, cat. no. LP-64851B), and finally the smears were dipped into Solution 2 (Lab-Aids, Australia, cat. no. LP-64851C) and then washed with running water for 1 - 3 sec.

2.3 Preparation of $CD2^+$-lymphocyte-enriched populations

(a) **Ficoll-Paque gradient**: 5 ml of fresh human peripheral blood or buffy-coat cells (diluted 1/2 in Ca^{++}-Mg^{++}-free PBS (PBS)), which were kindly provided by the Red Cross Blood Transfusion Centre, Adelaide, were layered onto 3 ml of Ficoll-Paque gradient (Pharmacia, Sweden, cat. no. 17-0840-02) in 10 ml polystyrene tubes. The Ficoll-Paque was allowed to reach room temperature (RT) prior to the layering of the blood. The tubes were then centrifuged at 400g for 25 min at RT in a Mistral 3000i centrifuge. This procedure separated the red blood cells and the granulocytes from the mononuclear cells (MNC). The MNC were collected via a plastic pasteur pipette and washed 3 times with PBS (see appendix A2.2.2 for preparation of PBS). The cells were then resuspended to approximately 1 x 10^7/ml in RPMI 1640 + 10% FCS.

(b) **Adherence to plastic (removal of monocytes)**: Up to 5 x 10^7 MNC in 5 ml were added to 100 mm tissue culture grade plastic petri dishes (Costar, USA, cat. no. 3100) and were incubated at 37°C for 1 hr in 5% CO_2 in air. The non-adherent cells were then harvested, centrifuged (200g), resuspended in RPMI 1640 + 10% FCS and counted. The cells were then centrifuged and resuspended to no more than 10^8 cells/250 µl of RPMI 1640 + 10% FCS.

(c) **Nylon-wool separation**: A sterile 10 ml nylon-wool column, containing 0.6 g nylon-wool (see appendix A2.2.1 for preparation), was saturated with RPMI 1640 +

10% FCS and incubated for at least 1 hr at 37°C, 5% CO_2, with top and bottom of column being sealed with parafilm. The column was then opened at both ends and a few ml of RPMI 1640 (+ 10% FCS) were allowed to pass through. Then 250 µl of the cell suspension was added to the top of the column (no greater than 10^8 cells were applied to each column). A further 750 µl of RPMI 1640 (+ 10% FCS) was added to the column which was sealed with parafilm once more and allowed to stand at 37°C for 1 hr. Following this incubation, 12 ml of RPMI 1640 (+ 10% FCS) was added, dropwise, to the column and the effluent containing the $CD2^+$-lymphocyte-enriched population (LEP) was collected, the cells were counted and then cryopreserved until required.

2.4 Panning of lymphocytes

(a) Preparation of GαM-coated dish

Polystyrene bacteriological grade 100 mm x 15 mm petri dishes (Johns, Victoria) were coated with sterile, affinity-purified goat antibody to mouse immunoglobulin (GαM) by adding 8 ml of PBS containing 10 µg/ml GαM and incubating at 4°C overnight, or longer, in a humidified atmosphere. The fluid was then decanted and the dishes were washed 2 times with PBS and incubated at RT for 30 min in the presence of 8 ml of PBS containing 0.2% bovine serum albumen (BSA). The dishes were then washed 2 times with PBS and were ready for the addition of Ig-coated lymphocytes.

(b) Preparation of Ig-coated lymphocytes and panning

0.5 - 1 x 10^7 lymphocytes, or LEP, were incubated with 200 µl sterile hybridoma supernatant for 1 hr at 4°C. The cells were then washed 2 times with RPMI 1640 (+ 10% FCS) and resuspended in PBS (+ 0.2% BSA). Aliquots of 3 - 4 ml containing up to 4 x 10^7 cells were added to each GαM-coated dish and incubated for 70 min at 4°C on a level table. The dishes were gently swirled midway through the incubation and rotated 180°. After the 70 min incubation the nonadherant cells were

collected by gently swirling the contents and aspirating the PBS using a plastic pasteur pipette.

2.5 Cell sorting

Indirect immunofluorescent (IIF) staining was performed (see section 2.11.2 for details) on LEP on ice in the absence of sodium azide (Az) using either an anti-CD4 IgG_{2b} mAb, OKT4, or an anti-MHC class II IgG_1 mAb, FMC 14. Isotype controls, 1A6.11 (IgG_{2b}) and 3D3.3 (IgG_1), were used respectively. The labelled cells were resuspended at approximately 1 - 2 x 10^7/ml with ice cold RPMI 1640 (+ 10% FCS), run through the FACSTAR (Becton-Dickinson cell sorter) and lymphocytes expressing no detectable levels of either CD4 or MHC class II molecules were collected. Approximately 99% of cells collected were either $CD4^-$ or MHC class II^- as determined by analysis of populations post-sorting.

2.6 Mixed leucocyte cultures (MLC)

Responder population: Aliquots (0.1 ml) of LEP in RPMI 1640 (+ 10% FCS) containing 1 μg/ml Indomethacin (Sigma, USA, cat. no. 17378) were added to the wells of 96 well (round bottomed) tissue culture trays (Linbro, USA, cat. no. 76-042-05) to give 1 x 10^5 cells/well.

Stimulator population: Various cell types were used as stimulators. Cells in log phase growth were incubated at 10^6/ml in the presence of 0.02 mg/ml mitomycin C (Sigma, USA, Cat. No. M-0503) in RPMI 1640 (+ 10% FCS) for 1 hr at 37°C and in a 5% CO_2 air atmosphere. The cells were then washed 3 times with RPMI 1640 (+ 2% FCS), then resuspended in RPMI 1640 (+ 10% FCS + Indomethacin) and 0.1 ml aliquots were added to the wells of the 96 well tissue culture tray to give 1 x 10^5 cells/well, unless otherwise stated.

The responder and stimulator populations were mixed together in the wells of the tissue culture tray to a final volume of 200 μl and cultured for 5 days at 37°C in a

humidified chamber containing 8% CO_2, 7% O_2, balance N_2. After 5 days the cultures were pulsed with 1 μCi (50 μl) of tritiated (^3H) thymidine (ICN, CA, cat. no. 2406605; Specific activity: 6.7 Ci/mmole) per well for 18 hr and then harvested using a semi-automatic cell harvester (Skatron MS.5, Norway), onto filter paper (ICN, UK, cat. no. 78-115-05), which was then dried and placed into 2 ml of scintillation fluid (Linbrook International) in 6 ml screw cap vials (Beckman, USA, cat. no. 592928) and the amount of incorporated ^3H-thymidine determined using a β-counter (Beckman, USA).

MLC were performed in quadruplicate and results were expressed as the mean ± the standard error of the mean (SEM) of the 4 values.

2.7 MLC inhibition assay

MLC were set up as described above. Additionally, mAb were added at the initiation of the assay (day 0). In the majority of assays sterile supernatant derived from hybridoma cultures were added to the MLC at a 1/4 final dilution. The appropriate isotype controls were used. These included the *Salmonella*-specific 3D3.3 (IgG_1), 1D4.5 (IgG_{2a}), 1A6.11 (IgG_{2b}) and 1A6.12 (IgM).

Exceptions to the above description include those occasions when purified mAb were added to the MLC such as: the anti-TNF-α mAb, BC-7 (IgG_1) (Serotec, U.K., cat. no. MCA 747); the anti-IL-12 mAb, B72 (IgG_{2a}) (a gift from Dr. M. Gately, Hoffman-LaRoche, Nutley, N. J., USA); and the anti-ICAM-2 mAb, CBR-IC2/2 (IgG_{2a}), and anti-ICAM-3 mAb, CBR-IC3/1 (IgG_1) (both were kindly provided by Dr. T. Springer of the Centre for blood research, Boston, MA, USA); and the anti-c-kit mAb, SR-1 (IgG_{2b}). Purified 3D3.3 was added at the appropriate concentration as a control. Also, a rabbit polyclonal antiserum shown to specifically inhibit the function of human monocyte-derived IL-1 (Cistron, USA, cat. no. 02-1100) was used in MLC inhibition assays. The amount used (12 μl) was capable of neutralizing 6000 half-maximal units of human monocyte IL-1 in a thymocyte proliferation assay.

2.8 Thymocyte proliferation assay

A single cell suspension, in HBSS, was prepared from the fresh thymus of a 6 week old BALB/C x C57BL/6 F1 mouse using a loose-fitting glass homogeniser followed by filtration through cotton wool. The cells were washed 2 times in HBSS (+ 2% FCS) and resuspended at 10^7 cells/ml in RPMI 1640 (+ 10% FCS) and Indomethacin (1 μg/ml). Aliquots of 100 μl of this cell suspension were added to the wells of a 96 well flat-bottomed tray. Phytohaemagglutanin-P (PHA) (Wellcome, U.K., cat. no. HA 16) was added to each well to give a final concentration of 5 μg/ml. Additionally, either recombinant human IL-1β (a gift from Dr. Jenny Gamble, Hanson Centre, Adelaide) or a crude preparation of mouse IL-1 (derived from the supernatant of murine spleen cells cultured with PHA (10 μg/ml) for 4 days; a gift from Dr. Melissa Pope, Adelaide University), was added to each well except control wells, which included 1) thymocytes alone and 2) thymocytes plus PHA (5 μg/ml). Anti-human interleukin-1 rabbit polyclonal serum (Cistron, USA, cat. no. 02-1100) was added, at the recommended dose (6 μl) to the assay to test the activity. The final volume in each of the wells was 200 μl. The trays were incubated for 3 days at 37°C in a humidified chamber containing 8% CO_2, 7% O_2, balance N_2. The wells were then pulsed for 4 hr with 1 μCi of ^{3}H-thymidine, harvested and counted as previously described (section 2.6).

2.9 Proliferation assay for IL-12 and anti-IL-12 activity

This procedure was based on the method described by Stern *et al*, 1990. Human MNC (5 x 10^5/ml) were incubated (37°C, in 8% CO_2, 7% O_2, balance N_2) in RPMI 1640 (+ 10% FCS) in flat-bottomed microtitre wells for 3 days in the presence of 0.1% PHA. The cultures were then split 1:1 with fresh culture medium plus recombinant IL-2 (Boehringer-Mannheim, Germany, cat. no. 799 068) at a final concentration of 50 U/ml and then cultured for 24 hours at 37°C. The cells were then harvested, washed and resuspended at 4 x 10^5/ml and cultured for 2 days in the

presence of specified amounts of IL-12 with or without anti-IL-12 (7B2) or the isotype control mAb 1D4.5. The wells were pulsed for 16 hr with 1 μCi/well of ^{3}H-thymidine and then harvested and counts incorporated were measured as previously described in section 2.3.

2.10 Cytotoxicity assay

(a) Target cells

Target cells were incubated at 1 x 10^{6} - 1 x 10^{7}/ml in 0.5 ml RPMI 1640 (+ 10% FCS) with 50 μCi of $Na_2{}^{51}CrO_4$ (^{51}Cr) (Amersham, USA, cat. no. CJ54) for 1 hr at 37 °C in an atmosphere of 8% CO_2, 7% O_2, balance N_2. Subsequently, the cells were underlayed with 1 ml of FCS, centrifuged (200g), and the supernatant was discarded. The cells were then resuspended in 1 ml of RPMI 1640 (+ 10% FCS) and incubated at 37°C under the aforementioned atmospheric conditions for a further 30 min and then the underlaying procedure with FCS was repeated. The cells were washed once more and were then ready for addition to the culture tray.

(b) Effector cells

The effector cells were derived from 96 well round-bottomed microtitre trays after 6 days of MLC conditions.

(c) The assay

Target cells were added to 96 well round-bottomed trays at 10^{4}/well and effectors were added at effector:target ratios of 50:1 in a total volume of 200 μl RPMI 1640 (+ 10% FCS). As a measure of total releasable ^{51}Cr, 0.5 N HCl was added to separate wells containing target cells alone. The trays were then centrifuged at 400g (Hettich Universal 2S) for 5 min and then incubated for 4 hr at 37°C in a humidified atmosphere with 8% CO_2, 7% O_2, balance N_2.

After the incubation the trays were centrifuged again and 100 μl of supernatant was harvested from each well, transferred to counting tubes and the ^{51}Cr

released was determined using a γ-counter (Packard, Model 5630). This assay was performed in triplicate.

The percentage cytolysis was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{(\text{CPM released by targets} - \text{CPM released spontaneously})}{(\text{CPM released by HCl-lysed targets} - \text{CPM released spontaneously})} \times 100$$

2.11 Immunofluorescence techniques

2.11.1 Monoclonal antibodies - details

The majority of mAb used in the indirect immunofluorescence (IIF) technique and other assays are listed in table 2.11.1(a). Details of other Ab are given in relevant sections of this text.

All mAb listed were produced in this laboratory, with the following exceptions; FMC 4, FMC 14, FMC 15, FMC 19, FMC 32 and FMC 63 were purchased from, or donated by, Dr. Heddy Zola, Flinders Medical Centre; VIM-13, VIL-A1, VIB-E3, VIM-D5, VIP-1, anti-CD36 (a cocktail of CD36-specific mAb) and VIFcRIII were generously provided by Dr. Stephen Gadd, University of Vienna, Austria; TS1/22 was kindly donated by Dr. Paul Simmons, Leukaemia Research Unit, Hanson Centre; W-CAM-1 was kindly donated by Dr. Jenny Gamble, Division of Human Immunology, I.M.V.S.; CBR-IC2/2 and CBR-IC3/1 were gifts from Dr. Timothy Springer, Centre for Blood Research, Boston, MA, USA; TS2/9 was a gift from Dr. Andrew Boyd, Melbourne, Victoria; OKT11, OKT3, OKT4 and OKT8 were kindly provided by the Division of Human Immunology, I.M.V.S; RM 3.170 was a gift from Dr. Graham Russ, Queen Elizabeth Hospital, Adelaide.

Table 2.11.1(a): **Monoclonal antibodies.** Details of mAb used in these studies.

mAb	Isotype	Specificity	Reference
6c6b	IgG_1	MHC class I	Cole and Ashman (unpublished)
7b6c	IgG_{2a}	MHC class I	Gesche and Ashman, 1985
1B4.B12	IgG_1	MHC class I	Cole and Ashman, (unpublished)
FMC 4	IgG_1	MHC class II	Brooks *et al*, 1980
FMC 14	IgG_1	MHC class II	Brooks *et al*, 1980
FMC 15	IgG_{2b}	HLA-DR	Brooks *et al*, 1980
FMC 63	IgG_1	CD19	Zola *et al*, 1991
5A4.C5	IgG_1	CD11b	Lyons *et al*, 1988
B2B1.F9	IgG_1	CD31	Ashman *et al*, 1991
W-CAM-1	IgG_{2b}	ICAM-1	Boyd *et al*, 1988
CBR-IC2/2	IgG_{2a}	ICAM-2	de Fougerolles *et al*, 1991
CBR-IC3/1	IgG_1	ICAM-3	de Fougerolles *et al*, 1992
VIM13	IgM	CD14	Knapp, 1984
TS2/9	IgG_1	LFA-3	Sanchez-Madrid *et al*, 1982
TS1/22	IgG_1	LFA-1	Sanchez-Madrid *et al*, 1982
VIL-A1	IgM	CD10	Knapp, 1982
VIB-E3	IgM	CD24	Sugita, 1986
VIMD5	IgM	CD15	Majdic, 1981
VIP-1	IgG_1	CD71	Holter, 1985
VIFcRIII	IgM	CD16	Gadd *et al*, 1990
OKT11	IgG_1	CD2	Cotner *et al*, 1981
OKT3	IgG_{2a}	CD3	Kung *et al*, 1979

Table 2.11.1(a): *(continued)*

OKT4	IgG_{2b}	CD4	Kung *et al*, 1979
OKT8	IgG_{2a}	CD8	Thomas *et al*, 1980
RM 3.170	IgG_3	HLA-A2/B17	Russ, unpublished
YB5.B8	IgG_1	c-kit	Gadd and Ashman, 1985
17F11	IgM	c-kit	Buhring *et al*, 1991
SR-1	IgG_{2a}	c-kit	Broudy *et al*, 1992
UCHL1	IgG_{2a}	CD45RO	Smith *et al*, 1986
3D3.3	IgG_1	*Salmonella*	O'Connor & Ashman, 1982
1D4.5	IgG_{2a}	*Salmonella*	O'Connor & Ashman, 1982
1A6.11	IgG_{2b}	*Salmonella*	O'Connor & Ashman, 1982
1A6.12	IgM	*Salmonella*	O'Connor & Ashman, 1982
FMC 19	IgG_3	*Toxoplasma*	Johnson *et al*, 1981

2.11.2 Indirect immunofluorescence

Target cells were washed twice in ice cold PBS containing 0.1% bovine serum albumin and 0.1% sodium azide (PBS-BSA-Az) and resuspended at 10^7/ml in PBS-BSA-Az (see appendix A3.2.1 for preparation) supplemented with 10% heat inactivated normal rabbit serum (NRS) in order to block the binding of mAb to Fc receptors, especially FcγRI. Aliquots of 50 µl were dispensed into conical-bottomed plastic tubes (Sardstedt, Monomed, cat. no. 57477) and were placed on ice. Culture supernatant mAb or purified mAb (5 - 30 µg/ml) was added in 50 µl aliquots to the cell suspension, mixed and then incubated for 1 hr at 4°C.

When it was necessary to detect dead cells, 15 µl of 100 µg/ml (in PBS) ethidium monoazide (EMA) (Molecular probes, cat. no. E-1374) was added to each tube prior to the 1 hr incubation. The tubes were incubated on ice in the dark for the first 30 min and then placed directly under a fluorescent light for the subsequent 30 min. The cells were washed three times with 1 ml PBS-BSA-Az (4°C). With each washing the cells were centrifuged at 200g in the Mistral 3000i, MSE and after the last wash the supernatant was aspirated such that approximately 50 µl of PBS-BSA-Az was remaining. Each tube was then vortexed and to the resuspended cells was added 50 µl of a 1/10 dilution (in PBS-BSA-Az) of affinity-isolated FITC-labelled $F(ab')_2$ sheep antibody to mouse Ig (Silenus, Australia, cat. no. DDAF). The cells were then vortexed and incubated at 4°C for 45 min. The cells were then washed three times using 1 ml of PBS-BSA-Az each time. After the last wash the supernatants were aspirated, the cells vortexed and 0.5 - 1 ml of 1% paraformaldehyde (4°C)(see appendix A2.3.2 for preparation) was added to each tube. These cells were stored in the dark at 4°C for up to 3 weeks prior to analysis by flow cytometry.

2.11.3 Direct immunofluorescence

Target cells were washed twice in ice cold PBS-BSA-Az and resuspended at 10^7/ml in PBS-BSA-Az supplemented with 10% heat-inactivated NRS. Aliquots of 50 µl were dispensed into conical-bottomed plastic tubes (Sardstedt, Monomed, cat. no. 57477) and were placed on ice. 50 µl of antibody (5 - 30 µg/ml), directly labelled with either Fluorescein-isothiocyanate, 5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) (Boehringer-Mannheim, Germany, cat. no. 1055 089), phyco-erythrin (PE) or biotin, was added to the cell suspension and vortexed. The cells were then incubated for 1 hr at 4°C. mAb used in this assay included; a FITC-labelled IgG_1 mAb, "IL-2R", specific for the β chain of the IL-2R (Becton-Dickinson, USA, cat. no. 7643); a PE-labelled IgG_1 mAb, "Leu11c", specific for CD16 (Becton-Dickinson, USA, cat. no. 7617); biotinylated OKT4 and OKT8 (biotinylation was performed by Steve Cole of this laboratory).

Note: When it was necessary to check for the presence of dead cells, EMA was added, as previously described (section 2.11.2), to the cells prior to the addition of the fluorescent-labelled mAb.

Cells incubated with biotinylated mAb were washed 3 times with PBS-BSA-Az and then incubated 45 min, 4°C with 50 µl of streptavidin-PE (Tago inc., USA, cat. no. 6966) diluted 1/20 in PBS-BSA-Az. Subsequent to the 45 min incubation the cells were washed three times, and after the last wash the supernatant was aspirated, the cells vortexed and 0.5 - 1 ml of 1% paraformaldehyde (4°C) was added. In the case of the cells treated with directly fluoresceinated mAb the second incubation step was bypassed.

2.11.4 Purification of mouse IgG monoclonal antibodies

Antibodies were purified as described by Ey *et al*, 1978. A 10 ml plastic column (Linbro, Surgical and Medical supplies, cat. no. 612 4407) was packed with 5 ml of Protein A-sepharose (Pharmacia, Australia, Cat. No. 17-0780-01) and was

equilibrated with 0.1 M phosphate buffer (Na^+, pH 8.2) + Az. The column was stored in this form at 4°C until required. The column was then loaded with antibody solution. If ascites fluid was used, the solution was centrifuged at 100 000g (40 000 rpm, Beckman L8.80) for 30 min, filtered through a 0.45 μM filter (Sartorius, Germany, Cat. No. 175 98 K) and then diluted 1:1 with 0.1 M Tris-HCl, pH 8.6 prior to addition to the column. Hybridoma culture supernatants were adjusted to pH 8.2 and filtered through a 0.45 μM filter prior to loading on to the column. When working with ascites solutions of less than the void volume of the column, the entire antibody solution was allowed to enter the column and then the flow was halted and the antibody was left for 30 min. Otherwise the antibody solution was loaded onto column and the flow was started immediately The column was then washed with 0.1 M phosphate (Na^+, pH 8.2; see appendix A2.4.1 for preparation) until the $O.D._{280}$ of the effluent was less than 0.05 as measured by a spectrophotometer (DU-65 Beckman, USA, part number 596791). The antibodies were then eluted using 0.1 M citrate buffer at the appropriate pH (i.e. for IgG_1 used pH 5.5; for IgG_{2a} used pH 4.5; for IgG_{2b} used pH 3.5) and the eluate was collected in 1 - 2 ml fractions until $O.D._{280}$ was less than 0.05. The eluate was then immediately adjusted to pH 7.0 with the appropriate volume of Tris-HCl pH 8.6 (i.e. to change pH 5.5 to pH 7, 1 vol Tris-HCl was added to 10 vol eluate; from pH 4.5 to pH 7, 1 vol:4 vol; from pH 3.5 to pH 7.0, 1 vol:1 vol). Fractions with an $O.D._{280}$ of greater than 0.2 were pooled and the purity of the eluate was checked using the spectrophotometer to scan $O.D._{220}$ - $O.D._{320}$. The protein concentration (mg/ml) was determined according to the formula $O.D._{280}$ x 0.74. The column was washed with 0.1 M phosphate (Na^+, pH 8.2) until the column was neutralized. The purified antibody was then dialysed against PBS and could be stored at -20° C in the presence of 50% v/v glycerol.

2.11.5 Fluorescein-labelling of monoclonal antibodies

(a) Preparation of the monoclonal antibody

The labelling of mAb with FLUOS was carried out according to the manufacturers instructions. The mAb was purified, as described in section 2.11.4, and then dialysed against 0.1 M $NaHCO_3$ (Ajax Chemicals, Australia, cat. no. 475) pH 8.4. The mAb was then concentrated to 0.5 mg/ml with a centricon 10 concentrator (Amicon, USA, cat. no. 4205). 0.8 g of FLUOS was added to 800 µl of DMSO immediately prior to addition to reaction mix. Then varying ratios of mAb (0.5 mg/ml) to FLUOS (1 mg/ml in DMSO) were mixed in a final volume of 140 µl in wheaton vials (Wheaton, cat. no. 224 891) as follows;

mAb (0.5 mg/ml)	FLUOS (1 mg/ml)	0.1 M $NaHCCO_3$
100 µl	2 µl	38 µl
100 µl	4 µl	36 µl
100 µl	8 µl	32 µl
100 µl	20 µl	20 µl
100 µl	40 µl	0 µl

These mixtures were then shaken vigorously, using a tray-shaker, for 4 hr at RT in the dark.

Each reaction mix was then loaded onto a 1.5 ml Sephadex G-25 column (Sephadex G25: Pharmacia, Sweden, cat. no. 01-900-1-1854-05) and aliquots of approximately 100 µl aliquots were collected into glass tubes via a fraction collector (ISCO, USA). The 100 µl samples were monitored for the presence of mAb by measuring the absorbence at 280 nm for a 1 cm light path. Those samples containing sufficient quantities (> 30 µg/ml) of protein (mAb) were scanned on the spectrophotometer using a wavelength range of 220 - 550 nm such that the peak absorbence for both protein (280 nm) and fluorescein (495 nm) could be assessed. The amount of mAb (µg/ml) present in each sample was calculated via the following equation:

Concentration of mAb (μg/ml) = $0.74(Abs_{280} - 0.25 \times Abs_{495})$

Note: 25% of the absorbence value at 495 nm was subtracted from the absorbence value at 280 nm as this approximately correlated to the contribution that fluorescein alone consistently made to the 280 nm peak, as determined by measuring the Abs_{495} and Abs_{280} values of different concentrations of FLUOS. Protein alone however was not found to detectably absorb light at 495 nm.

If one assumed that all of the fluorescein was linked to antibodies then the ratio of fluorescein:mAb could be ascertained. The fluorescein-labelled antibody samples were then diluted, if necessary, to 20 μg/ml in PBS-BSA-Az and then dialysed against PBS-BSA-Az (1 litre) three times. Each sample was checked for activity by direct immunofluorescence and compared to the activity of the non-fluoresceinated version of the same mAb with the latter requiring indirect immunofluorescence to assess its activity. The sample exhibiting the greatest fluorescence intensity indicated that the ratio of mAb:FLUOS used in that reaction mix was the most effective and thus a scaled up version of the fluoresceination was performed using that ratio.

2.11.6 Enzyme-linked immunosorbent assay

The wells of an ELISA tray (Costar, USA, cat. no. 2595) were coated with G αM Ig by adding 0.1 ml/well of 5 μg/ml GαM and incubating overnight in a humid chamber at 4°C. Wells were then washed 3 times with 0.2 ml/well PBS-Az. Next, 0.2 ml of PBS-Az containing 1% BSA was added to each well and incubated for 2 hr at RT, then the washing procedure was repeated. Then 0.1 ml of test antisera was added in duplicate, making 1/2 serial dilutions, across the row of 12 wells of the ELISA tray where PBS-Az containing 1% BSA (0.1 ml/well) was used as the diluent, and then the tray was incubated for 30 min at 37°C followed by a 90 min incubation at 4°C in a humidified chamber. The washing procedure was repeated and 0.1 ml of rabbit antibody to mouse Ig conjugated to horse-radish peroxidase (Dako, Denmark, cat. no. P260) diluted 1/2000 in standard ELISA enzyme diluent (20 mM Tris-HCl, 137 mM

NaCl, 200 µg/ml BSA, 1 mM $MgCl_2$, 2.5 µM $ZnCl_2$, 7.7 mM NaN_3, pH 7.5) was added to each well. The tray was then incubated for 30 min at 37°C, then 90 min at 4° C in a humidified chamber and then washed 3 times using 0.1 ml/well PBS (no Az). Dissolved 1 tablet of o-Phenylenediamine.2HCl (OPD) (Abbott lab., USA, cat. no. 7181E) per 5 ml OPD diluent (Abbott lab., USA, cat. no. 5695) and added 0.1 ml/well and then incubated the tray in dark for 10 - 30 min at 37°C. The reaction was stopped with 50 µl/well 1 *N* H_2SO_4 (Abbott lab., USA, cat. no. 7212). Read $O.D._{490nm}$ using an ELISA reader.

2.12 Molecular biology

2.12.1: Small scale preparation of plasmid DNA.

This technique was derived from Sambrook *et al*, 1989. The bacteria harbouring the desired plasmid DNA (see section 2.12.4 for details) were grown overnight (37°C) on Luria-broth (L-B) agar plates (see appendix A2.5.2 for preparation) supplemented with 50 µg/ml of ampicillin (amp) (Sigma, USA, cat. no. A-9518). Single colonies were picked and grown independently in L-B (+ amp) (see appendix A2.5.1 for preparation) at 37°C overnight whilst being constantly shaken. 1.4 ml of culture was then transferred to an Eppendorf tube (Eppendorf, Germany, cat. no. 3810), microfuged for 5 min, and the supernatant was discarded. 1.4 ml of fresh L-B were added, microfuged 5 min and the supernatant was removed. The pellet was vortexed thoroughly and 100 µl of 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetra-acetic acid (EDTA) (Ajax Chemicals, Australia, cat. no. 181) pH 8.0 was added. Left at RT for 5 min and then added 200 µl 0.2 M NaOH, 1% SDS (see appendix A2.5.4 for preparation) and incubated at 4°C for 5 min. Then 150 µl of ice cold 3 M potassium 5 M acetate pH 4.8 (see appendix A2.5.5 for preparation) was added and the suspension was left on ice for 5 min, microfuged for 5 min and then removed and retained supernatant and transferred to a new Eppendorf tube. To 500 µl of supernatant was added 500 µl phenol, followed by 200 µl of chloroform (Ajax

Chemicals, Australia, cat. no. 152) which was then vortexed and microfuged for 5 min at 4°C. The upper aqueous phase was retained and mixed with two volumes of ice cold absolute ethanol and then incubated at 4°C for 20 min and microfuged for 5 min. The supernatant was removed and 1 ml of 70% ethanol was added, mixed (not vortexed), microfuged for 5 min, the supernatant aspirated and the pellet was dried under vacuum (Speedvac, Savant) for 5 min. This DNA was resuspended in 50 µl Tris-EDTA (1x) (TE) and could be stored at 4°C.

2.12.2 Large scale plasmid preparation.

This technique was derived from Sambook *et al*, 1989. If the plasmid DNA obtained from the small scale plasmid preparation proved to be that desired, as determined by electrophoretic characterization of restriction enzyme digests, then single colonies were made from a derivation of the clone of cells originally used for the small scale procedure. A single colony was picked and used to inoculate a 10 ml culture of L-B (+ 50 µg/ml amp) which was then incubated (shaking) at 37°C overnight. Unless otherwise stated, all plasmids possessed the ampicillin resistance gene which codes for an enzyme that is secreted into the bacterial periplasmic space and catalyzes the hydrolysis of the β-lactam ring of ampicillin rendering the drug inactive (Sykes and Mathew, 1976).

2 ml of the overnight culture was added to 50 ml of L-B (+ 50 µg/ml amp) and was incubated with shaking for 5 hr at 37°C. The 50 ml culture was then added to 500 ml L-B (+ 50 µg/ml amp) and incubated (shaking) at 37°C, for approximately 3 hr. At this point 2.5 ml of chloramphenicol (34 mg/ml in absolute ethanol) was added and the culture was left to incubate overnight at 37°C, whilst shaking. Chloramphenicol is an antibiotic to which the bacterial species was susceptible. This drug has the ability to prevent protein synthesis by binding to the 50S ribosomal subunit. The plasmids used in these experiments relied on stable proteins for their replication, as opposed to the

bacterial chromosome, thus multiple copies of plasmid should theoretically be present in one bacterium (Clewell, 1972).

Bacteria were harvested from the 500 ml overnight culture by centrifugation at 2500g, 4°C for 15 min, in a RC5 Super Speed Refrigerated Sorvall centrifuge (PN136) using a GSA rotor. The supernatant was discarded and the cells were resuspended in 14 ml of "Solution 1" (50 mM glucose, 25 mM Tris-HCL, 10 mM EDTA, pH 8.0), then 2 ml lysozyme (Sigma, No. L-6876; 20 mg/ml in "Solution 1"), was added and the mixture was left to stand at RT for 10 min. This procedure results in the breaking up of the outer membrane and cell wall. Next the cells were lysed by the addition of 26 ml of "Solution 2" (0.2 M NaOH and 1% SDS) and a further incubation for 5 min on ice. The chromosomal DNA was precipitated by adding 14 ml of ice cold "Solution 3" (3 M Potassium 5 M Acetate) and left on ice for 15 min. This mixture was then centrifuged using a RC5, 2500g, at 4°C for 20 min or longer depending on how well the chromosomal DNA had pelleted. The supernatant was harvested using a plastic 10 ml pipette, transferred to 50 ml Falcon tubes (Corning, cat. no. 25331-50) and was extracted twice with an equal volume, each time, of phenol:chloroform:isoamylalcohol (25:24:1) at RT. This involved the vigorous mixing together of the aqueous and the organic phases followed by a 5 min centrifugation at 1000 rpm (200g) in a MSE centrifuge.

After the second extraction the upper aqueous phase was transferred to centrifuge buckets to which was added 0.6 volume of propan-2-ol (BDH, Australia, cat. no. 10224). This was then mixed and left at RT for 10 min resulting in the precipitation of the plasmid DNA. The plasmid DNA was subsequently pelleted by centrifugation at 20000g for 20 min at 4°C in a RC5 centrifuge and using a SS-34 rotor. The supernatant was removed, the pellet was washed with 70% ethanol and centrifuged for 10 min (RC5, 20000g, 4°C). The pellet was then dried under vacuum. The plasmid DNA obtained from a 500 ml culture was then dissolved in 2.4 ml of 1x TE (pH 8.0) and stored at 4°C overnight.

The following day precisely 4.2 g of caesium chloride (BDH, U.K., cat. no. 810709) was added to 2.4 ml of the plasmid solution and left to dissolve for 15 min at 37°C, with intermittent shaking. Then 0.4 ml of 10 mg/ml ethidium bromide was added. Caesium chloride solution, 8 ml, (18.23 g/25 ml TE; refractive index = 1.3783) was added to Beckman 80Ti quick-seal tubes (Beckman, USA, cat. no. 342413) and the DNA solution was gently layered beneath the CsCl solution. The tubes were topped up with the CsCl solution (18.23 g/25 ml) such that there were no air-bubbles. The tubes were then sealed (Beckman appliance) and centrifuged at 40000g (65000 rpm) for 5 hr at 20°C using a Beckman L8-80 ultracentrifuge and an 80Ti rotor (slow acceleration, no brake). After centrifugation, two bands were generally visible; the upper band was considered to be nicked plasmid DNA, and the lower band was supercoiled plasmid DNA. This lower band was removed using a 19 gauge needle and a 1 - 3 ml syringe and then extracted 4 - 5 times with 1 M NaCl-saturated butanol to remove the ethidium bromide (butanol was the top layer). The DNA solution was then dialysed twice against 2 litres TE (pH 8.0).

2.12.3 RNase protection assay

(a) Linearization of purified plasmid

The techniques used in the RNase protection assay were based on the protocol described by Yisraeli and Melton, 1989. Details of plasmids containing cDNA inserts are given in section 2.12.4. 2 μg of plasmid (in TE) were used per digestion reaction which included the specified restriction enzyme (1 - 4 units/μg of DNA) and appropriate buffer (1x) (see section 2.12.4 for details). Each digestion reaction was carried out in a volume of 20 - 40 μl comprising DNA, buffer, restriction enzyme and Milli Q-purified H_2O which had been treated with diethyl pyrocarbonate (DEPC) (Sigma, USA, cat. no. D-5758) to inactivate RNases (see appendix A2.6.1 for preparation). Restriction reactions were performed at 37°C for 2 - 3 hr and checked to ensure the plasmid was completely linearized by taking 3 - 5 μl of the reaction mixture,

mixing 5 µl of loading buffer and electrophoresing on an agarose mini-gel. Appropriate molecular weight markers were also used, e.g. SPP1, which was a bacteriophage digested with EcoR 1 and was provided by Chris Cusaro (Dept. of Microbiology and Immunology, Adelaide University).

After digestion of the DNA, the reaction mix was diluted to 200 µl with DEPC-treated H_20 and an equal volume of phenol(TE-saturated):chloroform:iso-amyl alcohol (25:24:1) was added. This was then vortexed, microfuged 10 min, 4°C and the aqueous phase was retained. One more extraction was performed followed by an extraction with chloroform. The plasmid was precipitated with 1/10 volume 3 M Acetate (Na^+, pH 4.6) and 2 volumes absolute ethanol and incubated -20°C for at least 15 min, but usually overnight. The precipitated suspension was then microfuged 15 min, 4°C, washed three times with 70% ethanol, dried 5 min under vacuum, resuspended in 25 - 50 µl TE and stored at -20°C. The linearized plasmid was then ready for use in the transcription of the ribo-probe.

(b) Preparation of probe by transcription from linearized template with RNA polymerase.

The reaction mix containing: 1x transcription optimized buffer (Promega, USA,cat. no. P118B), 25 mM DTT (Cleland's Reagent, cat. no. 27-4416-01), 50 µM UTP (Bresatec, cat. no. 27-2086-01), 0.5 mM CTP (Bresatec, cat. no. 27-2066-01), 0.5 mM ATP (Bresatec, cat. no. 27-2056-01), 0.5 mM GTP (Bresatec, cat. no. 27-2076-01), 2 U/ml RNasin (Pharmacia, cat. no. 27-0815-01), 600 Ci/mmol α-^{32}P-UTP (Bresatec, cat. no. ARU-2; specific activity 3000 Ci/mmol), 0.125 mg/ml BSA (Pharmacia, cat. no. 27-8914-02), 1.5 U/ml SP6 polymerase (Amersham, USA, cat. no. SP-1) and 15% v/v of solution containing linearized plasmid DNA, was incubated 37°C for 1 hr. A total volume of 10 µl was used for the reaction mix. Then 1 µl of 1 U/µl RQ-DNase (Promega, cat. no. M610A) was added and incubated at 37°C, 15 min. Added 100 µl TE, 1 µl tRNA (10 mg/ml) (Sigma, USA, cat. no. R-8759) (see appendix A2.6.9 for preparation), 67 µl 5 M NH_4Ac, 420 µl ethanol to tube and briefly mixed.

Incubated -20°C for at least 15 min. Then the mixture was microfuged 25 min, 4°C and decanted supernatant with a 0 - 200 µl tip. The pellet was washed with 1 ml cold 70% ethanol and dried for 5 min under vacuum. The pellet was then dissolved in 5 µl formamide loading solution (see appendix A2.6.2 for preparation) and incubated at 100 °C for 3 min, loaded onto a 6% polyacrylamide gel which had been pre-run for at least 30 min to heat up the gel (640 V and 21 mAMP).

The gel was run until the xylene cyanole had travelled 10 cm. As molecular weight markers, ^{32}P-labelled Lambda DNA (cut with Pst 1) were also ran in a separate lane (see appendix A2.6.8 for labelling procedure). After electrophoresis, the gel was autoradiographed for 5 min and then the X-ray film was developed. The autoradiograph was placed under the gel and used to determine the position of the probe. Then a gel slice containing the probe was cut out, using a scalpel blade, and transferred to an Eppendorf tube. The gel slice was mashed well with the melted end of a 0 - 200 µl tip. Added 300 µl elution buffer (see appendix A2.6.5 for preparation), vortexed vigorously and shook for 3 hr.

The polyacrylamide was pelleted by centrifuging for 5 min. Transferred 200 µ of the supernatant to an Eppendorf tube already containing 84 µl 5 M NH_4Ac and 1 µ l tRNA (10 mg/ml). Then added 696 µl ethanol (-70°C) and microfuged tube at 4°C for 15 min. The supernatant was discarded with a 0 - 200 µl tip and the pellet was washed with 1.5 ml 70% ethanol, dried for 5 min under vacuum and resuspended in 100 µl DEPC-treated Milli Q-purified H_2O. A 5 µl sample was then assessed for level of radioactive incorporation through the use of a β-counter (Cerenkov ^{3}H channel) for 1 min.

(c) Hybridization of the probe with sample RNA

Mixed probe (2×10^4 - 5×10^4 cpm) with sample RNA (1 - 20 µg) and dried under vacuum. Also added 10 µl of hybridization solution (see appendix A2.6.6 for preparation), incubated at 80°C for 5 min (destroying 2°y structures) and then incubated 45°C for 16 hr (to hybridize). After incubation 100 µl RNase A (Boehringer-

Mannheim, Germany, cat. no. 109169) (40 µg/ml in RNase buffer) was added and tubes were incubated at 26°C for 40 min. Added 2 µl of 10% SDS and 3 µl of proteinase K (Merck, Germany, cat. no. 24568) (10 mg/ml in Milli Q-purified H_2O). Vortexed and microfuged at 37°C for 15 min and then transferred 110 µl of this mixture to a fresh Eppendorf tube containing 1 µl of tRNA (10 mg/ml) and 100 µl of TE-saturated phenol:chloroform (1:0.8). Vortexed well, microfuged for 5 min, transferred aqueous phase to a Eppendorf tube already containing 250 µl absolute ethanol and incubated at -20°C for 15 min. Microfuged at 4°C for 30 min, aspirated supernatant with drawn out pasteur pipette and then washed the pellet with 1 ml of 70% ethanol. Dried pellet briefly under vacuum, and then dissolved pellet in 4 µl formamide loading solution. This solution was microfuged (pulsed) and incubated at 100°C for 3 min. The contents of each tube was then electrophoresed on a pre-run (30 min) 6% polyacrylamide sequencing gel (8 M Urea (BDH, England, cat. no. 45204)) (see appendix A2.6.3 for preparation) at 1650 V and 53 mAMP until xylene cyanole had run 10 cm. Used ^{32}P-labelled pUC 19 which had been digested with Hpa1 (see appendix A2.6.10 for preparation) Then treated gel with a freshly made fixing solution (10% HAc, 10% ethanol) 3 times, 2 - 3 min each time. Layed 3 mm of Whatman paper (prewet with the ethanol-HAc fixing solution) on top of gel and pressed gently over the gel using a roller. Absorbed excessive moisture using a dry Whatman paper, then lifted up Whatman paper plus gel and placed on gel dryer for 40 min under vacuum at 80°C. The gel was then autoradiographed, usually overnight, and the X-ray film was then developed.

2.12.4 Details of plasmids containing cDNA inserts

pSP65 + β-actin cDNA: A plasmid containing approximately 130 base pairs (bp) of cDNA encoding a segment of the human β-actin protein inserted into the multiple cloning site of the pSP65 vector (Promega) was kindly provided by Greg Ryan, (IMVS, Adelaide) and was grown in E. *coli* (DH5α). The insertion was at the

Xma I and Pst I restriction sites such that anti-sense RNA could be transcribed from the SP6 promoter. A map of the vector, with insert, is shown in figure 2.12.4(a). Pvu II (Promega, USA, cat. no. R6332), which cut within the pSP65 sequence was used to linearize the plasmid to create a template of 368 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the protected probe was expected to be 130 bp.

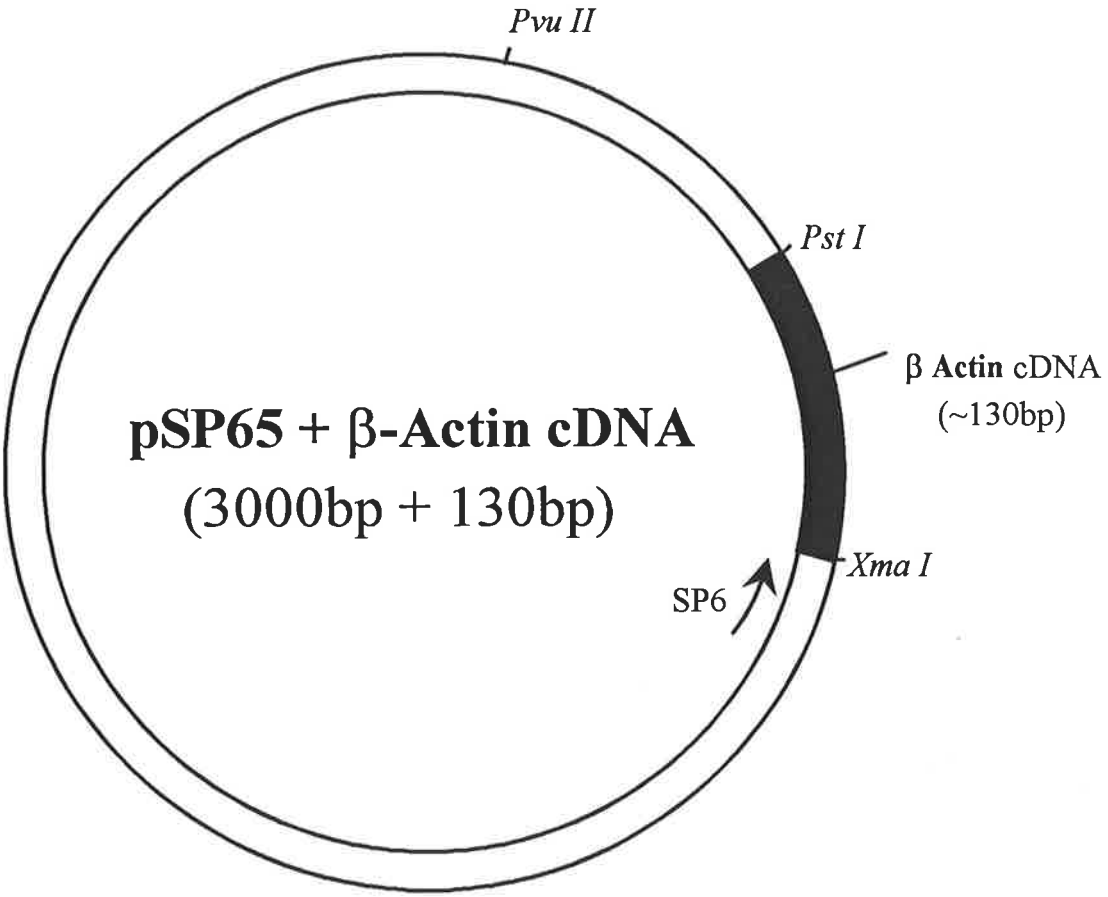
pSP72 + IL-1α: A plasmid containing a 350 bp insert which possessed approximately 250 bp of cDNA encoding a segment of the human IL-1α protein, and which was inserted into the multiple cloning site of pSP72 (Promega) was kindly provided by Dr. Malcolm Smith (Flinders Medical Centre, Adelaide) and was grown in E. *coli* (HB101). The insertion was at the EcoR I and Hind III restriction sites, such that antisense RNA could be transcribed from the SP6 promoter. A map of the vector, with insert, is shown in figure 2.12.4(b) EcoR I (Boehringer-Mannheim, Germany, cat. no. 703 737) was used to linearize the plasmid to create a template of 350 bp for transcribing anti-sense probe RNA where the size of the protected probe was expected to be 250 bp.

pSP72 + IL-1β cDNA: A plasmid containing 669 bp of cDNA encoding a segment of the IL-1β protein, including the leader sequence inserted into the multiple cloning site of pSP72 (Promega) was kindly provided by Dr. Malcolm Smith (Flinders Medical Centre, Adelaide) and was grown in E. *coli* (HB101). The insertion was at the Pst I and Pvu II restriction sites, such that anti-sense RNA could be transcribed from the SP6 promoter. A map of the vector, with insert, is shown in figure 2.12.4(c). The restriction enzyme, Dde I (Boehringer-Mannheim, Germany, cat. no. 835 293), which cut within the cDNA sequence, was used to linearize the plasmid to create a template of 339 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the unprotected probe was expected to be 323 bp.

pGEM-1 + IL-6 cDNA: A plasmid containing 1130 bp of cDNA encoding the entire IL-6 protein inserted into the multiple cloning site of pGEM-1 was

Figure 2.12.4(a): Map of pSP65 containing 130 bp of β-actin cDNA. Probe was produced using the SP6 promoter (Pvu II was used to linearize the plasmid).

Figure 2.12.4(b): Map of pSP72 containing a 350 bp of which 250 bp was IL-1α cDNA. Probe was produced from using SP6 as the promoter (EcoR 1 was used to linearize the plasmid).


Pvu II
Pst I
β Actin cDNA
(~130bp)
pSP65 + β-Actin cDNA
(3000bp + 130bp)
Xma I
SP6

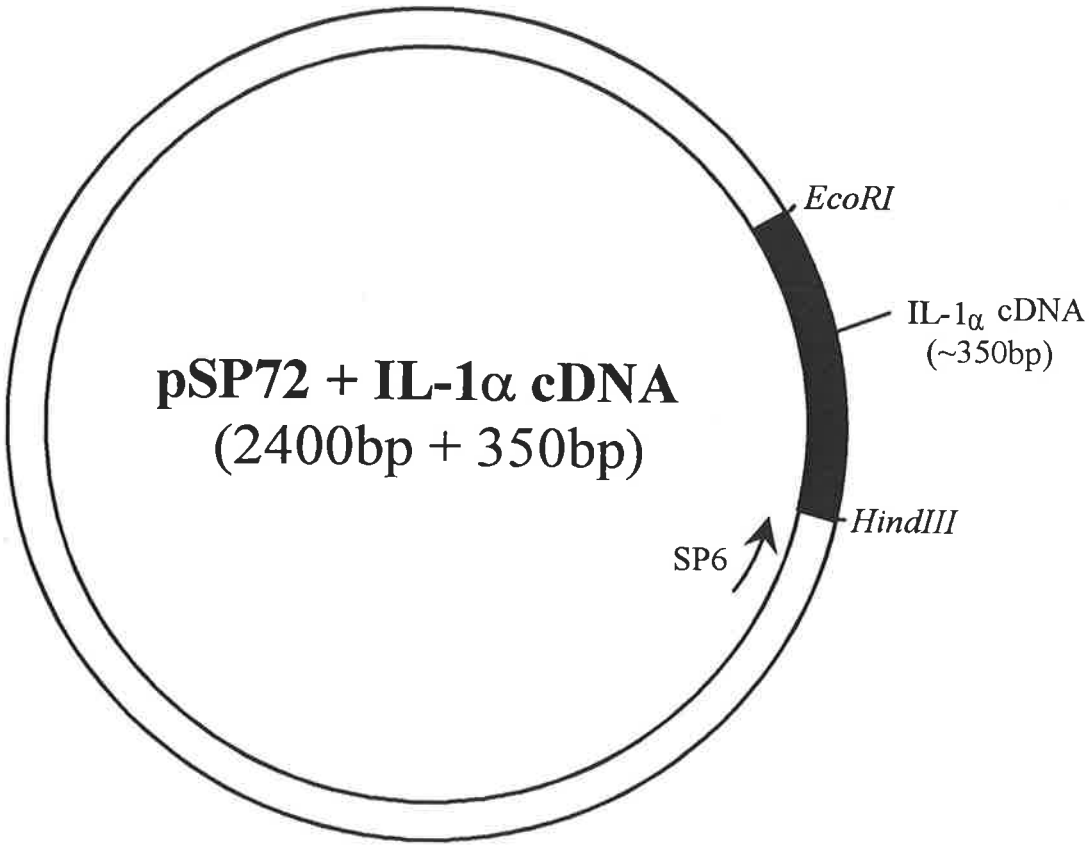
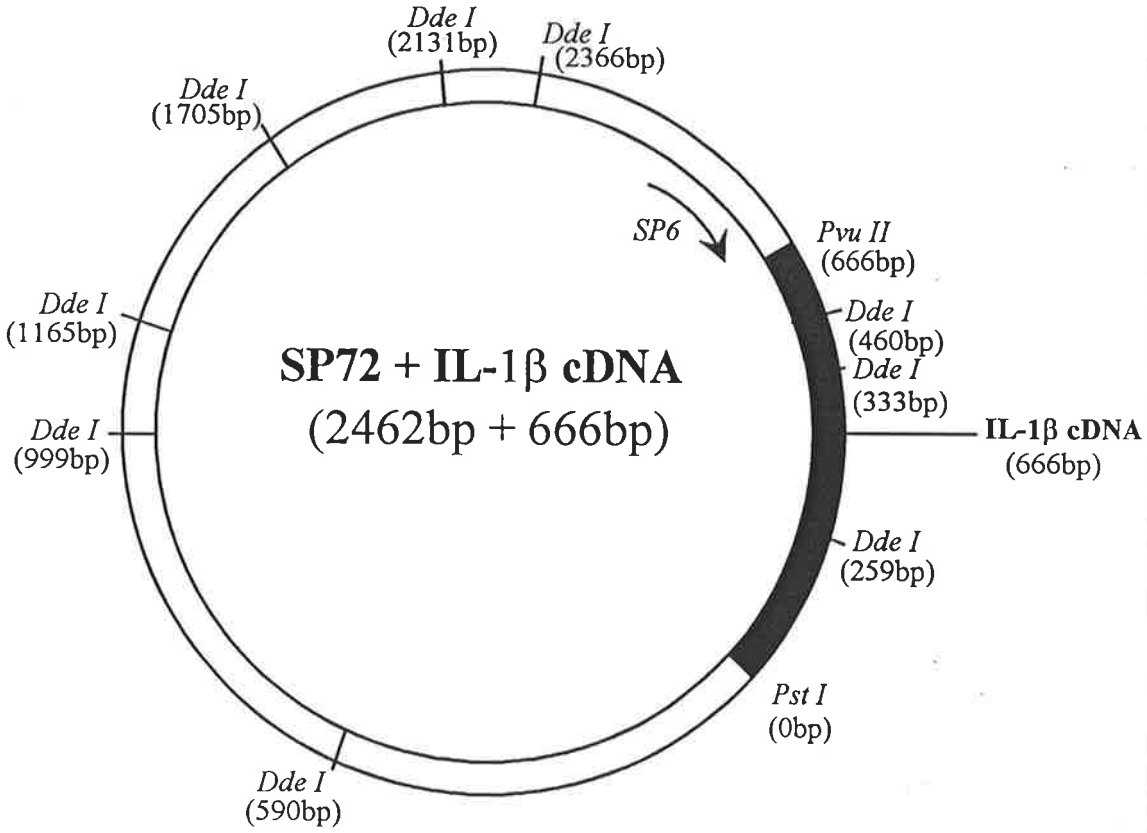
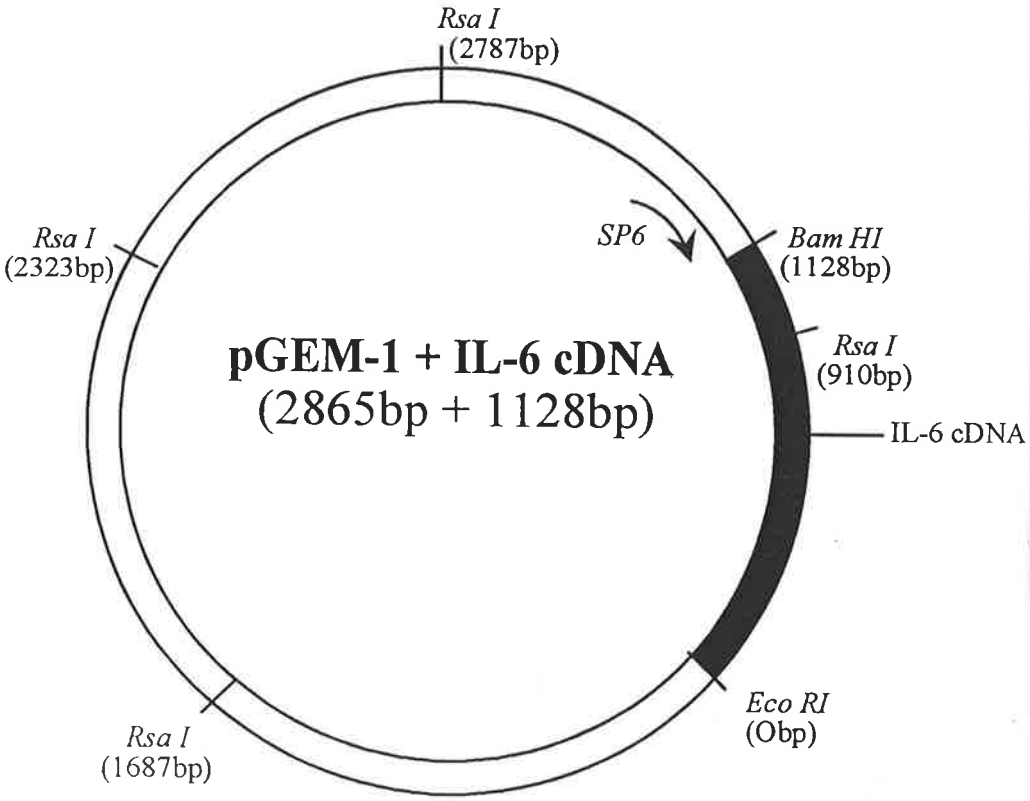

EcoRI
IL-1α cDNA
(~350bp)
pSP72 + IL-1α cDNA
(2400bp + 350bp)
HindIII
SP6

Figure 2.12.4(c): Map of pSP72 containing 669 bp of IL-1β cDNA. Probe was produced using SP6 as the promoter (Dde 1 was used to linearize the plasmid).

Figure 2.12.4(d): Map of pGEM-1 containing 1130 bp of IL-6 cDNA. Probe was produced using SP6 as the promoter (Rsa 1 was used to linearize the plasmid).


Dde I
(2131bp)
Dde I
(2366bp)
Dde I
(1705bp)
SP6
Pvu II
(666bp)
Dde I
(1165bp)
Dde I
(460bp)
Dde I
(333bp)
SP72 + IL-1β cDNA
(2462bp + 666bp)
Dde I
(999bp)
IL-1β cDNA
(666bp)
Dde I
(259bp)
Pst I
(0bp)
Dde I
(590bp)


Rsa I
(2787bp)
SP6
Rsa I
(2323bp)
Bam HI
(1128bp)
Rsa I
(910bp)
pGEM-1 + IL-6 cDNA
(2865bp + 1128bp)
IL-6 cDNA
Eco RI
(Obp)
Rsa I
(1687bp)

generously provided by Dr. Greg Goodall (Hanson Centre, Adelaide) and was grown in E. *coli* (DH5α). The insertion was at the BamH I and EcoR I restriction sites, such that anti-sense RNA could be synthesized from the SP6 promoter. A map of the vector, with insert, is shown in figure 2.12.4(d). The restriction enzyme, Rsa I (Promega, USA, cat. no. R6371), which cut within the cDNA sequence, was used to linearize the plasmid to create a template of 261 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the protected probe was expected to be 218 bp.

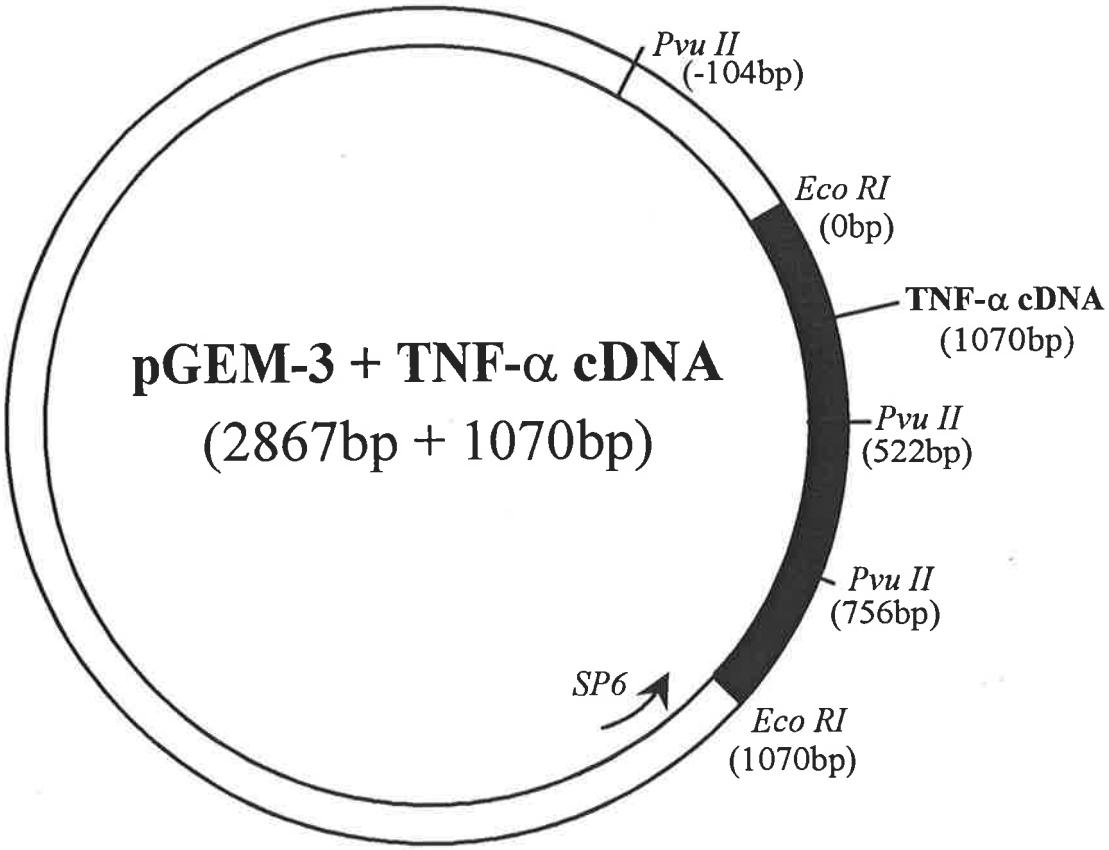
pGEM-3 + TNF-α cDNA: A plasmid containing 840 bp of cDNA encoding a segment of the TNF-α protein inserted into the multiple cloning site of pGEM-3 was kindly provided by Keril Blight (Dept. of Microbiology and Immunology, Adelaide University) and was grown in E. *coli* (DH5α). The insertion was at the EcoR I restriction site. A map of the vector, with insert, is shown in figure 2.12.4(e). The restriction enzyme, Pvu II, which cut within the cDNA sequence, was used to linearize the plasmid to create a template of 370 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the protected probe was expected to be 314 bp.

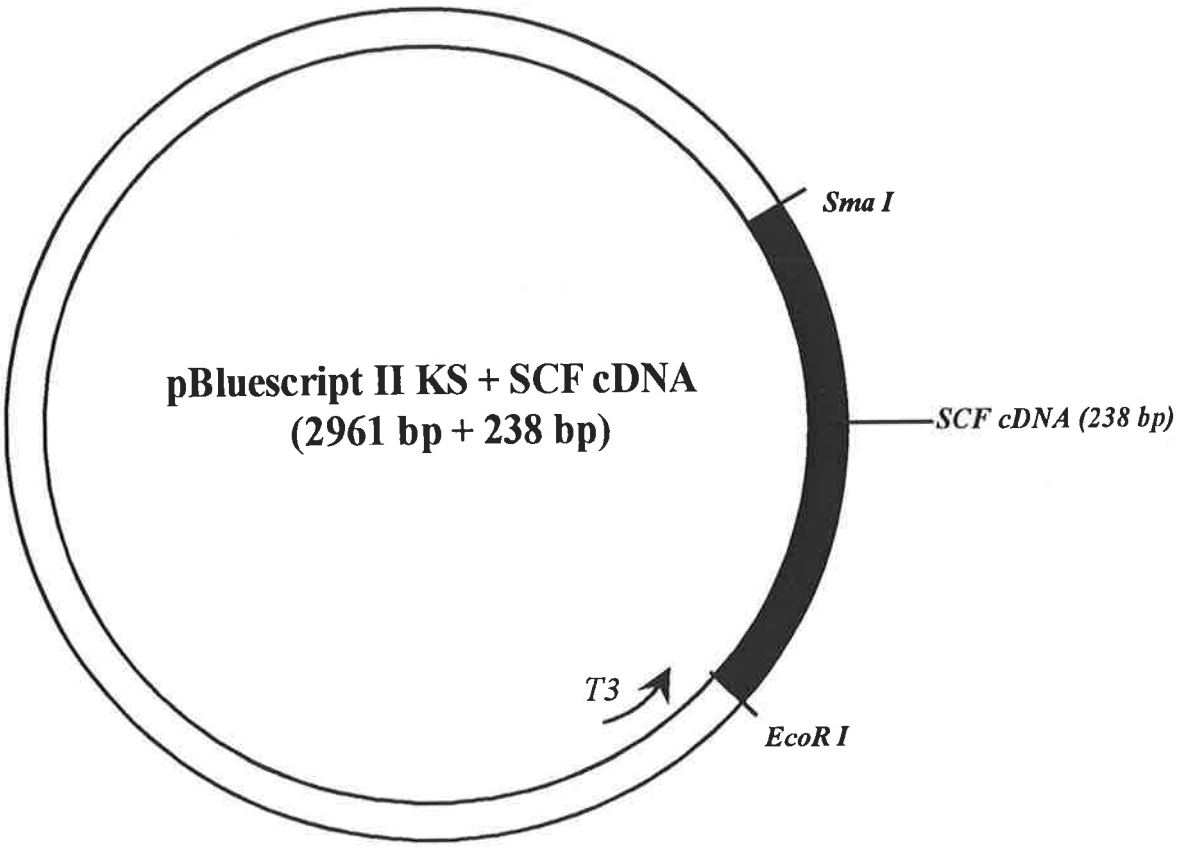
pBluescript II KS plus SCF cDNA: A plasmid containing 238 bp of cDNA encoding an internal segment of the SCF protein inserted into the multiple cloning site of pBluescript II KS was generously provided by Steve Cole (of this laboratory) and was grown in E. *coli* (DH5α). The insertion was at the Sma I and EcoR I restriction sites, such that anti-sense RNA could be synthesized from the T3 promoter. A map of the vector, with insert, is shown in figure 2.12.4(f). The restriction enzyme, Ecl 136 II (New England Biolabs, USA, cat. no. ERO251S), was used to linearize the plasmid to create a template of 344 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the protected probe was expected to be 238 bp.

pBluescript II KS plus GAPDH cDNA: A plasmid containing 144 bp of cDNA encoding an internal portion of the glyceraldehyde 3-phosphate dehydrogenase

Figure 2.12.4(e): Map of pGEM-3 containing 840 bp of TNF-α cDNA. Probe was produced using SP6 as the promoter (Pvu II was used to linearize the plasmid).

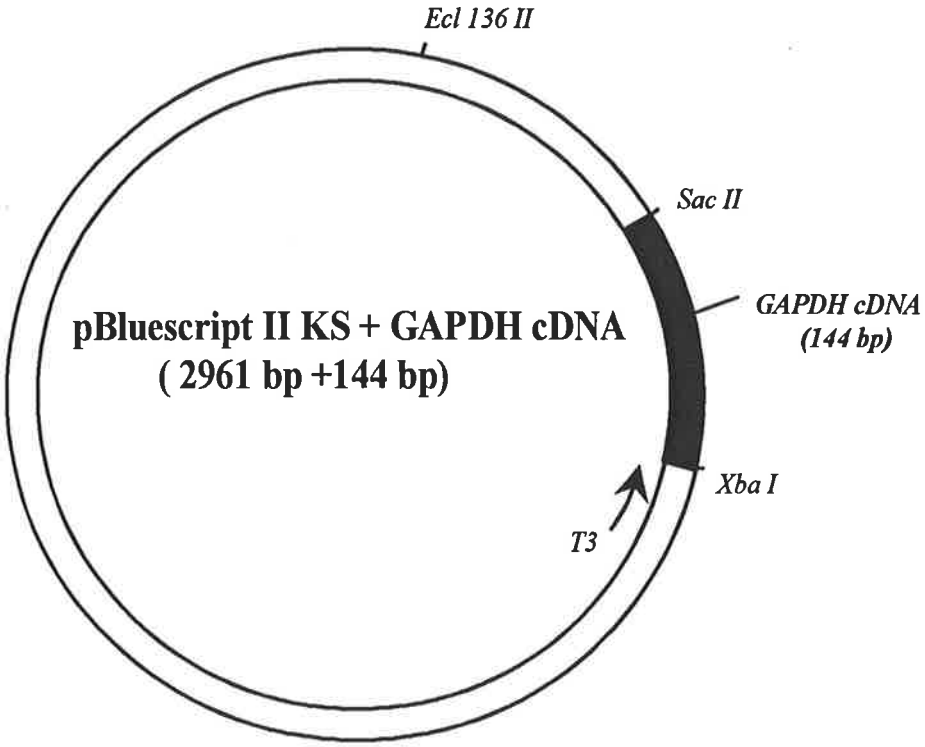
Figure 2.12.4 (f): Map of pBluescript II KS containing 238 bp of SCF cDNA. Probe was produced using T3 as the promoter (Ecl 136 II was used to linearize the plasmid).


pGEM-3 + TNF-α cDNA
(2867bp + 1070bp)
Pvu II
(-104bp)
Eco RI
(0bp)
TNF-α cDNA
(1070bp)
Pvu II
(522bp)
Pvu II
(756bp)
SP6
Eco RI
(1070bp)


pBluescript II KS + SCF cDNA
(2961 bp + 238 bp)
Sma I
SCF cDNA (238 bp)
T3
EcoR I

(GAPDH) protein inserted into the multiple cloning site of pBluescript II KS was generously provided by Steve Cole (of this laboratory) and was grown in E. *coli* (DH5 α). The insertion was performed via the Xba I and Sac II restriction sites, such that anti-sense RNA could be synthesized from the T3 promoter. A map of the vector, with insert, is shown in figure 2.12.4(g). The restriction enzyme, Ecl136 II, was used to linearize the plasmid to create a template of 242 bp for transcribing anti-sense probe RNA for use in RNase protection assays where the size of the protected probe was expected to be 144 bp.

Figure 2.12.4 (g): Map of pBluescript II KS containing 144 bp of GAPDH cDNA. Probe was produced using T3 as the promoter (Ecl 136 II was used to linearize the plasmid).


Ecl 136 II
Sac II
GAPDH cDNA
(144 bp)
pBluescript II KS + GAPDH cDNA
(2961 bp +144 bp)
Xba I
T3

CHAPTER 3

The effect of culturing HL-60 cells with all-*trans* retinoic acid

3.1 Introduction

The initial aim of this section was to induce HL-60 cells, with physiological factors, to become immunostimulatory for $CD2^+$-lymphocyte-enriched cell populations (LEP) derived from normal human peripheral blood. This chapter mainly focuses upon the effects of culturing HL-60 cells with the vitamin A derivative, all-*trans* retinoic acid (RA).

HL-60 cells are recognized as being poor stimulators of peripheral blood T lymphocytes in MLC (O'Keefe and Ashman, 1982) and, although there have been many reports of HL-60 cells being induced to differentiate into monocytes using a variety of physiological factors (Grant *et al*, 1985; Trinchieri *et al*, 1986; Rigby *et al*, 1985), relatively few researchers have studied the immunostimulatory nature of these induced cells. It has recently been demonstrated that the sequential treatment of HL-60 cells with Vit D_3 (1×10^{-7} M for 4 days) and interferon-γ (IFN-γ) (500 U/ml for 2 days) resulted in an up-regulation of cell surface expression of both MHC class II molecules and ICAM-1 (Shinbori *et al*, 1992). These cells failed to stimulate purified peripheral blood T cells under MLC conditions, but possessed an enhanced capacity to act as accessory cells in the activation of T lymphocytes treated with anti-CD3 mAb coupled to latex beads, a function which was inhibitable by mAb specific for ICAM-1 and HLA-DR antigen.

In the studies performed here, RA was the physiological agent used to induce differentiation of HL-60 cells, and the rationale behind using this factor stemmed from

previous work performed in other laboratories showing that a combination of 100 - 1000 U/ml of IFN-γ and 10 nM RA could induce HL-60 cells to become monocyte-like (Hemmi and Breitman, 1987). IFN-γ has been shown to possess only a modest ability to induce monocytic differentiation in HL-60 cells, however when used in combination with RA, the two factors were reported to act synergistically to induce a recognizably more advanced stage of monocyte maturation as defined by a variety of morphological and functional criteria (Ball *et al*, 1984; Hemmi and Breitman, 1987; Dayton *et al*, 1984).

3.2 Preliminary studies

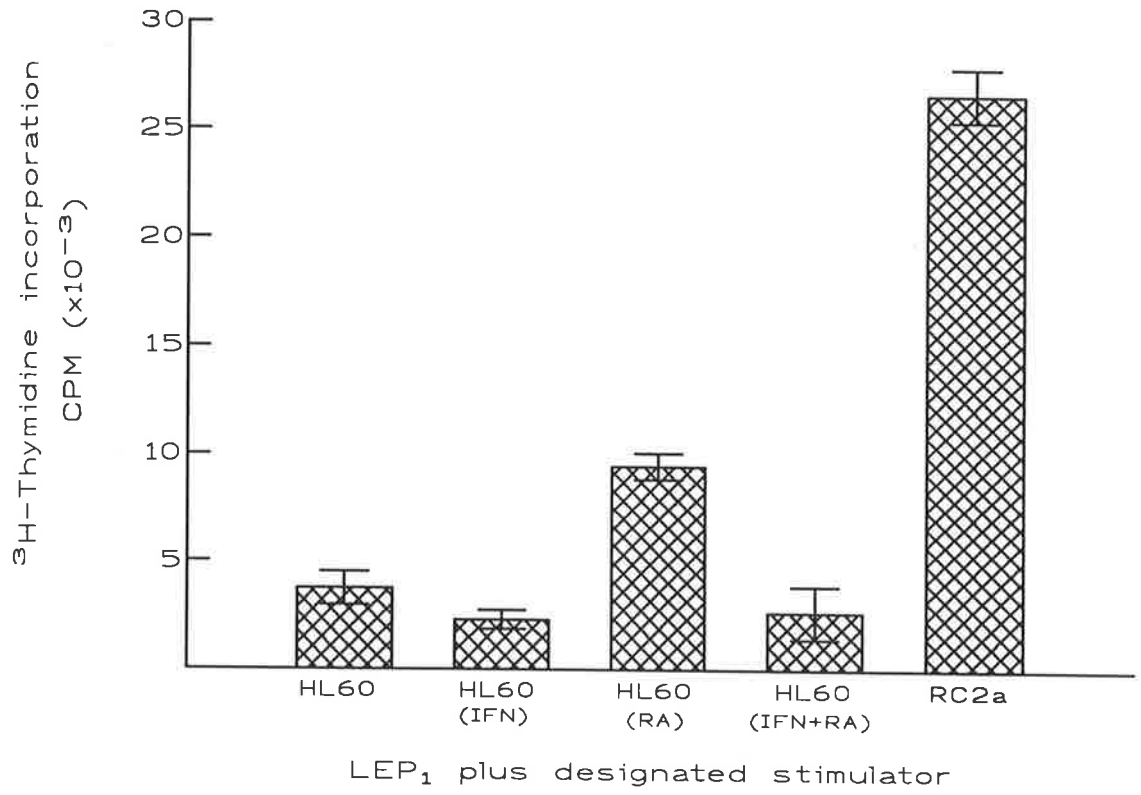
Since HL-60 cells were reported to travel along what appeared to be the monocyte pathway when cultured with a combination of IFN-γ and RA (Hemmi *et al*, 1987), it was thought possible that such cells may well have a greater capacity to activate LEP than their undifferentiated counterparts. Such a possibility prompted the performance of a MLC involving IFN-γ/RA-treated HL-60 cells and LEP.

In short, HL-60 cells were cultured with IFN-γ (1000 U/ml) and/or RA (10 nM) for seven days and then mixed with LEP in a MLC and the degree of proliferation was ascertained via uptake of tritiated thymidine (^{3}H-thymidine) in a 6 day assay. The outcome of the experiment performed with LEP_1 is displayed in figure 3.2.1 and it indicates that the treatment of HL-60 cells with a combination of IFN-γ and RA did not result in increased immunostimulatory activity, whilst treatment with RA alone, did*. These results were shown to be reproducible for five other LEP. The IFN-γ was shown to be active through its ability to up-regulate the expression of a number of cell surface markers, such as MHC class I, and is discussed in more detail in chapter 6.

The results obtained with HL-60 cells cultured for 7 days with RA only (HL-60-R7), indicated that these cells possessed an enhanced immunostimulatory capacity, a phenomenon which was unexpected since HL-60 cells have, on numerous occasions, been reported to travel along the neutrophil pathway when treated with RA (Breitman

*These findings are discussed further in chapter 6

Figure 3.2.1: **Proliferation of LEP in MLC in response to HL-60 cells cultured for 7 days with IFN-γ (1000 U/ml), IFN-γ + RA (10 nM) or RA alone**. RC-2a and untreated HL-60 cells were also independently used as stimulators. Counts incorporated by the stimulator populations were each < 300 cpm, and for LEP_1 alone; 1600 ± 500 cpm.


30
25
20
15
10
5
^{3}H-Thymidine incorporation
CPM (x10^{-3})
HL60
HL60 (IFN)
HL60 (RA)
HL60 (IFN+RA)
RC2a
LEP$_1$ plus designated stimulator

et al, 1983; Santoli *et al*, 1983; Hemmi and Breitman, 1987), and neutrophils are not well recognized for their capacity to activate lymphocytes. Indeed, when freshly isolated peripheral blood neutrophils were used as stimulators in an allogeneic MLC involving LEP (n = 2) they were found to be non-stimulatory (data not shown).

The enhanced immunostimulatory capacity of HL-60-R7 cells was shown to be reproducible. HL-60-R7 cells were, on average, 11 times more stimulatory than untreated HL-60 cells with a range of 1.5 - 70 and a median of 6 (n = 32). In comparison, cells of the myelomonocytic line, RC-2a, which have previously been reported to be potent stimulators in a MLC ((O'Keefe and Ashman, 1982), were shown here to be, on average, 24 times more stimulatory than HL-60 cells with a range of 5 - 115 and a median of 13 (n = 31). Figure 3.2.2 is a compilation of 8 different MLC involving 6 different LEP, showing the reproducibility of the enhanced proliferative response. However, the variability with respect to the relative proliferative responses to HL-60-R7 and RC-2a cells was noted, even for the same LEP (LEP_3).

As described in chapter 2, MLC were pulsed with ^{3}H-thymidine on day 5. This decision was made on the basis of the results obtained from MLC performed with 4 different LEP, where the uptake of ^{3}H-thymidine by the proliferating lymphocytes was measured on days 3 - 7 of the culture. Results demonstrated that the proliferative peak for 3 of the 4 LEP responding to HL-60-R7 cells was on day 5, with the remaining LEP peaking on day 4. The responses to RC-2a cells peaked on day 5 for two of the four LEP, with one peaking on day 4 and the other on day 6 (data not shown).

It was also of interest to determine whether only a small percentage of HL-60-R7 cells were immunostimulatory, especially since a minority of the cells possessed the morphological features of monocytes or expressed the monocyte marker CD14 (see later). Whether such a situation was likely was tested by means of a MLC where decreasing numbers of stimulator cells (RC-2a, HL-60 or HL-60-R7) were added to a constant number of LEP. Results for LEP_{17} are shown in figure 3.2.3 and suggest that

Figure 3.2.2: **Proliferation of LEP in MLC in response to HL-60, HL-60-R7 or RC-2a cells.** A collage of 8 different MLC performed with 6 independent LEP is shown. Counts incorporated by stimulator populations were each < 300 cpm.

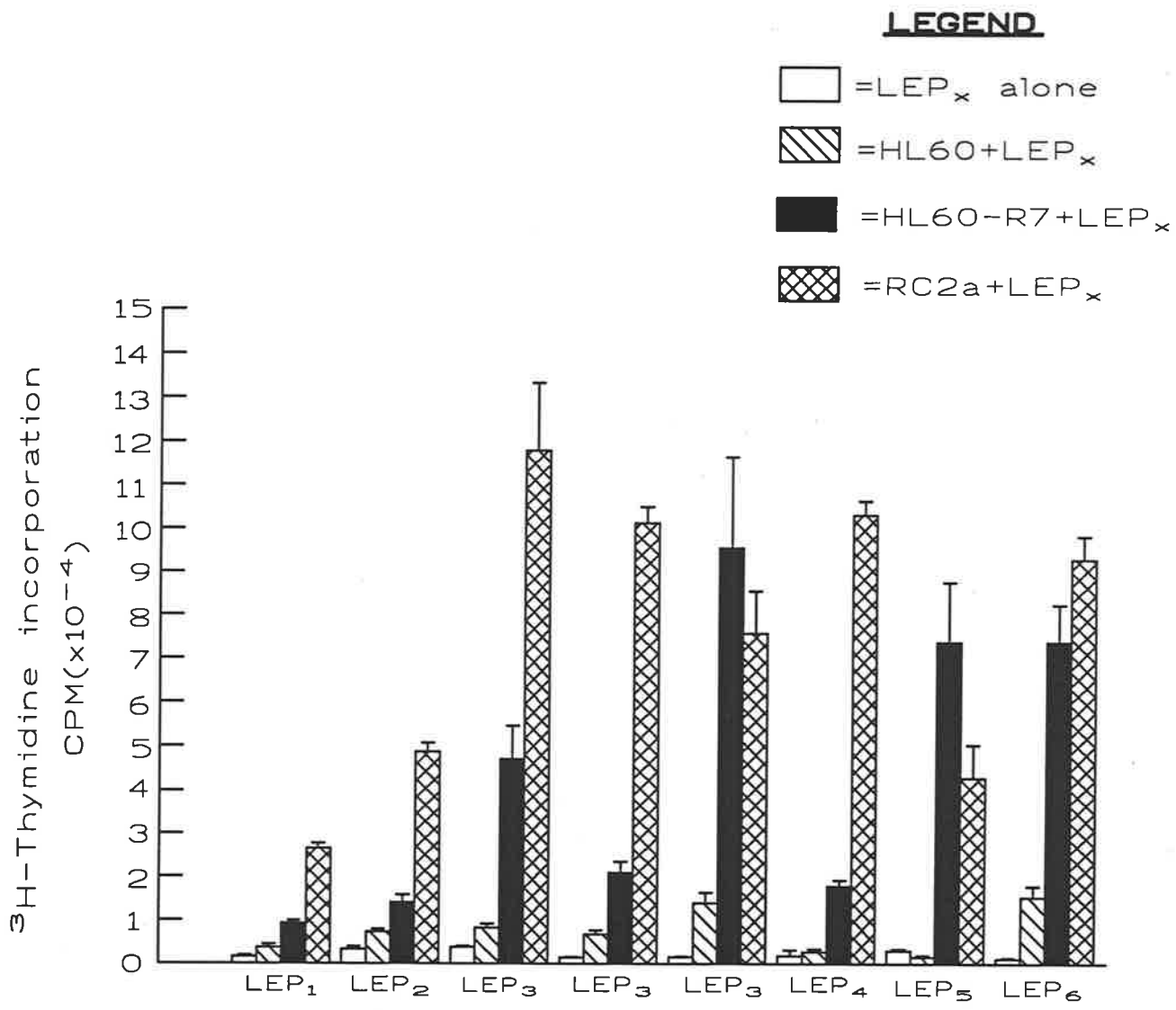
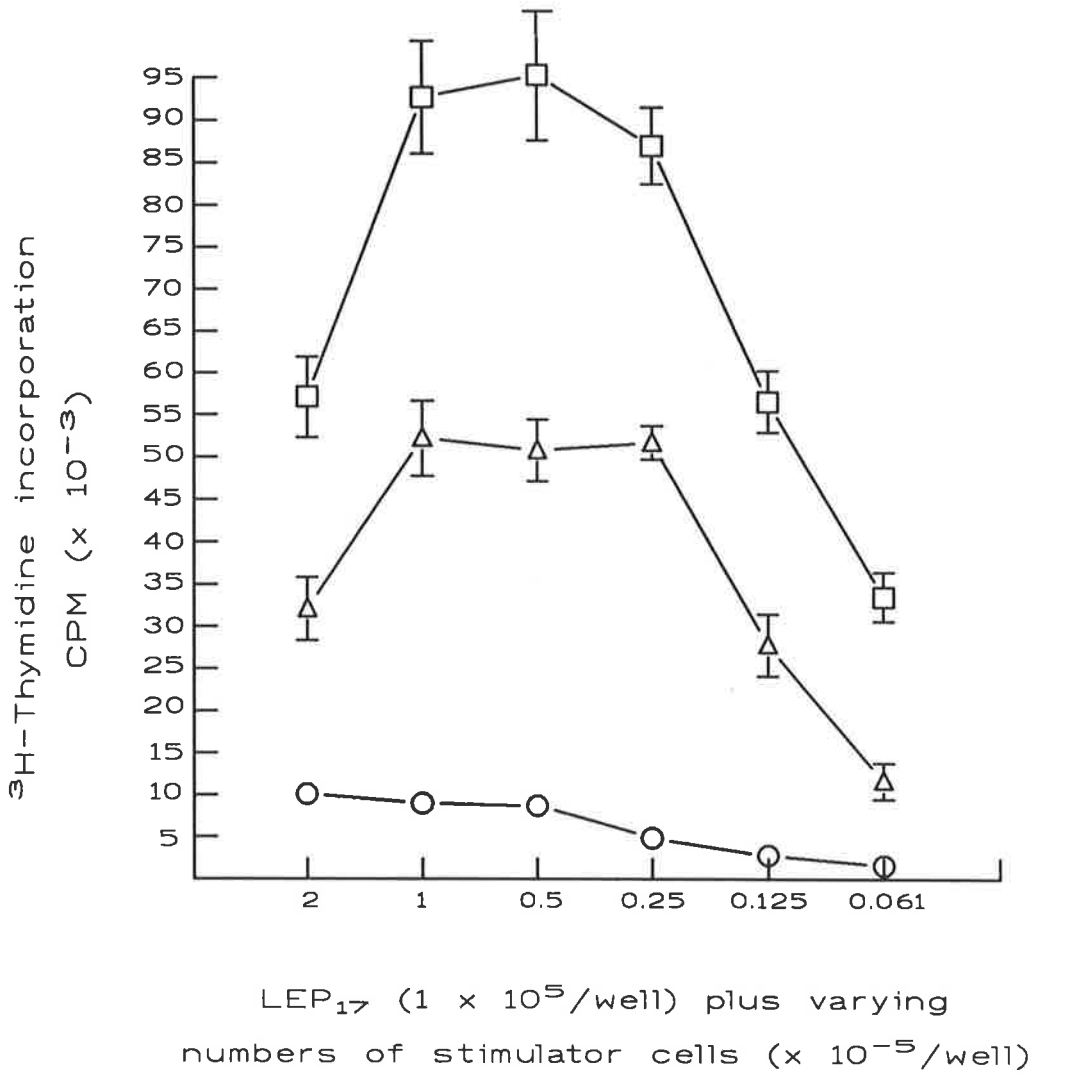

LEGEND
=LEP$_x$ alone
=HL60+LEP$_x$
=HL60-R7+LEP$_x$
=RC2a+LEP$_x$
^{3}H-Thymidine incorporation
CPM(x10^{-4})
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
LEP$_1$
LEP$_2$
LEP$_3$
LEP$_3$
LEP$_3$
LEP$_4$
LEP$_5$
LEP$_6$

Figure 3.2.3: **LEP response to varying stimulator concentrations.** MLC was set up where decreasing numbers of HL-60, HL-60-R7 or RC-2a cells were added to 1 x 10^5 cells of LEP_{17}. Counts incorporated by stimulators alone were < 200 cpm, and by LEP_{17}, < 2000 cpm.


O = HL60 cells
Δ = HL60-R7 cells
□ = RC2a cells
^{3}H-Thymidine incorporation
CPM (x 10^{-3})
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
2
1
0.5
0.25
0.125
0.061
LEP_{17} (1 x 10^5/well) plus varying
numbers of stimulator cells (x 10^{-5}/well)

a large proportion of HL-60-R7 cells were immunostimulatory since small numbers of HL-60-R7 cells (2.5×10^4 cells/well) were capable of triggering substantial lymphoproliferation, a result which paralleled the result obtained with the highly stimulatory RC-2a cells. Similar results were also yielded from 3 other LEP tested.

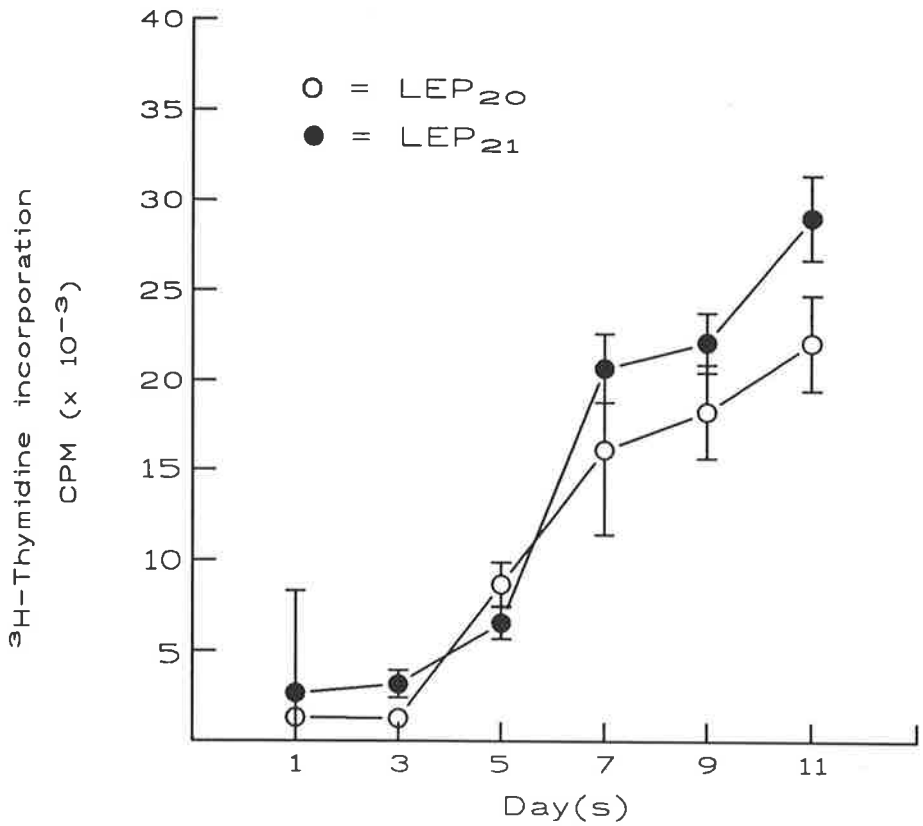
In order to determine the stage at which RA-treated HL-60 cells acquired an enhanced immunostimulatory capacity, an MLC was performed involving HL-60 cells cultured with 10 nM RA for 0, 1, 3, 5, 7, 9 and 11 days, and the results are shown in figure 3.2.4. It can be seen that RA-treated HL-60 cells were not immunostimulatory for the first 3 days of culture, however, by day 5, an increase in immunopotency was noted, and further increases occurred on days 7 through to 11. The 7 day cultures were chosen for future studies as the viabilities of the day 9 and 11 cultures would often drop below 90%.

The enhanced capacity of HL-60-R7 cells to stimulate lymphocyte proliferation warranted further investigation. In order to gain a better understanding of this phenomenon, two areas of investigation were initiated. One of these concerned the maturation pathway of RA-treated HL-60 cells, and thus an attempt was made to determine whether the cells were being induced along the neutrophil, monocyte or some other pathway or combination of pathways. This involved analysis of their nuclear morphology, cytochemistry and cell surface antigen expression. The second area of investigation involved the analysis of the responding lymphocyte populations and is dealt with in chapter 4.

3.3 The effect of varying the concentration of RA upon the proliferative capacity and viability of HL-60 cells.

Haemopoietic cells tend to lose their proliferative ability the further they differentiate along their respective maturation pathways, with the obvious exception of the lymphocyte lineages (Sachs, 1980; Greaves 1982). Therefore, if RA was inducing HL-60 cells to differentiate, one would expect a drop in their proliferation rate. To

Figure 3.2.4: Immunopotency of HL-60 cells cultured for 0 - 11 days with RA. MLC were set up involving LEP_{20} or LEP_{21} (1 x 10^5/ml) plus HL-60 cells which had been cultured with 10 nM RA for 0, 1, 3, 5, 7, 9 and 11 days. Counts incorporated for stimulators alone were < 200 cpm, and for LEP_{20} and LEP_{21}, < 1000 cpm.



LEP_{20} or LEP_{21} plus HL-60 cells

cultured for x days with RA

determine whether this was the case, a study was performed involving the culturing of HL-60 cells with varying concentrations of RA (0 - 300 nM) over a period of 12 days. Cultures were initiated in triplicate in 25 cm^2 tissue culture flasks at a density of 4 x 10^5 cells/ml. Every second day the cell cultures were monitored for their densities and viabilities, and were then subcultured at 5 x 10^5 cells/ml if the cell density was found to be $\geq$ 1 x 10^6 cells/ml.

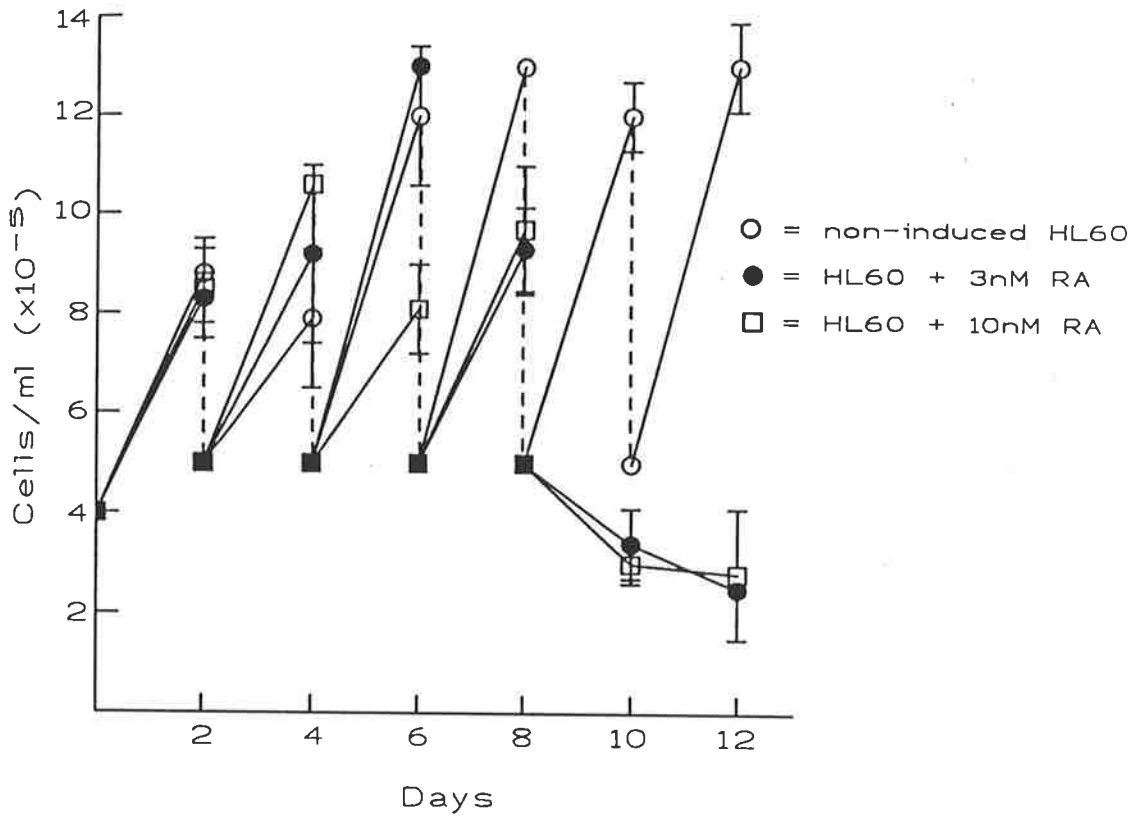
The results of these studies are shown in figures 3.3.1 and 3.3.2, and it would appear that all concentrations of RA used in this assay resulted, eventually, in a marked reduction in cell proliferation, although it was noted that the proliferation rate of RA-treated cells often exceeded that of untreated HL-60 cells during the first 4 days of the culture, which was shown to be a reproducible phenomenon. The kinetics of the proliferative inhibition appeared to be concentration-dependent.

Although these results supported the idea that RA can induce HL-60 cells to differentiate, more definitive analyses were required to be thoroughly convincing, especially when one considers the results of the viability assay displayed in figures 3.3.3 and 3.3.4. The cells had become substantially less viable the longer the period of culture, which could be the result of cells succumbing to the toxic effects of the RA, and possibly contributing to a decrease in their proliferative capacity. Alternatively, or concomitantly, it may be that the cells reached an end-point in the differentiation pathway and were only short lived. If this drop in viability was due to the toxicity of the RA, it is unlikely to have been a non-specific effect, since the cell line K562 appeared to be completely unaffected in terms of its proliferative capacity and its viability when cultured with 10 nM RA for up to 14 days (data not shown), an observation corroborated by others (Suedhoff *et al*, 1990).

In subsequent studies it was found that the viabilities of the induced cultures varied depending on the mode of handling. For instance, replenishing the cultures from day 6 onwards with fresh medium once they had attained or exceeded 1 x 10^6 cells/ml, was performed such that their cell densities were reduced by not much more than 1/5,

Figure 3.3.1: **Effect of culture with RA on proliferation of HL-60 cells.** Density (cells/ml) assay of HL-60 cells cultured with 0, 3 and 10 nM RA over a period of 12 days. Dashed line indicates the passage of a culture.

Figure 3.3.2: Density (cells/ml) assay of HL-60 cells cultured with 30, 100 and 300 nM RA over a period of 12 days. Dashed line indicates the passage of a culture.


14
12
10
8
6
4
2
Cells/ml (x10⁻⁵)
2
4
6
8
10
12
Days
○ = non-induced HL60
● = HL60 + 3nM RA
□ = HL60 + 10nM RA

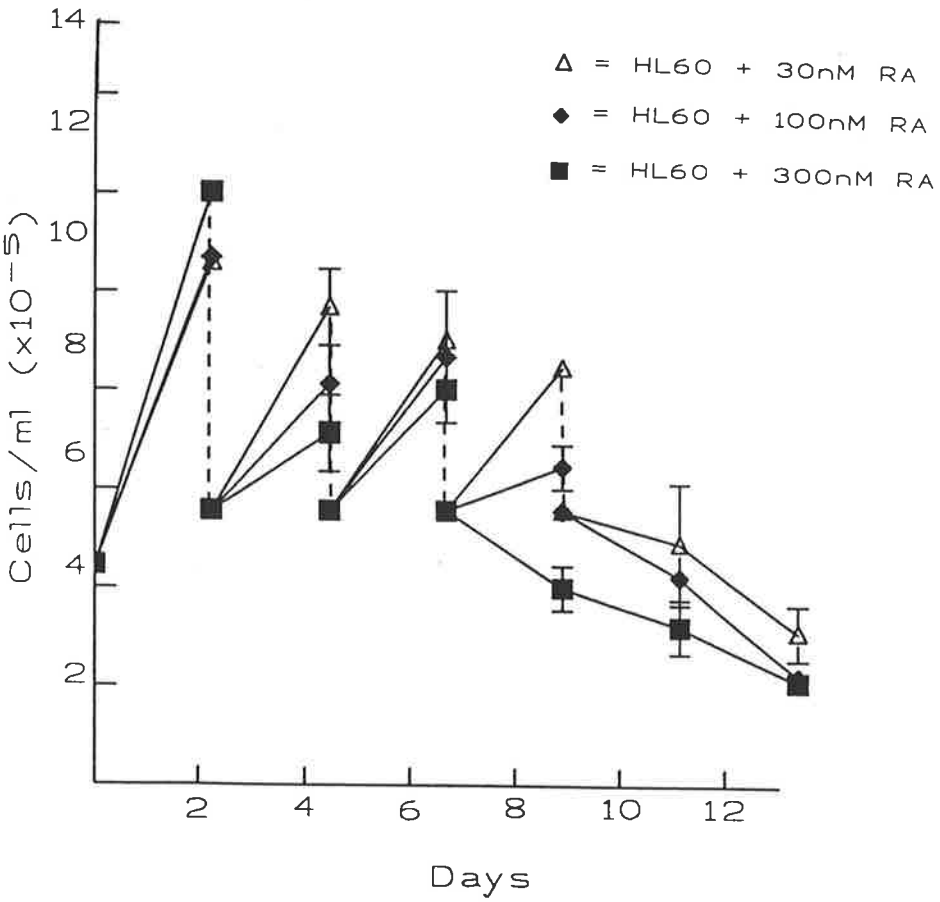
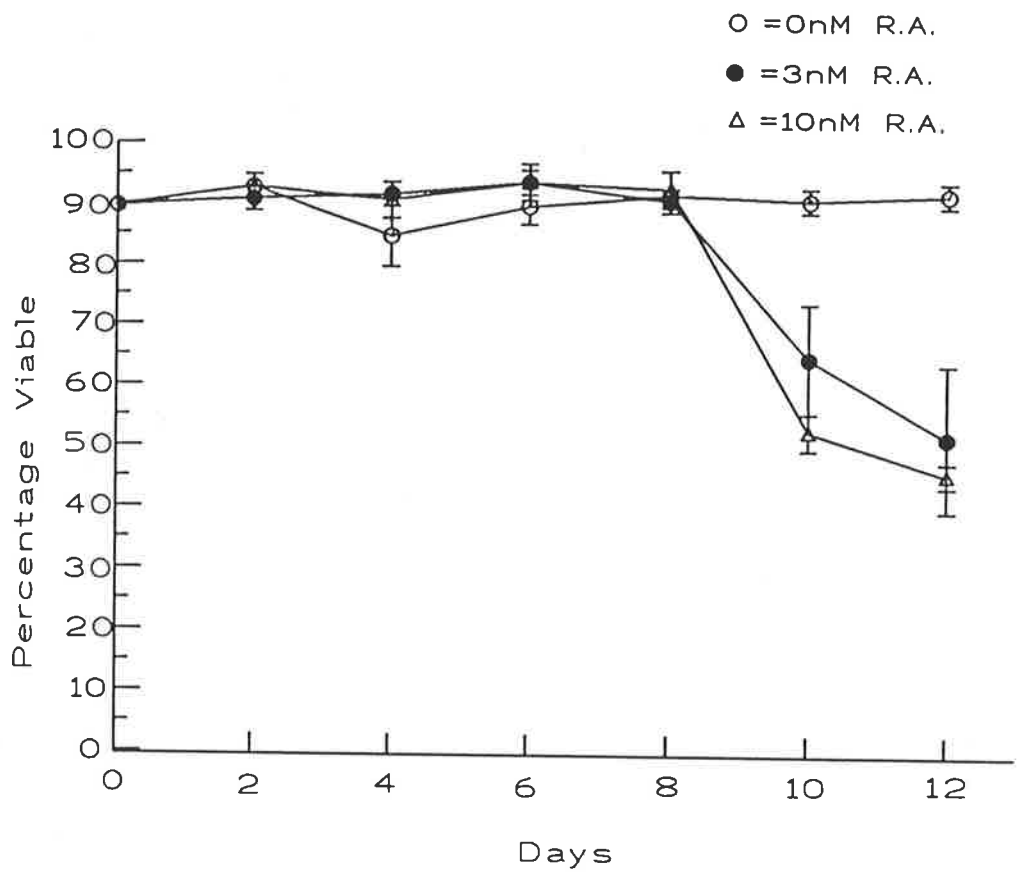
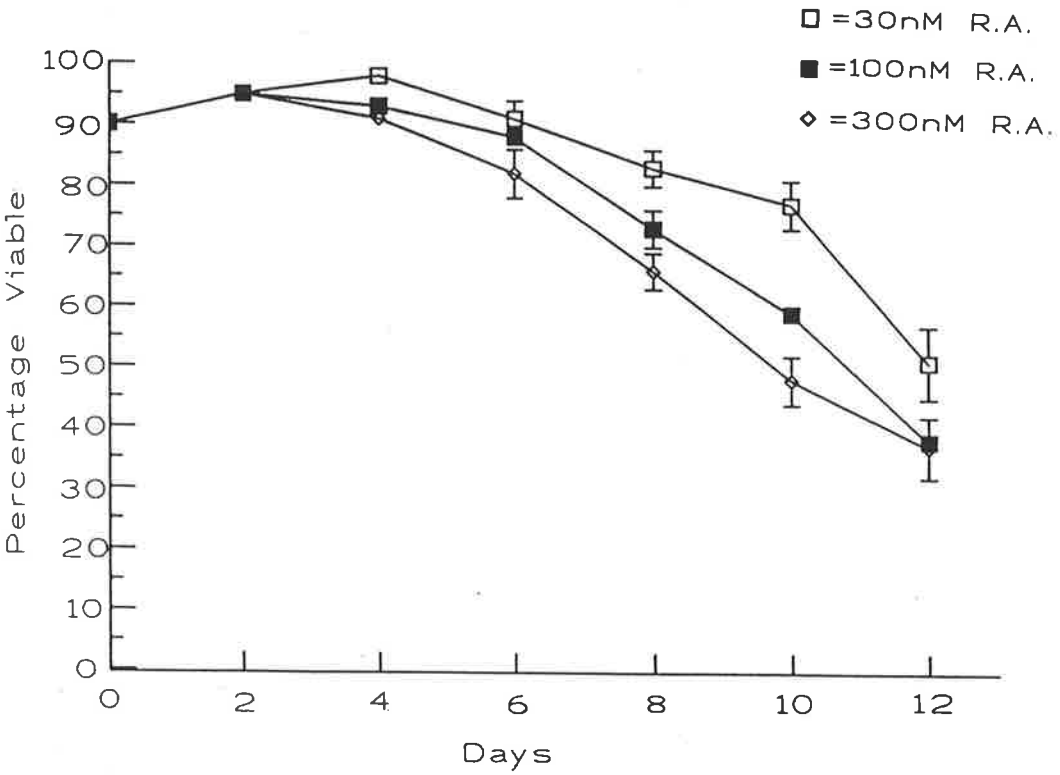

14
12
10
8
6
4
2
Cells/ml (x10⁻⁵)
2
4
6
8
10
12
Days
△ = HL60 + 30nM RA
◆ = HL60 + 100nM RA
■ = HL60 + 300nM RA

Figure 3.3.3: **Viability assay of RA-treated HL-60 cells**. Viabilities of HL-60 cells cultured with 0, 3 and 10 nM RA over a period of 12 days calculated by trypan blue exclusion.

Figure 3.3.4: Viability assay involving HL-60 cells cultured with 30, 100 and 300 nM RA over a period of 12 days.


O =0nM R.A.
● =3nM R.A.
Δ =10nM R.A.
Percentage Viable
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Days


□ =30nM R.A.
■ =100nM R.A.
◇ =300nM R.A.
Percentage Viable
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Days

as compared to 1/2 dilutions performed in the above experiment. This resulted in RA-treated HL-60 cells (10 nM RA) maintaining a higher viability (> 80%) for a longer period of time (up to 12 days). This phenomenon was further investigated in chapter 7, resulting in some interesting findings.

The concentration of 10 nM RA was decided upon for use in future experiments as this approximately corresponded to physiological levels (Tang and Russel, 1988), thus rendering viable any therapeutic implications which may be derived from these studies. Otherwise, any of the other concentrations would have been acceptable, since they all had an anti-proliferative effect, tentatively suggesting the induction of differentiation, and all RA-induced cultures were found to possess enhanced lymphocyte activating capacity by day 7, as judged through MLC (data not shown).

3.4 Morphology of HL-60-R7 cells

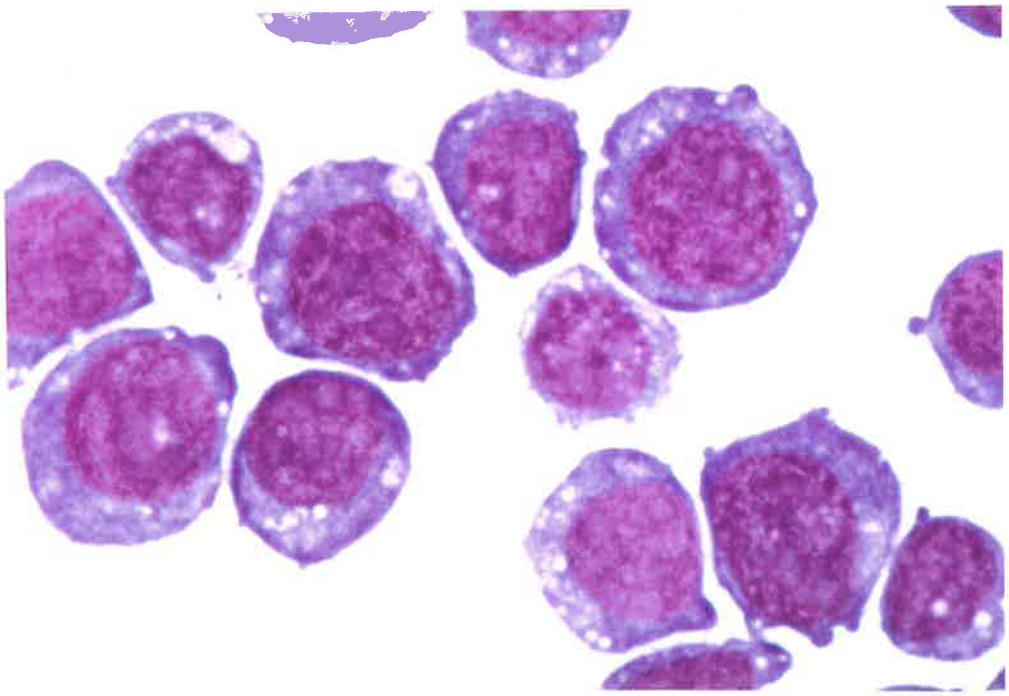
The morphology of HL-60-R7 cells was determined using the Diff-Quick method, which is a modification of the Wright staining technique. This allows determination of not only the shape of the nucleus, but also the cytoplasmic to nuclear ratio, the number of nucleoli, granularity, the density of the chromatin and the relative levels of RNA within the cytoplasm. All this information combined often aids in determining the type of cell that is under scrutiny.

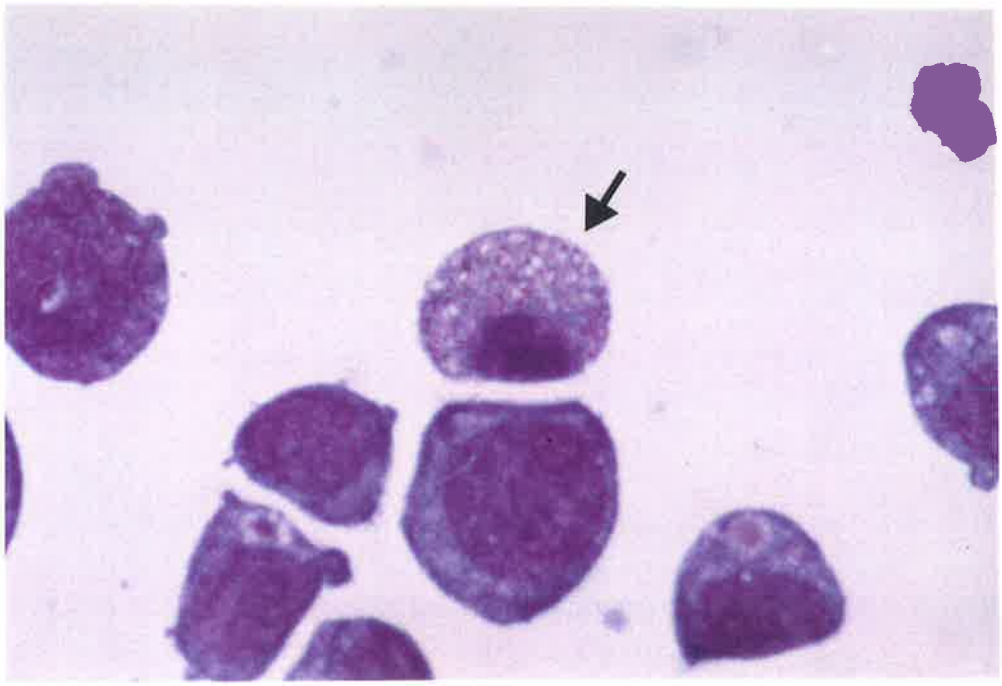
HL-60 cells are considered to be representative of a stage in haemopoiesis between the myeloblastic and the promyelocytic phases of differentiation (Dalton *et al*, 1988), and results obtained here through the Diff-Quick treatment were consistent with this and are displayed in figure 3.4.1. To illustrate, the nuclei were roughly spherical and took up approximately 70 - 80% of the volume of the cell. Nucleoli were faint, but were generally present in numbers ranging from 1 - 5. The cytoplasm of most cells was densely stained, a phenomenon which is apparently indicative of the cell producing substantial levels of RNA.

Figures 3.4.1-5: **Effect of RA on the morphology of HL-60 cells.** Untreated HL-60 cells and RA-treated HL-60 cells were stained via the Diff-Quick method. Magnification was x1000.

Figure 3.4.1: A typical field of untreated HL-60 cells.

Figure 3.4.2: Untreated HL-60 cells. The black arrow indicates a basophilic cell.





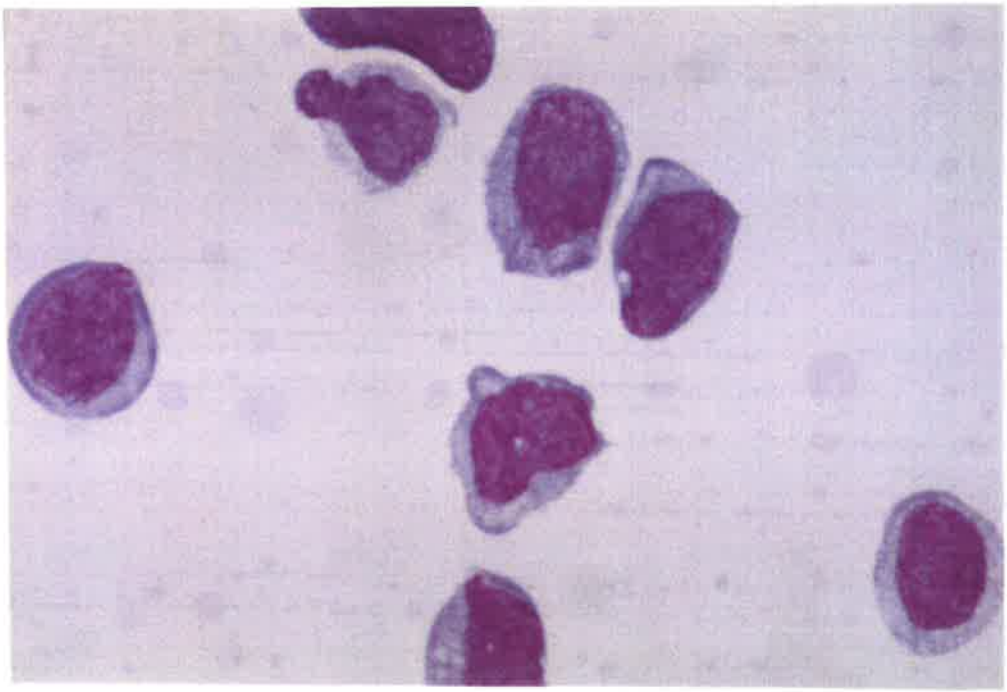
Often present within this population of HL-60 cells was another subset of phenotypically distinct basophilic cells which usually consisted of approximately 1 - 2% of the population (figure 3.4.2). The nuclear to cytoplasmic ratio of these cells was approximately 40/60, and the cytoplasm appeared not to stain, but instead contained many large spherical granules, some of which stained metachromatically. In general, the nuclei were more condensed and the nucleoli were very hard to distinguish. There were occasions, however, when these cells appeared to be completely absent from the HL-60 cultures suggesting that subtle variations in culture conditions may have played a part in determining whether or not they were present. Interestingly, cells of similar appearance were found amongst the primary leukaemic cells of the patient from which HL-60 was derived (Dalton *et al*, 1988)

Diff-Quick staining was also performed on HL-60 cells cultured with 10 nM RA for 2, 4, 6, 8, 10 and 12 days. Over this period the induced cells underwent marked morphological changes. By day 6 the chromatin appeared to become more condensed and the nucleoli were a lot harder to distinguish (figure 3.4.3). The nuclei of the cells were transformed from being uniformly spherical into many varied and irregular shapes by days 8, 10, and 12.

The cytoplasm retained its densely staining texture for the first 2 days, but from then on became lighter, until the staining was barely visible on day 12. Also by day 12, one could detect cells whose nuclei resembled monocytes in that they were kidney-shaped ($\approx$ 10%) (figure 3.4.4), as well as nuclei resembling those of neutrophils in that they were banded, or banded and segmented ($\approx$ 10%) (figure 3.4.5). Granules also became less prominent except in the basophilic cells which did not seem to be affected by the RA treatment and whose frequencies remained fairly constant (1 - 2%) over the 12 days.

Figure 3.4.3: HL-60 cells cultured for 6 days with 10 nM RA.

Figure 3.4.4: HL-60 cells cultured for 12 days with 10 nM RA. This figure displays two monocyte-like cells.



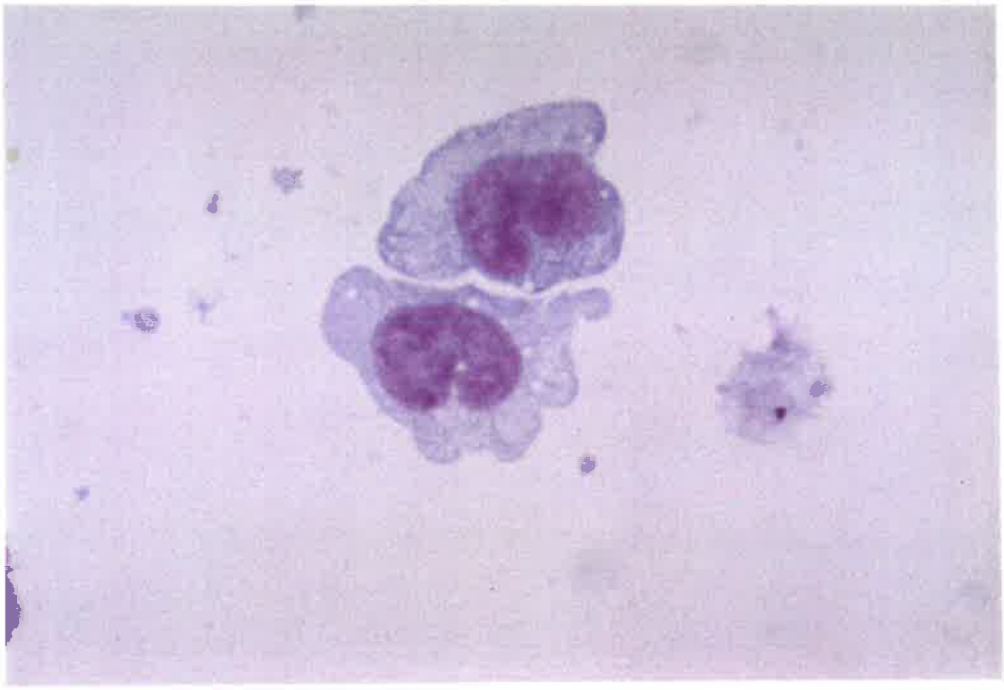
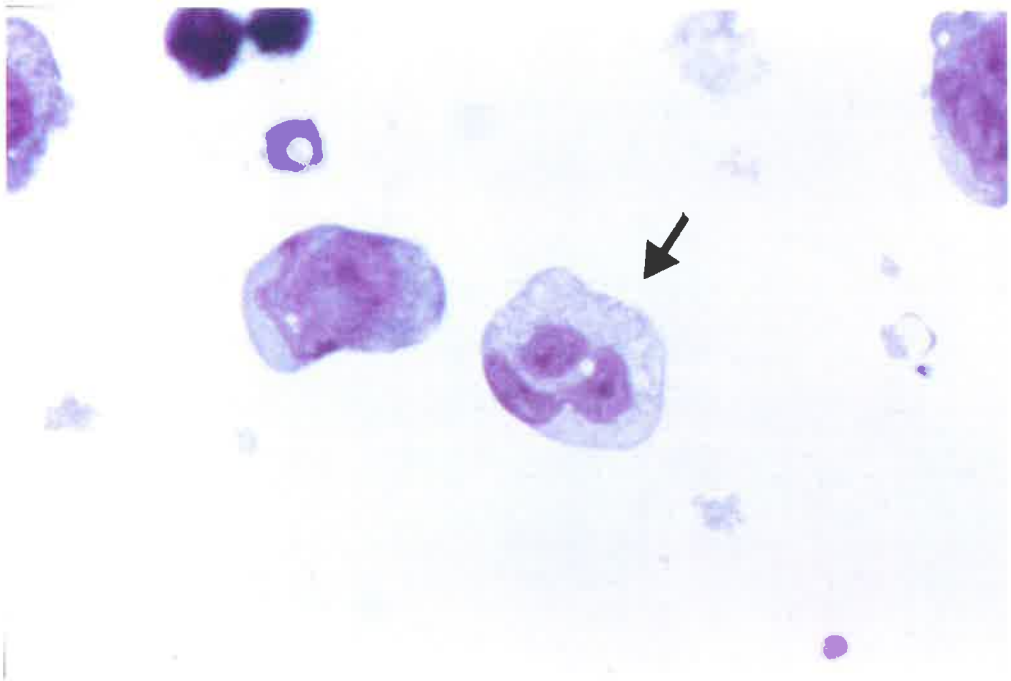


Figure 3.4.5: HL-60 cells cultured for 12 days with 10 nM RA. The black arrow indicates the presence of a granulocytic cell.



3.5 Staining for the presence of non-specific esterase

Non-specific esterase (NSE) is an enzyme diagnostic for monocytes/macrophages (Yam *et al*, 1971) and, considering that RA-induced HL-60 cells possessed an enhanced capacity to activate lymphocytes, it was considered possible that monocyte-like cells were present in these induced cultures. Thus a staining procedure was used to detect the presence of NSE in HL-60 cells that had been induced to differentiate for 0, 2, 4, 6, 8, 10, and 12 days (see section 2.2.2 for method). It was found that approximately 2 - 5% of control HL-60 cells expressed detectable levels of NSE (figure 3.5.1), as previously reported (Mangelsdorf *et al*, 1984). After 6 days of culture with RA approximately 1% of the population expressed low levels of NSE and, by day 12, no positively staining cells were detected (figure 3.5.2). The staining procedure was shown to be functional through its ability to detect NSE expression in peripheral blood monocytes (figure 3.5.3) as well as in RC-2a cells of which 5 - 10% expressed low through to high levels of the enzyme (figures 3.5.4)).

3.6 Detection of chloro-acetate esterase expression

Chloro-acetate esterase (CAE) is an enzyme characteristically expressed by neutrophils and their precursors, but not by monocytes (Yam *et al*, 1971). HL-60 cells have been reported to express this enzyme at generally low levels (Gallagher *et al*, 1979), and this was also shown to be the case here, with the majority of cells expressing low levels of CAE while approximately 5 - 10% had medium levels (figure 3.6.1). However, staining of the RA-treated cells revealed a gradual loss of CAE expression such that, by day 12, all cells expressed CAE levels comparable to those of RC-2a cells (figures 3.6.2 and 3.5.3). As a positive control, human peripheral blood leucocytes were stained for the presence of CAE (figure 3.5.4).

Figures 3.5.1 - 4: **Assaying for the presence of non-specific esterase.** Untreated HL-60 cells, HL-60 cells cultured with 10 nM RA, RC-2a cells and peripheral blood leukocytes were assayed for expression of NSE. The brown cytoplasmic staining is indicative of a cell expressing NSE. Magnification was x1000 for all photos.

Figure 3.5.1: HL-60 cells not treated with RA.

Figure 3.5.2: HL-60 cells cultured for 12 days with 10 nM RA.

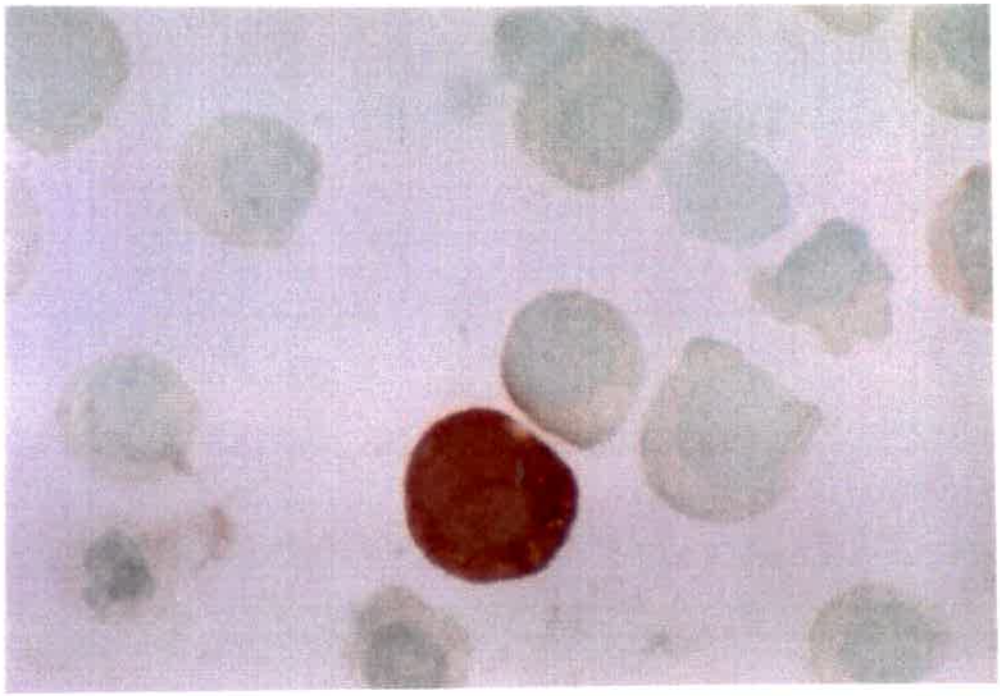
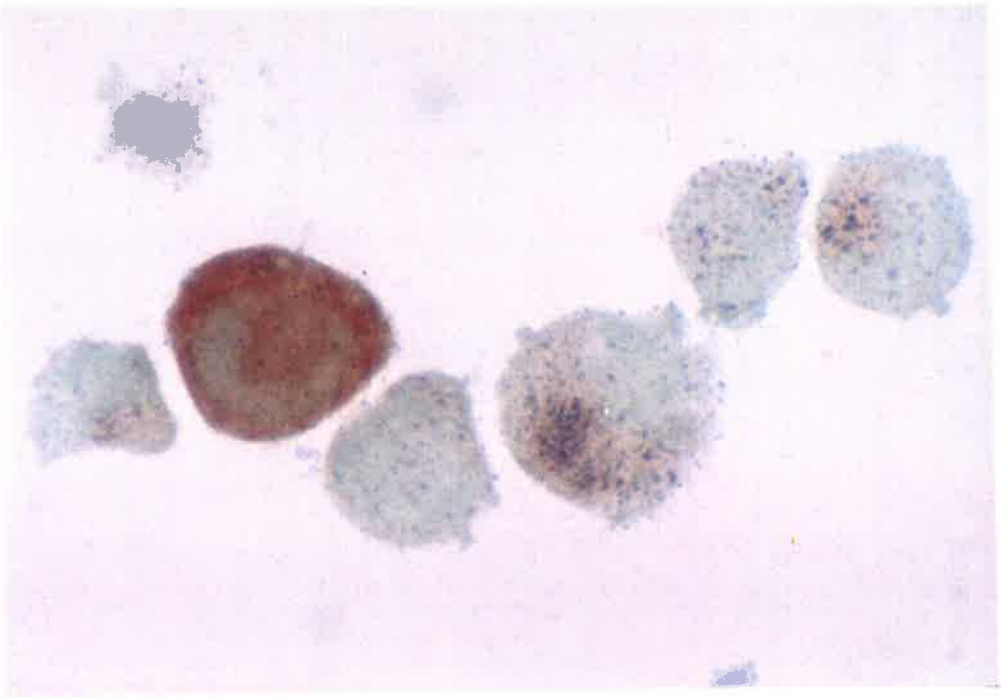
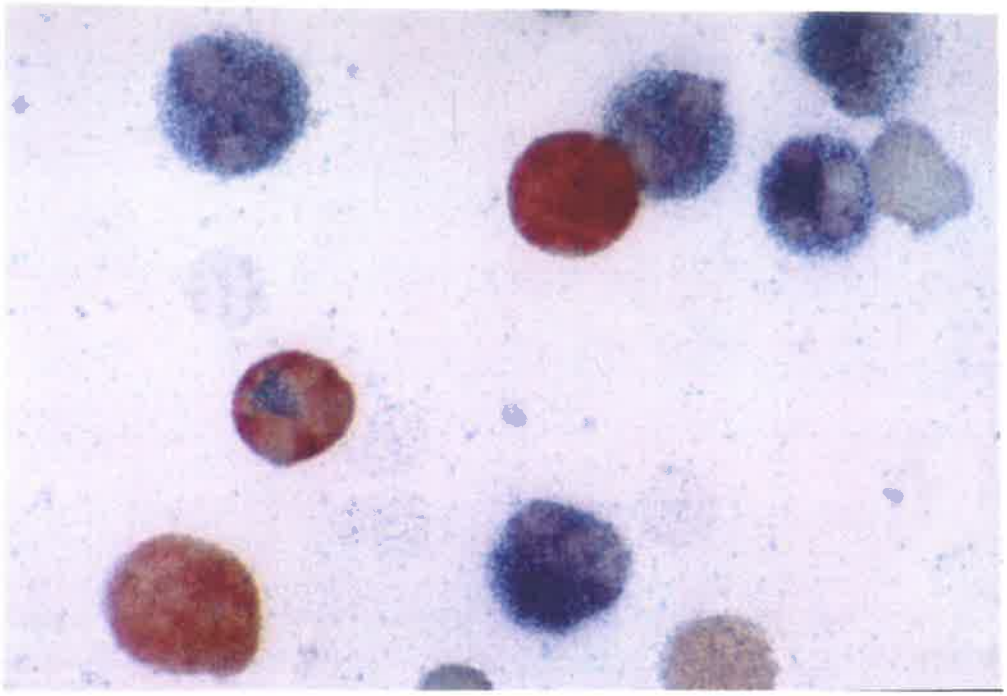


Figure 3.5.3: A typical field of RC-2a cells. The cells were simultaneously stained for the presence of NSE and CAE.

Figure 3.5.4: Peripheral blood polymorphonuclear cells as a positive control for the NSE and CAE staining techniques. Brown staining is indicative of NSE expression and blue staining is indicative of CAE expression.

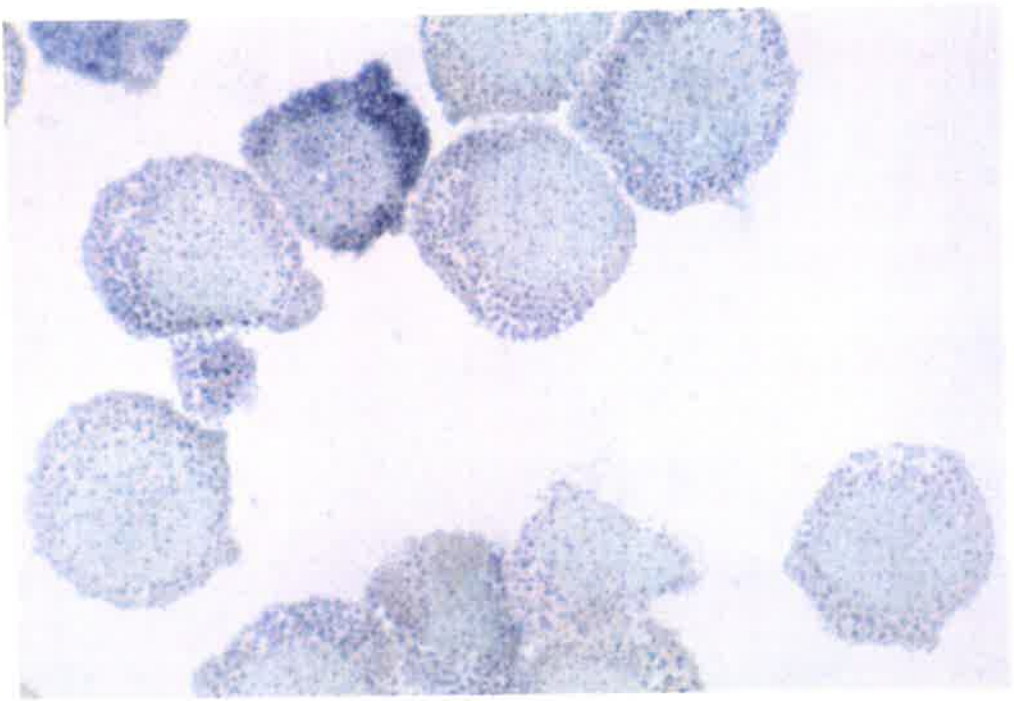


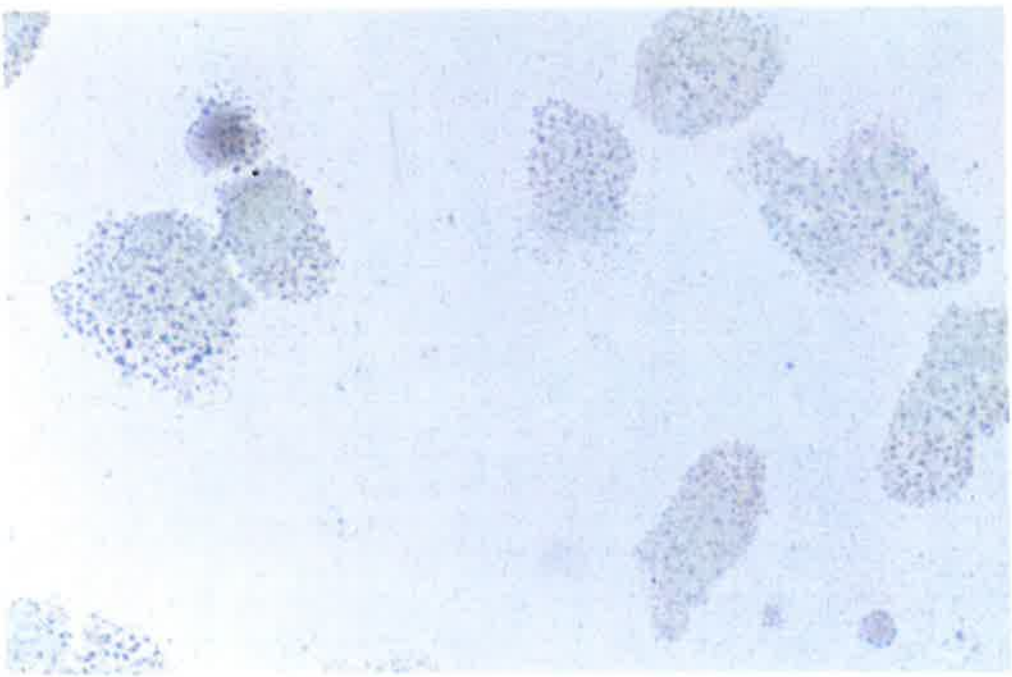


Figures 3.6.1 - 2: **Assaying for the presence of chloroacetate esterase.** RA-treated (10 nM) and untreated HL-60 cells, RC-2a cells and peripheral blood polymorphonuclear cells were assayed for CAE expression. The blue staining is indicative of cells expressing CAE. Magnification was x1000.

Figure 3.6.1: Untreated HL-60 cells

Figure 3.6.2: HL-60 cells cultured for 12 days with 10 nM RA.





3.7 Toluidine blue and Luxol fast blue staining

Toluidine blue is well recognized for it's capacity to stain basophil and mast cell granules metachromatically (Moore and James, 1965). This simple procedure (see section 2.2.3 of materials and methods) was performed upon RA-induced and control HL-60 cells revealing no metachromatic staining of granules in any cells over the 12 day culture period, apart from the basophilic cells which were variably present. The staining technique was shown to be functional through its ability to detect peripheral blood basophils (figure 3.7.1).

Luxol fast blue stains eosinophil granules metachromatically, and it has been previously shown that a small percentage of HL-60 cells possess these eosinophilic granules (Dalton *et al*, 1988). As above, it was discovered that neither RA-induced nor control HL-60 cells stained positively, apart from the basophil-like cells which were sometimes present, possessing granules that stained metachromatically (blue) (see section 2.2.4 for method). Peripheral blood eosinophils were stained as a positive control (figure 3.7.2).

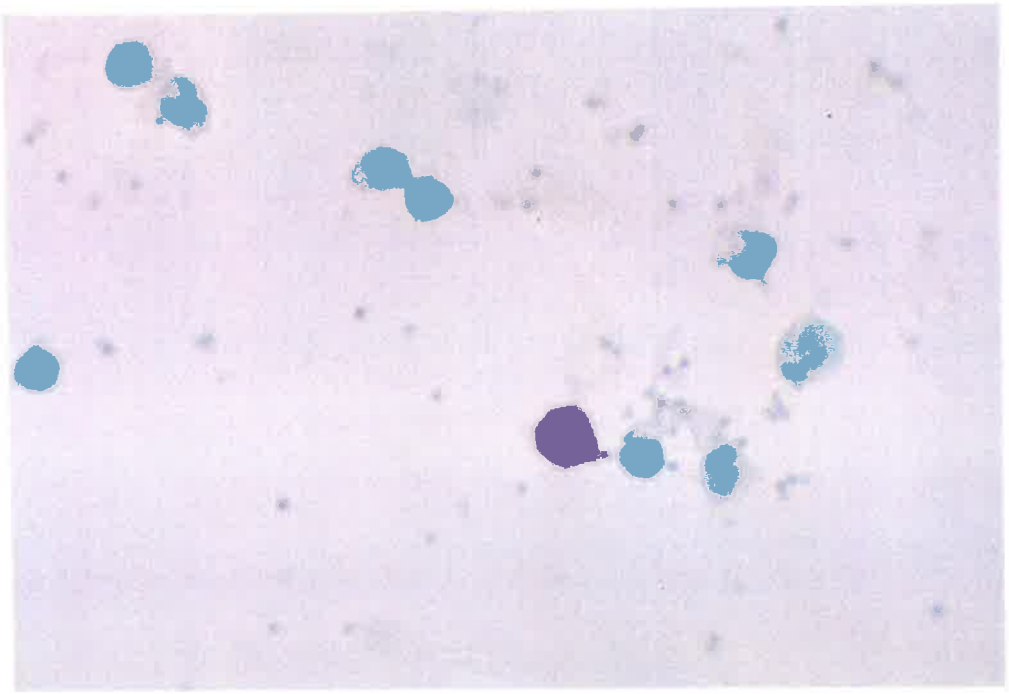
3.8 Analysis of surface antigen expression by HL-60 cells treated with 10 nM retinoic acid.

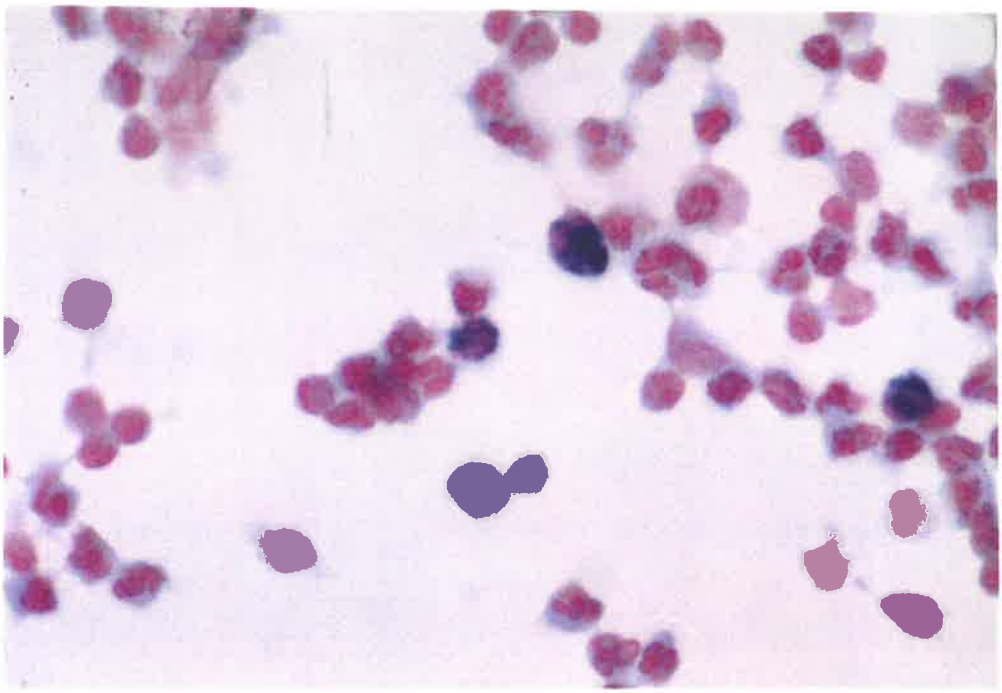
From the analyses performed on the RA-treated cells to this point, there still appeared to be a large amount of ambiguity with respect to the cell lineage, or lineages, that HL-60 cells were being induced along. Thus it was thought that a study of the expression of a number of cell surface markers could implicate the relevant lineage(s) as well as giving clues as to potential molecular participants in lymphocyte activation by RA-treated HL-60 cells.

In brief, HL-60 cells were cultured with 10 nM RA and indirect immunofluorescence (IIF) assays were performed on these cells on days 0, 1, 3, 5, 7

Figure 3.7.1: **Toluidine blue stained peripheral blood leukocytes.** Purple-blue staining of cytoplasm is indicative of basophils.

Figure 3.7.2: **Luxol Fast blue staining of peripheral blood leukocytes.** Dark blue cytoplasmic staining is indicative of eosinophils.





and 12 (see section 2.11.2 for method). For some antigens, expression was only assayed on days 0 and 7 and/or days 0 and 12. The mAb used included those regarded as being specific for antigens expressed by neutrophils, but not monocytes (CD10 and CD24), mAb specific for antigens generally found on monocytes, but not neutrophils (CD36, CD14 and CD64) as well as other antigens, generally unrelated to the lineage dilemma, such as the transferrin receptor, adhesion molecules and MHC antigens. The results of these IIF assays are summarized in table 3.8.1.

MHC class I

MHC class I molecules are expressed on the surface of virtually all cell-types and often at high levels, as is the case with HL-60 cells. However, after 7 days of culture with 10 nM RA a 10-fold decrease in MHC class I antigen expression was observed with the mAb 6c6b, which can recognize a non-polymorphic epitope on MHC class I molecules (Cole and Ashman, unpublished). This decrease in expression was noticeable by day 3, as the histogram overlay clearly displays in figure 3.8.1. It should be noted that the results for day 1 were not shown for this, or any of the subsequent antigens analysed, since the levels of expression were identical to those on day 0. Figure 3.8.2 shows the level of expression of MHC class I molecules by RC-2a cells.

The forward scatter (FSC) vs side scatter (SSC) dot-plots of HL-60 and RA-treated HL-60 cells revealed little change in cell size over the 7 days of culture with a marginal decrease by day 7, which alone could not have accounted for the reduction in fluorescence intensity seen here with anti-MHC class I mAb-treated cells (data not shown).

Further analysis of MHC class I antigen was performed using RM 3.170, a mAb specific for a polymorphic epitope present on HLA-A2 and HLA-B17 molecules only (Dr. G. Russ, Queen Elizabeth Hospital, Adelaide; personal communication). HL-60 cells have previously been shown to express HLA-B17 (Koeffler *et al*, 1980). It can be seen from figure 3.8.3 that normal HL-60 cells expressed at least one of the

VIL-A1	CD10	0 (0)	N.T.	0 (0)	0 (0)
VIB-E3	CD24	0 (0)	N.T.	0 (0)	0 (0)
VIMD5	CD15	99 (1068)	100*(291)	100*(417)	0 (0)
VIP-1	CD71	99 (516)	13#(318)	9#(288)	33*(15)
VIFcRIII	CD16	0 (0)	0 (0)	0 (0)	0 (0)
TS1/22	LFA-1	100 (30)	100 (81)	100 (6)	100 (78)
B2B1.F9	CD31	37*(42)	35*(39)	N.T.	100 (117)

Table 3.8.1: Surface antigen expression by HL-60, RA-treated HL-60 and RC-2a cells. An overview of the IIF assays performed on HL-60, HL-60-R7, HL-60-R12 and RC-2a cells are given in terms of percentage of cells positive for the relevant antigen. In brackets is given the relative mean fluorescence intensity (RMF) of the positive cells. The values obtained for the isotype control mAb ranged from 2 - 3 and were subtracted from the values obtained for the respective test antibodies. Where there was a homogeneous peak shift of fluorescence compared with cells treated with the isotype control mAb, the cells were considered to be 100% positive even though the histograms may have overlapped. Where the population was heterogeneous with respect to antibody binding, cells with fluorescence intensities > the 99^{th} percentile of the fluorescence intensity of the negative control were considered positive.

* = Asymmetric population

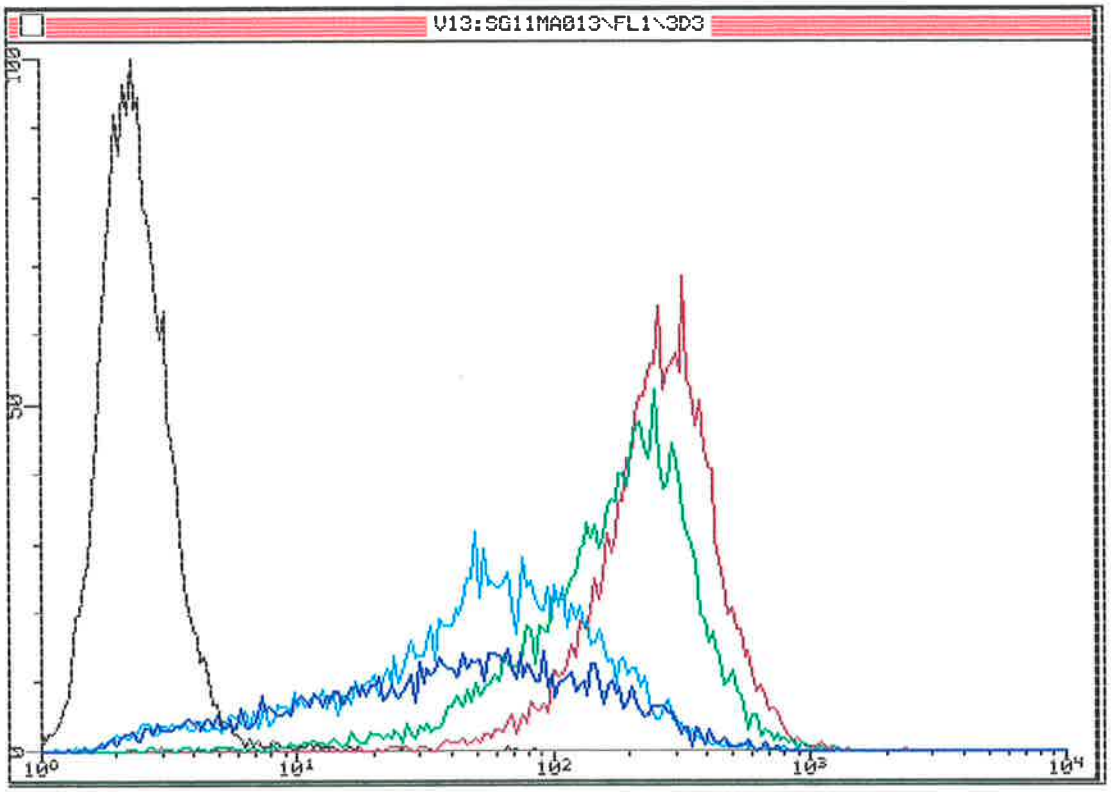
= Distinct populations

		HL60	HL60-R7	HL60-R12	RC2a
mAb	Ag	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)
6c6b	MHC Class I	100 (360)	92*(81)	90*(42)	100 (33)
RM 3.170	HLA-A2/ B17	98 (72)	25 (12)	N.T.	100 (12)
FMC 4	MHC Class II	0 (0)	0 (0)	17*(27)	100 (273)
OKT4	CD4	100 (33)	100 (30)	100 (30)	100 (12)
W-CAM-1	ICAM-1	0 (0)	100 (21)	100 (61)	100 (9)
CBR-IC2/2	ICAM-2	82*(21)	11*(15)	N.T.	93*(33)
CBR-IC3/1	ICAM-3	100 (93)	100 (214)	N.T.	100 (420)
VIM13	CD14	0 (0)	9*(9)	22*(54)	0 (0)
TS2/9	LFA-3	100 (27)	100 (87)	100 (3)	100 (66)
5A4.C5	CD11b	0 (0)	89*(75)	100*(87)	100 (15)
anti-CD36 cocktail	CD36	0 (0)	26#(261)	87#(276)	35*(129)

Figures 3.8.1 - 46: Analysis of cell surface antigen expression by HL-60, RA-treated HL-60 and RC-2a cells. IIF assays were performed on HL-60 cells treated with 10 nM RA for 0 - 12 days and RC-2a cells. Histograms are expressed as log fluoresence intensity (FL1) vs cell number.

Figure 3.8.1: **Cell surface MHC class I expression**. Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA where 6c6b was used in an indirect immunofluorescence (IIF) assay to detect MHC class I cell surface expression. Red/brown = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb, 3D3.3.

Figure 3.8.2: **Cell surface MHC class I expression by RC-2a cells.** 6c6b was used in an IIF assay. Pink = 6c6b-treated cells, black = cells treated with isotype control mAb, 3D3.3.


V13:SG11MA013\FL1\3D3
100
50
0
10⁰
10¹
10²
10³
10⁴

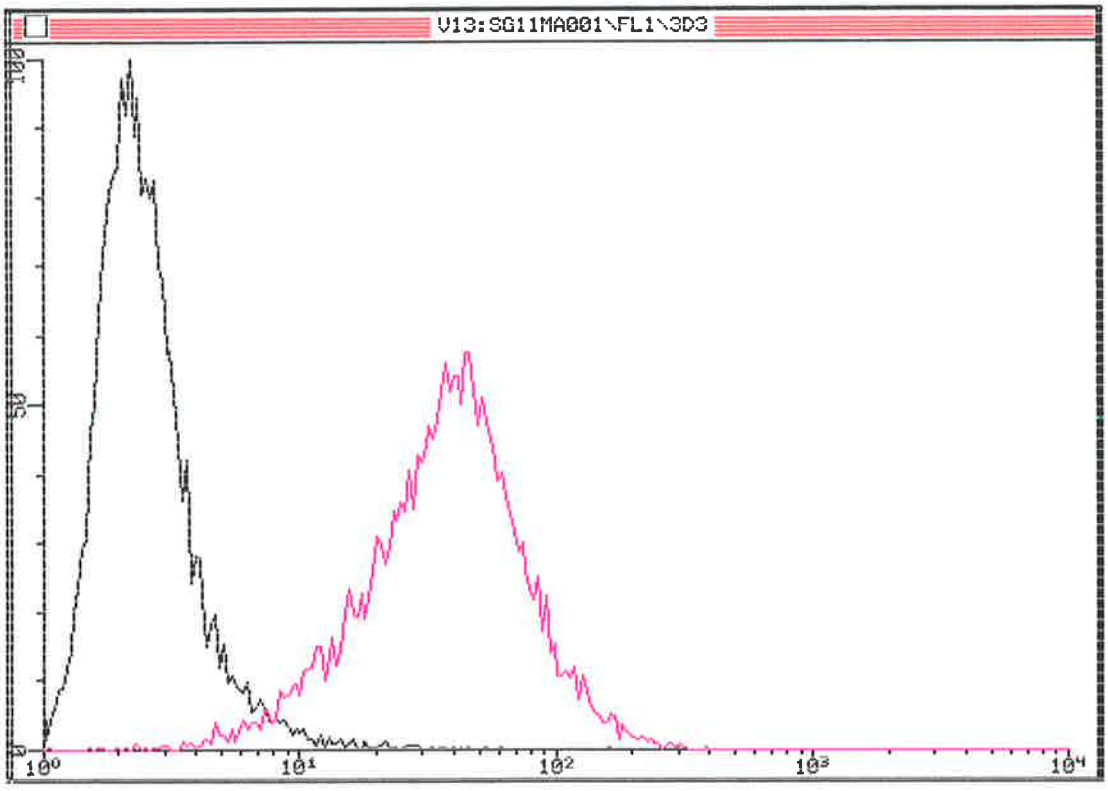
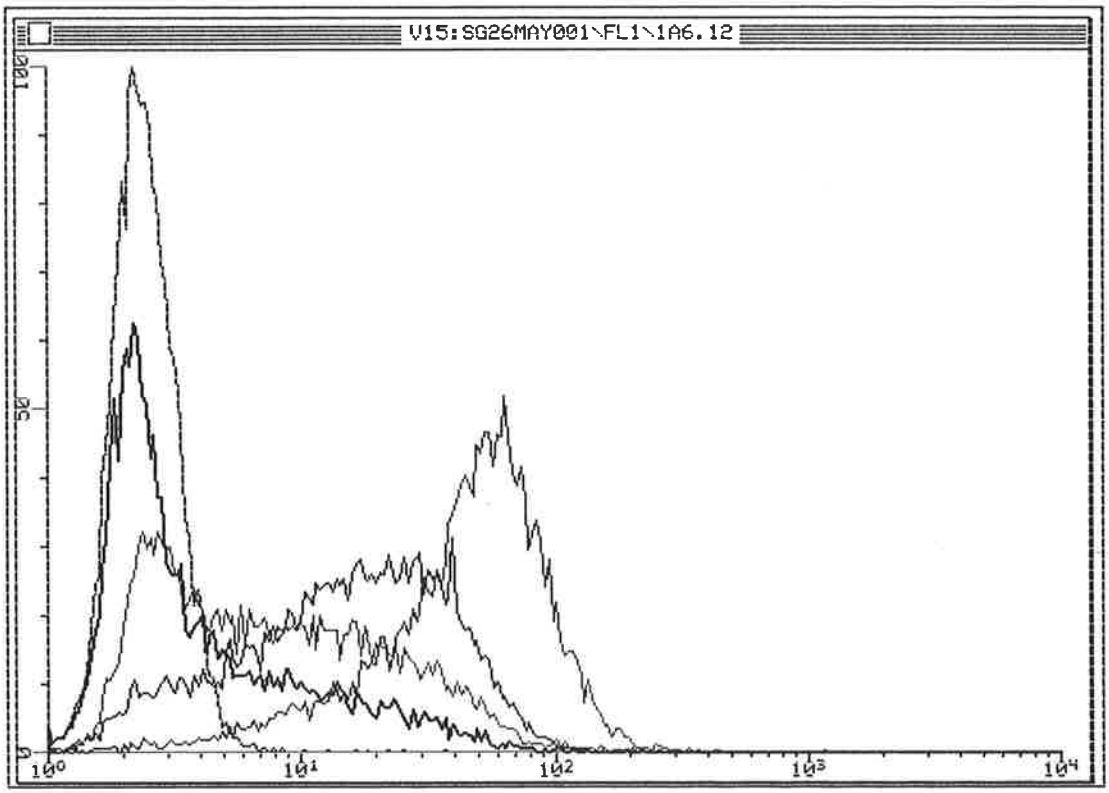
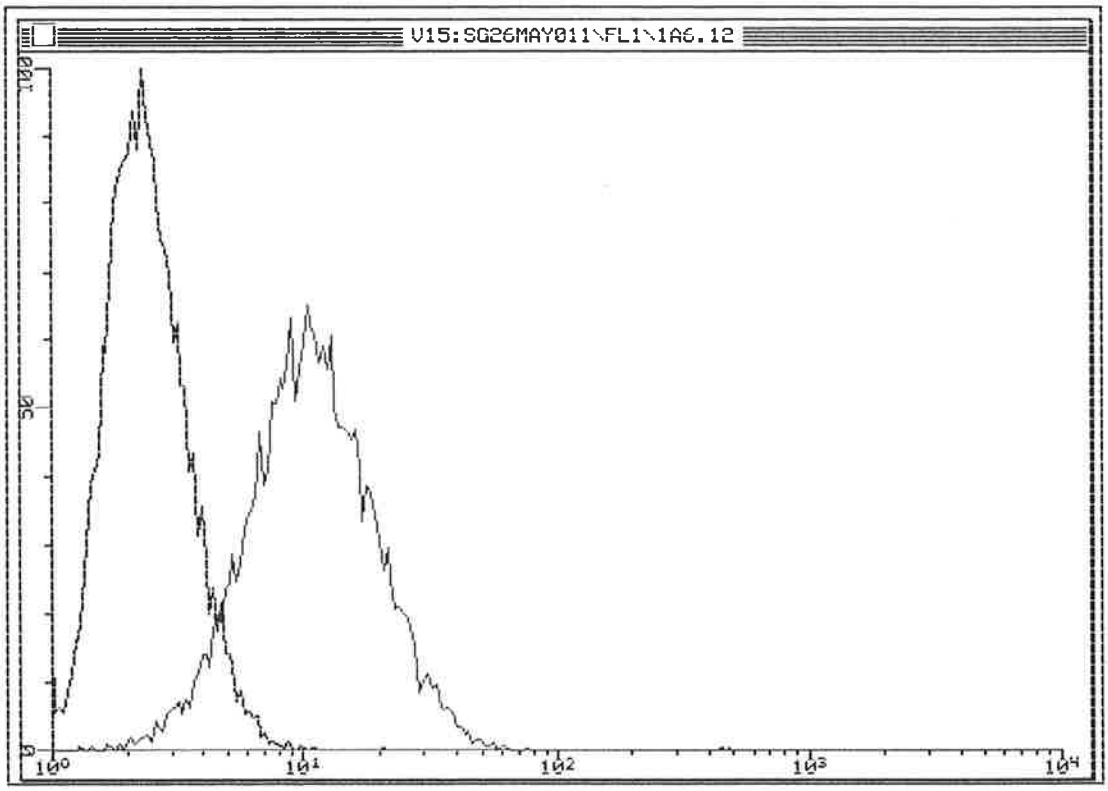

V13:SG11MA001\FL1\3D3
100
50
0
10⁰
10¹
10²
10³
10⁴

Figure 3.8.3: **Cell surface HLA-A2/HLA-B17 expression**. Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA. RM 3.170 was used in an IIF assay to detect HLA-A2 or HLA-B17 cell surface expression. Red = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb.

Figure 3.8.4: **HLA-A2 and/or HLA-B17 expression by RC-2a cells.** RM 3.170 was used in the IIF assay. Pink = 6c6b-treated cells, black = cells treated with isotype control mAb.


V15:SG26MAY001\FL1\1A6.12
100
101
102
103
104


V15:SG26MAY011\FL1\1A6.12
100
101
102
103
104

haplotypes detected by RM 3.170, and, as might be expected, this level of expression was a lot lower (1/5) than total MHC class I antigen expression. By day 7 the majority of the induced cells lacked detectable levels of HLA-A2 and/or HLA-B17. Figure 3.8.4 shows the level of expression by RC-2a cells of HLA-A2 and/or HLA-B17.

MHC class II

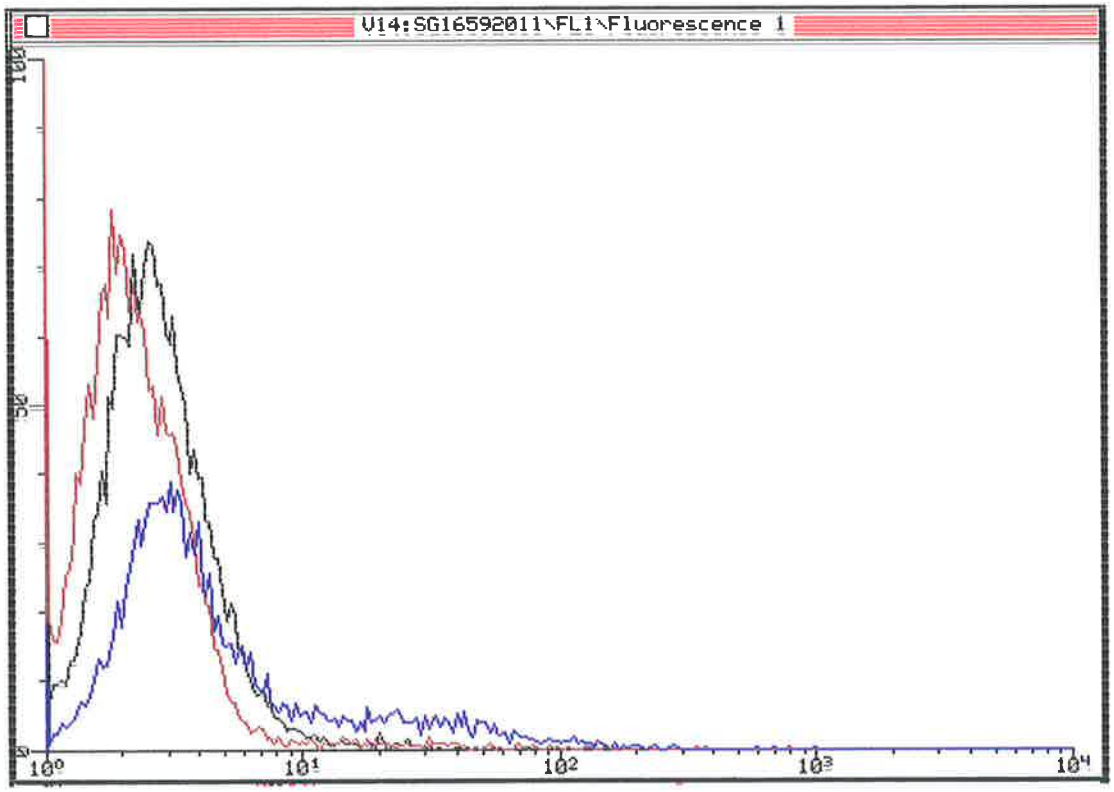
MHC class II antigen expression by RA-treated HL-60 cells was not detectable within the first 7 days of culture, however by day 12 a small percentage (approximately 17%) of the population possessed low to medium levels of this molecule on their cell surface. FMC 4, FMC 14 and FMC 15 (Brooks *et al*, 1980) were used in these assays, with FMC 4 and FMC 14 being specific for distinct non-polymorphic epitopes of MHC class II antigen whilst FMC 15 was specific for HLA-DR antigen (Beckman *et al*, 1984). Figure 3.8.5 displays the results obtained using FMC 14 on days 7 and 12, whilst the results for days 0, 1, 3 and 5 were not displayed as they were similar to day 7. Figure 3.8.6 displays the level of expression of MHC class II antigen by RC-2a cells. FMC 15 could not detect MHC class II expression by cells in the day 12 cultures (data not shown) suggesting that the MHC class II molecules that were detected were possibly coded for by either the HLA-DQ or HLA-DP loci.

CD4

Amongst the leukocytes, CD4 is found on most monocytes and macrophages as well as a subset of T lymphocytes and this molecule is responsible for recognizing a non-variable portion of MHC class II molecules. Thus it was somewhat surprising to find that HL-60 cells, which are considered to be myeloblastic/promyelocytic-like, expressed CD4 prior to RA treatment (figure 3.8.7), and at levels similar to that of the more mature RC-2a cells (figure 3.8.8), as detected by the mAb, OKT4. The treatment

Figure 3.8.5: **Cell surface MHC class II antigen expression.** Overlay of fluorescence histograms of HL-60 cells cultured for 0, 7 and 12 days with 10 nM RA. FMC 14 was used in an IIF assay to detect MHC class II cell surface expression. Brown = day 7, purple = day 12, black = cells treated with isotype control mAb, 3D3.3.

Figure 3.8.6: **MHC class II antigen expression by RC-2a cells.** FMC 14 was used in the IIF assay. Pink = FMC 14-treated cells, black = cells treated with isotype control mAb, 3D3.3.


V14:SG16592011\FL1\Fluorescence 1
100
50
0
10⁰
10¹
10²
10³
10⁴

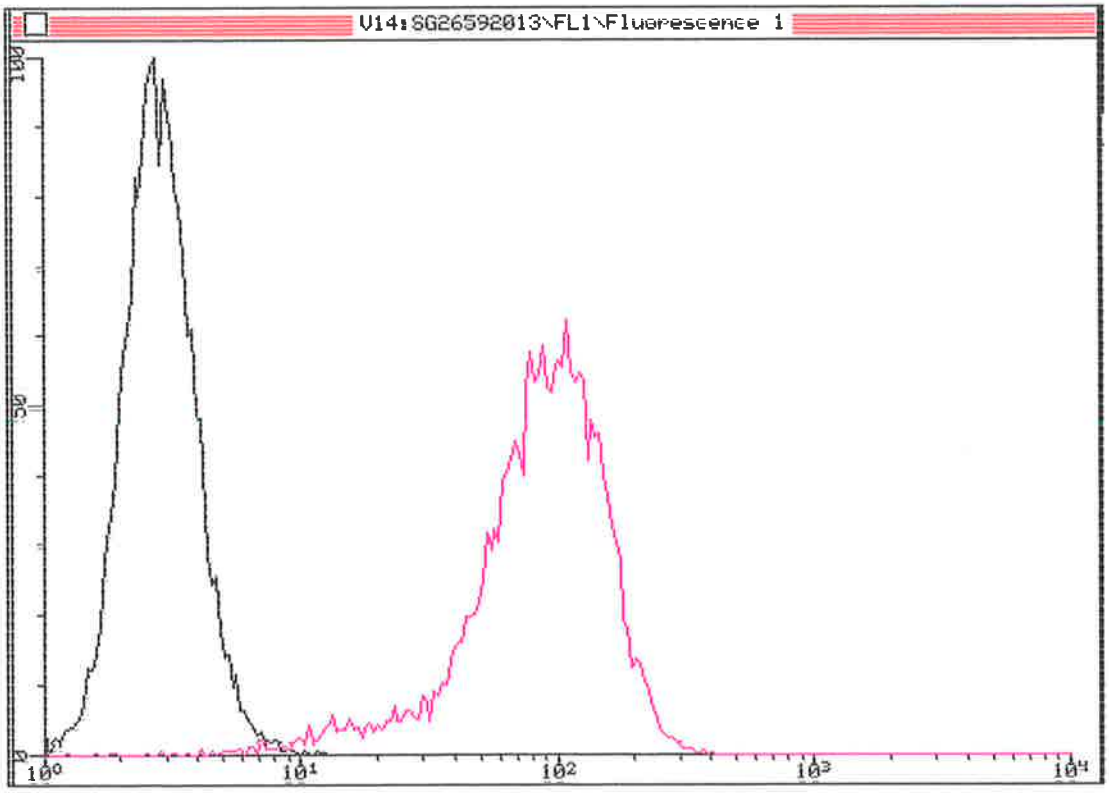
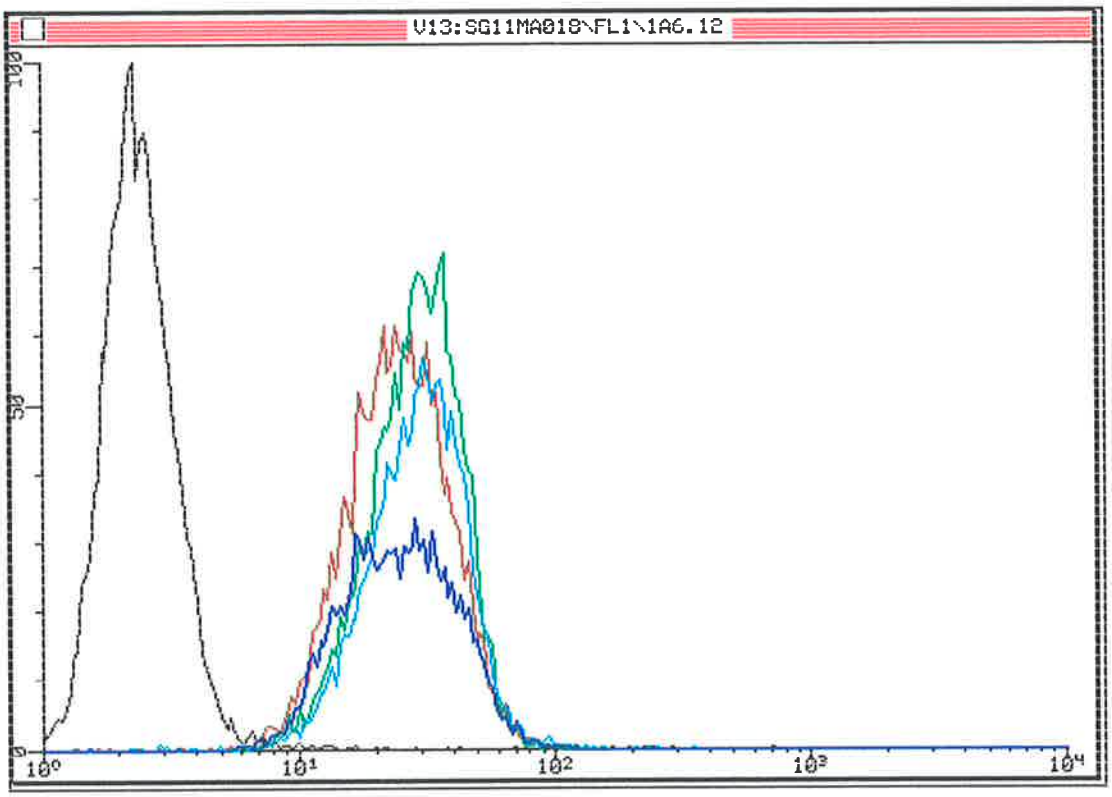

V14:SG26592013\FL1\Fluorescence 1
100
50
0
10⁰
10¹
10²
10³
10⁴

Figure 3.8.7: **Cell surface CD4 expression**. Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA using OKT4 in an IIF assay to detect CD4 cell surface expression. Red = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb, 1A6.11.

Figure 3.8.8: **CD4 expression by RC-2a cells.** OKT4 was used in the IIF assay. Pink = OKT4-treated cells, black = cells treated with isotype control mAb, 1A6.11.


V13:SG11MA010\FL1\1A6.12
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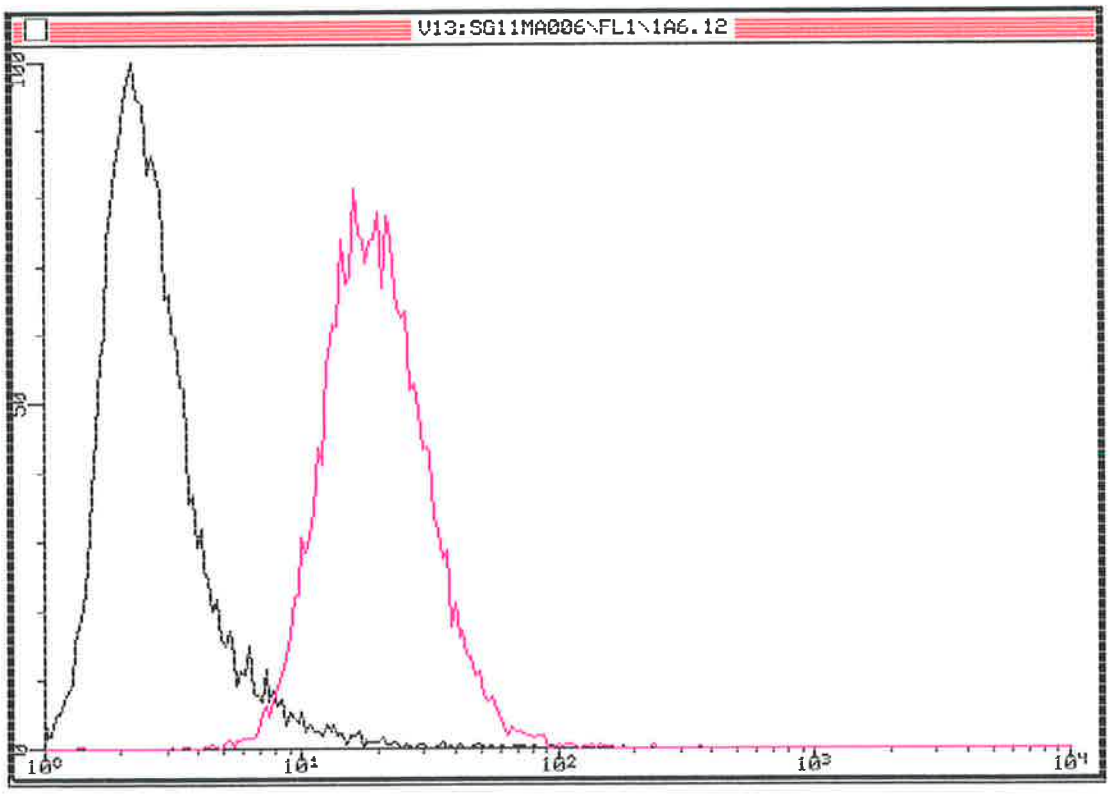
50
0
10⁰
10¹
10²
10³
10⁴


V13:SG11MA006\FL1\1A6.12
100
50
0
10⁰
10¹
10²
10³
10⁴

of HL-60 cells with RA had little effect on CD4 cell surface expression over the 7 days (figure 3.8.7). Even after twelve days of culture there was minimal change (figure not shown).

Adhesion molecules: ICAM-1, ICAM-2, ICAM-3, LFA-1, LFA-3, CD11b and CD31

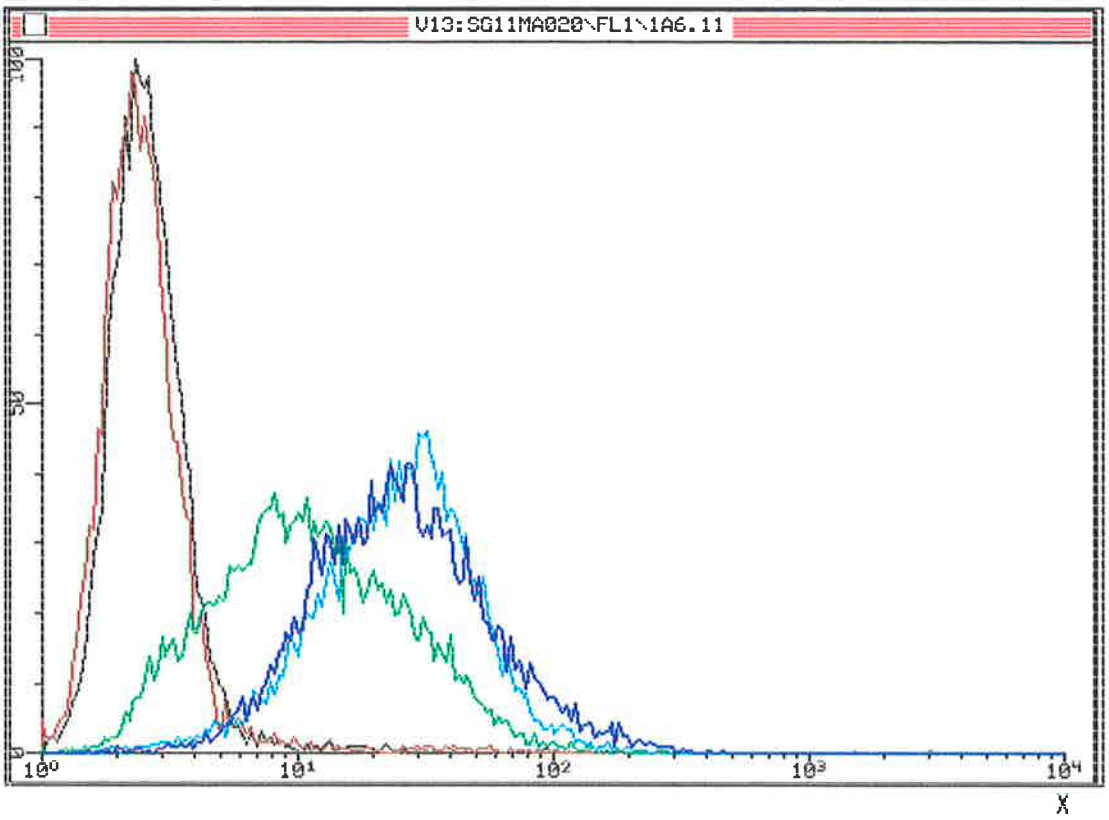
CD54, or intercellular adhesion molecule-1 (ICAM-1), has been shown to play a role in lymphocyte activation via its interaction with LFA-1 (Van Seventer *et al*, 1990). Control HL-60 cells possessed no detectable levels of ICAM-1 on their cell surface as detected by the mAb W-CAM-1 (Boyd *et al*, 1988). However, after seven days of RA treatment, ICAM-1 expression had increased dramatically, such that virtually all cells were positive for this antigen and the level of expression was higher than that of RC-2a cells (figures 3.8.9 and 3.8.10). Analysis of day 12 cultures revealed a further doubling in ICAM-1 levels (figure not shown).

Two other ligands for LFA-1 have been recently discovered (ICAM-2 and ICAM-3) and have been shown to be important in mediating lymphocyte adhesion as well as activation (de Fougerolles et al, 1991; de Fougerolles and Springer, 1992). The expression of both these molecules by HL-60, HL-60-R7 and RC-2a cells was examined using the mAb CBR-IC2/2 and CBR-IC3/1 which recognize epitopes on ICAM-2 and ICAM-3 respectively. Both molecules were shown to be expressed by control HL-60 cells, however ICAM-2 expression was down-regulated by RA to almost basal levels (figure 3.8.11), whereas ICAM-3 expression was up-regulated by approximately 2-fold (figure 3.8.13). RC-2a cells were found to express both ICAM-2 and ICAM-3 (figures 3.8.12 and 3.8.14).

LFA-1 has been shown to be a ligand for ICAM-1, 2 and 3 (Makgoba *et al*, 1988; de Fougerolles et al, 1991; de Fougerolles and Springer, 1992) and has been reported to be involved in signal transduction in lymphocytes (Van Noesel *et al*, 1988). TS1/22 was used to detect LFA-1 (Sanchez-Madrid, 1982) and control HL-60 cells

Figure 3.8.9: **Cell surface ICAM-1 (CD54) expression**. Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA using W-CAM-1 in an IIF assay to detect CD54 cell surface expression. Red/brown = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb, 1A6.11.

Figure 3.8.10: **Cell surface ICAM-1 expression by RC-2a cells.** W-CAM-1 was used in the IIF assay. Pink = W-CAM-1-treated cells, black = cells treated with isotype control mAb, 1A6.11.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100
SG
0
10⁰
10¹
10²
10³
10⁴
X

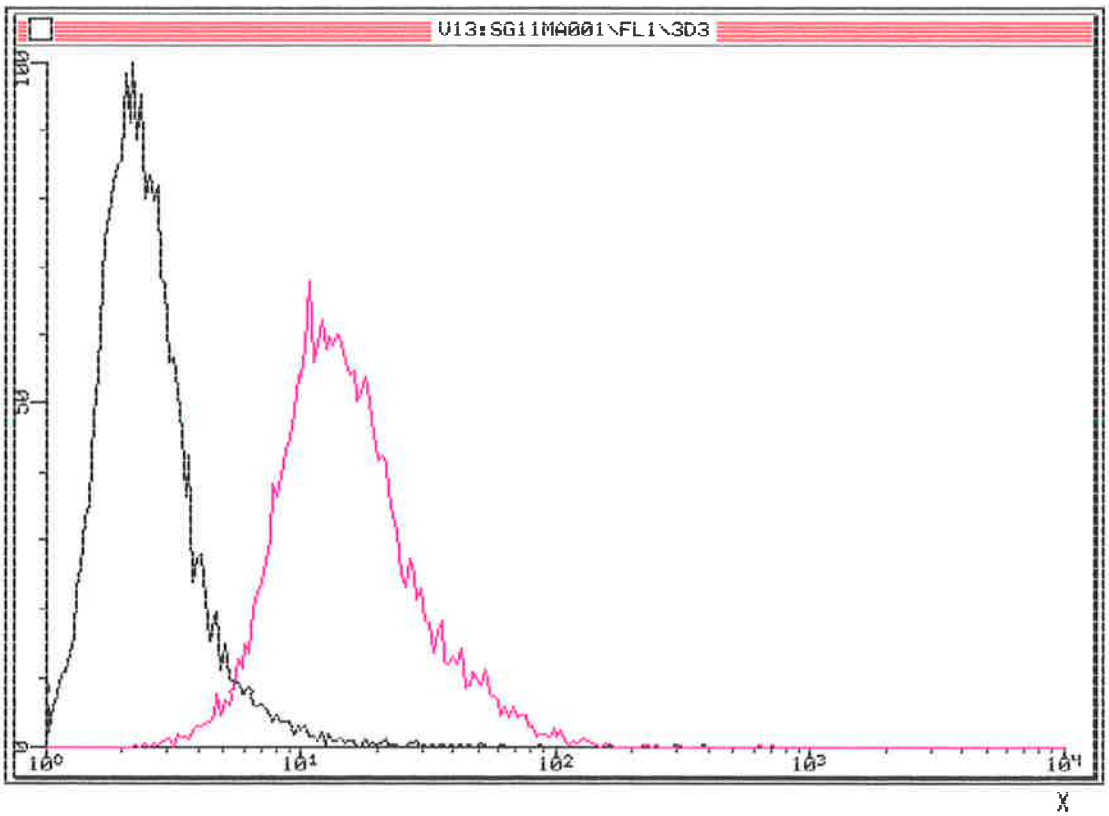
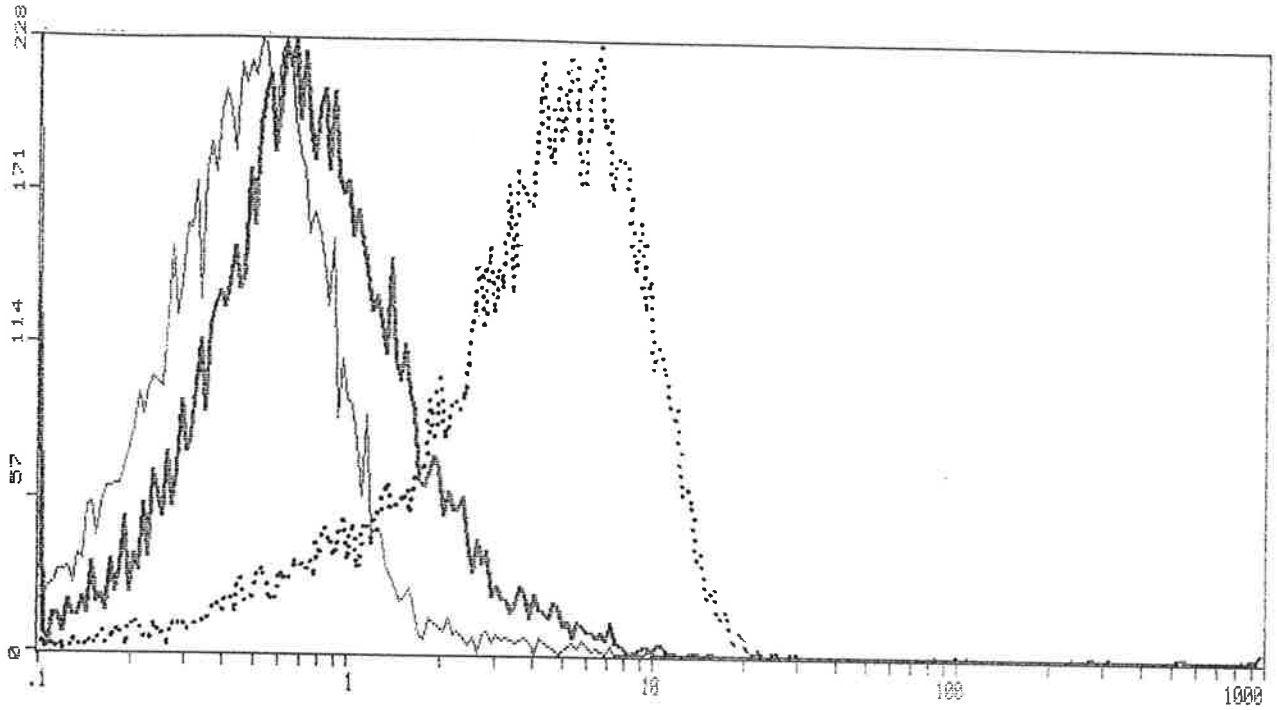

V13:SG11MA001\FL1\3D3
100
SG
0
10⁰
10¹
10²
10³
10⁴
X

Figure 3.8.11: **Cell surface ICAM-2 expression by HL-60 cells cultured with 10 nM RA for 0 and 7 days.** Dotted line = CBR-IC2-treated HL-60 cells, Thick line = CBR-IC2/2-treated HL-60-R7 cells, Thin line = isotype control-treated cells.

Figure 3.8.12: **Cell surface ICAM-2 expression by RC-2a cells.** Dotted line = CBRIC-2/2-treated cells, Thin line = isotype control-treated cells.


228
171
114
57
0
.1
1
10
100
1000

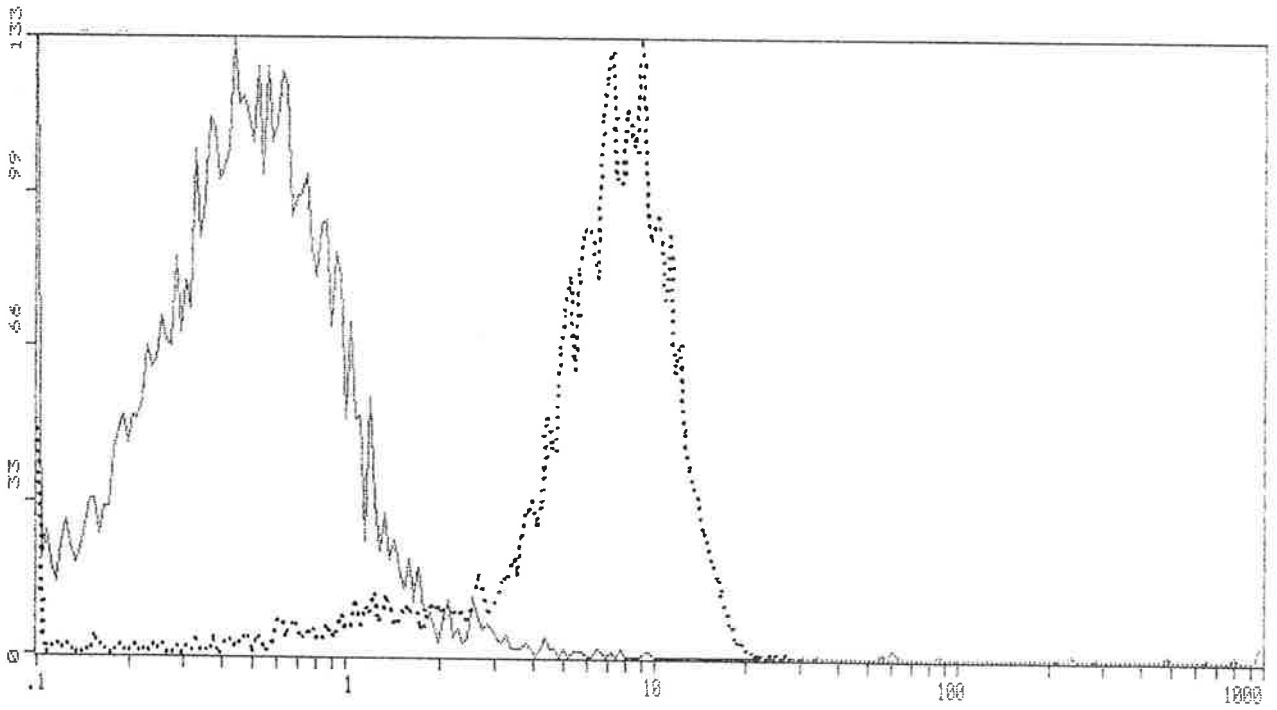
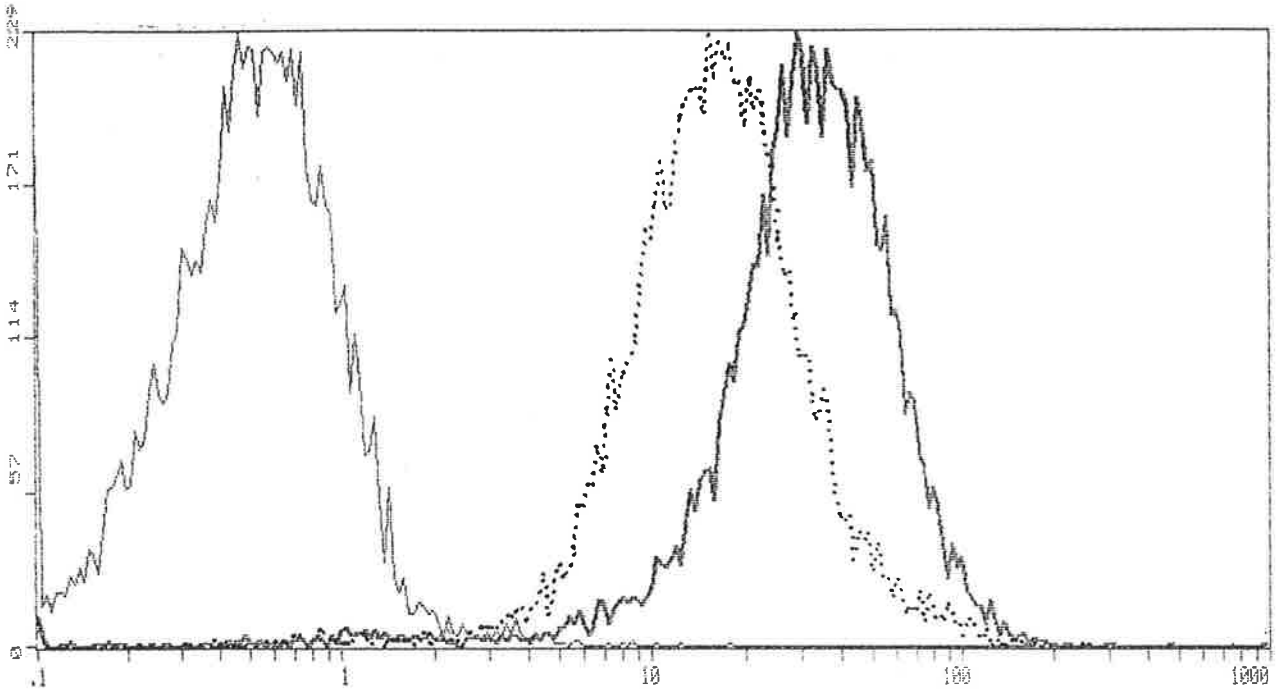
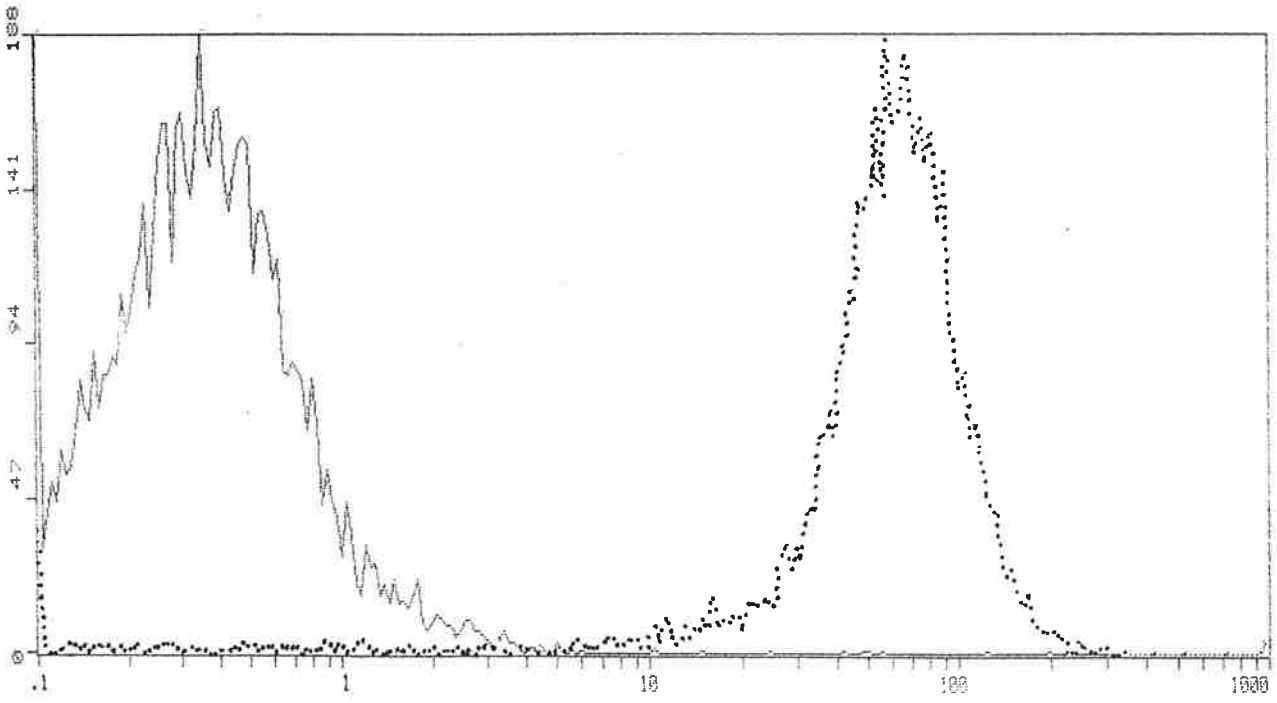

133
99
66
33
0
.1
1
10
100
1000

Figure 3.8.13: **Cell surface ICAM-3 expression by HL-60 cells cultured with 10 nM RA for 0 and 7 days.** Dotted line = CBR-IC3/1-treated HL-60 cells, Thick line = CBR-IC3/1-treated HL-60-R7 cells, Thin line = isotype control-treated cells.

Figure 3.8.14: **Cell surface ICAM-3 expression by RC-2a cells.** Thin line = CBR-IC-3/1-treated cells, Dotted line = isotype control-treated cells.


229
171
114
57
0
.1
1
10
100
1000


188
141
94
47
0
.1
1
10
100
1000

were found to express this antigen. After 7 days of culture with 10 nM RA a slight increase in LFA-1 expression was noted (figure 3.8.15). RC-2a cells were also shown to express LFA-1 (figure 3.8.16).

CD58, or LFA-3, is a ubiquitous adhesion molecule which was found to be the ligand for CD2 (Dustin *et al*, 1987). Perturbation of the CD2 molecule has been shown to induce signal transduction in T lymphocytes (Sanders *et al*, 1988). All control HL-60 cells were found to express LFA-3 (figure 3.8.17) but not CD2 (figure not shown). After 7 days of RA treatment, the levels of LFA-3 appeared to have increased slightly as detected by the mAb TS2/9 (Sanchez-Madrid, 1982). RC-2a cells were also found to express LFA-3 (figure 3.8.18).

CD11b is a receptor for C3bi, and is a good indicator of differentiation along both the neutrophil and monocyte pathways, since both these cell types are known to express CD11b (Anderson and Springer, 1987). Additionally CD11b has been shown to promote NK-target cell conjugation (Axberg *et al*, 1987) and has been implicated in cell signalling (Couturier *et al*, 1990). Figure 3.8.19 shows that HL-60 cells did not appear to express CD11b, but by day 7 the majority of them had become positive. After 12 days, virtually all cells were positive (figure not shown). RC-2a cells were shown to express CD11b (figure 3.8.20). 5A4.C5 was the mAb used to detect CD11b expression (Lyons *et al*, 1988).

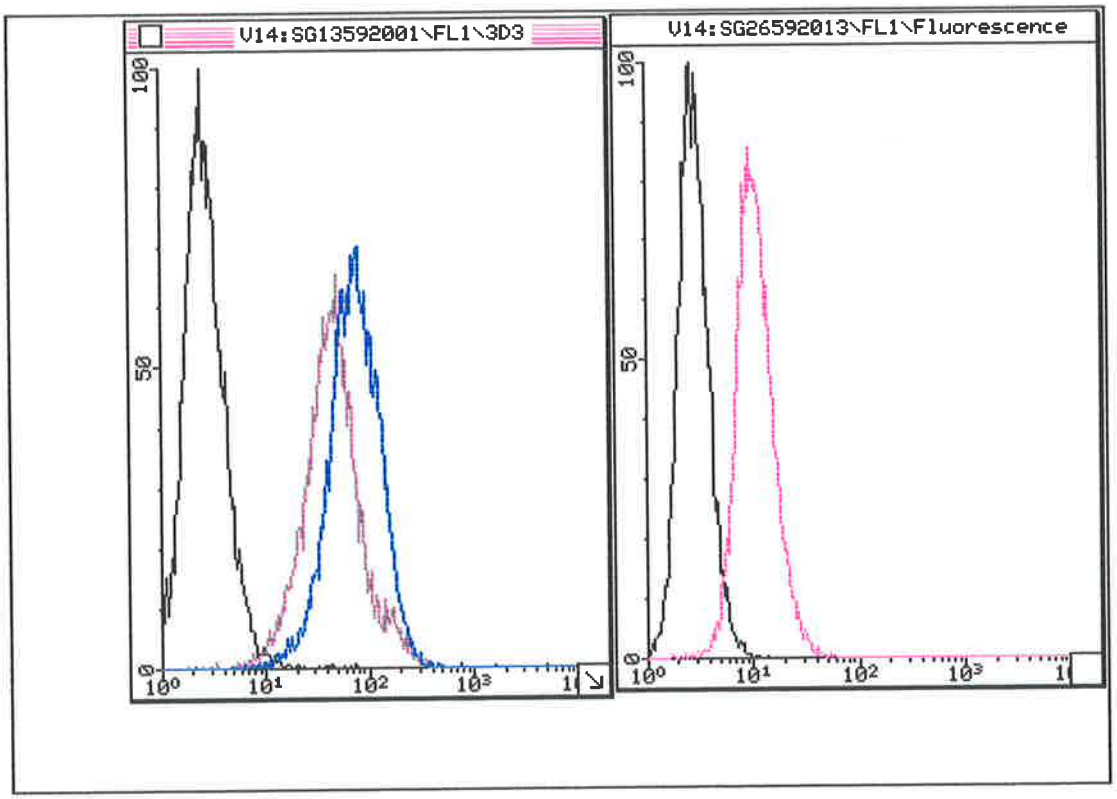
CD31 is a cell surface molecule which has recently been shown to have a role in adhesion and is expressed on macrophages, B and T lymphocytes, granulocytes and platelets (Muller *et al*, 1992). Expression of CD31, as detected by B2B1.F9 (Ashman *et al*, 1991), on control HL-60 cells (figure 3.8.21) occurred at relatively low levels, whilst both HL-60-R7 and HL-60-R12 cells (figure 3.8.22) displayed little or no increase in expression. Figure 3.8.23 displays the histogram of RC-2a cells treated with B2B1.F9, where it was shown that all cells expressed relatively high levels of CD31.

Figure 3.8.15: (Top, left) **Cell surface LFA-1 expression by HL-60 cells cultured with 10 nM RA for 0 and 7 days.** Brown = TS1/22-treated HL-60 cells, blue = TS1/22-treated HL-60-R7 cells, black = isotype control-treated cells.

Figure 3.8.16: (Top, right) **Cell surface LFA-1 expression by RC-2a cells.** Pink = TS1/22-treated cells, black = isotype control-treated cells.

Figure 3.8.17: (Bottom, left) **Cell surface LFA-3 (CD58) expression by HL-60 cells cultured with 10 nM RA for 0 and 7 days.** Brown = TS2/9-treated HL-60 cells, blue = TS2/9-treated HL-60-R7 cells, black = isotype control-treated cells.

Figure 3.8.18: (Bottom, right) **Cell surface LFA-3 expression by RC-2a cells.** Pink = TS2/9-treated cells, black = isotype control-treated cells.


V14:SG13592001\FL1\3D3
V14:SG26592013\FL1\Fluorescence
100
50
0
10⁰
10¹
10²
10³

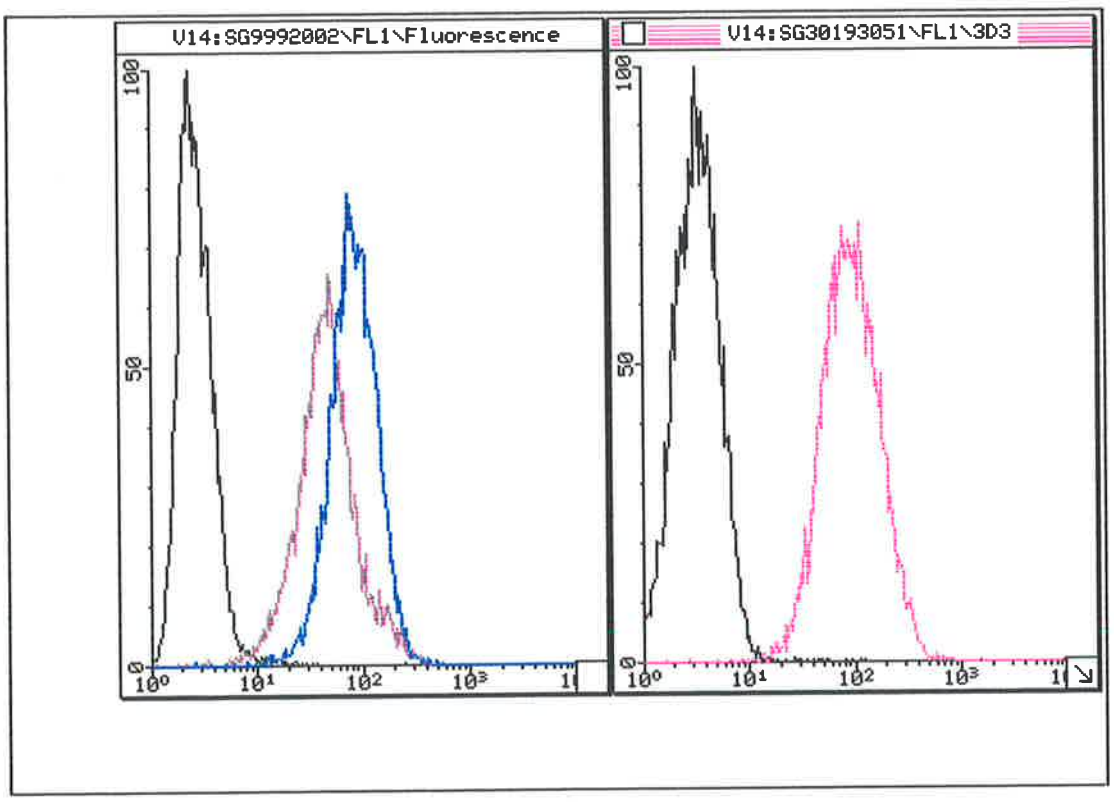
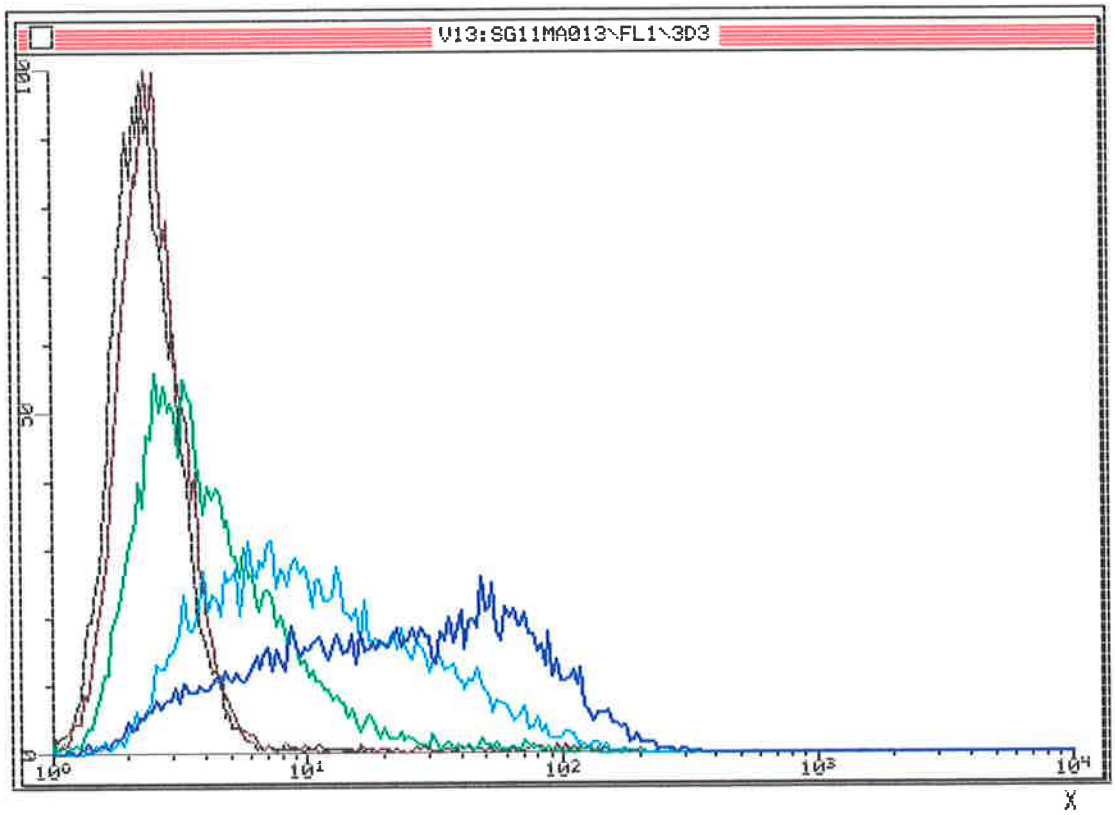

V14:SG9992002\FL1\Fluorescence
V14:SG30193051\FL1\3D3
100
50
0
10⁰
10¹
10²
10³

Figure 3.8.19: **Cell surface CD11b expression.** Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA using 5A4.C5 in an IIF assay to detect CD11b cell surface expression. Brown = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb.

Figure 3.8.20: **CD11b expression by RC-2a cells.** 5A4.C5 was used in the IIF assay. Pink = 5A4.C5-treated cells, black = cells treated with isotype control mAb.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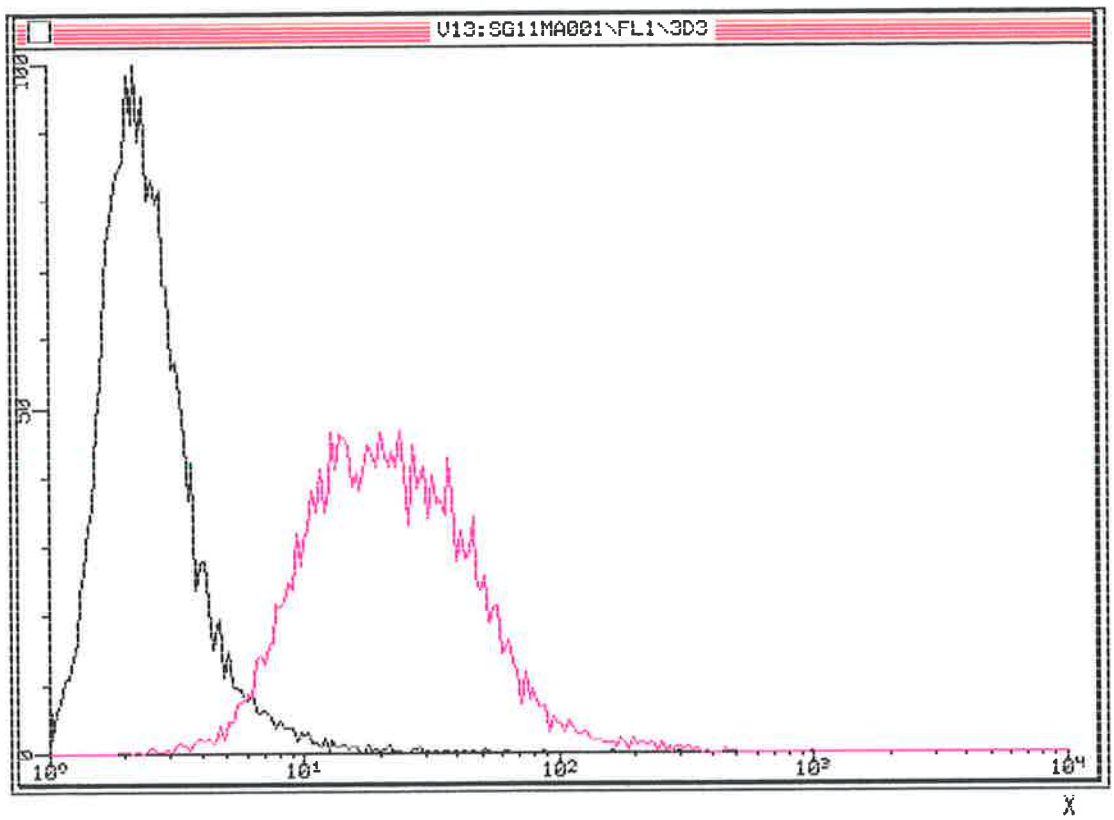
100
50
0
10⁰
10¹
10²
10³
10⁴
X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100
50
0
10⁰
10¹
10²
10³
10⁴
X

Figure 3.8.21: (Top, left) **Cell surface CD31 expression by HL-60 cells.** B2B1.F9 was the test mAb used in a IIF. Brown = B2B1.F9-treated cells, black = isotype control-treated cells.

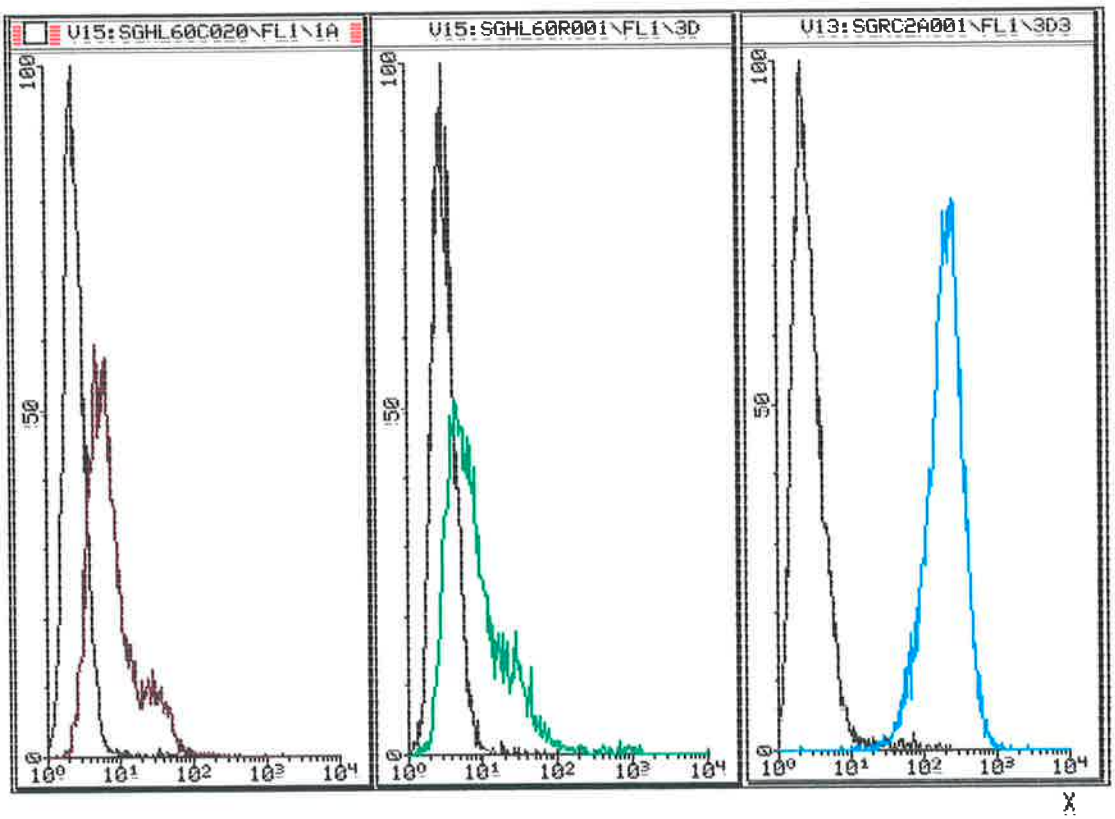
Figure 3.8.22: (Top, middle) **Cell surface CD31 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = B2B1.F9-treated cells, black = isotype control-treated cells.

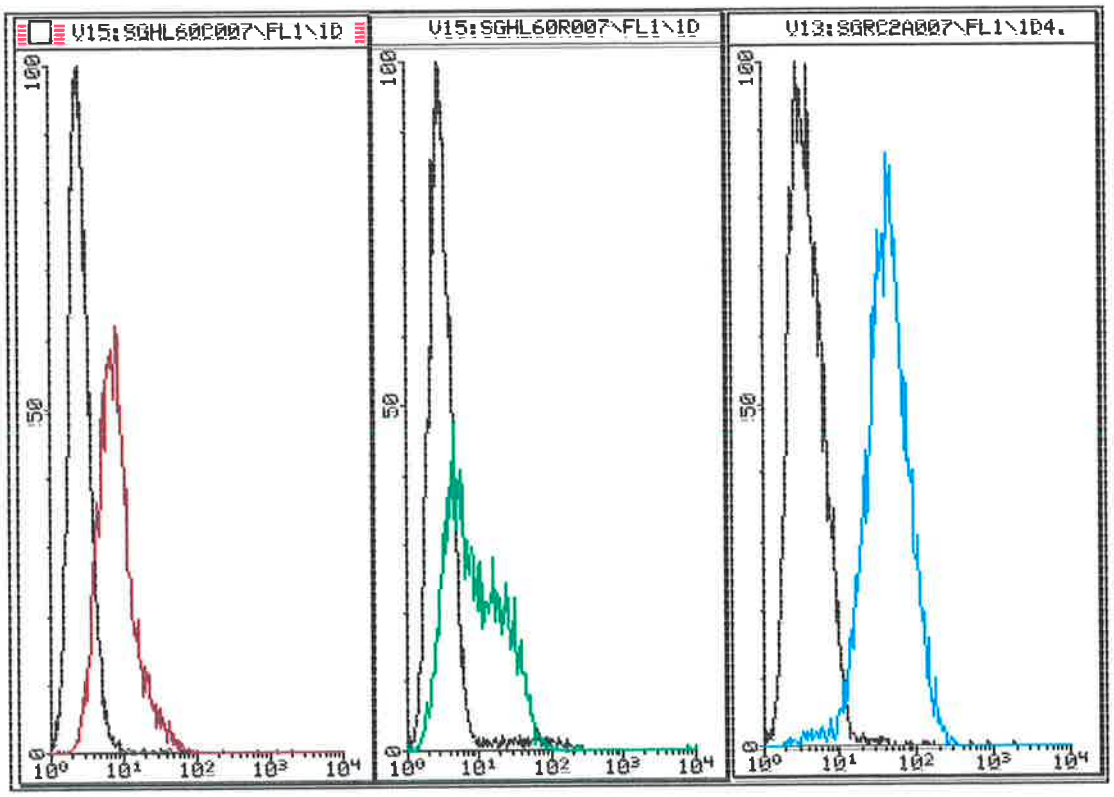
Figure 3.8.23: (Top, right) **Cell surface CD31 expression by RC-2a cells.** Cyan = B2B1.F9-treated cells, black = isotype control-treated cells.

Figure 3.8.24: (Bottom, left) **Cell surface CD64 expression by HL-60 cells.** Legend: Brown = 1D4.5-treated cells without NRS, black = 1D4.5-treated cells with NRS.

Figure 3.8.25: (Bottom, middle) **Cell surface CD64 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = 1D4.5-treated cells without NRS, black = 1D4.5-treated cells with NRS.

Figure 3.8.26: (Bottom, right) **Cell surface CD64 expression by RC-2a cells.** Cyan = 1D4.5-treated cells without NRS, black = 1D4.5-treated cells with NRS.


V15:SGHL60C020\FL1\1A
V15:SGHL60R001\FL1\3D
V13:SGRC2A001\FL1\3D3
100
50
0
10⁰ 10¹ 10² 10³ 10⁴
X


V15:SGHL60C007\FL1\1D
V15:SGHL60R007\FL1\1D
V13:SGRC2A007\FL1\1D4.
100
50
0
10⁰ 10¹ 10² 10³ 10⁴

Monocyte markers: CD64, CD14 and CD36

CD64, or FcγR1, is a high affinity receptor for monomeric IgG, and is expressed by monocytes and IFN-γ-stimulated granulocytes along with monocytic leukaemias and many myeloid cell lines (Majdic *et al*, 1989; Shen *et al*, 1987).

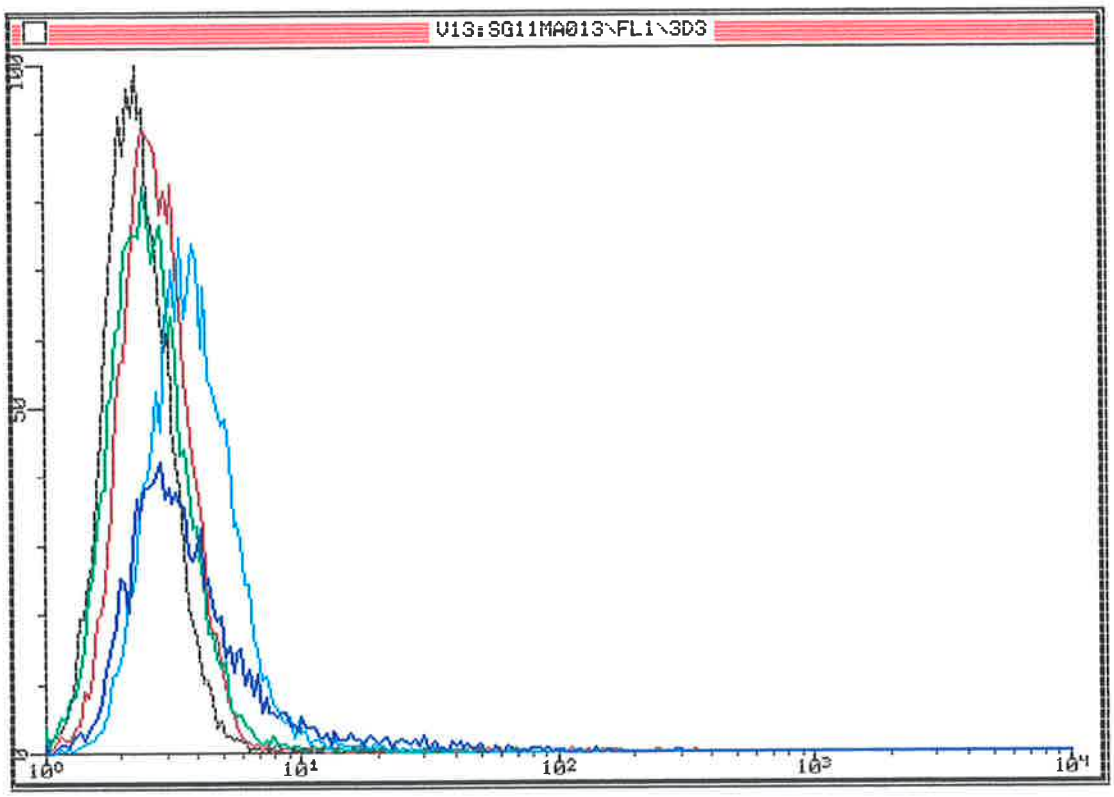
The fact that CD64 has high affinity for mouse IgG_{2a} (Gadd and Ashman, 1983) was exploited by using a *Salmonella*-specific mAb, 1D4.5 (O'Connor and Ashman, 1982), of the IgG_{2a} class, to detect the receptor in the absence of NRS, which was capable of blocking binding of 1D4.5 to cells. Untreated HL-60 cells were found to be already expressing CD64 and, by the end of 12 days, of culture with RA there appeared to be little change. Figures 3.8.24 - 26 show the histograms for untreated HL-60 cells, HL-60 cells after 12 days of RA treatment and RC-2a cells respectively.

CD14 is a molecule found predominantly on the surface of monocytes, macrophages and Langerhans cells (Goyert *et al*, 1989) and can function as a receptor for lipopolysaccharide (Wright *et al*, 1990). Although CD14 has been found on the cell surface of neutrophils, the mAb VIM13 is specific for an epitope on CD14 not expressed by neutrophils unless they are activated (Brooks *et al*, 1983; Goyert *et al*, 1989). Assays using this mAb showed that HL-60 cells were negative or possibly very weakly positive for this marker, as determined by visual examination of figure 3.8.27. The treatment with RA led to the gradual appearance over the seven days of a small percentage of positive cells. This increase was even more pronounced by day 12 (figure not shown). RC-2a cells were found to express CD14 at low levels (figure 3.8.28).

CD36, an antigen expressed by monocytes and platelets (Von dem Borne and Modderman, 1989), was not expressed by control HL-60 cells (figure 3.8.29) but its expression was induced in the majority of RA-treated cells on both days 7 (figure not shown) and 12 (figure 3.8.30) as detected by a cocktail of CD36-specific mAb. The

Figure 3.8.27: **Cell surface CD14 expression**. Overlay of fluorescence histograms of HL-60 cells cultured for 0, 3, 5 and 7 days with 10 nM RA using VIM13 in an IIF assay to detect CD14 cell surface expression. Brown = day 0, green = day 3, cyan = day 5, dark blue = day 7, black = cells treated with isotype control mAb.

Figure 3.8.28: **CD14 expression by RC-2a cells.** VIM13 was used in the IIF assay. Pink = VIM13-treated cells, black = cells treated with isotype control mAb.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50
0
10⁰
10¹
10²
10³
10⁴

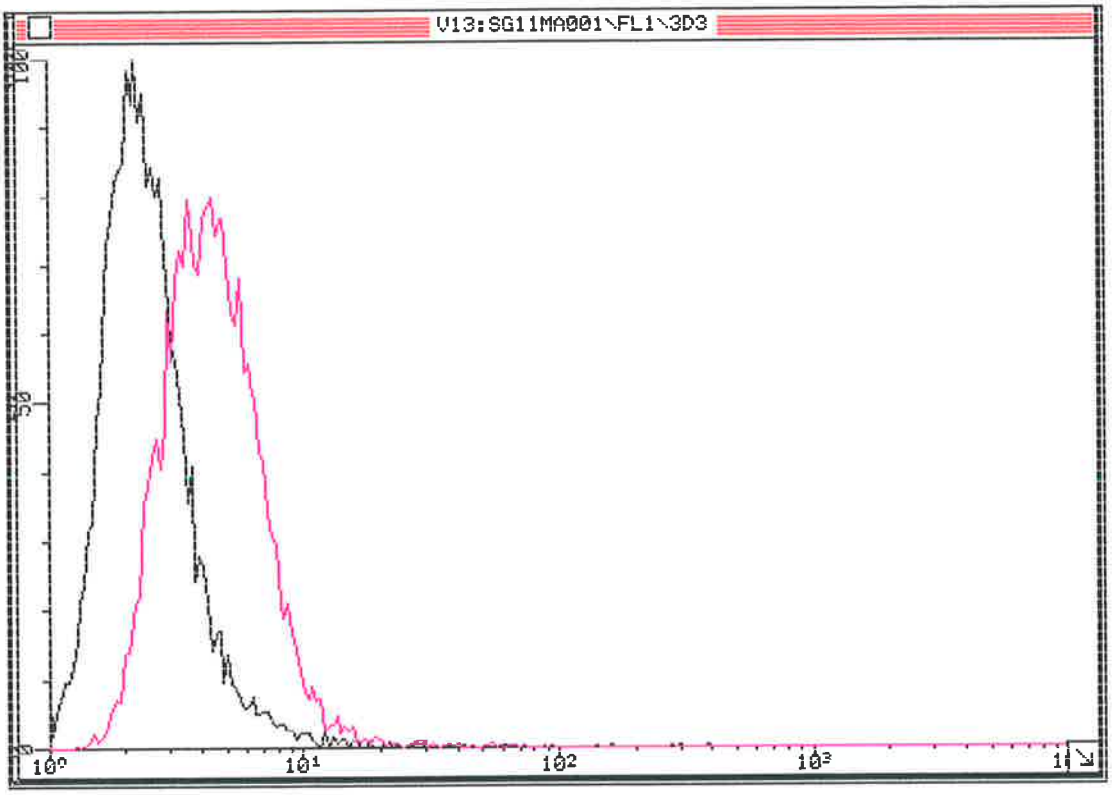

V13:SG11MA001\FL1\3D3
100
50
0
10⁰
10¹
10²
10³

Figure 3.8.29: (Top, left) **Cell surface CD36 expression by HL-60 cells.** Brown = anti-CD36 cocktail-treated cells, black = isotype control-treated cells.

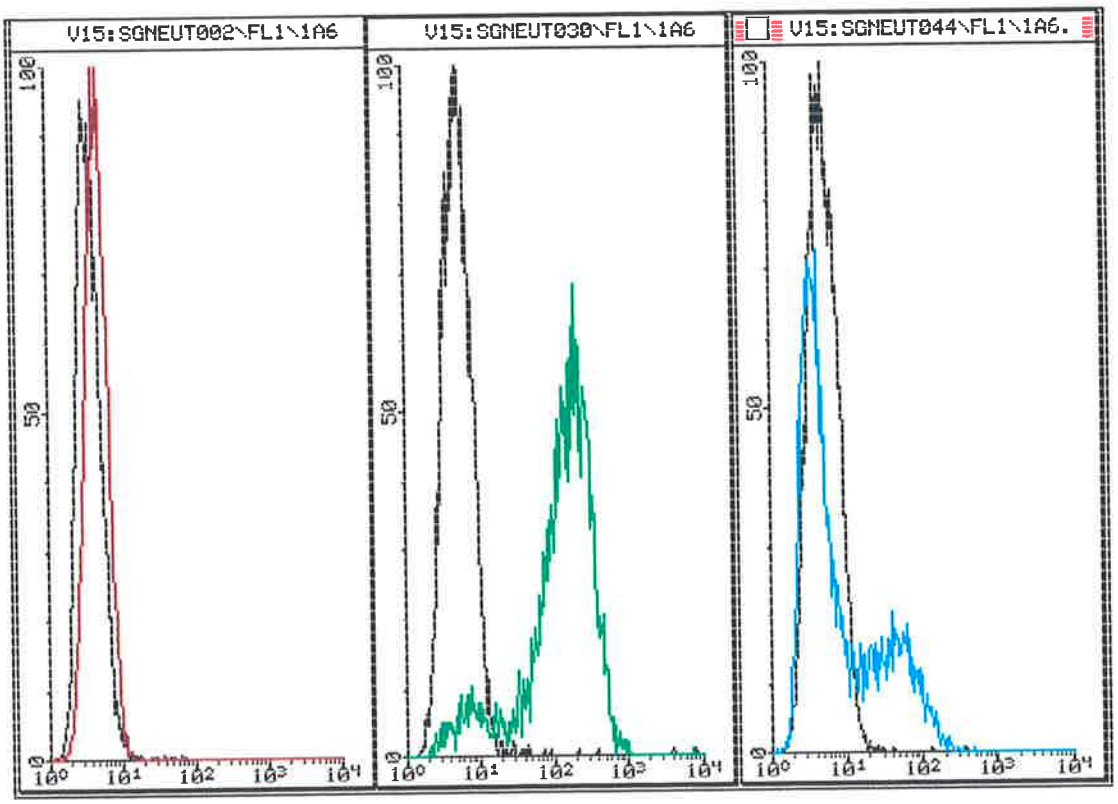
Figure 3.8.30: (Top, middle) **Cell surface CD36 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = anti-CD36 cocktail-treated cells, black = isotype control-treated cells.

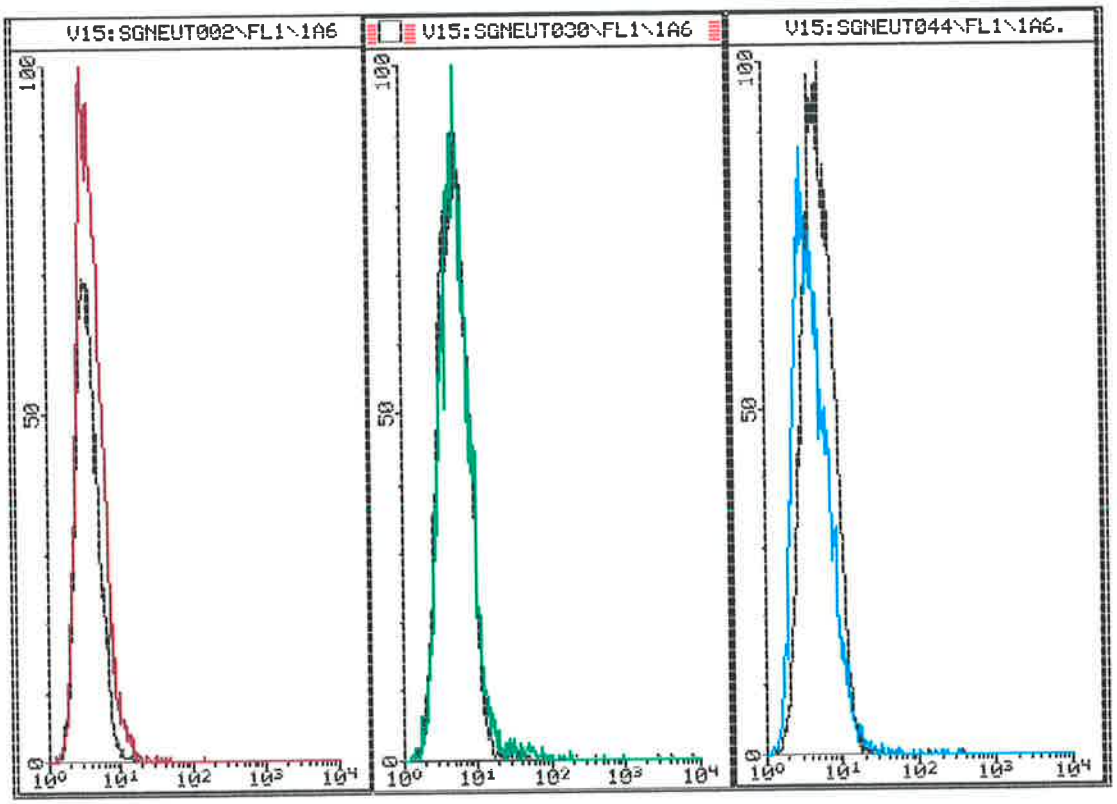
Figure 3.8.31: (Top, right) **Cell surface CD36 expression by RC-2a cells.** Cyan = anti-CD36 cocktail-treated cells, black = isotype control-treated cells.

Figure 3.8.32: (Bottom, left) **Cell surface CD16 expression by HL-60 cells.** Brown = VIFcRIII-treated cells, black = isotype control-treated cells.

Figure 3.8.33: (Bottom, middle) **Cell surface CD16 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = VIFcRIII-treated cells, black = isotype control-treated cells.

Figure 3.8.34: (Bottom, right) **Cell surface CD16 expression by RC-2a cells.** Cyan = VIFcRIII-treated cells, black = isotype control-treated cells.


V15:SGNEUT002\FL1\1A6
V15:SGNEUT030\FL1\1A6
V15:SGNEUT044\FL1\1A6.
100
50
0
10⁰ 10¹ 10² 10³ 10⁴


V15:SGNEUT002\FL1\1A6
V15:SGNEUT030\FL1\1A6
V15:SGNEUT044\FL1\1A6.
100
50
0
10⁰ 10¹ 10² 10³ 10⁴

two peaks observed, representing the positive and the negative populations, could well be indicative that there were at least two cell types, with respect to lineages, within the induced population. However, RC-2a cells also gave a heterogeneous pattern (figure 3.8.31).

CD16

CD16, or FcγRIII, which can be expressed by both neutrophils and monocytes (Schmidt and Perussia, 1989), appeared not to be expressed by HL-60 cells (figure 3.8.32), HL-60-R7, HL-60-R12 (figure 3.8.33) or RC-2a cells (figure 3.8.34). The mAb, VIFcRIII, was used to detect CD16 (Gadd *et al*, 1990).

CD71

CD71, the transferrin receptor, was strongly expressed by almost all control HL-60 cells (figure 3.8.35) as detected by the mAb VIP-1 (Holter *et al*, 1985), however, when induced to differentiate with RA for 12 days, there was a dramatic down-modulation of CD71 (figure 3.8.36) as might be expected since these cells had substantially lost their proliferative capacity. The majority (> 95%) of RC-2a cells did not express detectable levels of CD71 (figure 3.8.37).

CD15

Although found predominantly on neutrophils, CD15, or 3-fucosyl-N-acetyllactosamine, has also been found to be expressed by monocytes (Jayaram and Hogg, 1989; Ball and Schwarz, 1989). CD15 was present on control HL-60 cells (figure 3.8.38) and was retained on HL-60-R12 cells with what appeared to be a marginal decrease in cell surface expression (figure 3.8.39) as detected by the mAb VIMD5 (Majdic *et al*, 1981). This antigen was found to be absent from the cell surface of RC-2a cells (figure 3.8.40).

Figure 3.8.35: (Top, left) **Cell surface CD71 expression by HL-60 cells.** Brown = VIP-1-treated cells, black = isotype control-treated cells.

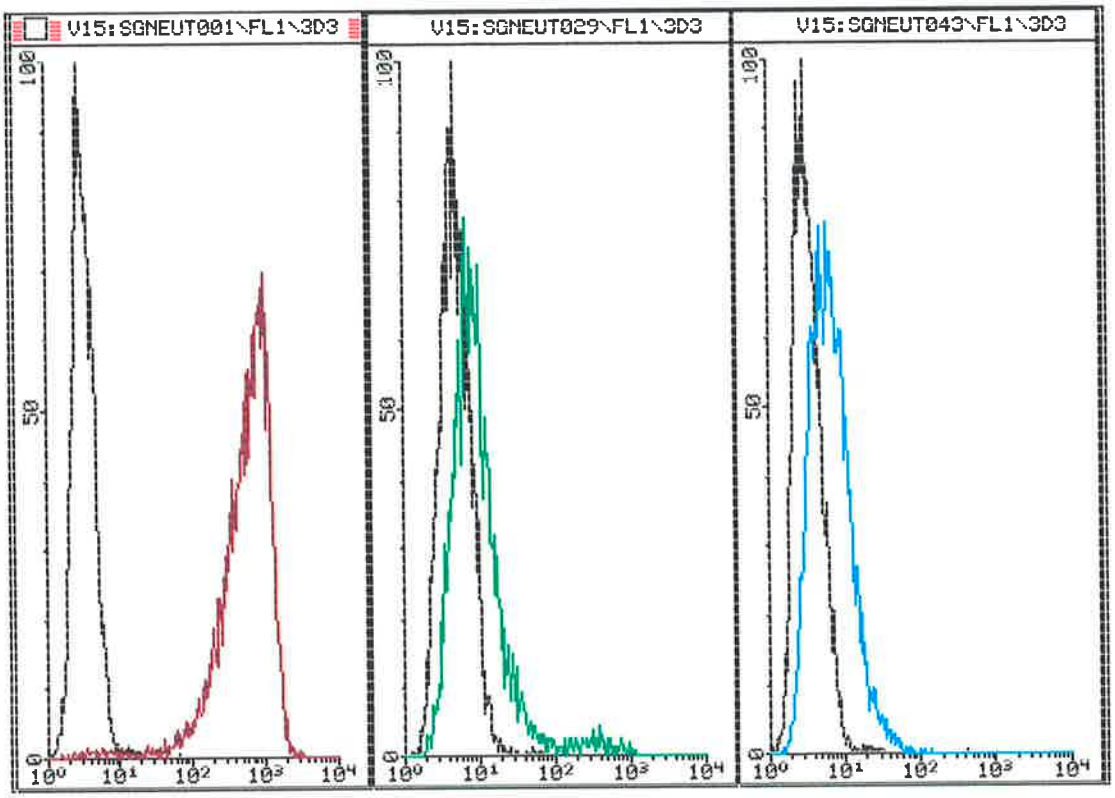
Figure 3.8.36: (Top, middle) **Cell surface CD71 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = VIP-1-treated cells, black = isotype control-treated cells.

Figure 3.8.37: (Top, right) **Cell surface CD71 expression by RC-2a cells.** Legend: cyan = VIP-1-treated cells, black = isotype control-treated cells.

Figure 3.8.38: (Bottom, left) **Cell surface CD15 expression by HL-60 cells.** Brown = VIMD5-treated cells, black = isotype control-treated cells.

Figure 3.8.39: (Bottom, middle) **Cell surface CD15 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = VIMD5-treated cells, black = isotype control-treated cells.

Figure 3.8.40: (Bottom, right) **Cell surface CD15 expression by RC-2a cells.** Cyan = VIMD5-treated cells, black = isotype control-treated cells.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V15:SGNEUT043\FL1\3D3
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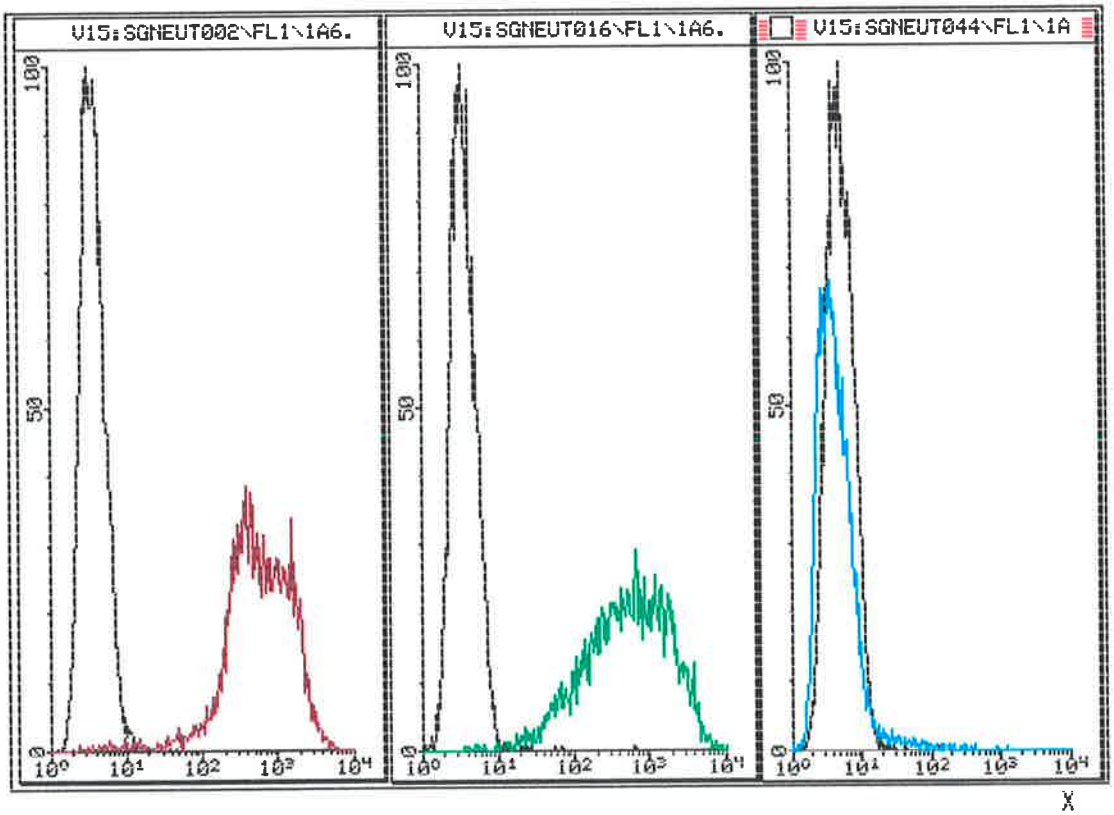
0
10⁰ 10¹ 10² 10³ 10⁴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V15:SGNEUT016\FL1\1A6.
V15:SGNEUT044\FL1\1A
100
50
0
10⁰ 10¹ 10² 10³ 10⁴

X

Neutrophil markers: CD10 and CD24

CD10, a neutral endopeptidase, and CD24 have both been recognized as being expressed by neutrophils as opposed to monocytes (Dorken *et al*, 1989a; Dorken *et al*, 1989b). Using the mAb VIL-A1 and VIB-E3 to detect CD10 and CD24 respectively (Majdic *et al*, 1981; Sugita *et al*, 1986) it was shown that neither of these antigens were expressed by control HL-60 cells, HL-60-R7, HL-60-R12 or RC-2a cells. The histograms for control HL-60, HL-60-R12 and RC-2a cells are displayed in figures 3.8.41 - 43 for CD10 and figures 3.8.44 - 46 for CD24.

Discussion

HL-60 cells cultured with 10 nM RA for 7 days (HL-60-R7) possessed an enhanced capacity to stimulate $CD2^{+}$-lymphocyte-enriched populations (LEP). Concomitantly, discernible changes occurred to the RA-treated cells with respect to their nuclear morphology, cell surface marker expression and cytoplasmic enzyme expression. All cells appeared to have been affected in some way by the RA treatment as evidenced by the results obtained with some of the mAb, such as 5A4.C5, where it was found that the expression of CD11b was up-regulated such that all cells eventually expressed this antigen for which they were initially negative.

That these cells were undergoing maturation seems likely since the RA-treated cells had a reduced proliferative capacity, down-modulated their expression of CD71, expressed detectable levels of a number of cell surface markers associated with maturing myeloid cells such as CD11b and CD14 and gained morphologically mature traits. However, defining which lineage, or lineages, these cells were being induced along remains problematic.

The results obtained here, superficially at least,. appear to challenge those obtained by other researchers where it has been reported that RA simply induced HL-60 cells along the neutrophil pathway (de Cremoux *et al*, 1991; Hemmi and Breitman, 1987; Breitman *et al*, 1980). Here the nuclear morphology of the HL-60 cells after 12

Figure 3.8.41: (Top, left) **Cell surface CD10 expression by HL-60 cells.** Brown = VIL-A1-treated cells, black = isotype control-treated cells.

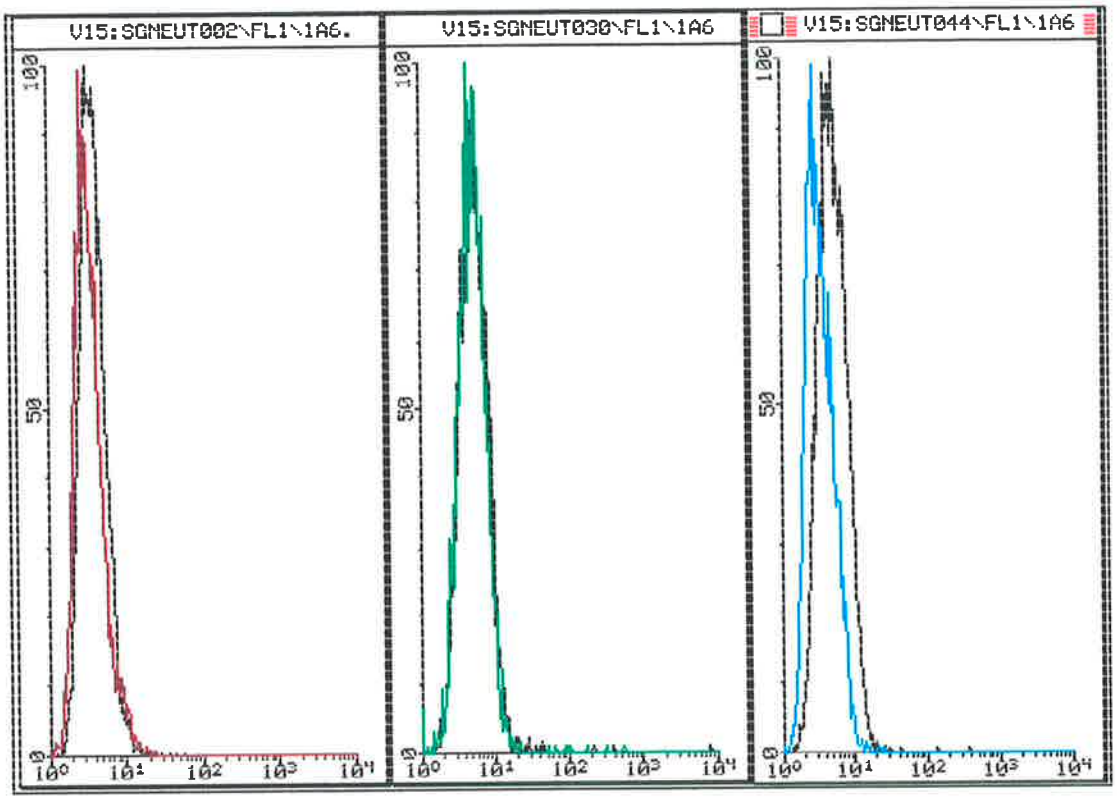
Figure 3.8.42: (Top, middle) **Cell surface CD10 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = VIL-A1-treated cells, black = isotype control-treated cells.

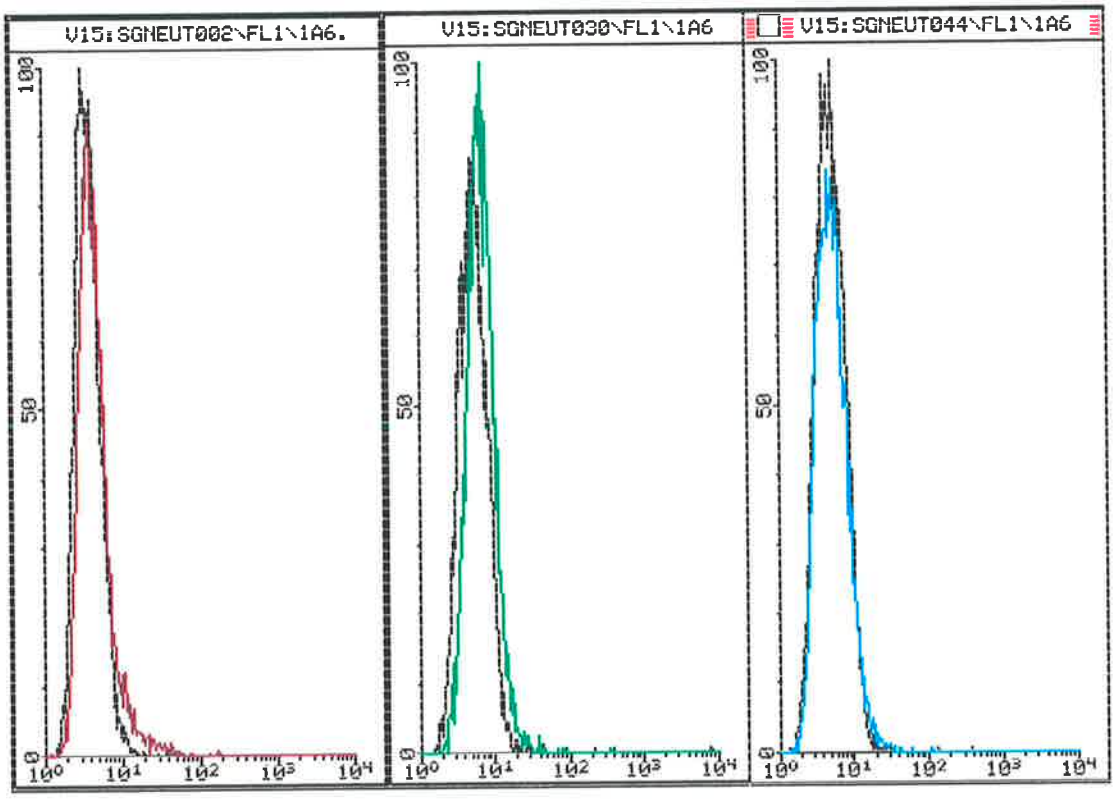
Figure 3.8.43: (Top, right) **Cell surface CD10 expression by RC-2a cells.** Cyan = VIL-A1-treated cells, black = isotype control-treated cells.

Figure 3.8.44: (Bottom, left) **Cell surface CD24 expression by HL-60 cells.** Brown = VIB-E3-treated cells, black = isotype control-treated cells.

Figure 3.8.45: (Bottom, middle) **Cell surface CD24 expression by HL-60 cells cultured for 12 days with 10 nM RA.** Green = VIB-E3-treated cells, black = isotype control-treated cells.

Figure 3.8.46: (Bottom, right) **Cell surface CD24 expression by RC-2a cells.** Cyan = VIB-E3-treated cells, black = isotype control-treated cells.


V15:SGNEUT002\FL1\1A6.
V15:SGNEUT030\FL1\1A6
V15:SGNEUT044\FL1\1A6
100
50
0
10⁰ 10¹ 10² 10³ 10⁴


V15:SGNEUT002\FL1\1A6.
V15:SGNEUT030\FL1\1A6
V15:SGNEUT044\FL1\1A6
100
50
0
10⁰ 10¹ 10² 10³ 10⁴

days of culture with RA indicated the presence of neutrophil-like cells (i.e. banded and segmented nuclei were present in some of the cells), whilst other cells appeared to be monocyte-like possessing kidney-shaped nuclei. Expression of the neutrophil-specific enzyme CAE was down-regulated to levels comparable to those expressed by the myelomonocytic cell line RC-2a. Meanwhile, the expression by a proportion of the cells of CD14, CD36 and MHC class II antigens was indicative of the presence of monocyte-like cells since these antigens are generally recognized as being associated with monocytes and not neutrophils. Finally, the observation that HL-60-R7 cells possessed an enhanced capacity to stimulate LEP also supports the idea that monocyte-like cells were present within the population since neutrophils are not well recognized for their capacity to stimulate lymphocyte proliferation.

One possible explanation for the apparent differences between the studies performed here, and those of other researchers is that we were observing a sub-line phenomenon where the HL-60 cells used in these experiments were different in some way from HL-60 cells used in other laboratories, perhaps as a result of genetic drift. Such a possibility is further reinforced by the results obtained by two independent researchers who karyotyped HL-60 cells and found significant chromosomal differences between the two populations (Koeffler *et al* 1980; Dalton *et al*, 1988). Another factor which may have led to discrepancies is the presence of mycoplasma, a micro-organism which was however shown not to be a contaminant of the HL-60 or RC-2a cells used in these experiments. Alternatively, differences in the constituents of the FCS in the culture medium may have been responsible for these discrepancies. Finally, perhaps these studies have merely highlighted subtle events which were either overlooked or not assayed for by others and thus the findings could be considered additional rather than contradictory.

Most functional traits indicative of maturation that have been assayed by other researchers, such as phagocytosis and superoxide anion production, are characteristic of both monocytes and neutrophils, whereas the more discerning CAE was generally

not tested for. So, whilst such studies indicate functional maturation, they do not enlighten us as to the specific lineage(s) of differentiation being followed. Here the histochemical stains were of little discernible value, although the loss of expression of CAE and NSE does not support the notion that the induced cells were travelling along the neutrophil or monocyte pathways respectively. However, based primarily on the morphological studies one could conclude that both neutrophil-like and monocyte-like cells were present in the cultures by day 12 of RA treatment. Such a conclusion is supported by the recent findings of Hsu *et al*, (1993) where HL-60 cells were shown to express mRNA for the macrophage colony-stimulating factor receptor (c-fms) which is capable of transducing signals that result in the activation of cells of the monocyte lineage (Stanley *et al*, 1983). Overall, the results presented here indicate that the differentiation of HL-60 cells in response to RA is abnormal, which is not surprising since the cells are leukaemic in origin.

The finding that HL-60 cells cultured with RA were capable of enhanced immunostimulatory ability was previously reported by Santoli *et al*, (1983) who showed that, after treatment for 5 days with 5×10^{-7} M RA, HL-60 cells were capable of stimulating the proliferation of mononuclear cells (MNC). Whether or not autologous APC played an important role in lymphocyte proliferation was not investigated and could be considered possible since; 1) no attempt was made to rid the MNC of APC and 2) the same researchers also found that allogeneic peripheral blood neutrophils were capable of stimulating lymphocyte proliferation. The LEP used in our assays were not able to proliferate in response to peripheral blood neutrophils. Of course this does not rule out the possibility that APC were playing a role in the activation of HL-60-R7-stimulated LEP. This issue was tackled in more depth in chapter 4.

CHAPTER 4

Characterizing the lymphocyte response to HL-60-R7 and RC-2a cells

4.1 Introduction

It was shown in the previous chapter that RA-treatment of HL-60 (HL-60-R7) cells resulted in a substantial increase in their capacity to activate $CD2^+$-lymphocyte-enriched cell populations (LEP) derived from human peripheral blood. Clues as to what cell surface antigens may or may not be involved in this activation process were given by means of the immunofluorescence studies performed upon the RA-treated and control HL-60 cells. For instance, the observed induction of ICAM-1 expression upon the cell surface of RA-treated HL-60 cells may well have been important as this molecule has been shown to play a role in cell-cell adhesion and subsequent T lymphocyte activation through it's interaction with LFA-1 (Dougherty *et al*, 1988). Another important observation derived from the cell surface marker analysis was that, for at least seven days after the initiation of RA-treatment, no detectable levels of MHC class II molecules were expressed, and that there was only marginal expression, by a small percentage of the population, induced over the succeeding 5 days.

The weight of current evidence has led to the general proposal that the initiation of an immune response requires helper T lymphocytes, usually $CD4^+$, which interact with MHC class II antigen upon the surface of an APC in order to become activated and produce IL-2, along with other lymphokines, which in turn can support the proliferation of other locally antigen-stimulated lymphocyte populations such as B lymphocytes or cytotoxic T cells. The capacity of HL-60-R7 cells to activate LEP, despite the virtual lack of observable cell surface class II antigen, suggested an alternative mode of lymphocyte activation to that classically described. Although rare,

such phenomena have been previously reported. The murine mastocytoma cell line, P815, along with murine Ia^- peritoneal macrophages have been demonstrated to trigger the proliferation of resting $CD8^+$ lymphocytes (Sprent and Schaefer, 1989; Inaba *et al*, 1987). It has also been reported that human cytotoxic T cell activation can occur without requiring $CD4^+$ T cells (Jooss *et al*, 1989).

Thus, it seemed possible that HL-60-R7 cells may have bypassed the T helper/$CD4^+$ step and directly activated other lymphocyte populations, the most likely candidates being $CD8^+$ T lymphocytes and/or natural killer (NK) cells since B lymphocytes were selected against in the purification procedure and, as will be shown, usually made up only a small percentage of the total LEP.

The experiments performed in this chapter were designed to provide some insight into the possible cellular mechanism of stimulation of the lymphocyte response to HL-60-R7 cells and to compare this with the LEP response to the highly stimulatory RC-2a cells which expressed abundant levels of cell surface MHC class II molecules. Hence cell surface marker analysis of the lymphocyte populations responding to HL-60-R7 and RC-2a cells was performed as well as a variety of manipulations of the MLC.

4.2 Phenotypic definition of the $CD2^+$-lymphocyte-enriched populations prior to their introduction to a MLC.

Unless otherwise stated, the ensuing experiments involved LEP that had undergone the following three purification steps: 1) Ficoll-Paque density gradient separation (to remove red blood cells and granulocytes), 2) Plastic adherence (to remove monocytes), 3) Nylon-wool fractionation (to remove monocytes and B cells). The cells were then cryopreserved until required. The process of cryopreservation appeared to have little effect, either qualitatively or quantitatively, upon the proliferative responses in the MLC and the lymphocyte populations were $\geq$ 98% viable

subsequent to thawing. It has been previously shown that T cell function is retained after cryopreservation (Ashman *et al*, 1987).

Before analysing the lymphocyte response to the various cell lines used in the MLC, it was thought important to have an insight into the general composition of the LEP used in these assays, as such information could help to determine the possible participants in the proliferative responses. Thus a number of LEP were tested for their expression of a wide variety of lineage-restricted antigens as well as a few immunologically relevant antigens such as MHC class II antigens and the α-chain of the IL2-R (CD25). These were examined by means of direct or indirect immunofluorescence. The antibodies used and their corresponding antigen and lineage specificities are listed in section 2.11.1 along with the isotype control mAb.

Many different LEP were independently analysed for percentages of cells expressing each of the antigens and a summary of the results obtained is shown in table 4.2.1. The details of the analysis of one of the lymphocyte populations (LEP_8) are illustrated in figures 4.2.1 - 16. In brief, initially a forward scatter (FSC) vs side scatter (SSC) dot-plot of the LEP was established, around which a gate was constructed to exclude some of the debris and/or dead cells (which were identified by their ability to incorporate ethidium monoazide, EMA) and to include any potentially contaminating monocytes and lymphoblasts, as well as the normal resting lymphocyte population (figures 4.2.1 and 4.2.2).

Using a cell sample treated with an isotype control mAb, the voltage sensitivity of the fluorescence 1 (FL1) detector was adjusted and the settings were maintained for the respective classes of test antibodies. Figure 4.2.3 shows a histogram representing LEP_8 treated with the negative control IgG_1 mAb 3D3.3, where a region was set between fluorescence channel number 60 and 255. The percentage yielded (3.4%) within the region was subtracted from the percentage values obtained for some tests, such as MHC class II (figure 4.2.5), where the fluorescence intensities overlapped, but not for others, such as CD3 (figure 4.2.11), where the fluorescence

Table 4.2.1: **Composition of LEP**: Summary of percentage of cells in LEP expressing various markers as determined by IIF assays

N. T. = not tested.

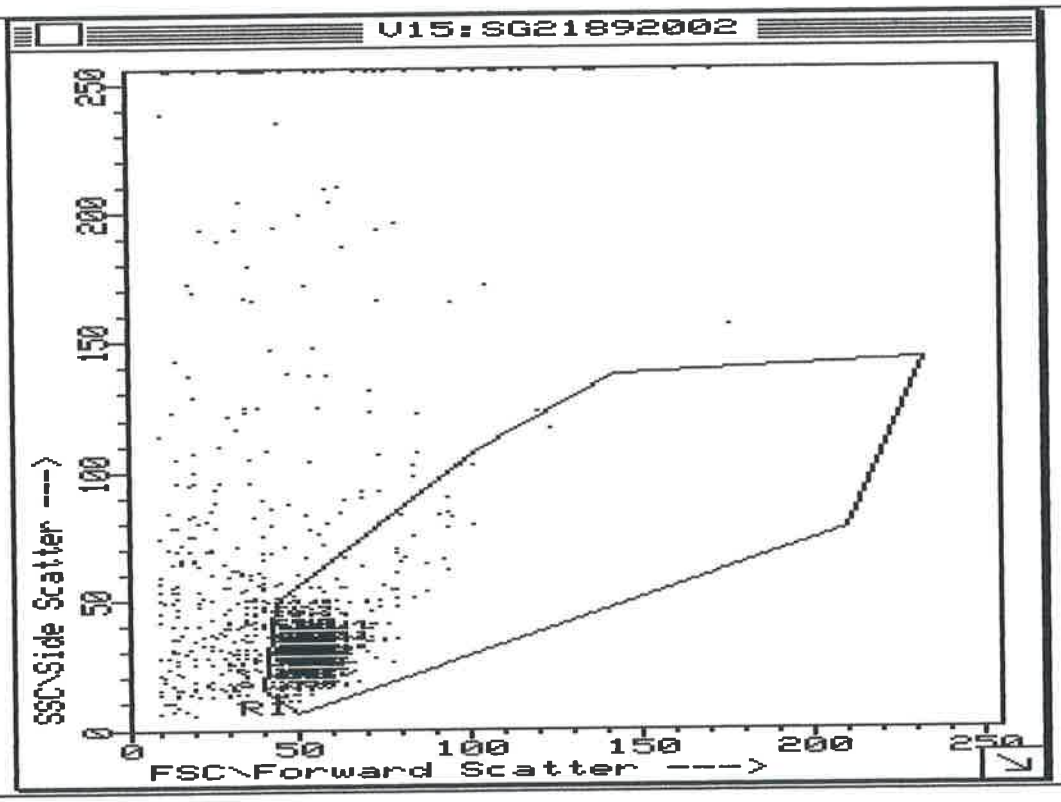
*An example of how total, and high, MHC class II expression by LEP was calculated is given in figures 4.2.5 and 4.2.6 respectively.

	CD19	HLA-D total	HLA-D high	HLA-DR total	HLA-DR high	CD25	CD2	CD3	CD4	CD8	CD16	CD14	CD45RO
LEP_2	9	20	9	9	9	1	90	79	51	21	8	< 1	N.T.
LEP_7	8	18	8	10	6	3	89	70	48	21	18	< 1	N.T.
LEP_8	2	54	< 1	6	< 1	2	96	88	46	53	6	1	89
LEP_9	< 1	33	3	19	3	7	96	83	54	35	9	1	63
LEP_{10}	2	33	3	5	3	12	94	76	61	22	13	1	79
LEP_{11}	4	27	7	19	5	10	92	73	50	34	16	3	66
LEP_{12}	2	20	3	6	2	1	95	87	68	21	1	< 1	N.T.
LEP_{13}	3	22	4	4	2	1	95	87	54	30	8	< 1	N.T.
LEP_{14}	3	17	4	7	4	1	95	87	47	32	4	< 1	N.T.
LEP_{15}	3	35	4	20	3	10	95	82	50	39	11	< 1	N.T.
LEP_{16}	1	19	4	10	1	3	94	86	62	30	4	< 1	51
LEP_{17}	2	33	6	21	6	3	83	71	33	41	26	< 1	48
LEP_{18}	N.T.	16	6	16	3	4	92	82	52	35	8	< 1	70
LEP_{19}	1	12	4	N.T.		3	90	77	56	20	14	< 1	68
LEP_{24}	2	N.T.		N.T.		3	N.T.	70	46	21	20	< 1	N.T.
LEP_{25}	1	N.T.		N.T.		2	N.T.	86	44	35	5	1	N.T.

Figure 4.2.1: **Gating of LEP**: FSC versus SSC dot-plot of LEP_8 with gate designating those events included in the analysis.

Figure 4.2.2: *Paint-a-gate* display defining, in red, the position of dead cells and debris for LEP_8 as determined by treatment with EMA. Window 0 (Bottom, left) = FSC vs SSC; Window 1 (Top, left) = FL3 vs cell number. Windows 2 - 5 can be ignored.

*FL3 represents EMA fluorescence



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RGYBVCWO gate; +- mode; <> size
0-5 plot; FZHXPMU$'"s

Dots = 2000
848 448

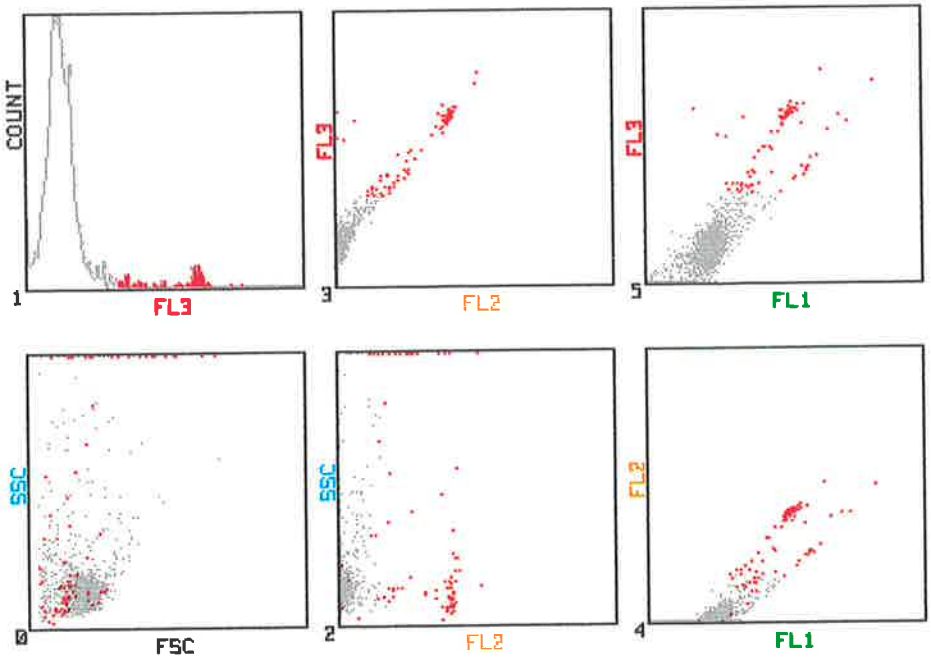


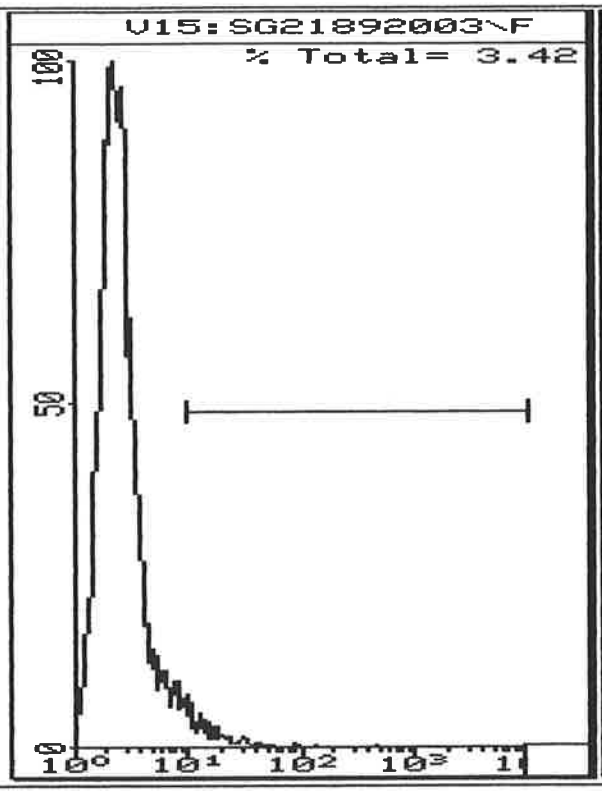
Figure 4.2.3 - 15: **Analysis of LEP**: Histograms of log fluorescence 1 intensity (FL1) vs frequency for LEP_8. Percentage total reflects the number of events within designated region.

Figure 4.2.3: (top, left) LEP_8 treated with 3D3.3 (IgG_1 isotype control) in an IIF assay.

Figure 4.2.4: (top, right) LEP_8 treated with VIM13 (anti-CD14) in an IIF assay.

Figure 4.2.5: (bottom, left) LEP_8 treated with FMC 14 in an IIF assay. Region was positioned to include all MHC class II^+ cells.

Figure 4.2.6: (bottom, right) LEP_8 treated with FMC 14 in an IIF assay. Region was positioned to include a discrete population of cells expressing high levels of MHC class II antigen.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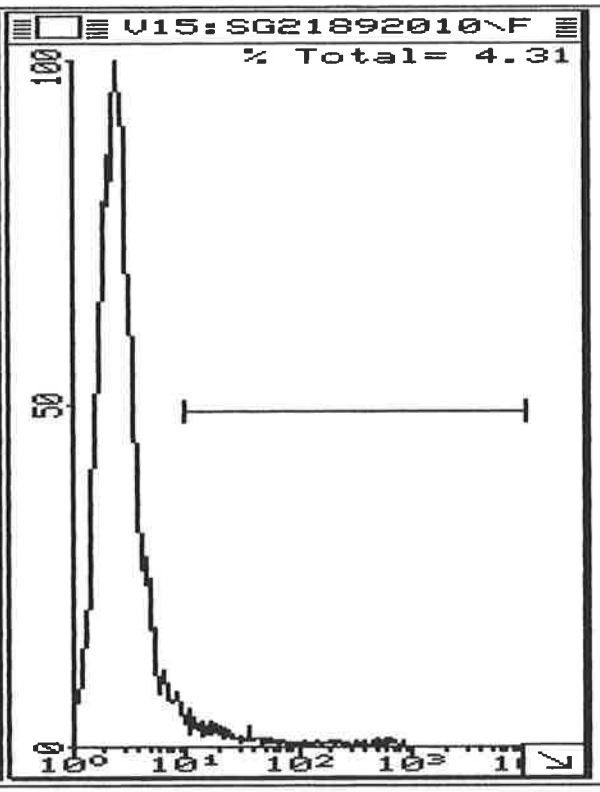
0
10⁰ 10¹ 10² 10³ 1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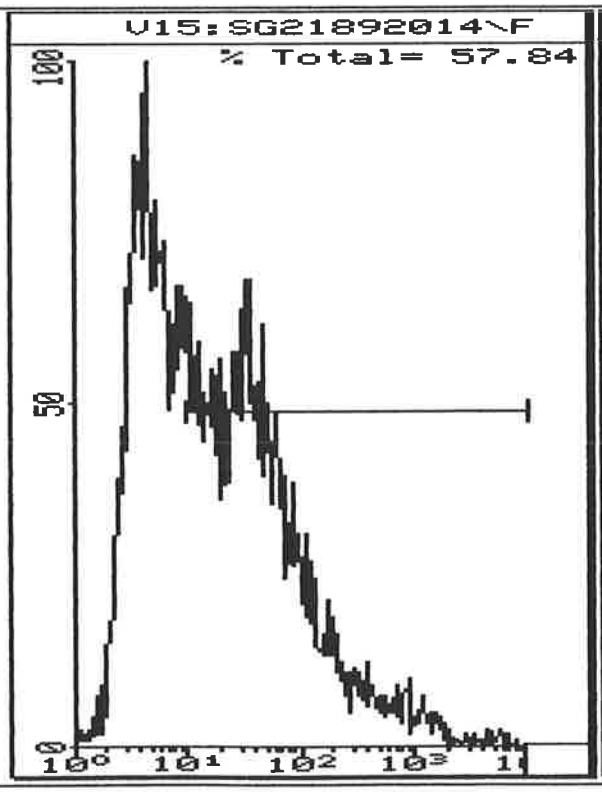
50
0
10⁰ 10¹ 10² 10³ 1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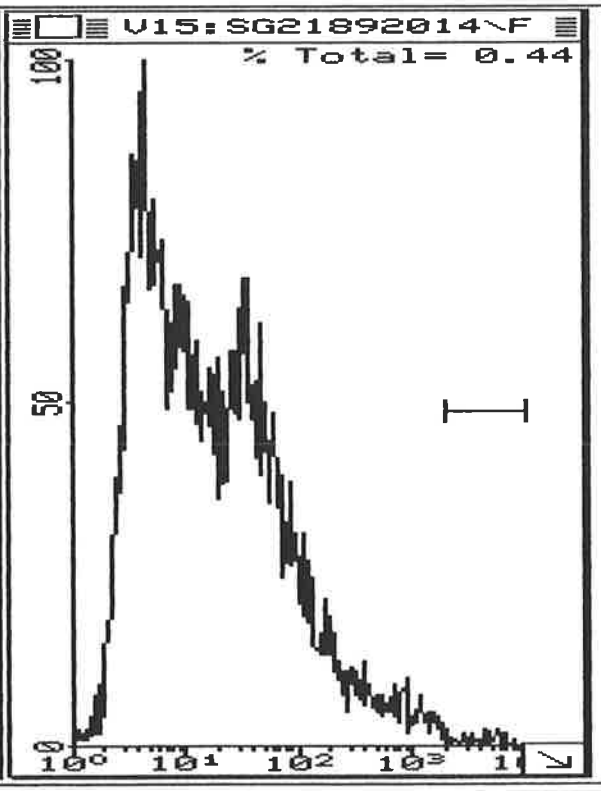
0
10⁰ 10¹ 10² 10³ 1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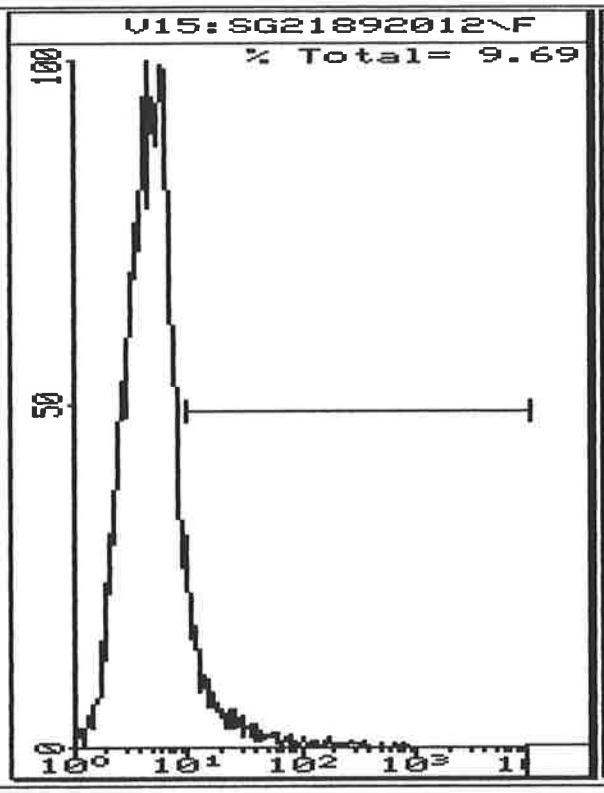
100
50
0
10⁰ 10¹ 10² 10³ 1

Figure 4.2.7: (top, left) LEP_8 treated with FMC 15 (anti-HLA-DR) in an IIF assay.

Figure 4.2.8: (top, right) LEP_8 treated with VIFcRIII (anti-CD16) in an IIF assay.

Figure 4.2.9: (bottom, left) LEP_8 treated with 3D3.3-Fl in a direct IF assay.

Figure 4.2.10: (bottom, right) LEP_8 treated with TAC (anti-CD25) in a direct IF assay.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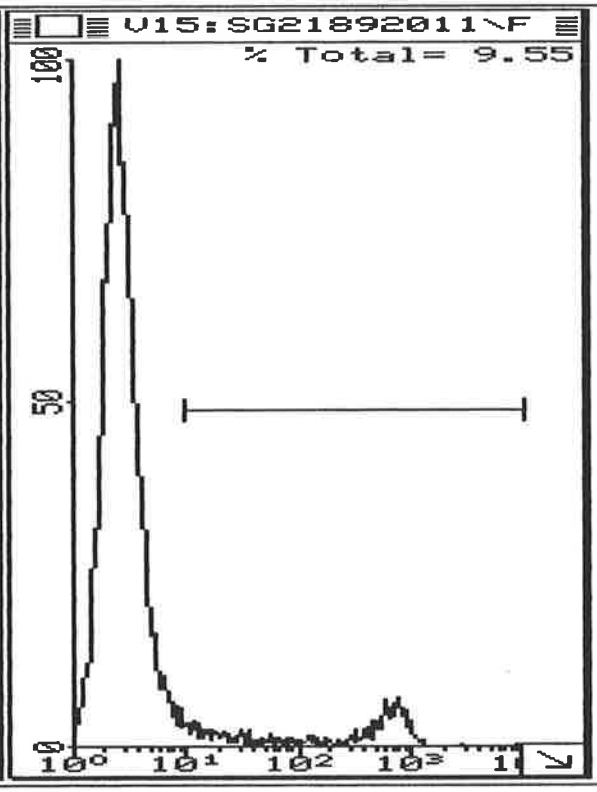
10⁰ 10¹ 10² 10³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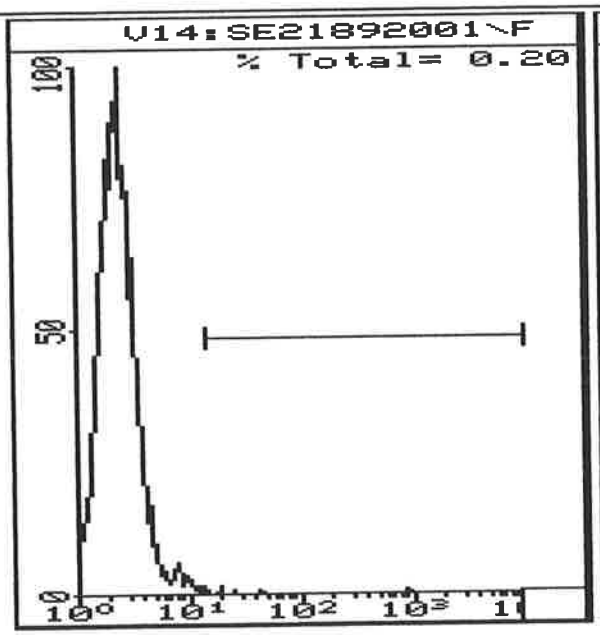
10⁰ 10¹ 10² 10³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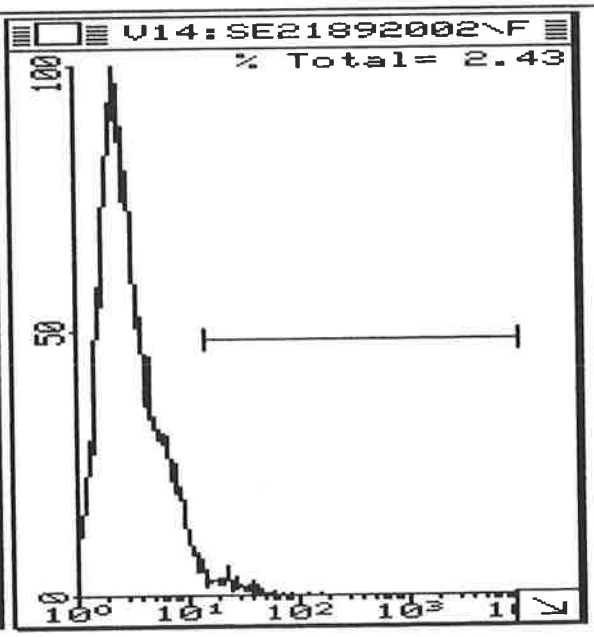
50
0
10⁰ 10¹ 10² 10³


V14:SE21892002\F
% Total= 2.43
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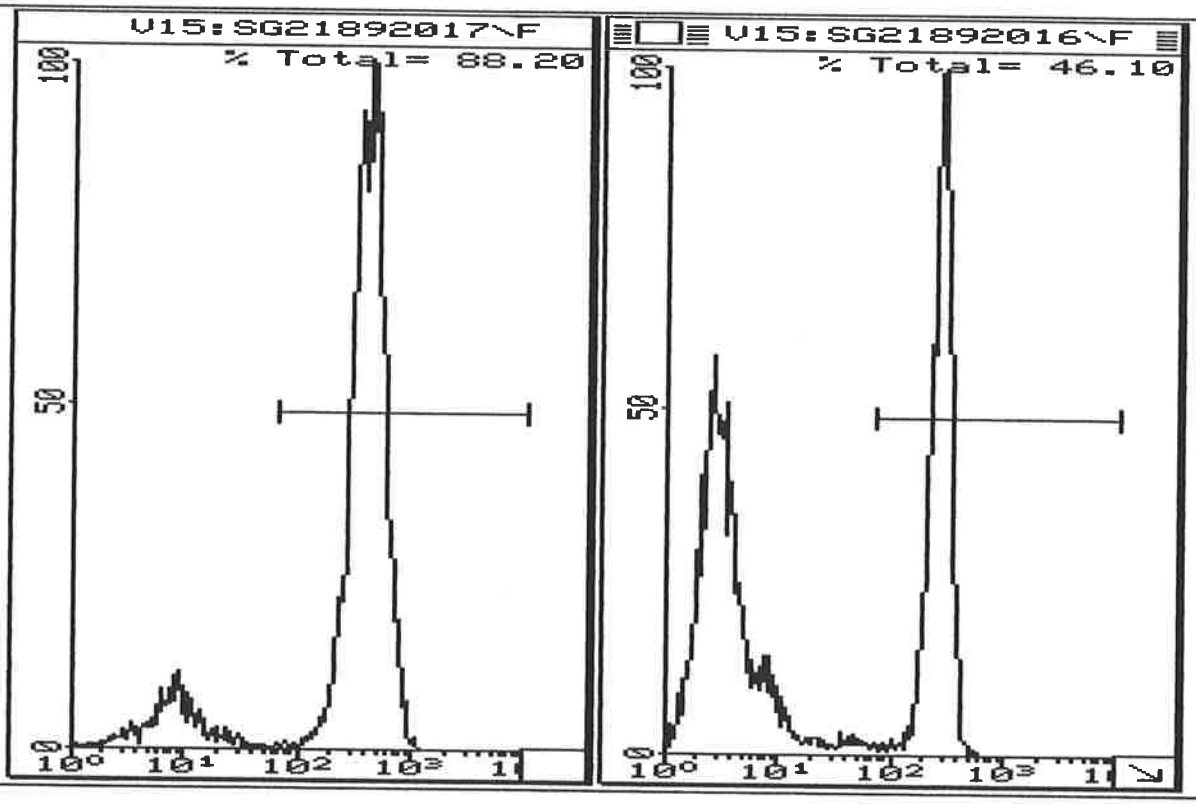
50
0
10⁰ 10¹ 10² 10³

Figure 4.2.11: LEP_8 treated with OKT3 (anti-CD3) in an IIF assay.

Figure 4.2.12: LEP_8 treated with OKT4 (anti-CD4) in an IIF assay.

Figure 4.2.13: LEP_8 treated with OKT8 (anti-CD8) in an IIF assay.

Figure 4.2.14: LEP_8 treated with UCHL1 (anti-CD45RO) in an IIF assay.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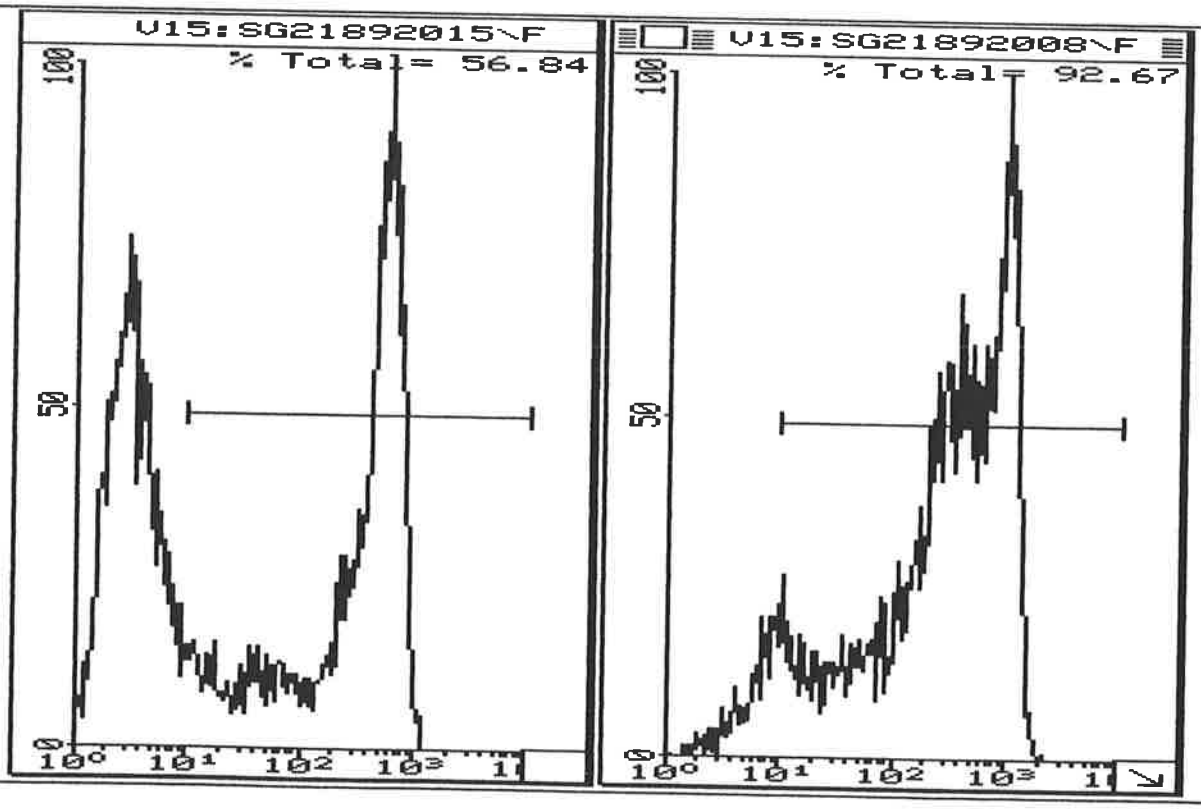
% Total= 88.20
100
50
0
10⁰ 10¹ 10² 10³ 1
V15:SG21892016\F
% Total= 46.10
100
50
0
10⁰ 10¹ 10² 10³ 1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10⁰ 10¹ 10² 10³ 1
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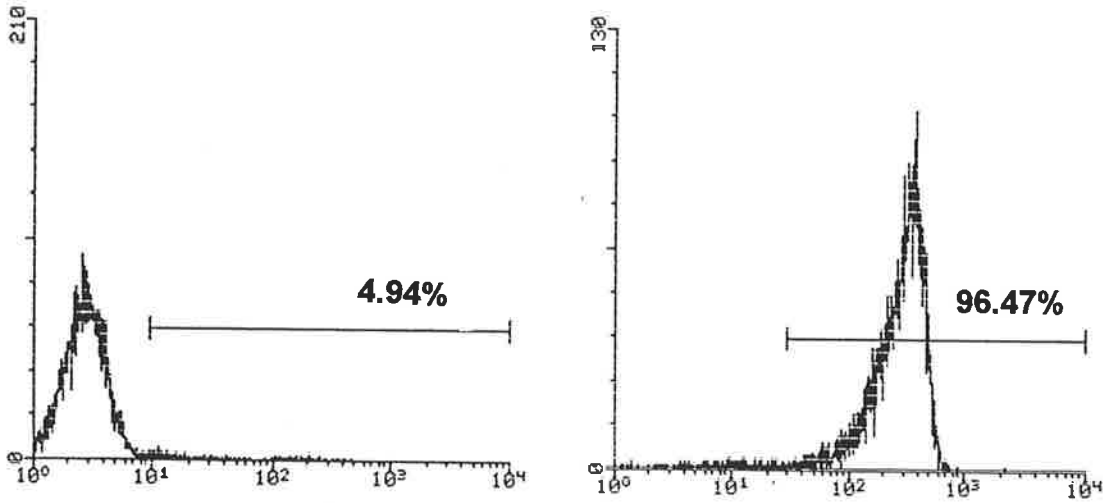
% Total= 92.67
100
50
0
10⁰ 10¹ 10² 10³ 1

Figure 4.2.15: (left) LEP_8 treated with FMC 63 (anti-CD19) in an IIF assay.

Figure 4.2.16: (right) LEP_8 treated with OKT11 (anti-CD2) in an IIF assay.


210
0
4.94%
10^0
10^1
10^2
10^3
10^4
130
0
96.47%
10^0
10^1
10^2
10^3
10^4

intensities did not overlap. The relevant isotype control antibodies were used for each of the various specific mAb, yielding results very similar to that shown for 3D3.3 (data not shown).

4.3 Preliminary analysis of LEP responding to HL-60-R7 or RC-2a cells

It was considered important to investigate the phenotype of the cells responding in the MLC, since it was thought that HL-60-R7 cells should not be capable of directly activating $CD4^+$ lymphocytes to any great extent, at least not via the classical pathway, as they expressed no detectable levels of MHC class II molecules at the initiation of the MLC. Hence an experiment was performed where the lymphocytes responding to HL-60-R7 cells were phenotypically analysed using probes for CD4, CD8 and CD25.

The high affinity IL-2R consists of a p75 protein chain, of intermediate affinity for IL-2, and a p55 (CD25) chain of low affinity for IL-2 (Robb *et al*, 1984; Waldmann, 1986). It has been reported that resting lymphocytes express very little discernible CD25 upon their cell surface and therefore very little high affinity IL-2R (Hatakeyama *et al*, 1989). However, upon receiving an appropriate signal, such as cell-associated allogeneic MHC class I or class II antigen, T lymphocytes can be induced to express CD25 which then associates with the generally concomitantly expressed p75 chain to form the high affinity receptor (reviewed by Rothenberg, 1992). If there is a local supply of IL-2 these lymphocytes could then proliferate. In this section cells were defined as being activated if they expressed CD25.

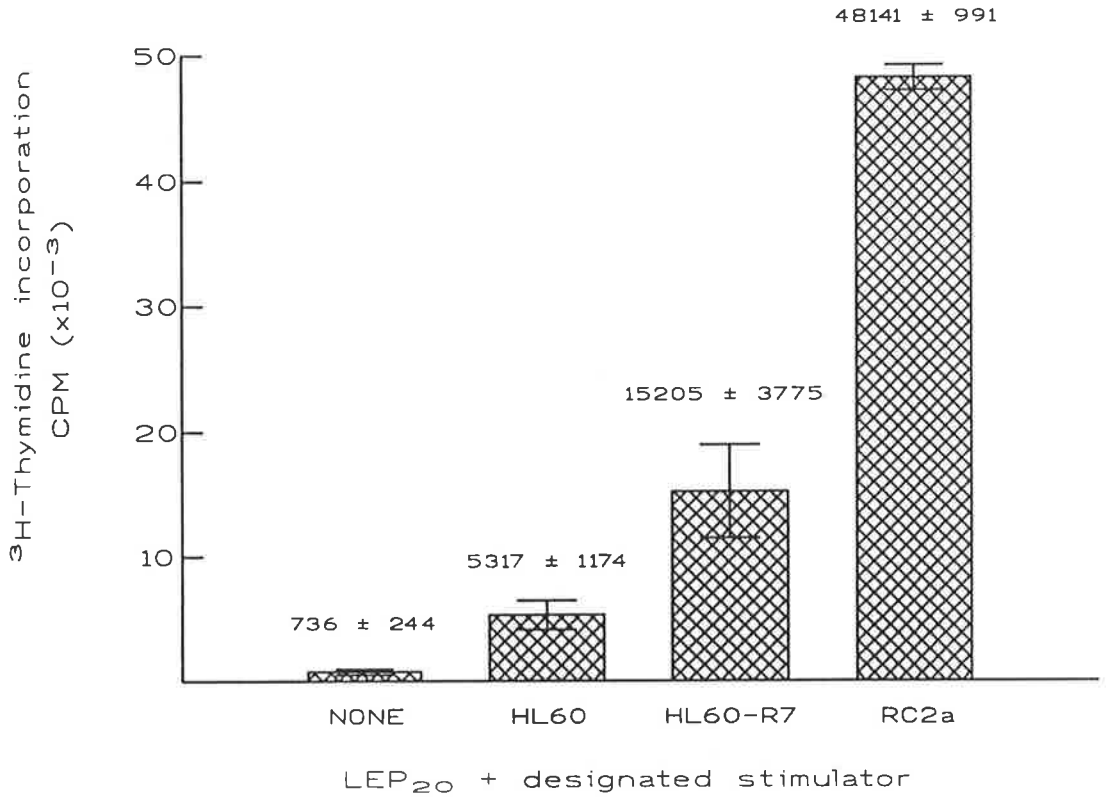
In order to determine the proportion of $CD4^+$ and $CD8^+$ lymphocytes responding to the various stimulator populations, MLC were set up in the fashion described in section 2.3 where untreated HL-60, HL-60-R7 and RC-2a cells were used as stimulators, at a concentration of 1×10^5 cells/well in round bottomed 96 well trays. The lymphocytes, or LEP, were freshly purified from normal human peripheral blood and were used at a concentration of 1×10^5/well.

To ensure that the cells were responding in the expected fashion a tritiated thymidine uptake assay was performed concomitantly, the results of which are displayed in figures 4.3.1 - 2. Along with the proliferation assay, a dual immunofluorescence assay was performed upon the cells harvested from the MLC after 5 days of culture. Lymphocytes alone, or with one of the three stimulator populations, were treated with the following combinations of reagents:

1) Anti-CD25-FITC + SPE
2) OKT4(Biot) + SPE
3) OKT8(Biot) + SPE
4) OKT4(Biot) + SPE + Anti-CD25-FITC
5) OKT8(Biot) + SPE + Anti-CD25-FITC

The dot-plots of FSC vs SSC of lymphocytes alone or with one of the three stimulators are shown in figures 4.3.3 - 6. Gates were constructed to include activated and resting lymphocytes, and to exclude dead cells and debris. At this stage ethidium monoazide (EMA) was not available and thus dead cells and debris were detected on the basis of non-specific fluorescence. To illustrate, figures 4.3.6 - 9 describe how the gate position was determined for lymphocytes cultured with RC-2a cells. Here, different sections of the FSC vs SSC dot-plot of an SPE-treated population were analysed for their ability to express red fluorescence (FL2) non-specifically. It was shown that cells of the same size but a higher SSC than the resting lymphocytes (gated region of figure 4.3.8), had increased fluorescence in a negative control treated with

Figure 4.3.1 - 2: **Proliferative response to HL-60, HL-60-R7 and RC-2a cells.** ^{3}H-thymidine uptake results of MLC with LEP_{20} or LEP_{21} plus designated stimulators. Incorporation by stimulator populations alone was < 300 cpm.


48141 ± 991
50
40
30
20
10
^{3}H-Thymidine incorporation
CPM (x10^{-3})
15205 ± 3775
5317 ± 1174
736 ± 244
NONE
HL60
HL60-R7
RC2a
LEP$_{20}$ + designated stimulator

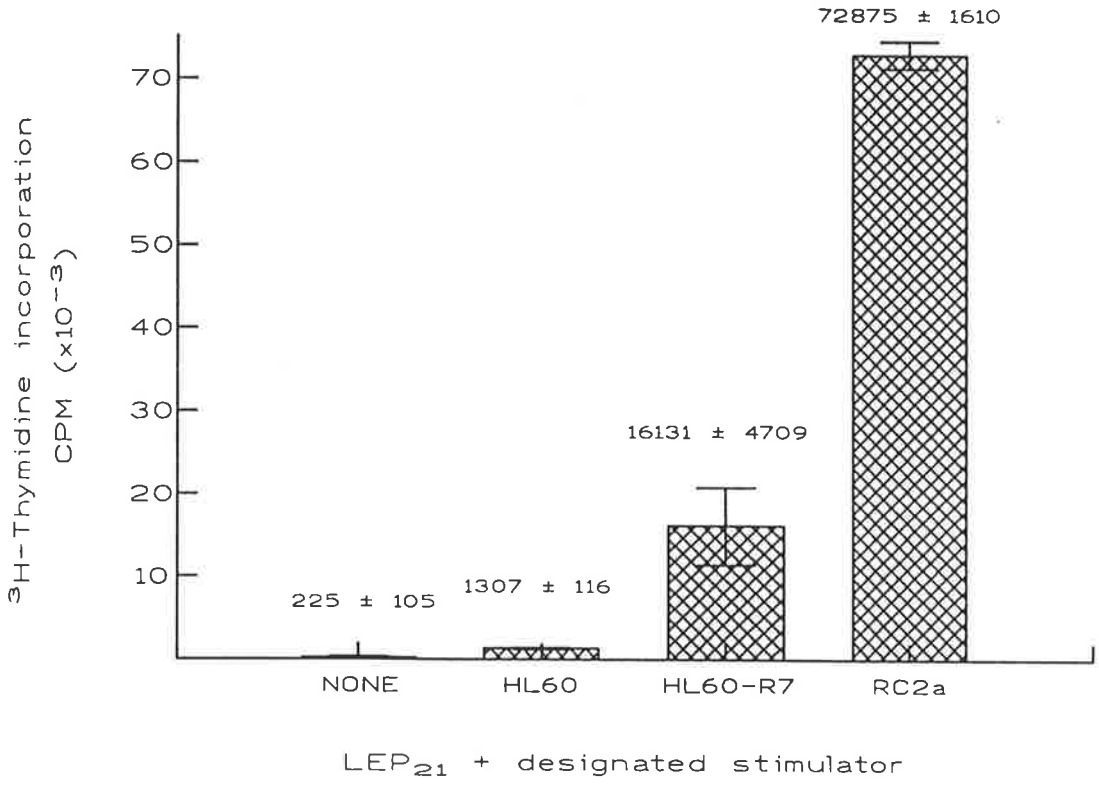
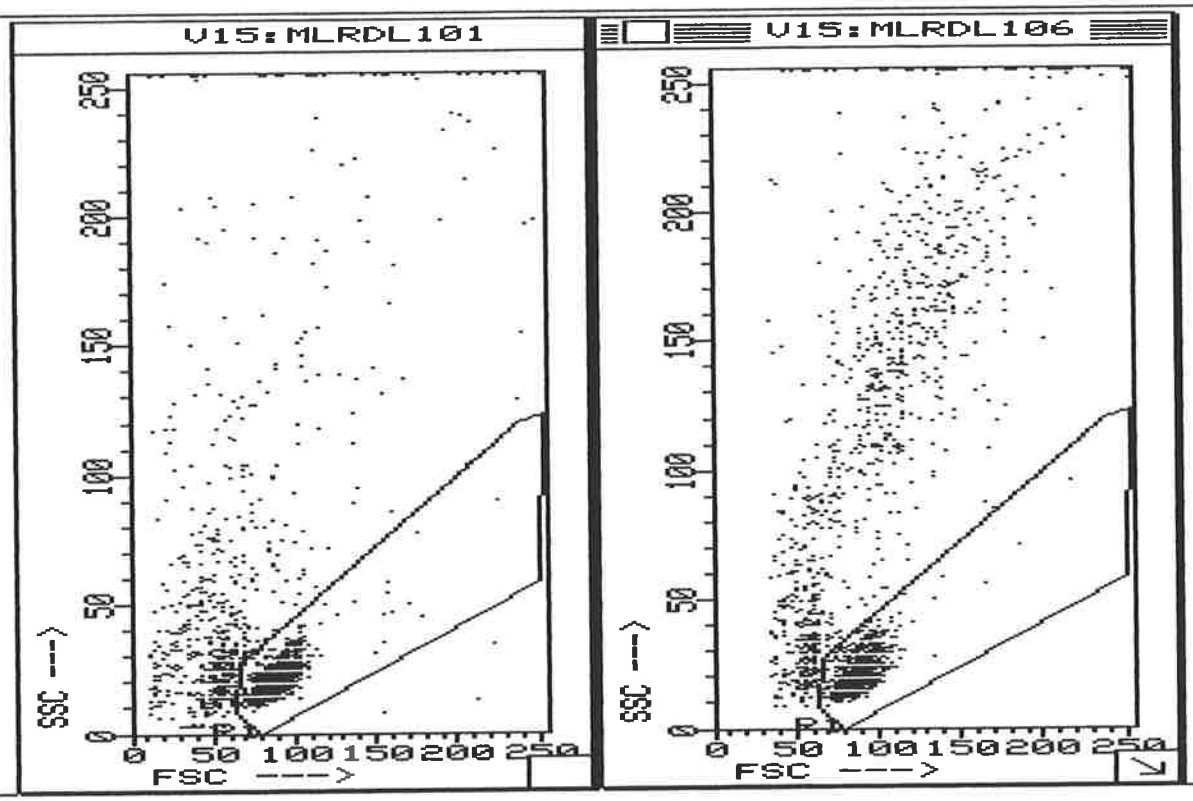

72875 ± 1610
70
60
50
40
30
20
10
^{3}H-Thymidine incorporation
CPM (x10^{-3})
16131 ± 4709
1307 ± 116
225 ± 105
NONE
HL60
HL60-R7
RC2a
LEP$_{21}$ + designated stimulator

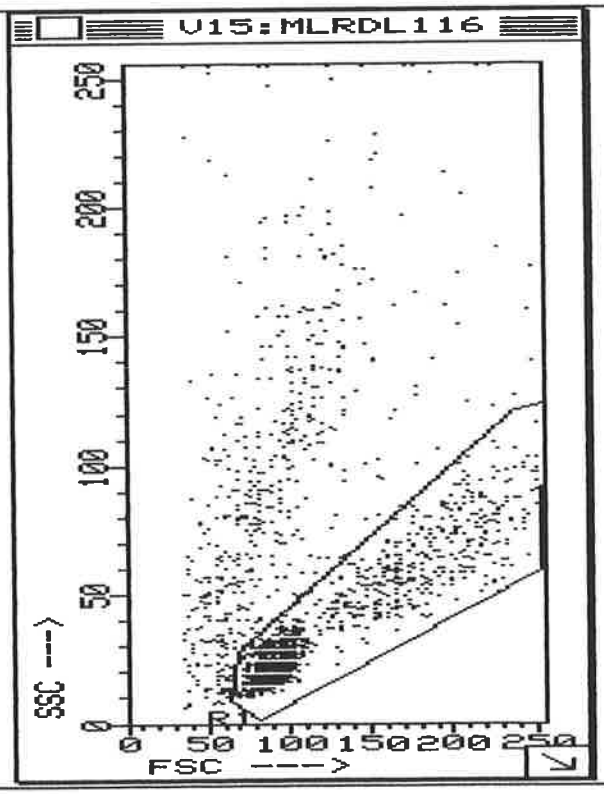
Figure 4.3.3 - 6: **FSC vs SSC dot-plots LEP_{20} in MLC**: LEP_{20} cultured alone or with designated stimulator cells for 5 days under MLC conditions. The gate indicates those cells which were analysed.

Figure 4.3.3: (top, left) LEP_{20} alone.

Figure 4.3.4: (top, right) LEP_{20} plus HL-60 cells.

Figure 4.3.5: (bottom) LEP_{20} plus HL-60-R7 cells.


V15:MLRDL101
V15:MLRDL106
SSC --->
FSC --->
0
50
100
150
200
250
R


V15:MLRDL116
SSC --->
FSC --->
0
50
100
150
200
250
R

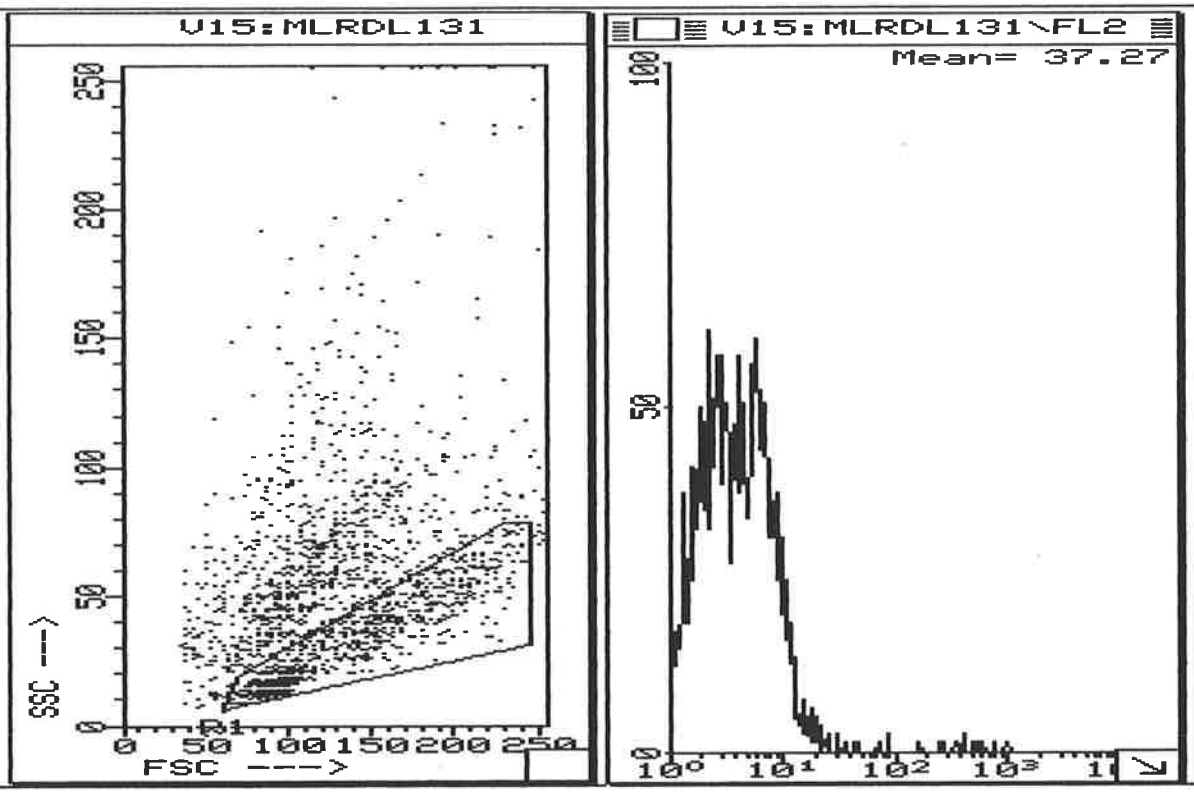
Figures 4.3.6 - 9: **Gating procedure for LEP_{20} cultured with RC-2a cells.**

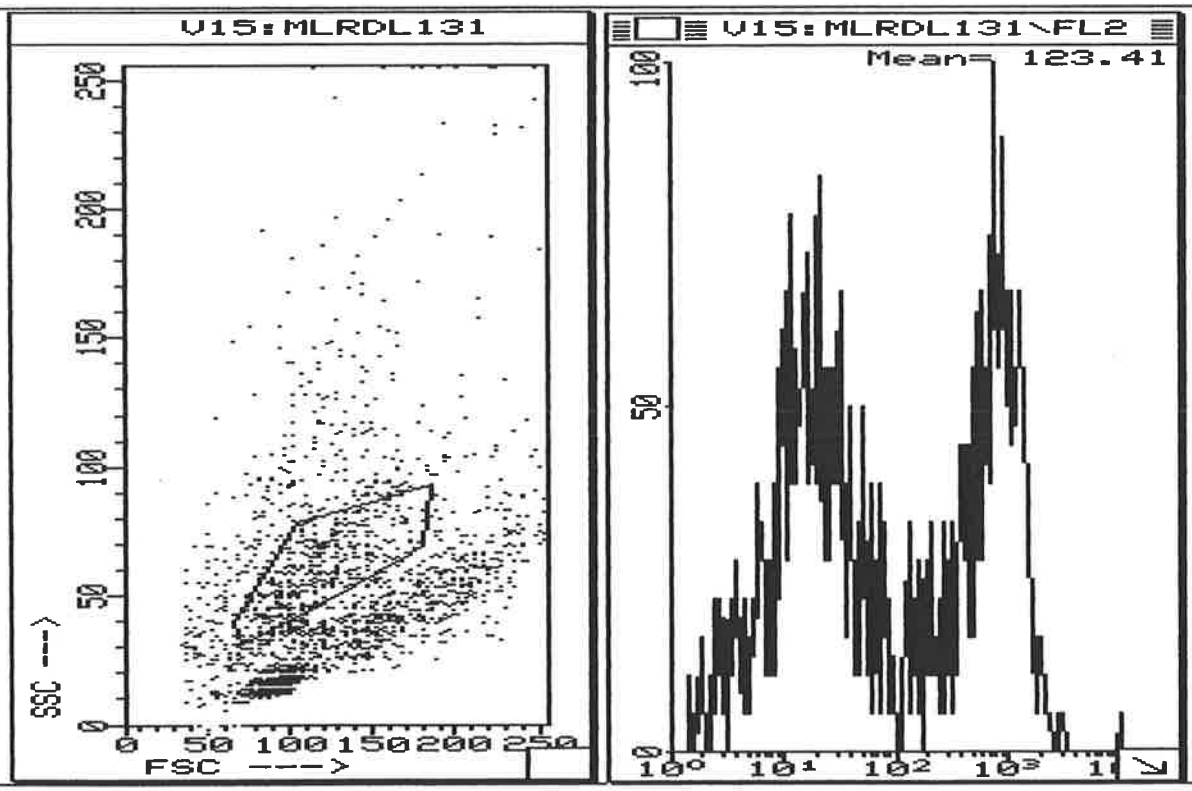
Figure 4.3.6: (top, left) LEP_{20} plus RC-2a cells.

Figure 4.3.7: (top, right) Histogram (log fluorescence 2 intensity (FL2) vs cell no.) of lymphocytes within the gate described in figure 4.3.6.

Figure 4.3.8: (bottom, left) Dot-plot of FSC vs SSC of LEP_{20} cultured with RC-2a cells for 5 days under MLC conditions.

Figure 4.3.9: (bottom, right) Histogram of FL2 vs cell no. of lymphocytes/debris within gate described in figure 4.3.8.


V15:MLRDL131
SSC --->
FSC --->
V15:MLRDL131^FL2
Mean= 37.27


V15:MLRDL131
SSC --->
FSC --->
V15:MLRDL131^FL2
Mean= 123.41

SPE alone (figure 4.3.9) compared to the fluorescence emitted by the resting population (figures 4.3.6 & 4.3.7) and thus it seemed legitimate to exclude those cells when constructing the gate as an increase in SSC does not generally result in an increase in fluorescence intensity when viable cells are involved, therefore suggesting that these events represent debris and/or dead cells. When a more direct method of detecting dead cells involving EMA was used in later experiments, the results obtained supported the gate positionings described above. To illustrate, the location of dead cells and debri, defined for LEP_{28} + HL-60-R7 or RC-2a cells after 5 days of MLC and treatment with EMA, is displayed in figures 4.3.10 and 4.3.11 respectively.

Two independent analyses were performed separately by Joseph Webster (Flinders Medical Centre, Adelaide) and myself using *consort-30* and *lysys* software respectively, producing very similar data with differences in percentage values varying from 0 - 5%. In ensuing experiments various programmes were used for analysis, such as *paint-a-gate, lysys (I and II), facscan* and *consort-30*.

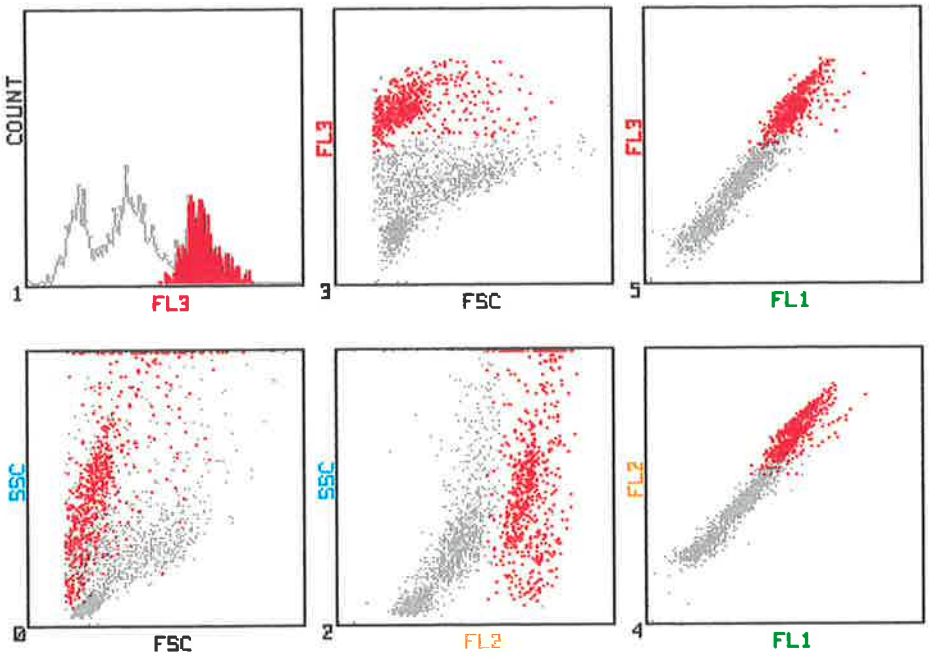
Results obtained for both LEP_{20} and LEP_{21} are summarized for each responder population in the form of histograms in figures 4.3.12 - 19. Lymphocytes cultured alone expressed very little detectable CD25, with virtually all cells being accounted for as either CD4 or CD8 positive, and much the same result was observed with lymphocytes cultured with control HL-60 cells. However, many of the lymphocytes cultured with HL-60-R7 cells expressed CD25, and the distribution of this marker was interesting, in that there was a relatively low level of activated $CD4^+$ cells that were $CD25^+$, whilst the majority of the activated cells were either $CD4^-$, $CD8^+$ or $CD4^-$, $CD8^-$ cells. The latter of which may have been $\gamma\delta$ cells, NK cells, B cells, or a combination of the three.

The low proportion of activated $CD4^+$ lymphocytes was not that surprising when one considers that HL-60-R7 cells did not express any detectable levels of MHC class II molecules upon their cell surface, at least at the initiation of the culture. The lymphocytes activated by RC-2a cells, however, were shown to be predominantly

Figure 4.3.10: (top) *Paint-a-gate* display of LEP_{28} + HL-60-R7 cells treated with EMA, with events in red designating dead cells or debris. Window 0 (bottom, left) = FSC vs SSC; Window 1 (Top, left) = FL3 vs cell number. Windows 2 - 5 can be ignored.

Figure 4.3.11: (bottom) *Paint-a-gate* display of LEP_{28} + RC-2a cells treated with EMA, with events in red designating dead cells or debris. Window 0 (bottom, left) = FSC vs SSC; Window 1 (Top, left) = FL3 vs cell number. Windows 2 - 5 can be ignored.

PAINT-A-GATE (TM) Mode File:#15:SG11192028 Dots = 2000
%= 31.6 0.0 0.0 0.0 0.0 0.0 0.0 656 272
RGYBVCWO gate; +- mode; <> size
0-5 plot; FZHXPMU$'"s



PAINT-A-GATE (TM) Mode File:#15:SG11192019 Dots = 2000
%= 27.3 0.0 0.0 0.0 0.0 0.0 0.0 896 544
RGYBVCWO gate; +- mode; <> size
0-5 plot; FZHXPMU$'"s

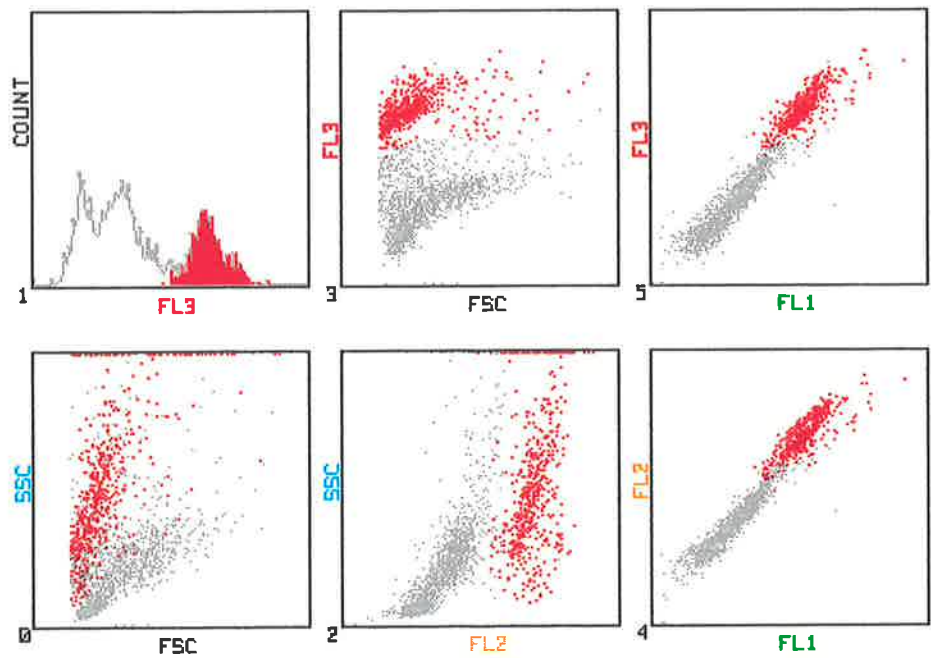
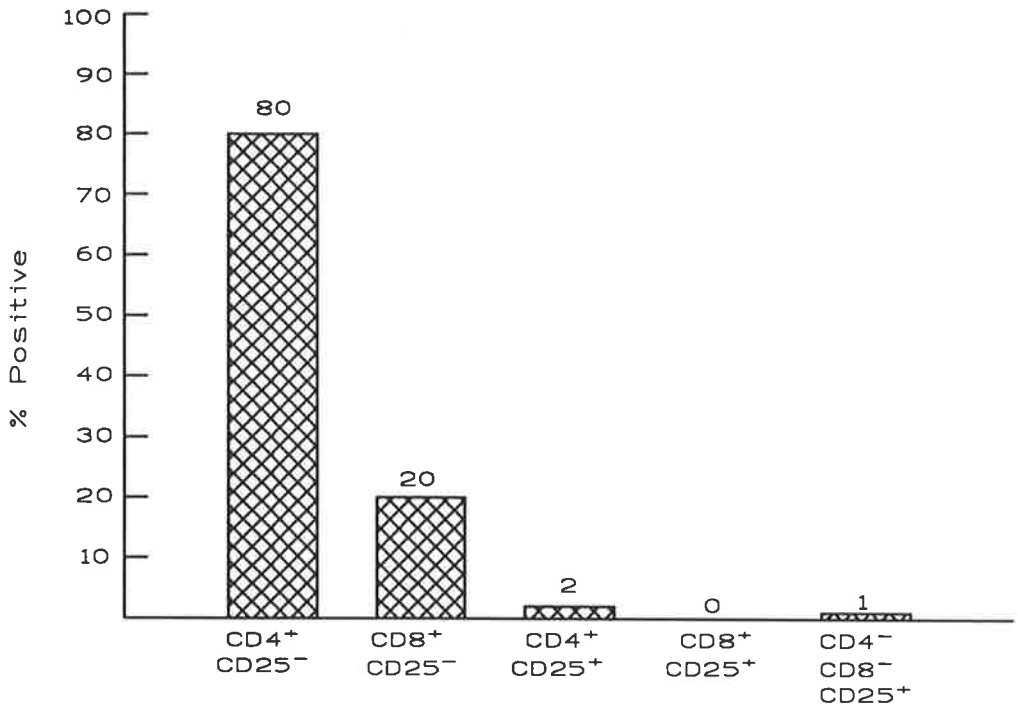


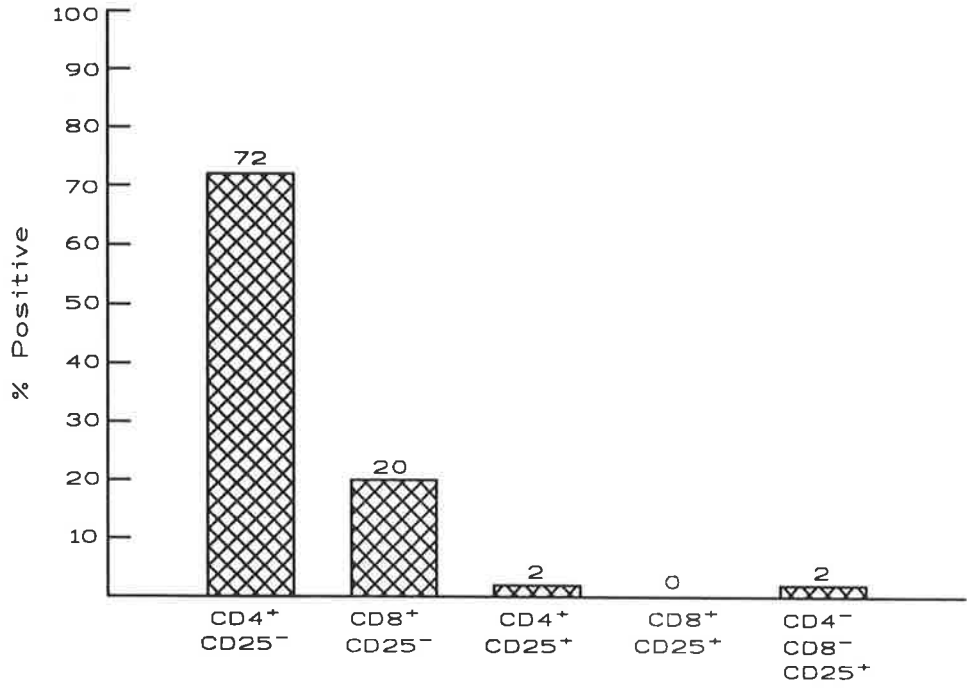
Figure 4.3.12 - 19: **Characterization of the LEP_{20} or LEP_{21} response to HL-60, HL-60-R7 or RC-2a cells.** Histograms of percentage phenotypes present in each of the lymphocyte populations responding to the designated stimulator cells as determined by a double direct immunuofluorescence assays performed on day 5 of the MLC. Biotinylated OKT4 and OKT8 were used to detect CD4 and CD8 respectively. A directly fluoresceinated anti-CD25 was also used.

Figure 4.3.12: (top) LEP_{20} alone.

Figure 4.3.13: (bottom) LEP_{20} + HL-60 cells.



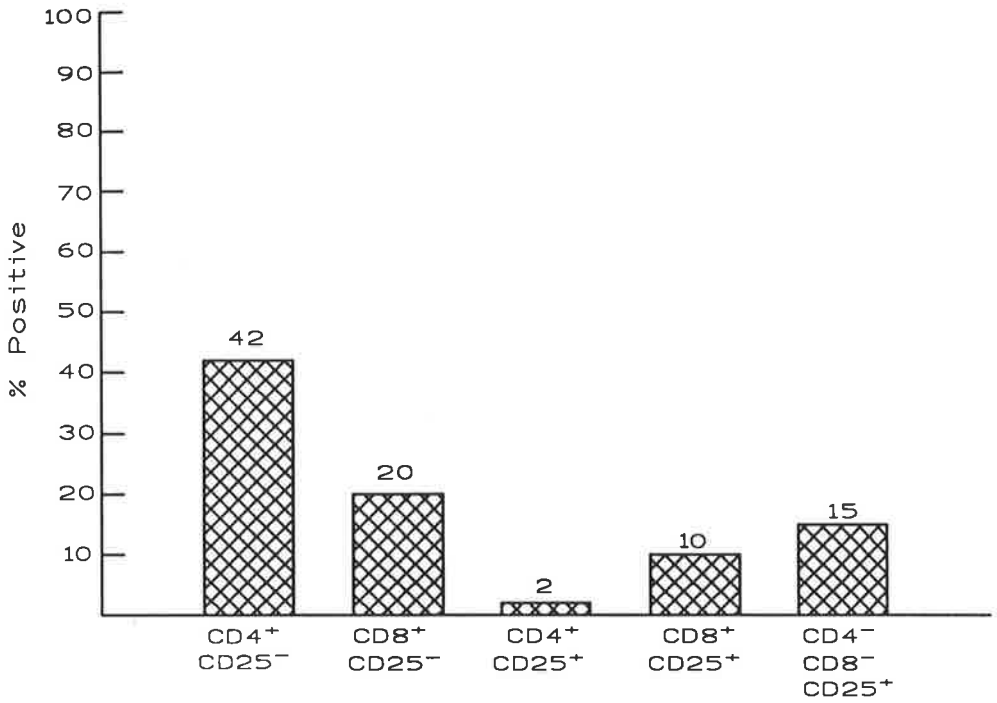
LEP_{20} Alone



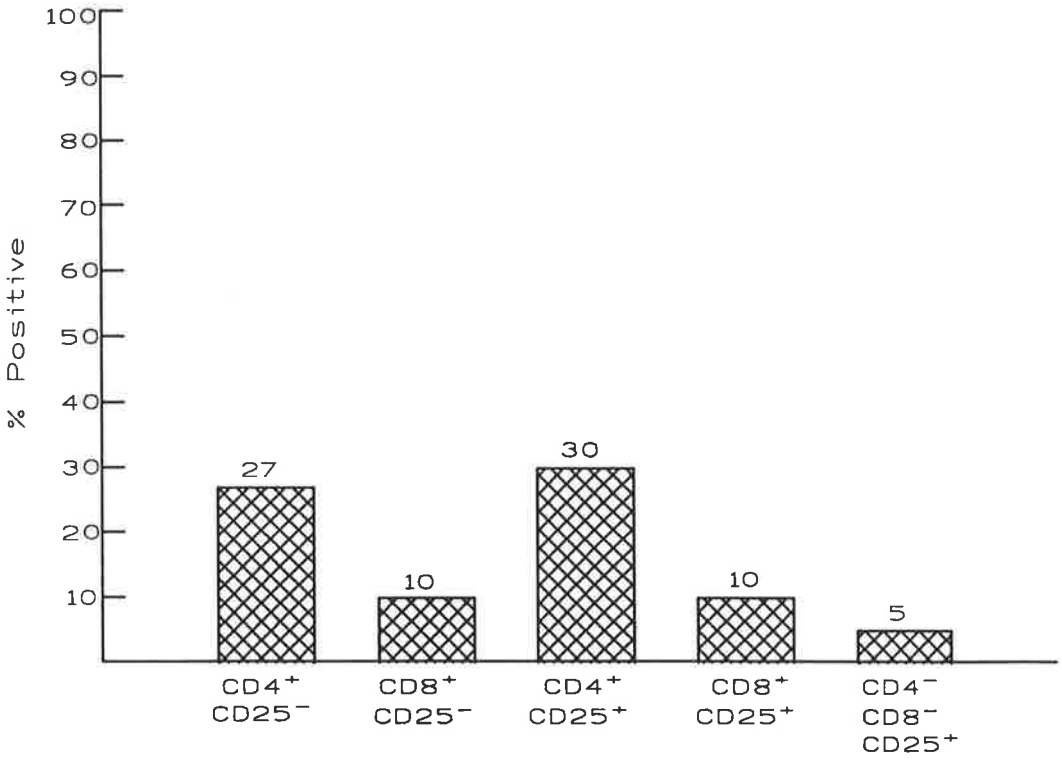
LEP_{20} + HL60

Figure 4.3.14: (top) LEP_{20} + HL-60-R7 cells.

Figure 4.3.15: (bottom) LEP_{20} + RC-2a cells.



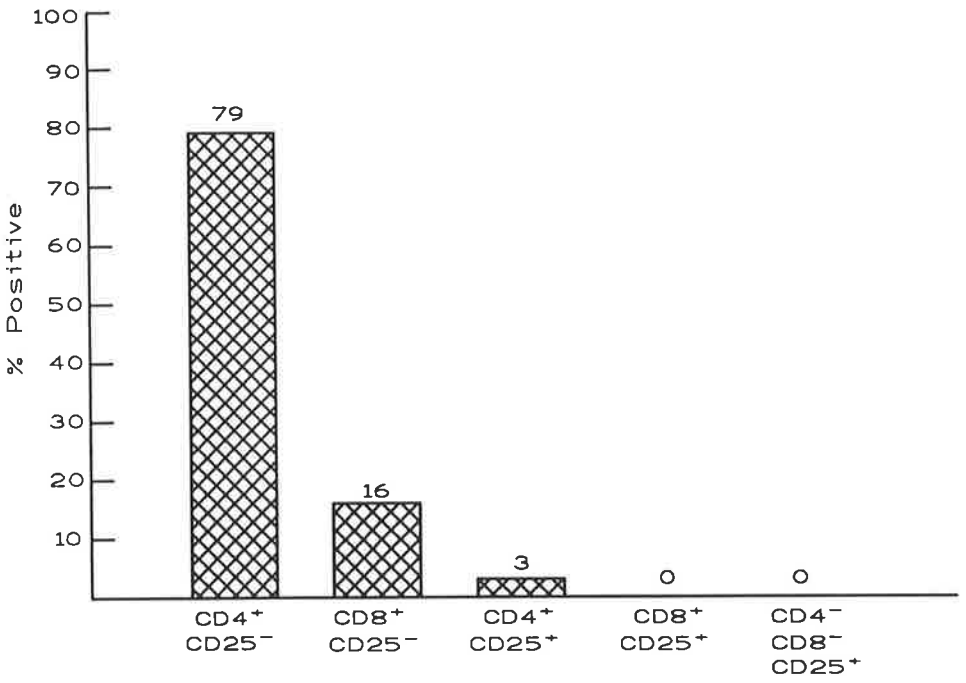
LEP_{20} + HL60-R7



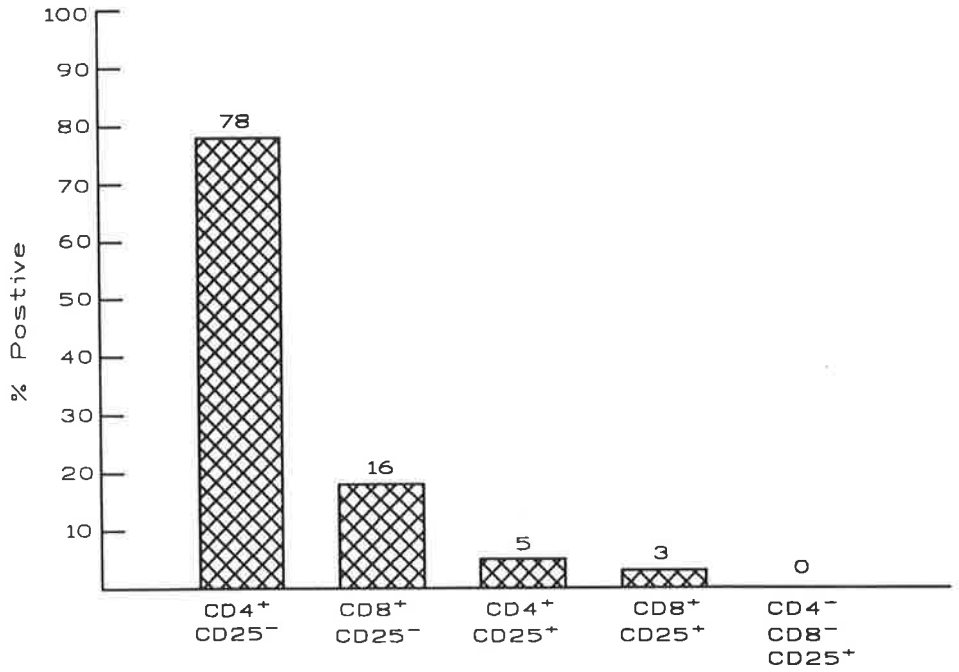
LEP_{20} + RC2a

Figure 4.3.16: (top) LEP_{21} alone.

Figure 4.3.17: (bottom) LEP_{21} + HL-60 cells.



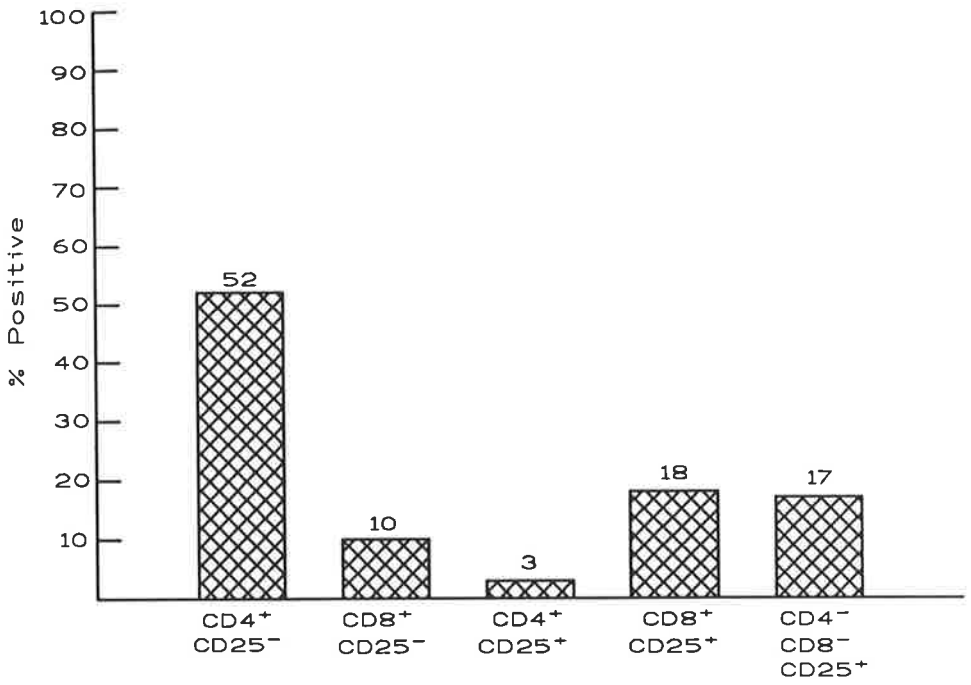
LEP_{21} Alone



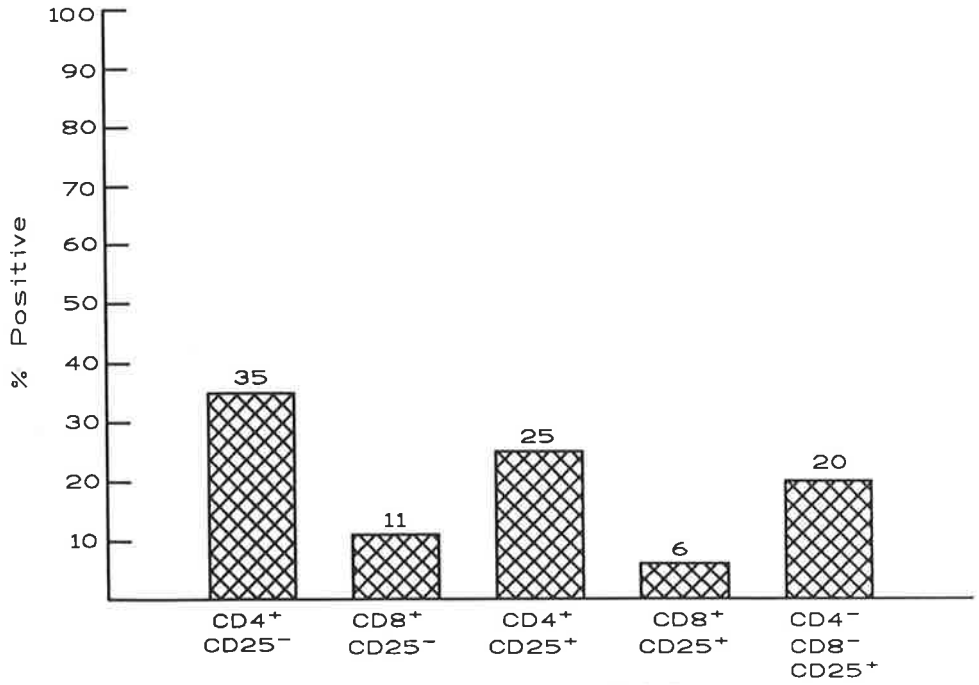
LEP_{21} + HL60

Figure 4.3.18: (top) LEP_{21} + HL-60-R7 cells.

Figure 4.3.19: (bottom) LEP_{21} + RC-2a cells.



LEP_{21} + HL60-R7



LEP_{21} + RC2a

comprised of $CD4^+$ cells, which was not unexpected considering RC-2a cells expressed high levels of MHC class II antigen upon their cell surface. Meanwhile, the responses by LEP_{21} were qualitatively very similar to LEP_{20}, with the most notable difference being that the lymphocytes responding to RC-2a cells possessed a high percentage (20%) of activated $CD4^-$, $CD8^-$ cells, a phenomenon which rarely occurred in subsequent assays performed with other LEP.

The above experiment was performed with freshly purified lymphocytes from normal human peripheral blood. Two pitfalls of using such cells are, 1) a limiting number of lymphocytes could be purified from 20 mls of blood, thus restricting the size of the experiment and making repetition of the experiment virtually impossible; 2) carrying out the purification of the lymphocytes as well as performing an MLC on the one day is a lengthy process. In order to avoid such problems, future experiments were performed with purified lymphocytes derived from buffy coats, thus, instead of a yield of $1.5 - 3 \times 10^7$ lymphocytes from 20 mls of blood, $2 - 5 \times 10^8$ lymphocytes could be obtained from each buffy coat. These lymphocytes were then frozen down in small aliquots (2×10^7/ampoule) and stored in liquid nitrogen until required.

4.4 Determination of proportion of responding lymphocytes positive for CD3

Here OKT3, an anti-CD3 mAb (Kung *et al*, 1979) was used as a probe for the determination of the proportion of $CD3^+$ cells in the responding populations. The reason for using this antibody was to gain an idea as to the possible phenotype of the responding lymphocytes since γδ lymphocytes express CD3 whereas NK cells do not. Dual colour immunofluorescence using OKT3 and anti-CD25 could not be done since a directly labelled OKT3 was not readily available.

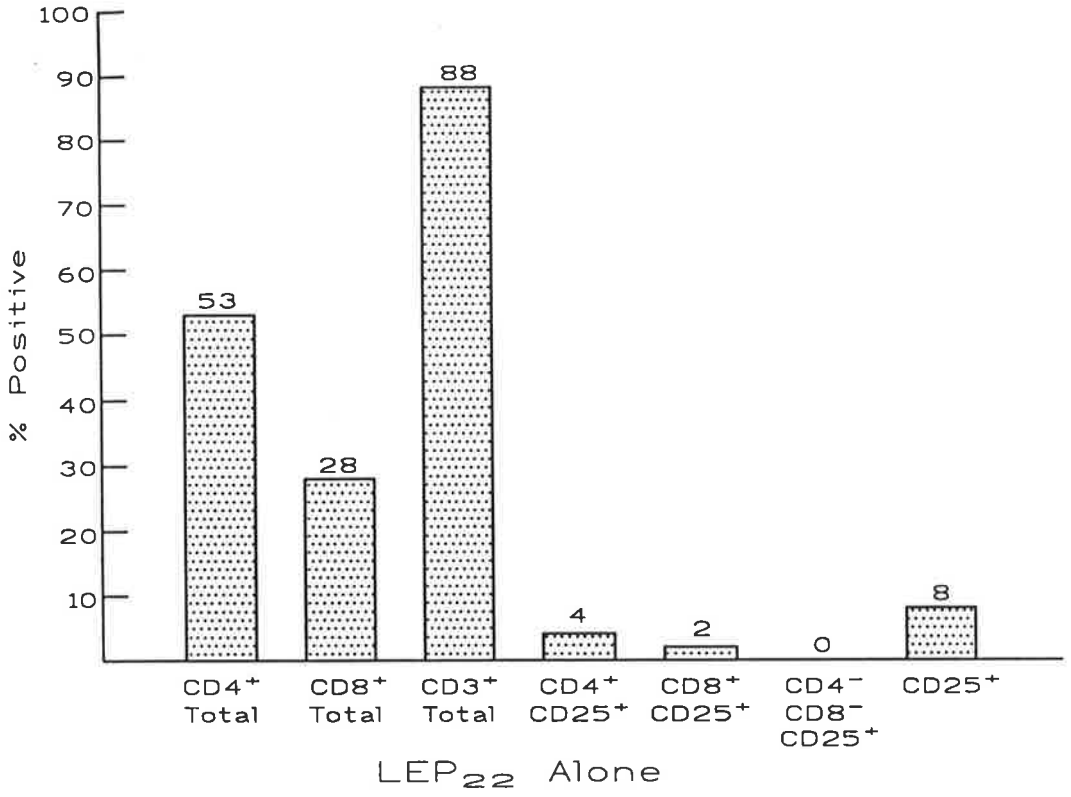
The results obtained with respect to the expression of CD4, CD8 and CD25 were much the same as the previous experiment, in terms of the relative phenotypic proportions of activated lymphocytes. Figures 4.4.1 - 4 show that with respect to the

Figure 4.4.1 - 4: **Characterization of the LEP_{22} and LEP_{23} response to HL-60, HL-60-R7 and RC-2a cells.** Results of dual direct immunofluorescence and IIF assays performed on LEP_{22} cultured alone or with HL-60, HL-60-R7 or RC-2a cells in MLC for 5 days. mAb used include those described in figure 4.3.12 - 19 as well as the CD3-specific OKT3.

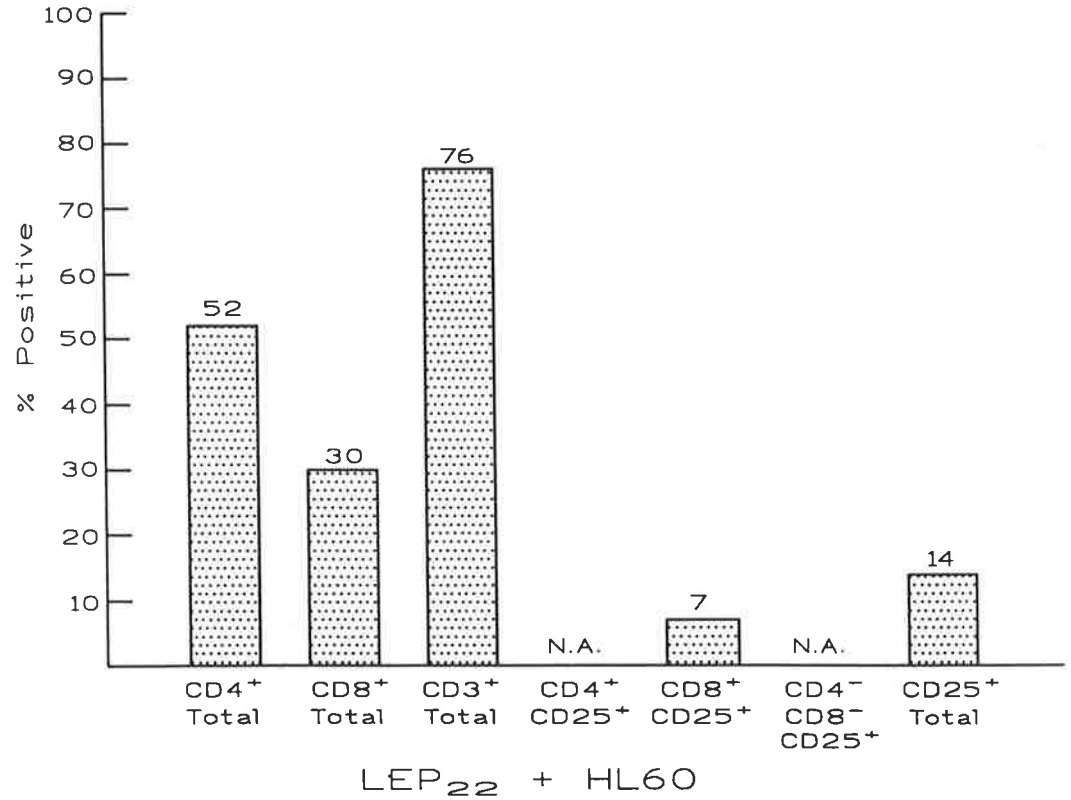
N. A. = not available

Figure 4.4.1: (top) LEP_{22} alone.

Figure 4.4.2: (bottom) LEP_{22} + HL-60 cells.



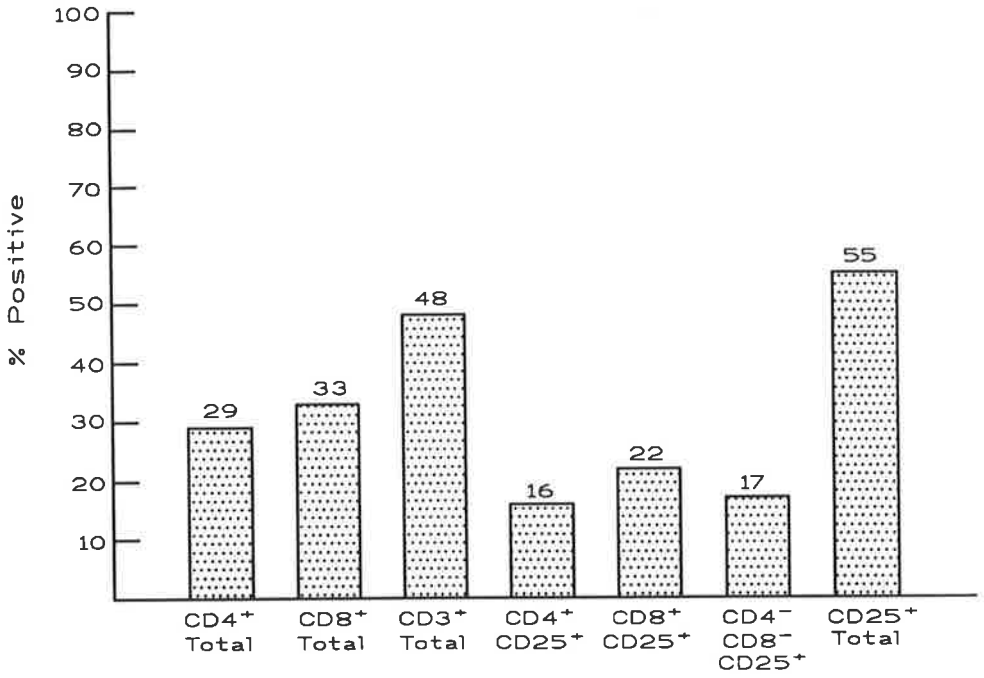
LEP_{22} Alone



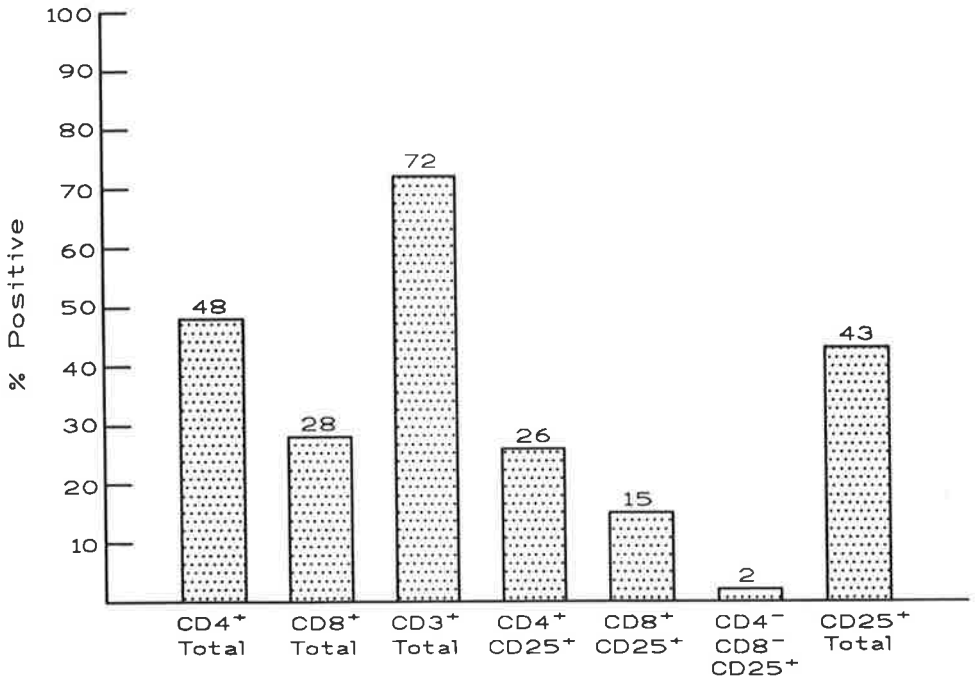
LEP_{22} + HL60

Figure 4.4.3: (top) LEP_{22} + HL-60-R7 cells.

Figure 4.4.4: (bottom) LEP_{22} + RC-2a cells.



LEP_{22} + HL60-R7



LEP_{22} + RC2a

other two stimulator populations, those LEP cultured with HL-60-R7 cells possessed a much lower proportion of $CD3^+$ lymphocytes. Similar results were obtained for another LEP (LEP_{23}) used in this experiment. This result steered us further toward the idea that the lymphocytes responding to HL-60-R7 cells may be partly comprised of NK cells, given that many of the activated lymphocytes are $CD4^-$, $CD8^-$ and that γδ lymphocytes generally express CD3 (Borst *et al*, 1987).

As a consequence of the results obtained here, the decision was made to purchase an anti-CD16 monoclonal antibody directly conjugated to PE (Leu11c) as opposed to an antibody specific for the γδ receptor. CD16 is a marker which, in the lymphocyte population, is expressed almost exclusively by NK cells (Perussia and Trinchieri, 1984).

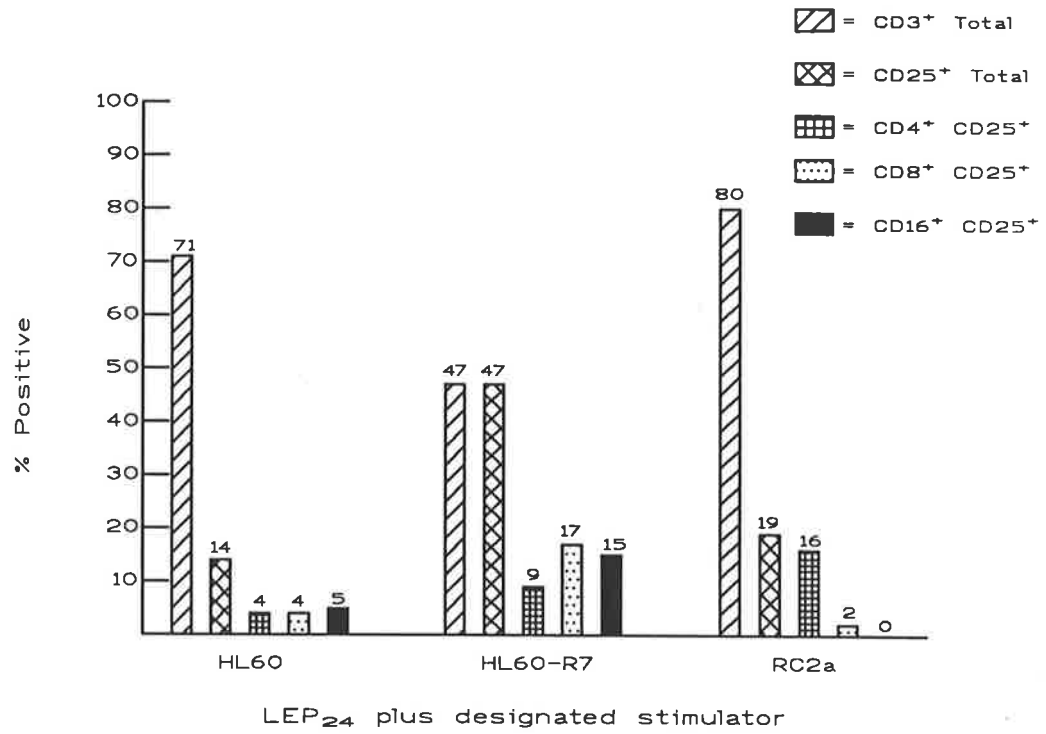
4.5 Detection of activated $CD16^+$ lymphocytes

A MLC similar to that performed previously was carried out on two new sets of responders, LEP_{24} and LEP_{25}, where a typical proliferative response was noted. A dual colour immunofluorescence analysis was performed on day 5, using the reagents described in section 4.4 as well as Leu11c, to detect NK cells. The results of this phenotypic analysis are shown in figures 4.5.1 and 4.5.2.

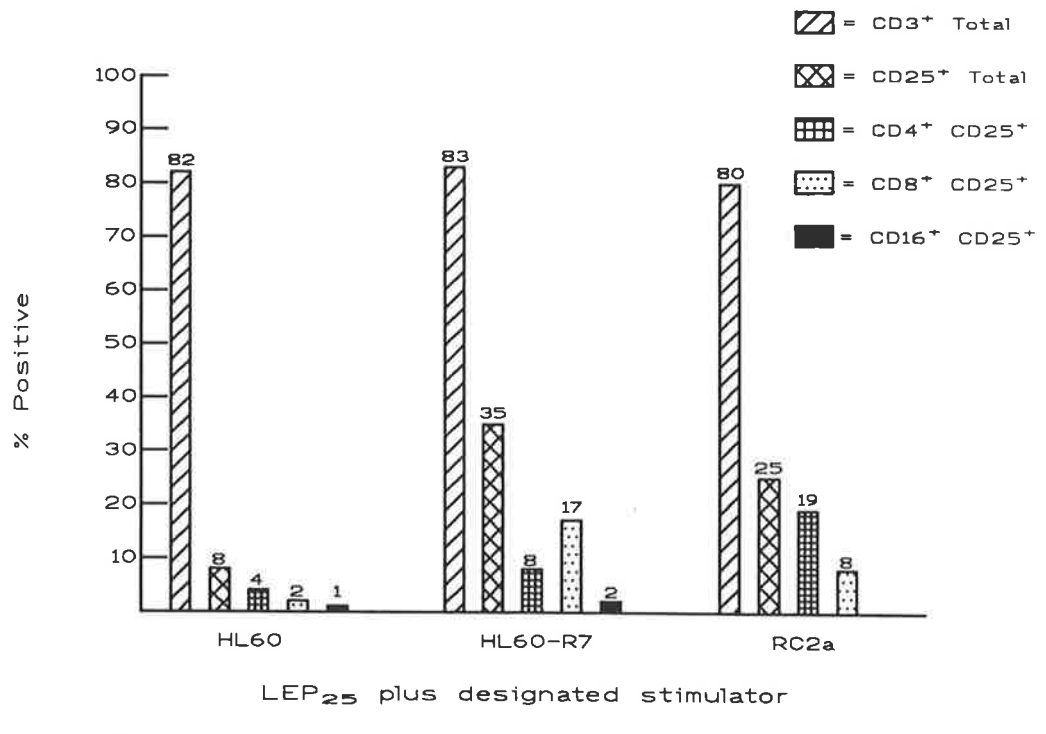
The results obtained for LEP_{24} revealed that a large percentage (15%) of cells were activated $CD16^+$ lymphocytes, reinforcing the notion derived from previous experiments that the $CD4^-$, $CD8^-$, $CD25^+$ populations induced by HL-60-R7 cells could have been largely composed of NK cells. Meanwhile, a smaller percentage (≈ 9%) were activated $CD4^+$ lymphocytes compared to 17% of $CD8^+$ lymphocytes expressing CD25. This experiment was repeated on LEP_{24}, yielding similar results to those described above.

Figure 4.5.1: **Characterization of response of LEP_{24} to HL-60, HL-60-R7 and RC-2a cells.** Results of dual direct immunofluorescence performed on LEP_{24} + HL-60, HL-60-R7 and RC-2a cells cultured for 5 days under MLC conditions. mAb used included biotinylated OKT4 and OKT8, directly fluoresceinated anti-CD25 and an anti-CD16 mAb directly labelled with phycoerythrin.

Figure 4.5.2: **Characterization of response of LEP_{25} to HL-60, HL-60-R7 and RC-2a cells.** Results of dual colour immunofluorescence performed on LEP_{25} + HL-60, HL-60-R7 and RC-2a cells cultured for 5 days under MLC conditions. mAb used are described in figure 4.5.1.



LEP_{24} plus designated stimulator



LEP_{25} plus designated stimulator

The response of LEP_{24} to RC-2a cells was almost solely comprised of $CD4^+$ lymphocytes, with a small percentage (2%) of responding $CD8^+$ lymphocytes and no detectable $CD16^+$ cells, activated or unactivated. FL1 (CD25) vs FL2 (CD4, CD8 or CD16) dot-plots of LEP_{24} responses to HL-60-R7 and RC-2a cells are shown in figures 4.5.3 - 8. The other responder population used in this experiment, LEP_{25}, gave only a meagre (2%) $CD16^+$ response to HL-60-R7 cells, however, activated $CD8^+$ lymphocytes once again predominated over activated $CD4^+$ lymphocytes (Figure 4.5.2). It was noted that the LEP_{24} possessed 20% $CD16^+$ cells at the initiation of the MLC whilst LEP_{25} only possessed 5% (see table 4.2.1).

A summary of the dual colour immunofluorescence studies performed on a number of other LEP is displayed in table 4.5.1. From the types of responses obtained thus far it would appear that a general pattern was being followed, which was largely independent of the responder lymphocyte population used. Specifically, the majority of the responses to the myelomonocytic cell line, RC-2a, were CD4 oriented, with often little or no CD16 response, although there was the exception of LEP_{21} in section 4.3. In contrast, HL-60-R7 cells preferentially activated $CD8^+$ and\or $CD16^+$ lymphocytes rather than $CD4^+$ lymphocytes. It is feasible that NK cells of the CD16- phenotype were also responding, however their presence was not tested for*. This general trend raised the question tentatively proposed at the beginning of this chapter; do $CD4^+$ lymphocytes play a crucial role in the activation process, or are they merely ineffectual and/or inadvertently activated bystanders? Sections 4.7 and 4.8 attempt to answer this question.

4.6 Cytolytic activity of LEP activated in the MLC.

Having discovered that the majority of the LEP activated by HL-60-R7 cells were $CD8^+$ and/or $CD16^+$ it was of interest to determine whether these lymphocytes had an enhanced capacity to lyse a variety of targets, which included the stimulating cells, HL-60, HL-60-R7 and RC-2a and the NK-susceptible K562 cells. This was

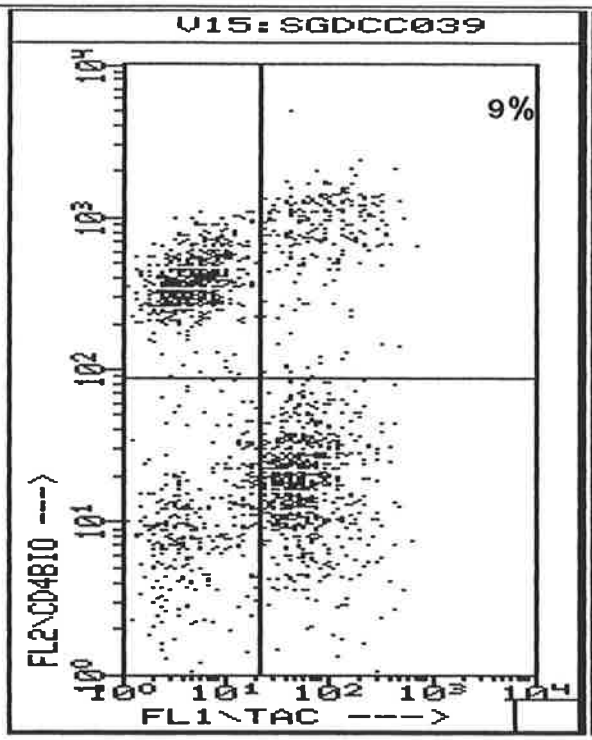
*(Nagler *et al*, 1989)

Figure 4.5.3 - 8: **Phenotypic percentages of activated LEP_{24}.** Dot-plots (log fluorescence 1 intensity (FL1) vs log fluorescence 2 intensity (FL2)) of LEP_{24} cultured with HL-60-R7 or RC-2a cells for 5 days in a MLC and subsequently treated with designated mAb in a dual direct IF assay.

Figure 4.5.3: (top, left) LEP_{24} plus HL-60-R7 cells treated with OKT4-biot plus SPE (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).

Figure 4.5.4: (top, right) LEP_{24} plus HL-60-R7 cells treated with OKT8-biot plus SPE (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).

Figure 4.5.5: (bottom) LEP_{24} plus HL-60-R7 cells treated with Leu11c (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).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FL2\CD4BIO --->
FL1\TAC --->
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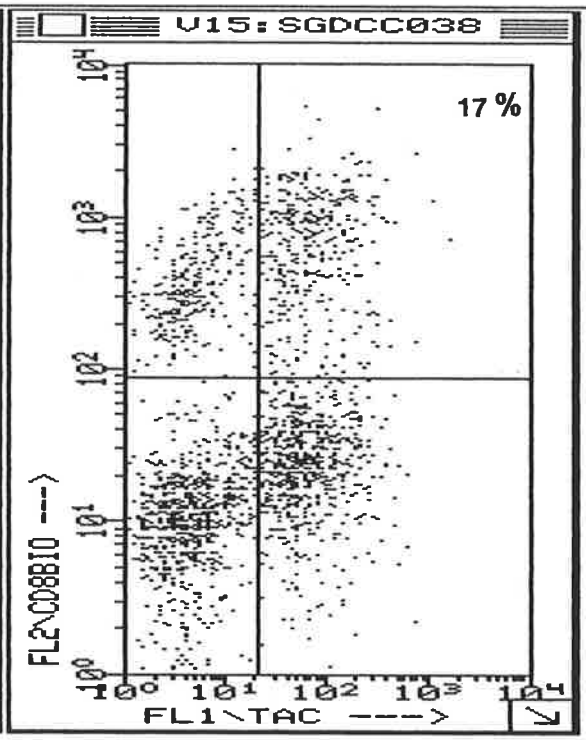
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17%
FL2\CD8BIO --->
FL1\TAC --->
10^0 10^1 10^2 10^3 10^4

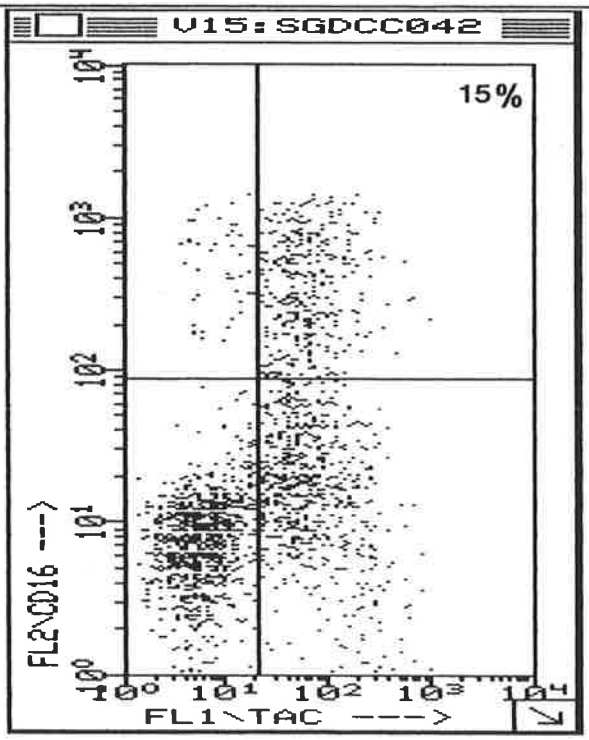
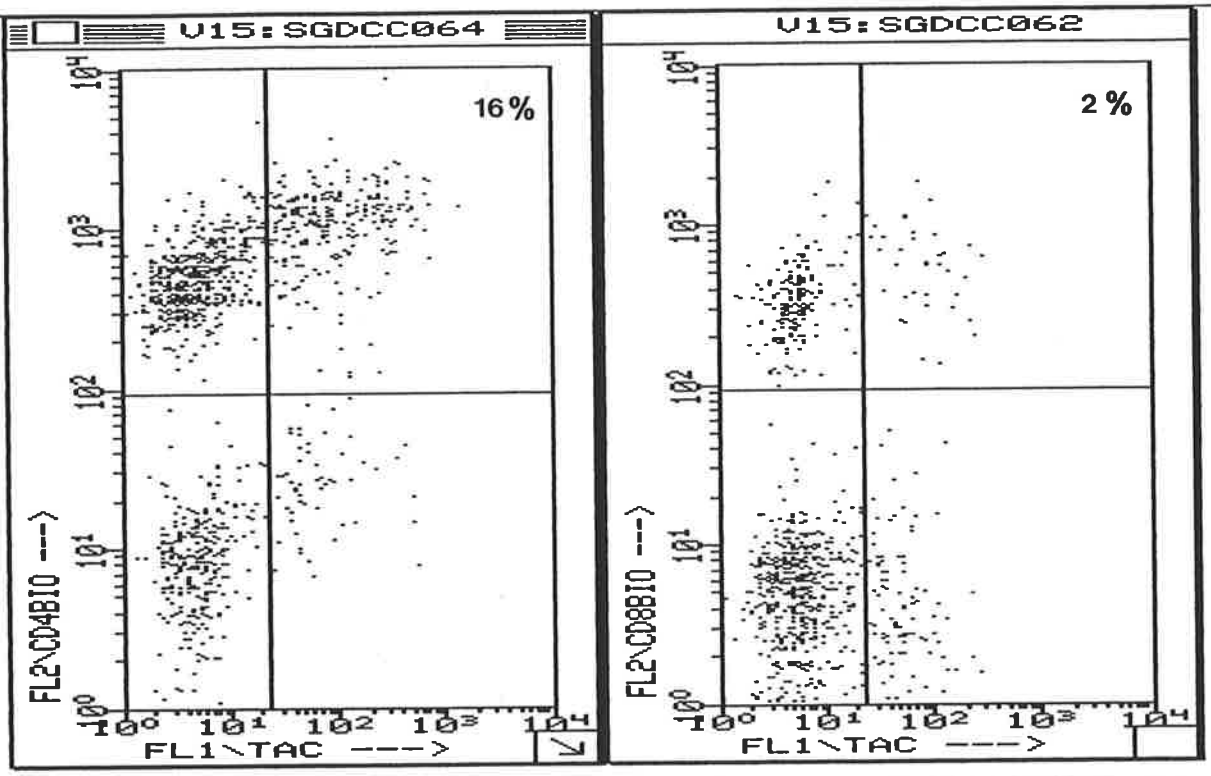

V15:SGDCC042
15%
FL2\CD16 --->
FL1\TAC --->
10^0 10^1 10^2 10^3 10^4

Figure 4.5.6: (top, left) LEP_{24} plus RC-2a cells treated with OKT4-biot plus SPE (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).

Figure 4.5.7: (top, right) LEP_{24} plus RC-2a cells treated with OKT8-biot plus SPE (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).

Figure 4.5.8: (bottom) LEP_{24} plus RC-2a cells treated with Leu11c (FL2) and a directly fluoresceinated anti-CD25 mAb (FL1).


V15:SGDCC064
16%
FL2\CD4BIO --->
FL1/TAC --->
V15:SGDCC062
2%
FL2\CD8BIO --->
FL1/TAC --->
V15:SGDCC065
<1%
FL2\CD16 --->
FL1/TAC --->

Mean percentage (and range) of $CD25^+$ cells expressing CD4, CD8 or CD16		
	Lymphocyte response to HL60-R7	Lymphocyte response to RC2A
CD4	16 (6 - 29) n = 9	73 (49 - 87) n = 8
CD8	39 (17 - 51) n = 9	27 (10 - 52) n = 9
CD16	32 (6 - 56) n = 5	< 1 (< 1 - 2) n = 5

n = number of experiments

Table 4.5.1: **Phenotype of activated LEP: A summary**. Summary of dual colour IF assays performed on LEP cultured with RC-2a or HL-60-R7 cells for 5 days under MLC conditions.

ascertained by means of a cytotoxicity assay, where lymphocytes and ^{51}Cr-labelled targets were cultured together for 4 hr at an effector:target ratio of 50:1. The results for LEP_8 and LEP_9, are shown in tables 4.6.1 - 2. These results were shown to be reproducible for the same LEP and two other LEP (LEP_{26} and LEP_{27}).

Lymphocytes cultured alone for 6 days were shown to possess no capacity to lyse any of the targets. However freshly thawed LEP, which were used at the initiation of the MLC, were capable of lysing approximately 70% of K562 cells implying the presence of active NK cells prior to the addition of LEP to the MLC. LEP cultured with HL-60, HL-60-R7 or RC-2a cells had, in general, a further enhanced capacity to lyse K562 cells. Importantly, LEP cultured for 6 days with HL-60-R7 had an enhanced capacity to lyse HL-60, HL-60-R7 and RC-2a cells compared to LEP cultured for 6 days with HL-60 or RC-2a cells. It was additionally noted that HL-60-R7 was more susceptible to cytolysis in general than HL-60 cells. Another observation was that LEP cultured with RC-2a cells in the MLC were particularly inefficient at lysing RC-2a cells when these leukaemic cells were used as targets.

4.7 Determining the role of $CD4^+$ lymphocytes and IL-2 in the LEP response to HL-60-R7 and RC-2a cells

In an effort to answer the question proposed at the conclusion of section 4.5 the removal of the $CD4^+$ cells from LEP was attempted by means of an indirect panning technique. In brief LEP were incubated with OKT4 (anti-CD4: IgG_{2b}) or 1A6.11 (isotype control mAb) prior to their addition to a panning dish coated with goat antibodies specific for mouse Ig. Analysis, via indirect immunofluorescence, of the cells which did not adhere to the dish revealed that < 2% were $CD4^+$. The results obtained for LEP_{16} are displayed, as an example, in figures 4.7.1 - 4. This result proved to be consistent for the majority of lymphocyte populations tested. Hence, some LEP were depleted of their $CD4^+$ fraction prior to cryopreservation.

	% Cytolysis			
	Target			
	K562	HL60	HL60-R7	RC-2a
LEP_8 alone (6 days in MLC)	< 1	< 1	< 1	< 1
LEP_8 alone (Freshly thawed)	72 ± 2	16 ± 5	27 ± 1	36 ± 10
LEP_8 + HL60	80 ± 3	35 ± 18	57 ± 1	48 ± 1
LEP_8 + HL60-R7	86 ± 1	61 ± 1	98 ± 8	90 ± 6
LEP_8 + RC-2a	88 ± 6	44 ± 2	71 ± 2	38 ± 3

	% Cytolysis			
	Target			
	K562	HL-60	HL60-R7	RC-2a
LEP_9 alone (6 days in MLC)	< 1	< 1	< 1	< 1
LEP_9 alone (Freshly thawed)	73 ± 4	11 ± 4	30 ± 6	13 ± 4
LEP_9 + HL-60	87 ± 1	36 ± 16	70 ± 6	30 ± 5
LEP_9 + HL60-R7	91 ± 9	70 ± 1	90 ± 6	42 ± 4
LEP_9 + RC-2a	72 ± 6	19 ± 2	45 ± 1	8 ± 1

Tables 4.6.1 - 2: **Cytotoxicity assay.** These assays were performed with LEP_8 or LEP_9 which had been cultured for 6 days alone or with HL-60, HL-60-R7 or RC-2a cells under MLC conditions prior to their addition to the cytotoxicity assay. Ratio of effector cells:target cells was 50:1. Freshly thawed LEP were also used in these assays. Percentage cytolysis was calculated as described in the materials and methods (section 2.10).

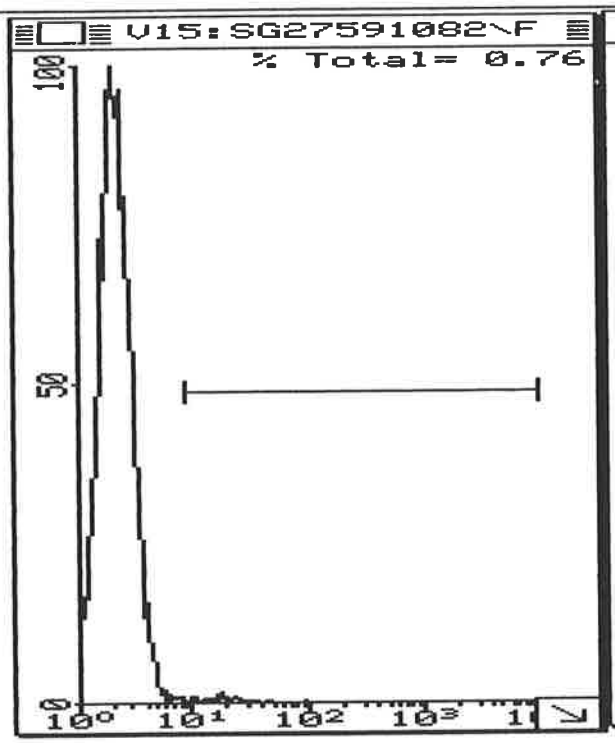
Figure 4.7.1 - 4: Removal of $CD4^+$ lymphocytes from LEP_{14} by the panning technique. Histograms (log fluorescence 2 intensity (FL2) vs cell no.) of dummy-panned and test-panned LEP_{16} subsequently treated with designated mAb (or SPE) in an IF assay. Positioning of the regions was consistent and percentage of events within each region is displayed in each figure.

Figure 4.7.1: (top, left) Dummy-panned LEP_{16} treated with SPE alone.

Figure 4.7.2: (top, right) Dummy-panned LEP_{16} treated with OKT4-biot plus SPE.

Figure 4.7.3: (bottom, left) Test-panned LEP_{16} treated with SPE alone.

Figure 4.7.4: (bottom, right) Test-panned LEP_{16} treated with OKT4-biot plus SPE.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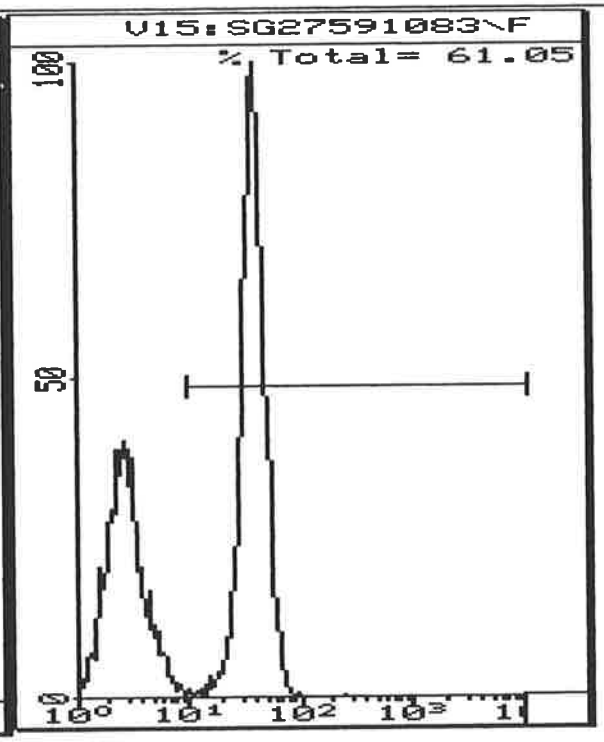
50
0
10⁰ 10¹ 10² 10³ 1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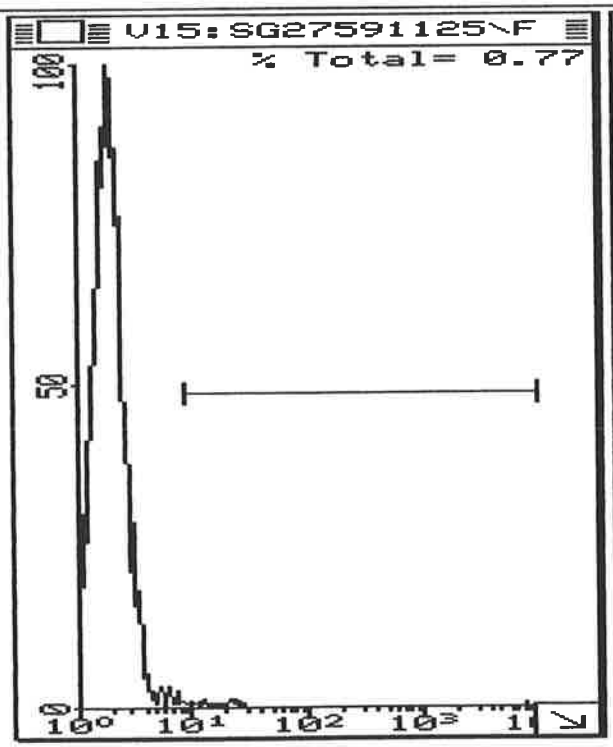
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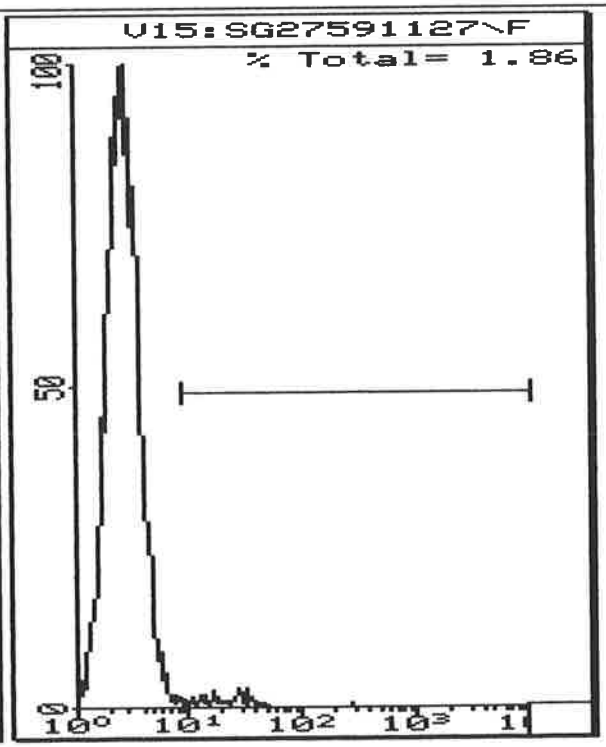
V15:SG27591127\F
% Total= 1.86
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50
0
10⁰ 10¹ 10² 10³ 1

The importance of $CD4^+$ lymphocytes in the responses to both HL-60-R7 and RC-2a cells was tested through MLC involving either CD4 depleted populations or control populations (panned using 1A6.11) as responders. As usual the stimulators used were HL-60, HL-60-R7, and RC-2a cells. The levels of proliferation of 4 different sets of lymphocyte populations (LEP 7, 14, 15 and 16) were measured and the results, for LEP_{14} and LEP_{15}, are displayed in figures 4.7.5 and 4.7.6 respectively. A summary of the results obtained with 4 different LEP is displayed in table 4.7.1.

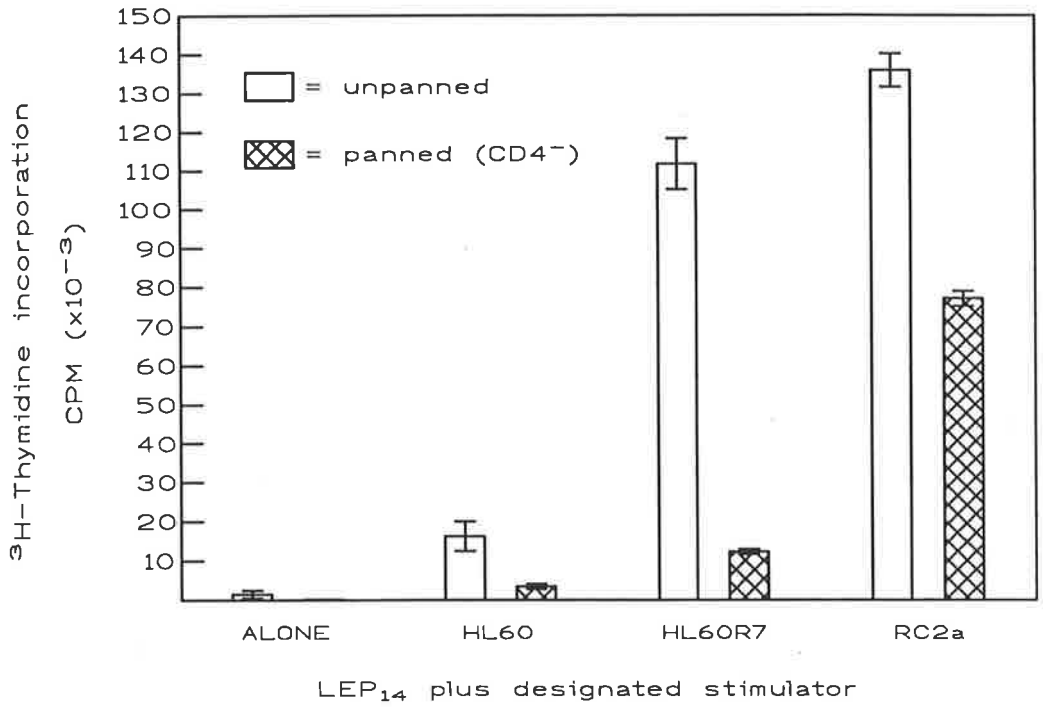
It can be deduced from these data, that $CD4^+$ lymphocytes played a major role with respect to the proliferative response to HL-60-R7 cells considering that up to 90% of the proliferative response was lost when $CD4^+$ cells were removed. The responses to RC-2a cells were not abrogated to the same extent as the responses to HL-60-R7 cells suggesting that RC-2a cells may have been capable of stimulating substantial proliferation of $CD4^-$ lymphocytes. Alternatively, the 1 - 2% of contaminating $CD4^+$ lymphocytes may have been triggered to produce sufficient IL-2 which in turn may have stimulated the proliferation of $CD4^-$ lymphocytes. Thus, in subsequent experiments $CD4^+$ lymphocytes were removed using a cell sorter which generally resulted in lymphocyte populations containing $\leq$ 1% $CD4^+$ lymphocytes. Similar results to those described above were yielded and a summary of these results is given in table 4.7.2.

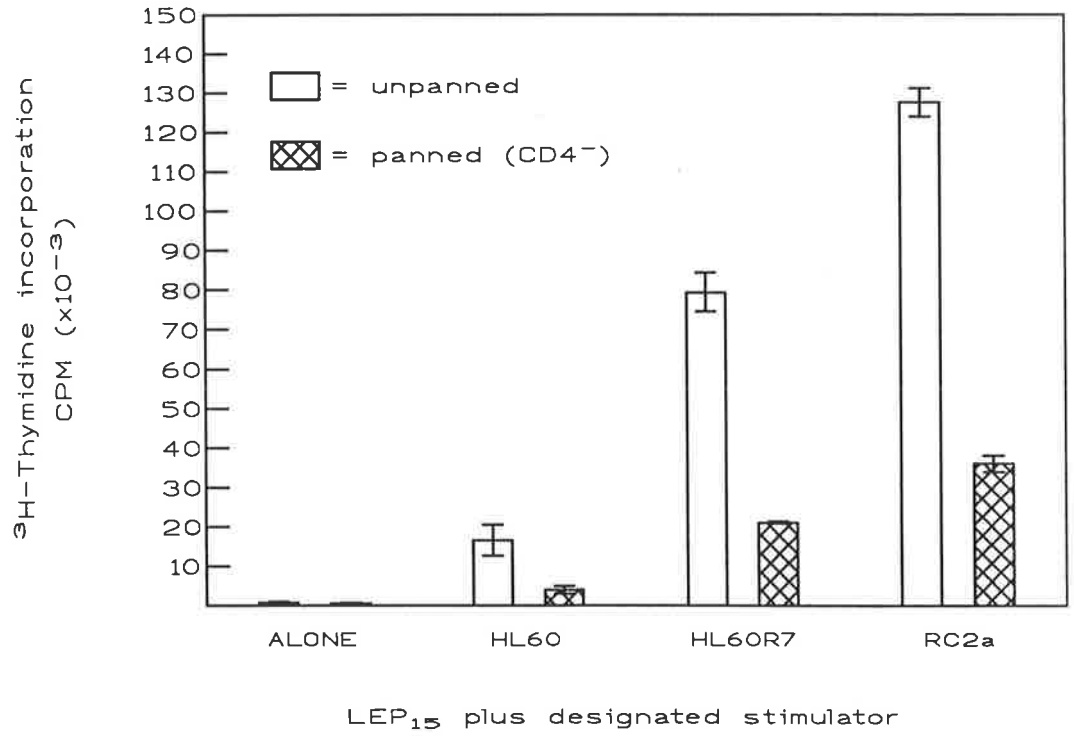
Since $CD4^+$ lymphocytes seemed to be of importance in the response to HL-60-R7 cells, it was of interest to investigate whether the primary function of these cells was to supply IL-2 to the neighbouring $CD8^+$ and $CD16^+$ lymphocytes. Thus, on day 3 of the MLC containing sorted $CD4^-$ lymphocytes, increasing doses (0.05 - 10 U/ml) of recombinant IL-2 were added and the levels of proliferation were subsequently measured by the usual ^{3}H-thymidine uptake technique where wells were pulsed for 16 hours on day 5 of the MLC. Results obtained for LEP_{30} and LEP_8 showed that 10 U/ml of IL-2 could adequately replenish the response to HL-60-R7 cells and almost for RC-2a cells as well (figures 4.7.7 - 8). However, on two occasions, even 0.05 U/ml led

Figure 4.7.5 - 6: **Effect of removing $CD4^+$ lymphocytes on LEP response in MLC.** ^{3}H-thymidine uptake assay involving dummy- and test-panned LEP_{14} or LEP_{15} cultured independently with HL-60, HL-60-R7 and RC-2a cells in an MLC for 5 days. Stimulator cells alone gave < 300 cpm.

Figure 4.7.5: (top) Effect of removing $CD4^+$ lymphocytes from LEP_{14}.

Figure 4.7.6: (bottom) Effect of removing $CD4^+$ lymphocytes from LEP_{15}.


150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
= unpanned
= panned (CD4⁻)
^{3}H-Thymidine incorporation
CPM (x10^{-3})
ALONE
HL60
HL60R7
RC2a
LEP$_{14}$ plus designated stimulator


150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
= unpanned
= panned (CD4⁻)
^{3}H-Thymidine incorporation
CPM (x10^{-3})
ALONE
HL60
HL60R7
RC2a
LEP$_{15}$ plus designated stimulator

Proliferation of panned LEP (expressed as a percentage of the proliferation of "dummy-panned" LEP)			
	Mean	Mode	Range
LEP + HL60	62 ± 39 (p = 0.446)	22	16 - 200
LEP + HL60-R7	21 ± 8 (p = 0.001)	15	5 - 49
LEP + RC2a	53 ± 16 (p = 0.032)	57	16 - 98

n = 4

Table 4.7.1: Effect of removing $CD4^+$ cells from LEP by panning: A summary.

Mean stimulation of proliferation of LEP, which had the $CD4^+$ lymphocytes removed by panning, relative to the stimulation of proliferation of the dummy-panned LEP. All MLC were performed using 1 x 10^5 responders/well. Probabilities (p) were calculated using the paired t test.

n = number of experiments

* Each experiment was performed with a different LEP

Proliferation of sorted LEP expressed as a percentage of the proliferation of "dummy-sorted" LEP			
	Mean	Mode	Range
LEP + HL60	63 ± 37 (p = 0.328)	50	3 - 150
LEP + HL60-R7	31 ± 9 (p = 0.005)	34	6 - 37
LEP + RC2a	51 ± 8 (p = 0.019)	50	36 - 70

n = 4

Table 4.7.2: Effect of removing $CD4^+$ cells from LEP by sorting: A summary.

Mean percentage stimulation of proliferation of LEP, which had the $CD4^+$ lymphocytes removed by sorting, relative to the stimulation of proliferation of the dummy-panned LEP. All MLC were performed using 1 x 10^5 responders /well. Probabilities (p) were calculated using the paired t test.

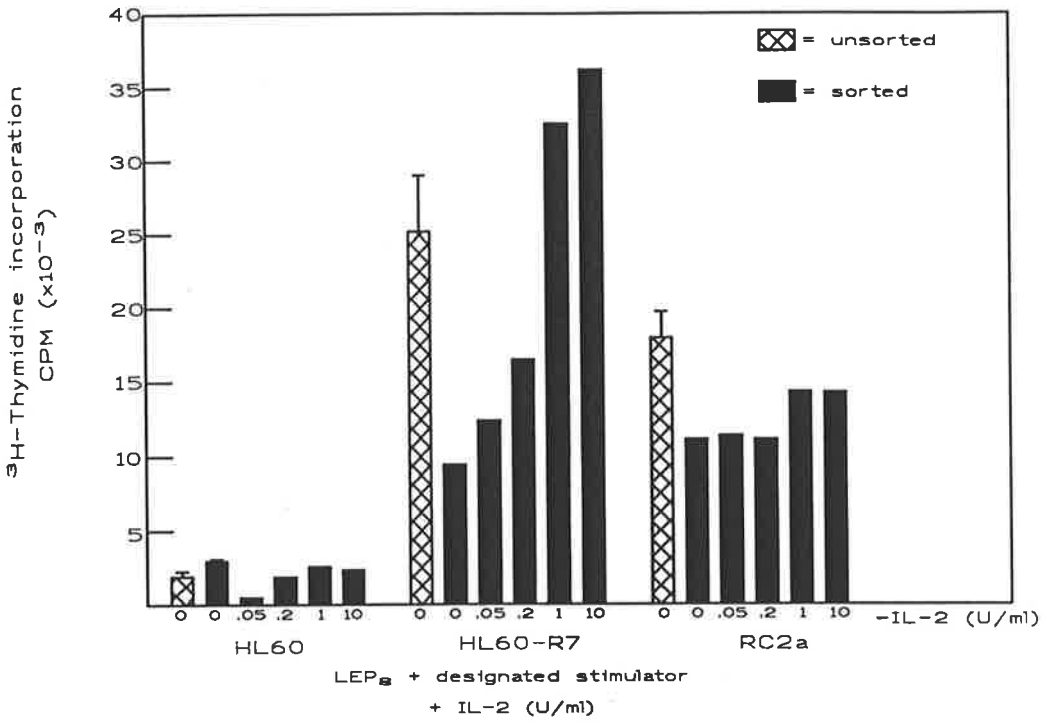
n = number of experiments

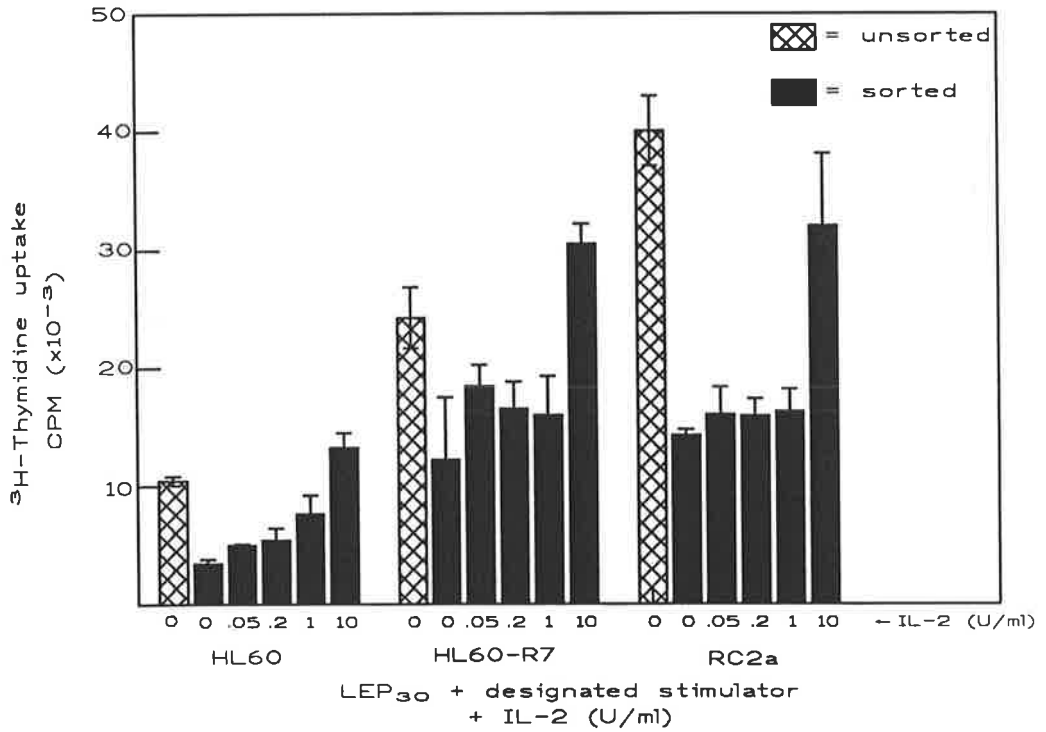
* Each experiment was performed with a different LEP

Figures 4.7.7 - 9: Effect of IL-2 on the response of CD4$^-$ LEP to HL-60, HL-60-R7 and RC-2a cells. Here ^{3}H-thymidine uptake assays show the effect of adding IL-2 at day 3 to MLC involving CD4$^-$ LEP in culture with HL-60, HL-60-R7 or RC-2a cells. All LEP cultured alone, or in the presence of the various concentrations of IL-2 yielded < 200 cpm.

Figure 4.7.7: (top) MLC with CD4$^-$ LEP_8 as responders. (There were not enough lymphocytes for replicate wells)

Figure 4.7.8: (bottom) MLC involved CD4$^-$ LEP_{30} as responders.


40
35
30
25
20
15
10
5
= unsorted
= sorted
^{3}H-Thymidine incorporation
CPM (x10^{-3})
0 0 .05 .2 1 10
0 0 .05 .2 1 10
0 0 .05 .2 1 10
-IL-2 (U/ml)
HL60
HL60-R7
RC2a
LEP$_8$ + designated stimulator
+ IL-2 (U/ml)


50
40
30
20
10
= unsorted
= sorted
^{3}H-Thymidine uptake
CPM (x10^{-3})
0 0 .05 .2 1 10
0 0 .05 .2 1 10
0 0 .05 .2 1 10
← IL-2 (U/ml)
HL60
HL60-R7
RC2a
LEP$_{30}$ + designated stimulator
+ IL-2 (U/ml)

to excessive proliferation to HL-60, HL-60-R7 and RC-2a cells and this is illustrated with the response of LEP_{31} in figure 4.7.9. On the occasions when the experiment was performed with panned, rather than sorted lymphocytes, this excessive response was always observed (n = 3).

4.8 The role of MHC class II molecules in the lymphocyte responses to HL-60-R7 and RC-2a cells

Having determined that $CD4^+$ lymphocytes were important mediators of the proliferative response of LEP to HL-60-R7 cells, and also had a role to play in the response to RC-2a cells, it would not be unreasonable to assume that MHC class II molecules were important contributors to the activation process in both circumstances. This prompted the following MLC inhibition assays involving the addition of a mAb supernatant, FMC 14, specific for a non-polymorphic epitope of MHC class II, to the MLC of LEP and HL-60-R7 or RC-2a cells. An example of the results obtained for HL-60-R7 and RC-2a cells is shown in figure 4.8.1. These results implicated MHC class II molecules in the activation of LEP by both HL-60-R7 and RC-2a cells, due to the substantial and, on occasions, almost complete, inhibitory effects of FMC 14. These inhibition assays were performed 9 times, using various responders, with an average percentage inhibition of 49% for responses to HL-60-R7 and 51% for responses to RC-2a cells. The smaller proliferative responses elicited by control HL-60 cells were also partially inhibited by FMC 14 (54 %).

Indirect evidence would suggest that the inhibitory effects of FMC 14 were not due to non-specific phenomena. Such evidence includes the observation that the isotype control mAb, 3D3.3, which was subsequently shown to have been added to the MLC at concentrations higher than FMC 14 (see appendix A1.2), was not able to inhibit proliferation in a MLC. Also, FMC 4, another mAb specific for a distinct non-polymorphic epitope of MHC class II antigen, possessed similar, in fact more potent, inhibitory effects to those of FMC 14. FMC 4 also potently inhibited the proliferative

Figure 4.7.9: MLC with $CD4^-$ LEP_{31} as responders.

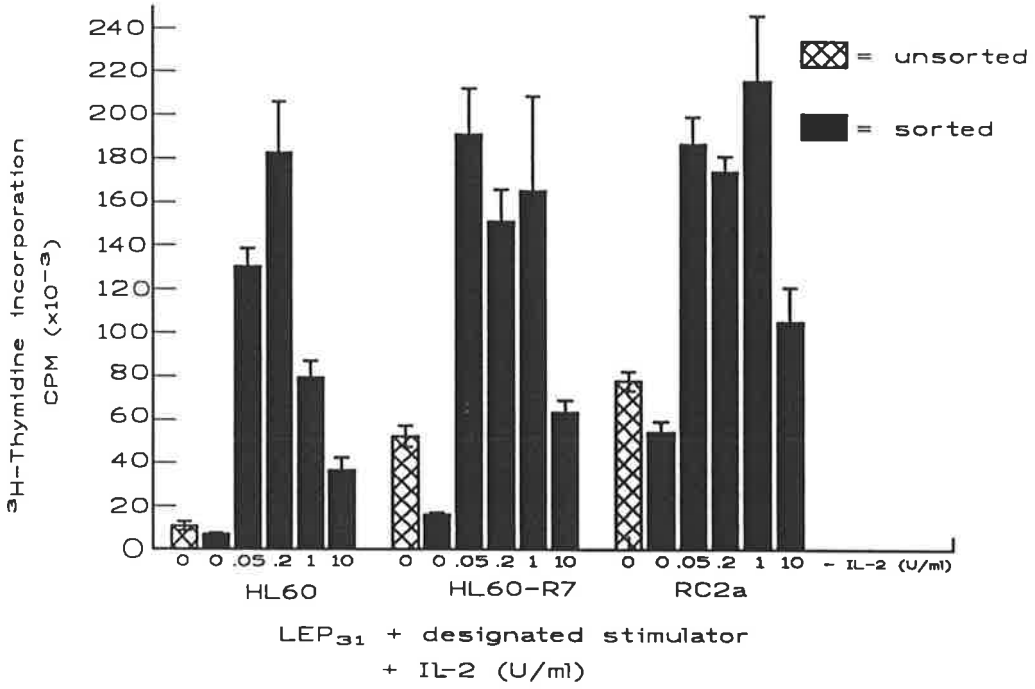
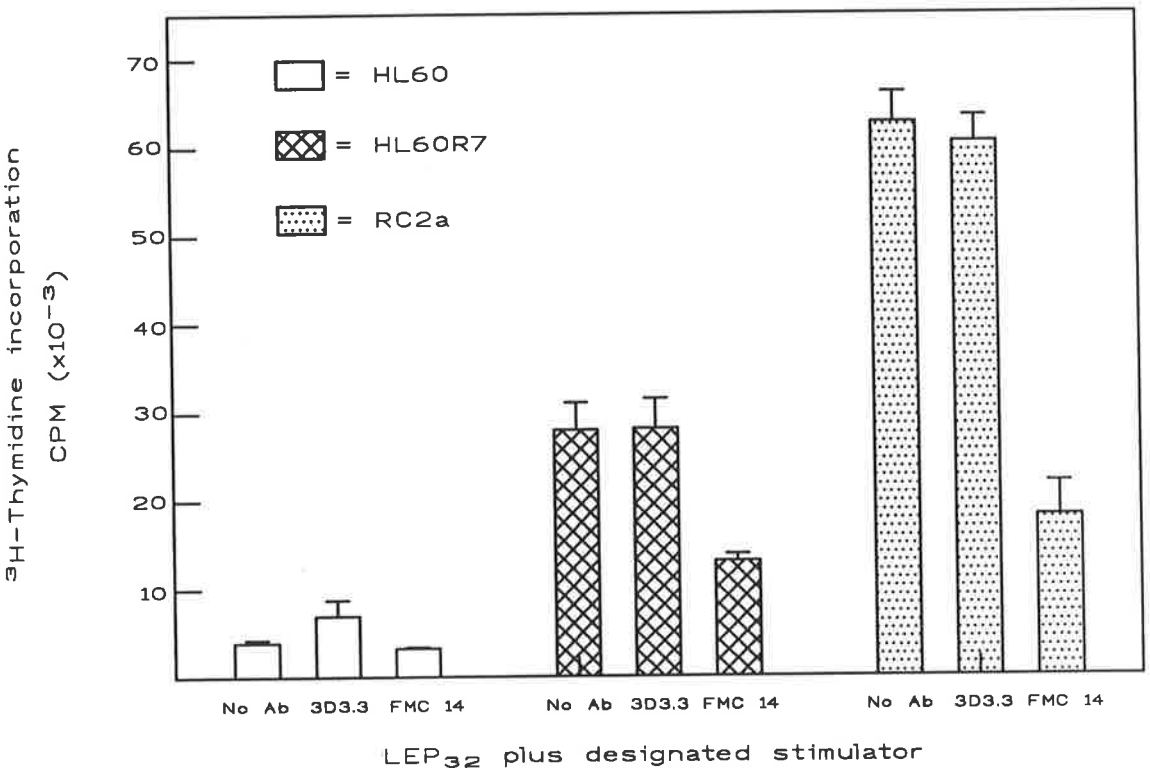

240
220
200
180
160
140
120
100
80
60
40
20
0
^{3}H-Thymidine incorporation
CPM (x10^{-3})
= unsorted
= sorted
0 0 .05 .2 1 10
HL60
0 0 .05 .2 1 10
HL60-R7
0 0 .05 .2 1 10
RC2a
- IL-2 (U/ml)
LEP$_{31}$ + designated stimulator
+ IL-2 (U/ml)

Figure 4.8.1: **Inhibition of LEP proliferation in MLC by mAb to MHC class II molecules.** The inhibitory effect of FMC 14 was tested on the lymphoproliferative responses of LEP_{32} to HL-60, HL-60-R7 or RC-2a cells in a MLC. The 3D3.3 and FMC 14 hybridoma supernatants were added to the cultures at a final dilution of 1/4. Stimulator cells alone gave < 300 cpm and LEP_{32} alone gave < 1000 cpm.

* 3D3.3 is an isotype (IgG1) control for FMC 14


70
60
50
40
30
20
10
= HL60
= HL60R7
= RC2a
^{3}H-Thymidine incorporation
CPM (x10^{-3})
No Ab
3D3.3
FMC 14
No Ab
3D3.3
FMC 14
No Ab
3D3.3
FMC 14
LEP$_{32}$ plus designated stimulator

response to control HL-60 cells. A summary of the experiments performed with FMC 4, FMC 14 and the HLA-DR-specific FMC 15 is given in table 4.8.1. FMC 15 inhibited responses to HL-60-R7 and RC-2a cells much less significantly, but it is possible that this mAb may not be capable of functionally inhibiting interactions with HLA-DR.

In the MLC inhibition assays described thus far, mAb were added at the initiation of the cultures. However, it was of interest to gain an insight as to the stage of the MLC that the anti-MHC class II antigen mAb could be added and still be inhibitory. Thus a MLC was set up where FMC 4 was added, on days 1, 2, 3, 4 and 5, to independent cultures of HL-60-R7 or RC-2a cells plus LEP_{12} or LEP_{33}. The results of this experiment are shown in figures 4.8.2 and 3 respectively, and demonstrate that FMC 4 dramatically inhibited the proliferative responses when added up to 2 days after initiation of the MLC. The inhibitory effect was reduced but still noticeable on days 3 and 4, whilst addition of FMC 4 on day 5 of the MLC had no effect.

Thus it would appear that MHC class II molecules had an important role to play in the responses of LEP to both HL-60-R7 and RC-2a cells. This raised the question as to whether it was MHC class II antigen expression by the responder, stimulator, or both, which was of consequence in these MLC. Since the responses to RC-2a cells were largely CD4 dominated, and considering that RC-2a cells were previously found to express MHC class II molecules in abundance, it was likely that MHC class II expression by the stimulator population was the major contributor to the response. However the situation with HL-60-R7 cells did not appear so clear cut, since these cells did not possess detectable levels of MHC class II antigen upon their cell surface on day seven and only after another 2 - 5 days (i. e. days 9 - 12) under normal culture conditions did a small percentage of cells start to express low to medium levels (see figure 4.8.4), implying that MHC class II expression by the LEP may be of greater relevance. Such a possibility was investigated by removing MHC class II^+ cells from

		Mean percentage inhibition of proliferation		
mAb	Specificity	HL60	HL60-R7	RC-2a
FMC 14	MHC class II (HLA-D)	54 ± 0 (p = 0.006) (n = 2)	49 ± 11 (p = 0.001) (n = 9)	51 ± 8 (p=0.0001) (n = 9)
FMC 4	MHC class II (HLA-D)	90 ± 2 (p = 0.0001) (n = 4)	86 ± 2 (p = 0.0001) (n = 4)	92 ± 5 (p=0.0001) (n = 4)
FMC 15	MHC class II (HLA-DR)	41 ± 16 (p = 0.223) (n = 4)	15 ± 7 (p = 0.456) (n = 4)	11 ± 1 (p= 0.058) (n = 2)

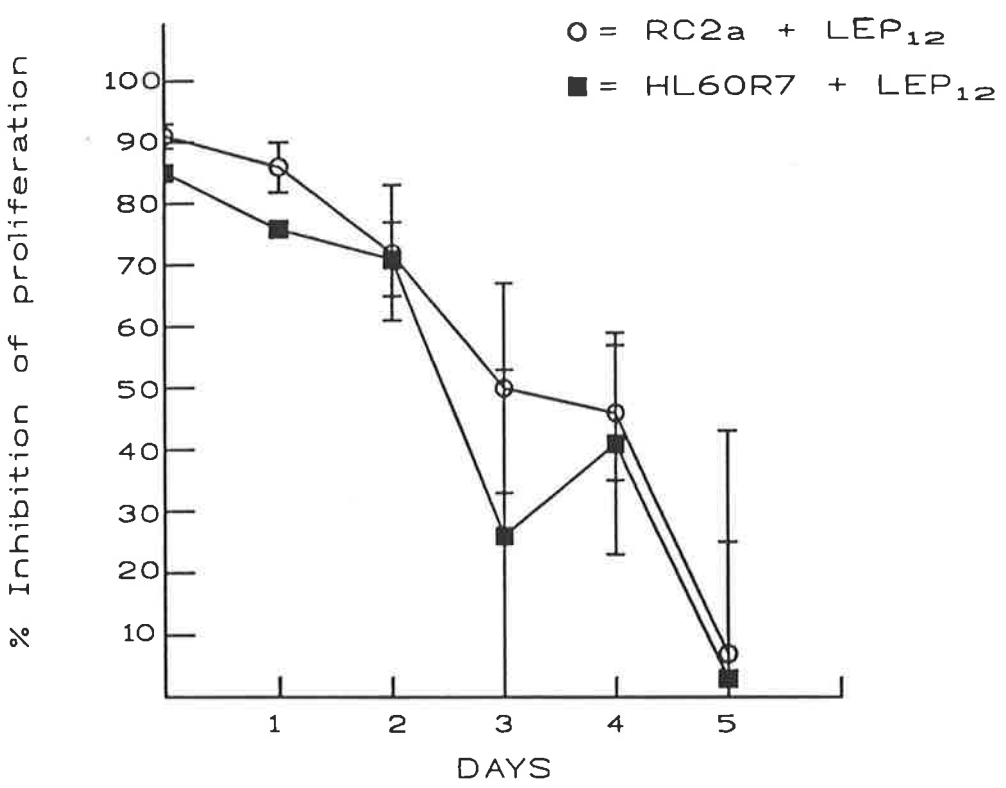
Table 4.8.1: Summary of MLC inhibition assays involving mAb specific for MHC class II molecules. Summary of the inhibitory effects of FMC 15, FMC 14 and FMC 4 upon the proliferative responses of LEP to HL-60, HL-60-R7 or RC-2a cells. Percentage inhibition was calculated by comparing results from the test mAb-treated sample to the results from the control mAb-treated sample. The probabilities (p) are displayed and indicate that the inhibition observed with FMC 4 and FMC 14 was statistically significant for all stimulators, as measured by the paired t-test, whilst the inhibition observed with FMC 15 was not statistically significant.

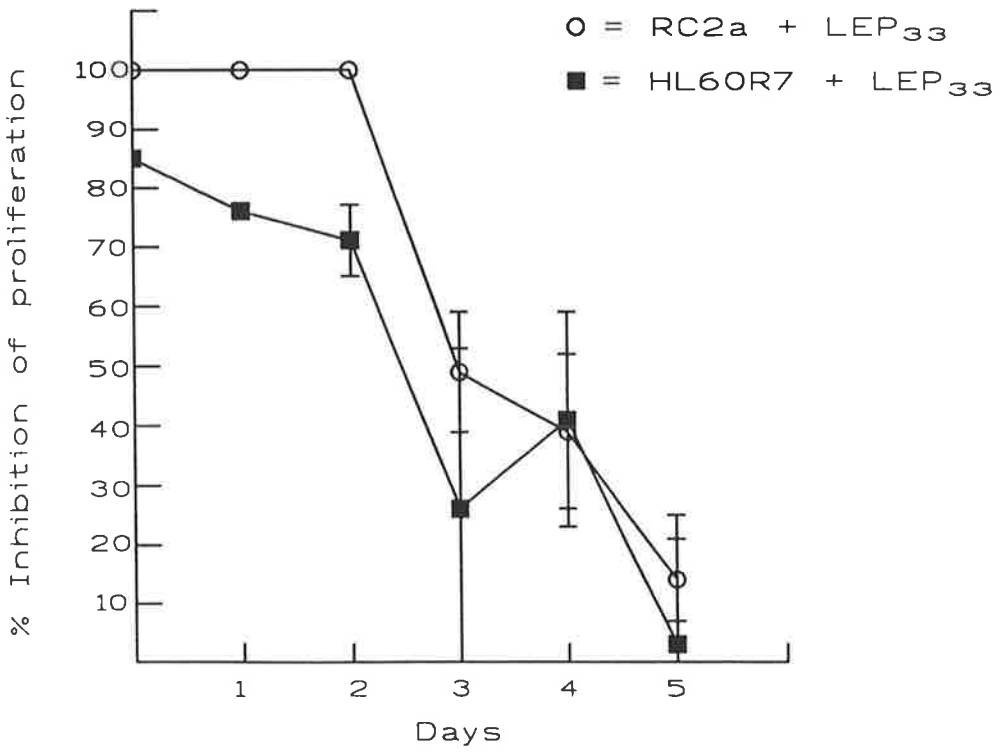
n = number of experiments

Figures 4.8.2 - 3: **Effect of delayed addition to MLC of mAb specific for MHC class II molecules.** ^{3}H-thymidine uptake assay showing the inhibitory effect of FMC 4 upon proliferative response of LEP_{12} or LEP_{33} to HL-60-R7 or RC-2a cells, where FMC 4 was added to MLC on days 0, 1, 2, 3, 4 or 5. The FMC 4 hybridoma supernatant was added to the MLC at a final dilution of 1/4.

Figure 4.8.2: (top) LEP_{12}

Figure 4.8.3: (bottom) LEP_{33}


o = RC2a + LEP12
■ = HL60R7 + LEP12
% Inhibition of proliferation
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
DAYS


o = RC2a + LEP33
■ = HL60R7 + LEP33
% Inhibition of proliferation
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
Days

the lymphocyte populations by cell sorting. The panning technique was only efficient at removing cells expressing medium to high levels of MHC class II molecules, whereas the sorting procedure resulted in the removal of virtually all MHC class II^+ cells such that ≥ 99% of the sorted population were MHC class II^-. Percentages of lymphocyte subsets for both LEP_{32} and LEP_{33} before and after sorting are shown in tables 4.8.2 and 3. From this small study it was evident that most of the MHC class II^+ cells were the $CD8^+$ lymphocytes, whilst $CD4^+$ lymphocytes appeared to express little or no MHC class II antigen.

The results of the MLC for sorted and unsorted, or "dummy-sorted" LEP_{33} are shown in figure 4.8.5 where it was seen that the responses to RC-2a and HL-60-R7 cells were not reduced by the removal of virtually all the MHC Class II^+ cells and this was shown to be reproducible for 5 other LEP. The inhibitory effect on the proliferative response to control HL-60 cells seen for LEP_{33} in this experiment was not observed for the 5 other LEP. FMC 14 was added to the MLC of MHC class II^- lymphocytes plus HL-60-R7 cells in order to determine whether this mAb could still inhibit the proliferative response. The results obtained for LEP_{33} show that FMC 14 still substantially inhibited the response to HL-60-R7, as well as to RC-2a cells (figure 4.8.6). These results were shown to be reproducible for 3 other LEP tested.

Having discovered that MHC class II^+ cells present in the responding population at the initiation of the cultures were apparently unimportant in the response to HL-60-R7 cells, it was deemed possible that MHC class II antigen expression by the stimulator cells themselves was important. Since HL-60-R7 cells cultured alone expressed no to little MHC class II upon their cell surface on days 7 - 9 of the RA induction, which paralleled days 1 - 3 of the MLC, it seemed possible that expression of this molecule was induced during the MLC. Thus MHC class II antigen expression may be induced more rapidly on HL-60-R7 cells under MLC conditions, perhaps because of the action of cytokines, such as IFN-γ, induced as a result of the leukocyte-lymphocyte interactions. In order to investigate this possibility, MLC were set up

Figure 4.8.4: **Expression of MHC class II antigen by RA-treated HL-60 cells.** The percentage of HL-60 cells treated with 10 nM RA expressing MHC class II antigen on days 7 - 12 of the culture, as determined by IIF using the MHC class II-specific mAb, FMC 4.

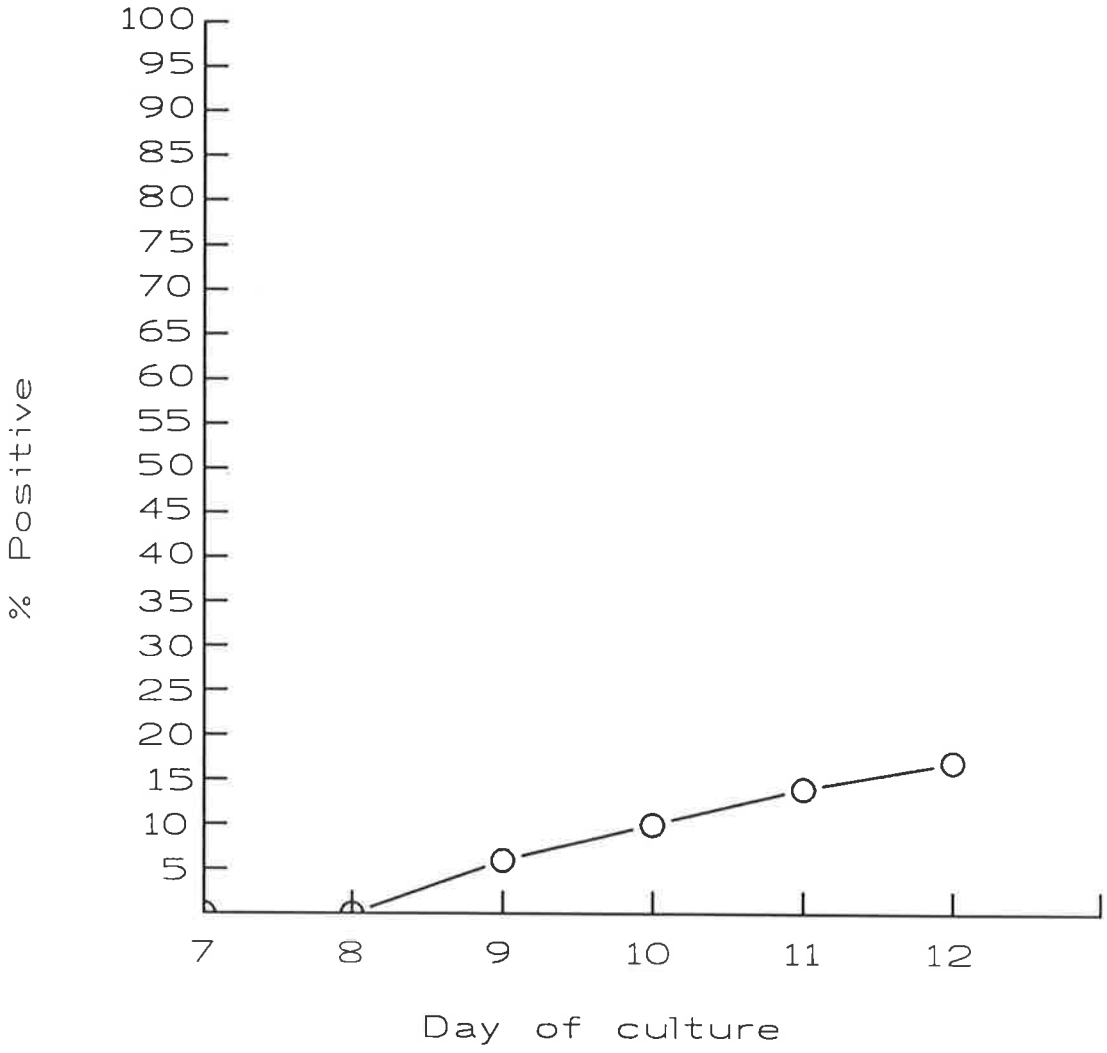

% Positive
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
7
8
9
10
11
12
Day of culture

Table 4.8.2: Removal of MHC class II^+ cells from LEP_{32}. An analysis of cell surface markers of LEP_{32} before and after the removal of MHC class II^+ cells by cell sorting. Percentages were determined through IIF assay using mAb specific for the indicated antigens, as previously described.

Table 4.8.3: Removal of MHC class II^+ cells from LEP_{33}. An analysis of cell surface markers of LEP_{33} before and after the removal of MHC class II^+ cells by cell sorting. Percentages were determined through IIF assay using mAb specific for the indicated antigens, as previously described.

	% positive	
Antigen	LEP_{32} unsorted	LEP_{32} sorted
MHC class II	24	0.5 - 1
CD2	85	92
CD3	78	85
CD4	47	64
CD8	33	22
CD14	1 - 2	< 0.5
CD16	14	13
CD19	1 - 2	< 0.5
CD25	6	1
CD45RO	73	76

	% positive	
Antigen	LEP_{33} unsorted	LEP_{33} sorted
MHC class II	20	< 0.5
CD2	93	95
CD3	77	86
CD4	60	72
CD8	33	17
CD14	0.5 - 1	< 0.5
CD16	14	20
CD19	1 - 2	< 0.5
CD25	8	0.5 - 1
CD45RO	79	73

Figure 4.8.5: **Effect of removal of MHC class II$^+$ cells from LEP$_{33}$.** ^{3}H-thymidine uptake assay showing the effect of removing MHC class II$^+$ cells from LEP$_{33}$ on the lymphoproliferative response to HL-60, HL-60-R7 or RC-2a cells in an MLC. Responders were added at 1 x 10^5/well. Stimulator cells alone gave < 200 cpm and LEP$_{33}$ gave less than < 1500 cpm.

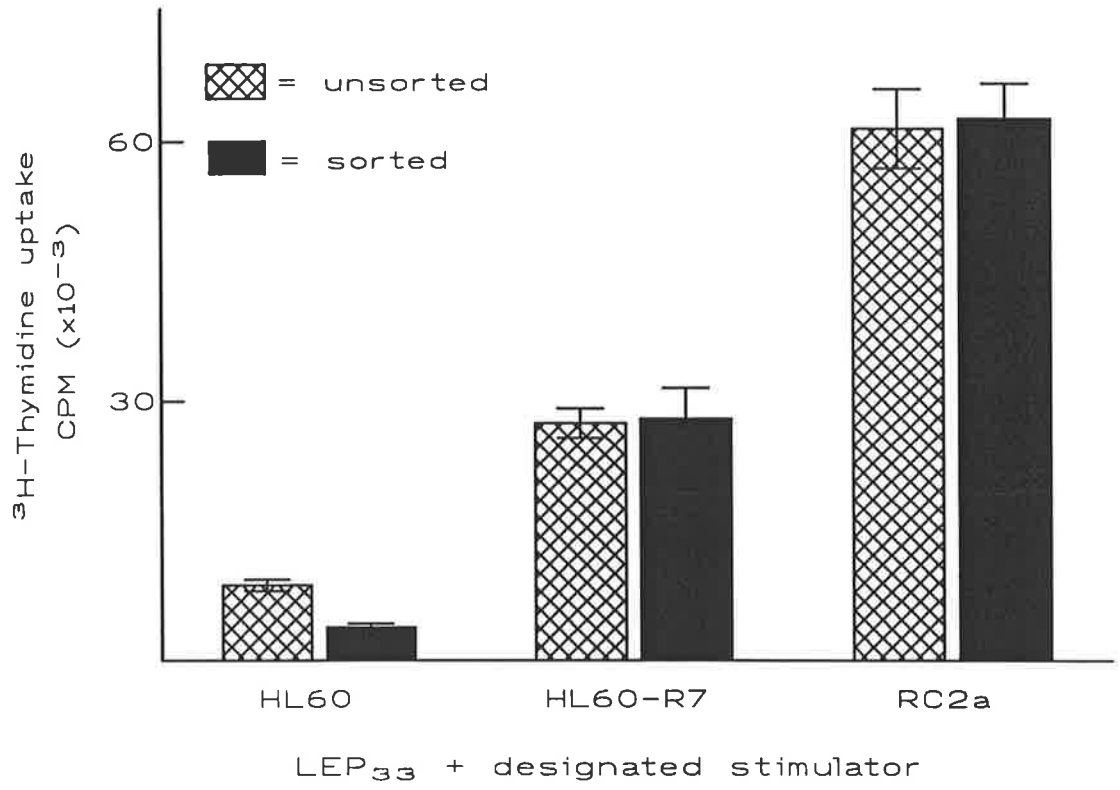
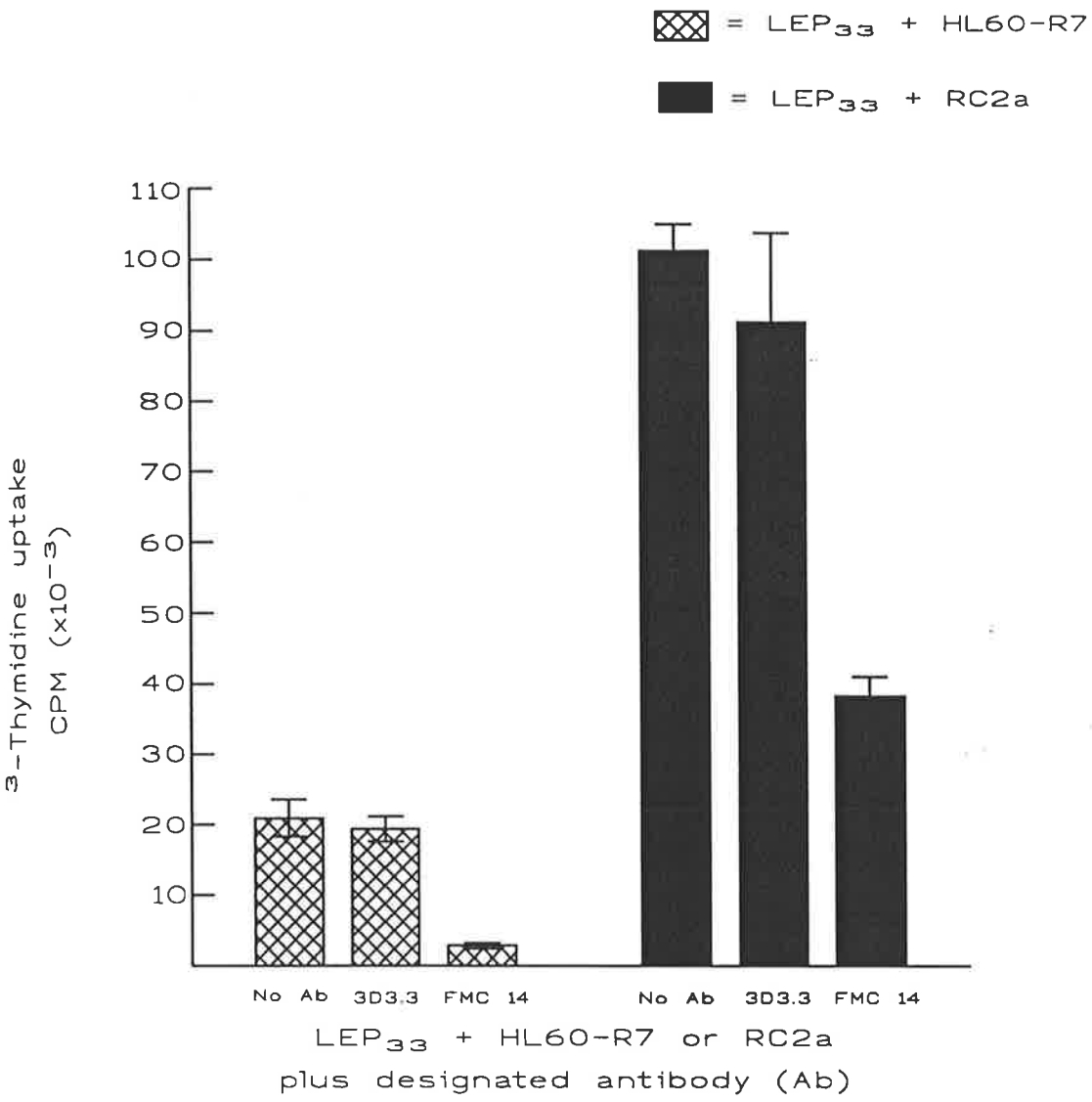

= unsorted
= sorted
60
30
^{3}H-Thymidine uptake
CPM (x10^{-3})
HL60
HL60-R7
RC2a
LEP$_{33}$ + designated stimulator

Figure 4.8.6: **MLC inhibition assay involving MHC class II$^-$ LEP$_{33}$ and FMC 14.** ^{3}H-thymidine uptake assay showing the effect of adding FMC 14 to a MLC of MHC class II$^-$ lymphocytes (LEP$_{33}$) with HL-60, HL-60-R7 or RC-2a cells. The 3D3.3 and FMC 14 hybridoma supernatants were added to the MLC at a final dilution of 1/4. LEP33 gave < 1500 cpm and stimulator cells alone gave < 300 cpm.


= LEP33 + HL60-R7
= LEP33 + RC2a
3-Thymidine uptake
CPM (x10-3)
110
100
90
80
70
60
50
40
30
20
10
No Ab
3D3.3
FMC 14
No Ab
3D3.3
FMC 14
LEP33 + HL60-R7 or RC2a
plus designated antibody (Ab)

involving the three stimulator types; HL-60, HL-60-R7 and RC-2a cells with two sets of responders (LEP_{19} and LEP_{34}), and the expression of MHC class II antigen, by both the LEP and stimulator populations, was monitored over days 0 - 4 by IIF using FMC 14. It was possible to analyse the lymphocyte and stimulator populations separately, since they possessed mutually exclusive FSC vs SSC traits. However the analysis of MHC class II antigen expression on the stimulator populations was really only credible for the first 3 days of the MLC for HL-60-R7 and the first 2 days for HL-60 and RC-2a since these stimulator populations eventually died and fragmented, as was determined by their decrease in FSC, increase in SSC as well as their increased uptake of EMA. Figure 4.8.7 indicates the gated population (HL-60 cells) which were analysed after 2 days of culture with LEP_{34} in a MLC. These cells were shown to be viable as they did not take up EMA, as illustrated in figure 4.8.8. A similar situation applied for LEP_{34} + HL-60-R7 cells (figures 4.8.9 and 4.8.10).

Expression of MHC class II antigen by HL-60 and HL-60-R7 cells in culture with LEP_{34} over the first 2 and 3 days respectively, was determined and the results displayed in figures 4.8.11 - 14 for HL-60 cells and figures 4.8.15 - 18 for HL-60-R7 cells. An illustrated overview of the results is displayed in figure 4.8.19.

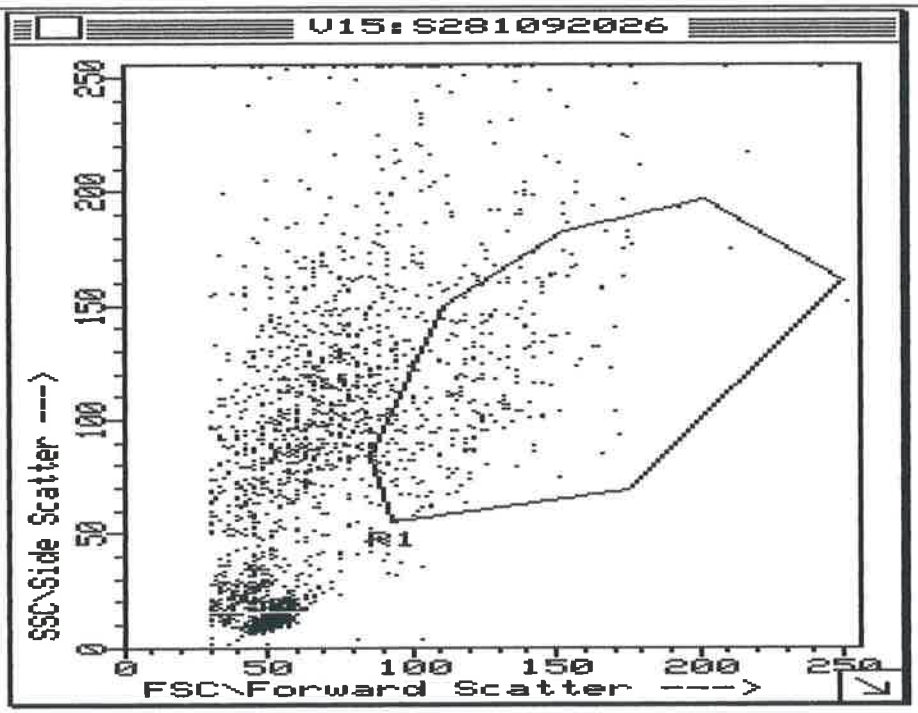
Using a gate encompassing lymphocytes only, the levels of expression of MHC class II antigen by the LEP_{34} over the first 4 days are illustrated in figure 4.8.20. Such a result was shown to be reproducible for this lymphocyte population (LEP_{34}) and for 4 other LEP, although the peak expression of MHC class II antigen by LEP cultured with RC-2a cells occurred at day 1 on 2 occasions.

4.9 The role of other molecules in the lymphocyte responses to HL-60-R7 and RC-2a cells

A variety of mAb specific for different cell surface molecules, including the adhesion molecules were added to the MLC containing LEP plus HL-60-R7 or RC-2a

Figure 4.8.7: **(top) Dot-plot of FSC vs SSC of HL-60 cells in culture with LEP_{34} on day 2 of the MLC.** Analysis was performed on the events within the gated region.

Figure 4.8.8: **(bottom) Location of viable HL-60 cells in MLC**. *Paint-a-gate* display of HL-60 cells plus lymphocytes (treated with EMA) on day 2 of MLC, indicating the location of dead cells (red) and the assumed position of viable HL-60 cells (green). Window 0 (bottom, left) = FSC vs SSC; Window 1 (top, left) = log fluorescence 3 intensity (FL3) vs cell number. Windows 2 -5 can be ignored.



PAINT-A-GATE (TM) Mode ++ File:#15:S281092026 Dots = 2000
%= 35.2 12.0 0.0 0.0 0.0 0.0 0.0 320 544
RGYBVCWO gate; +- mode; <> size
0-5 plot; FZHXPMU$'"s

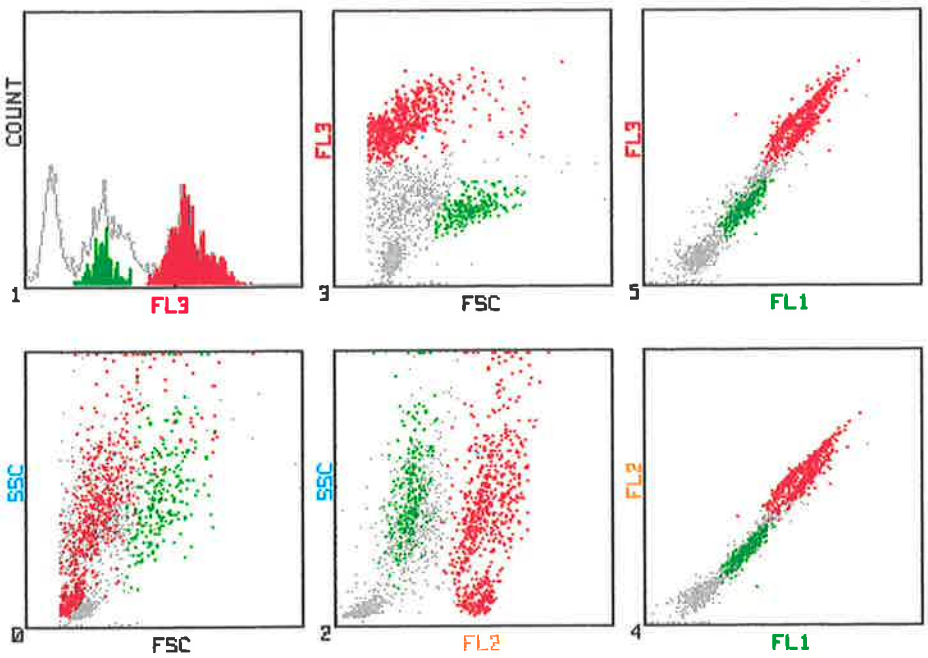
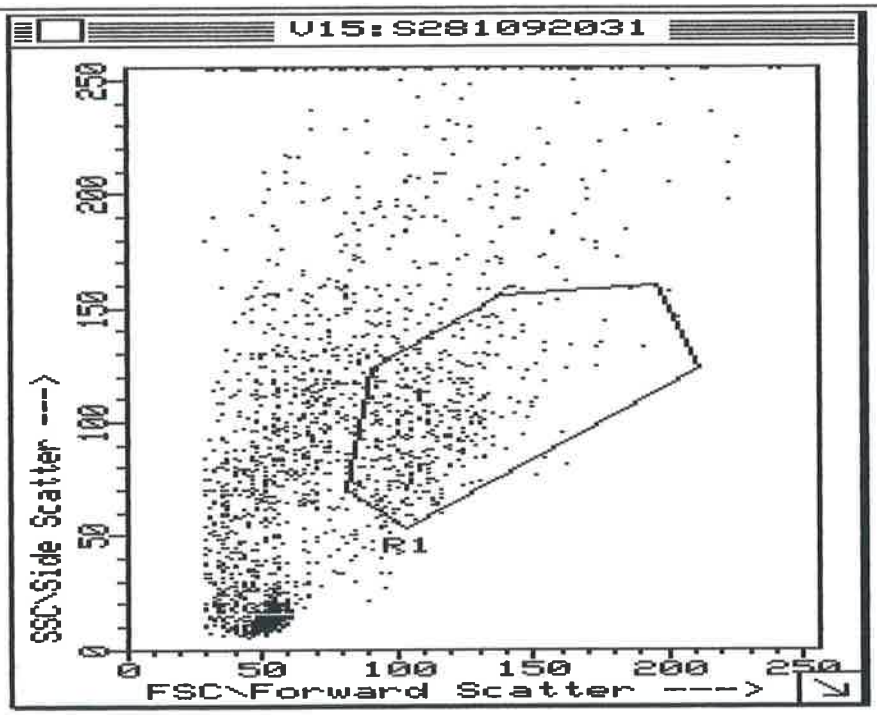
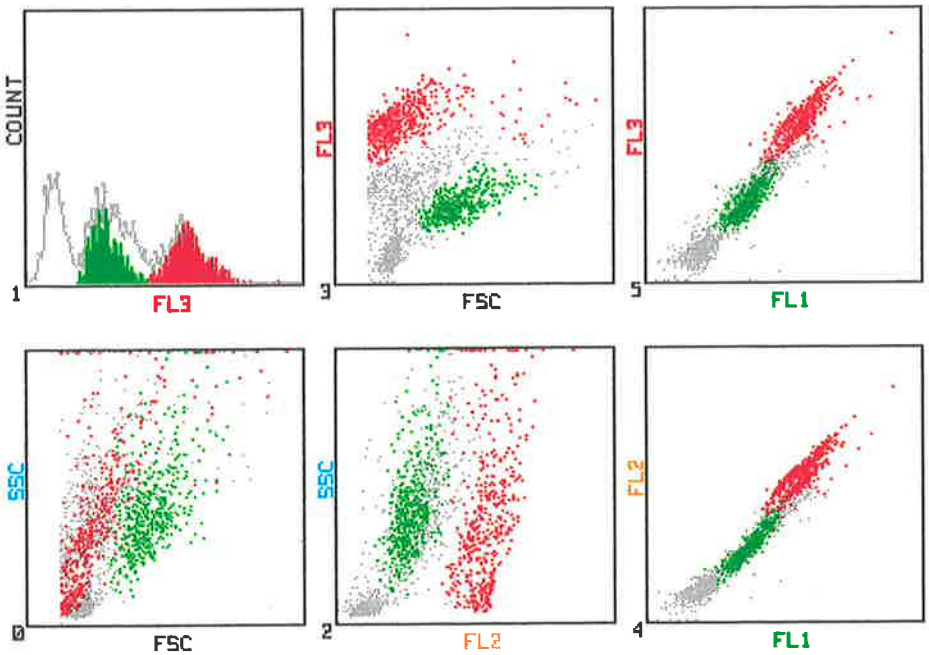


Figure 4.8.9: **(top) Dot-plot of FSC vs SSC of HL-60-R7 cells in culture with LEP_{34} on day 2 of the MLC.** Analysis was performed on the events within the gated region.

Figure 4.8.10: **(bottom) Location of viable HL-60-R7 cells.** *Paint-a-gate* display of HL-60-R7 cells plus lymphocytes (treated with EMA) on day 2 of MLC, indicating the location of dead cells (red) and the assumed position of viable HL-60-R7 cells (green). Window 0 (bottom, left) = FSC vs SSC; Window 1 (top, left) = log fluorescence 3 intensity (FL3) vs cell number. Windows 2 -5 can be ignored.



PAINT-A-GATE (TM) Mode ++ File:#15:S281092031 Dots = 2000
%= 23.9 23.3 0.0 0.0 0.0 0.0 0.0 728 704
RGYBVCWO gate; +- mode; <> size
0-5 plot; FZHXPMU$'"s



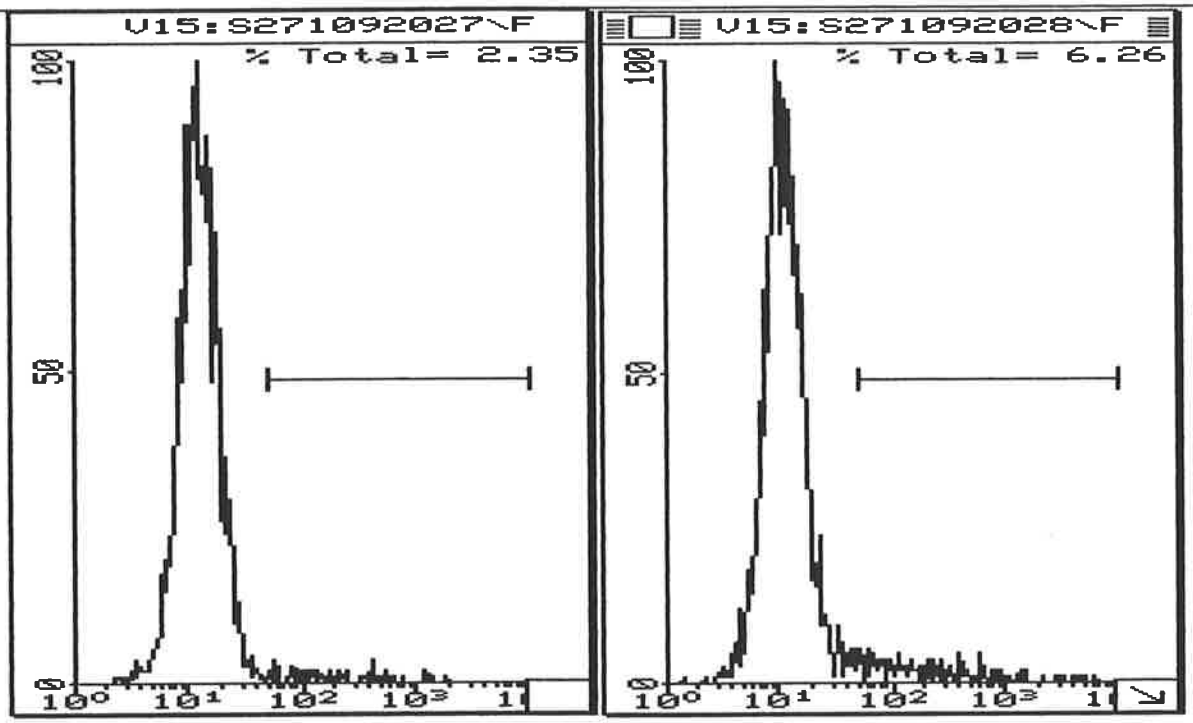
Figures 4.8.11 - 14: **Expression of MHC class II antigen by HL-60 cells in the MLC.** Histograms of log fluorescence 1 intensity (FL1) vs cell number showing profile of HL-60 cells that were in culture with LEP_{34} for 1 or 2 days of a MLC, and then treated with 3D3.3 (isotype control) or FMC 14 in an immunofluorescence assay.

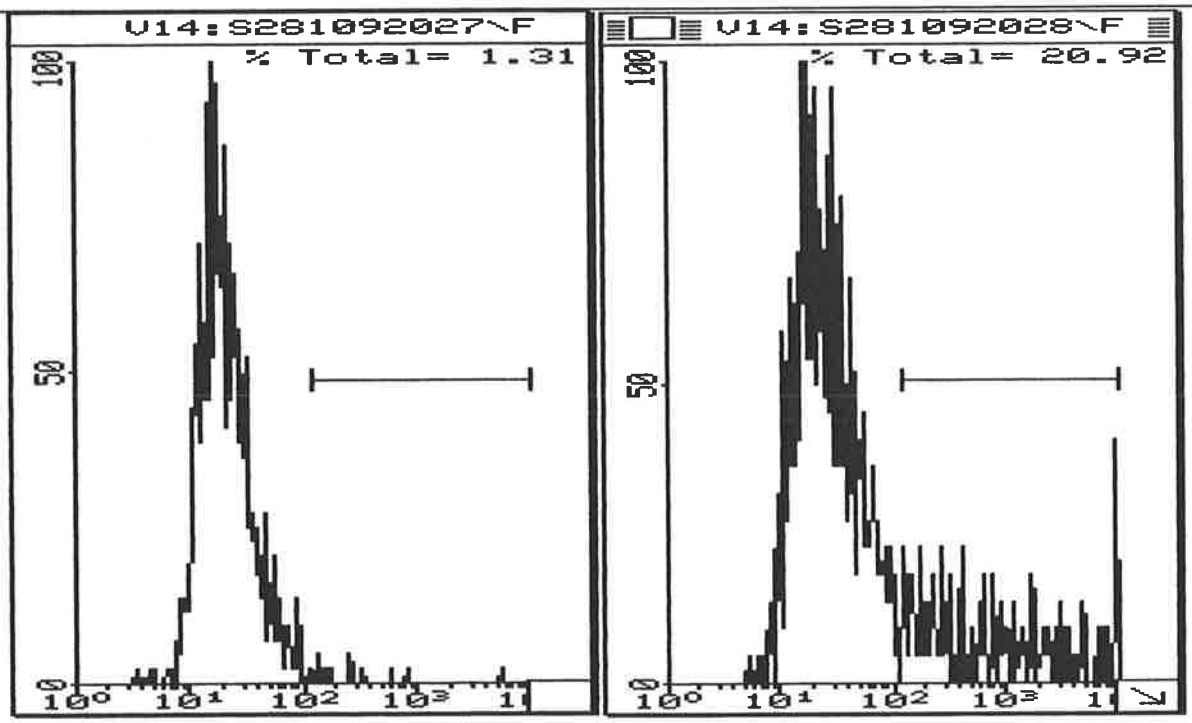
Figure 4.8.11: (top, left) HL-60 cells, on day 1 of MLC, treated with 3D3.3.

Figure 4.8.12: (top, right) HL-60 cells, on day 1 of MLC, treated with FMC 14.

Figure 4.8.13: (bottom, left) HL-60 cells, on day 2 of MLC, treated with 3D3.3.

Figure 4.8.14: (bottom, right) HL-60 cells, on day 2 of MLC, treated with FMC 14.


V15:S271092027\F
% Total= 2.35
V15:S271092028\F
% Total= 6.26
100
50
0
10⁰ 10¹ 10² 10³


V14:S281092027\F
% Total= 1.31
V14:S281092028\F
% Total= 20.92
100
50
0
10⁰ 10¹ 10² 10³

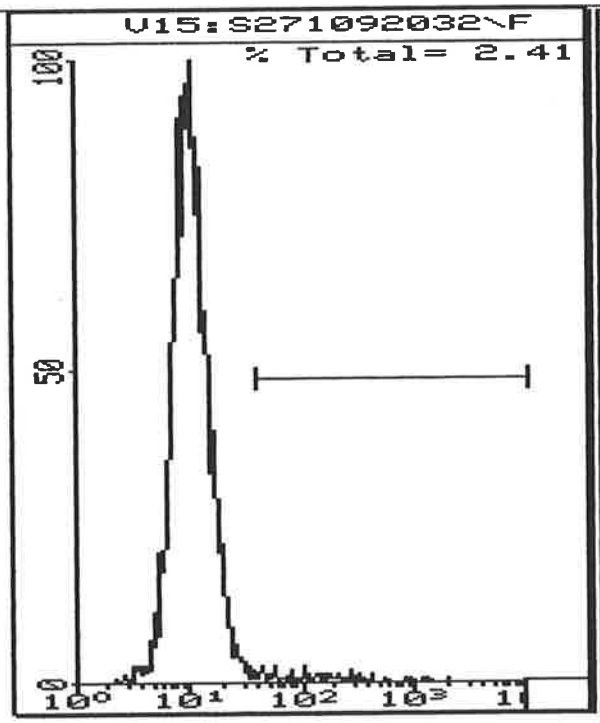
Figures 4.8.15 - 18: **Expression of MHC class II antigen by HL-60-R7 cells in the MLC.** Histograms of log fluorescence 1 intensity (FL1) vs cell number showing profile of HL-60-R7 cells that were in culture with LEP_{34} for 1 or 2 days of a MLC, and then treated with 3D3.3 (isotype control) or FMC 14 in an immunofluorescence assay.

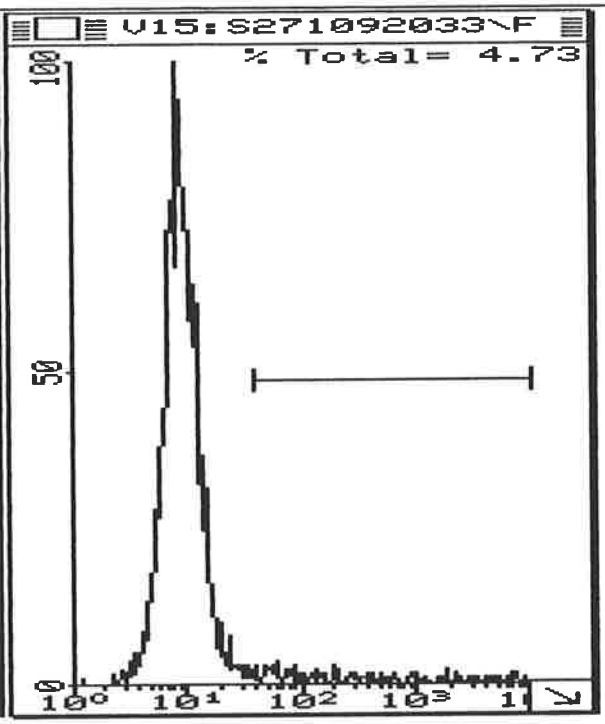
Figure 4.8.15: (top, left) HL-60-R7 cells, on day 1 of MLC, treated with 3D3.3.

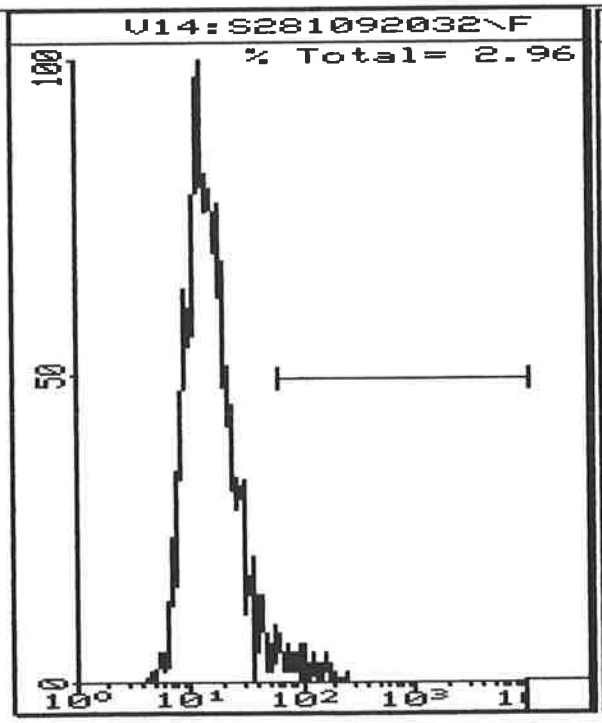
Figure 4.8.16: (top, right) HL-60-R7 cells, on day 1 of MLC, treated with FMC 14.

Figure 4.8.17: (bottom, left) HL-60-R7 cells, on day 2 of MLC, treated with 3D3.3.

Figure 4.8.18: (bottom, right) HL-60-R7 cells, on day 2 of MLC, treated with FMC 14.


V15:S271092032\F
% Total= 2.41
100
50
0
10⁰ 10¹ 10² 10³


V15:S271092033\F
% Total= 4.73
100
50
0
10⁰ 10¹ 10² 10³


V14:S281092032\F
% Total= 2.96
100
50
0
10⁰ 10¹ 10² 10³

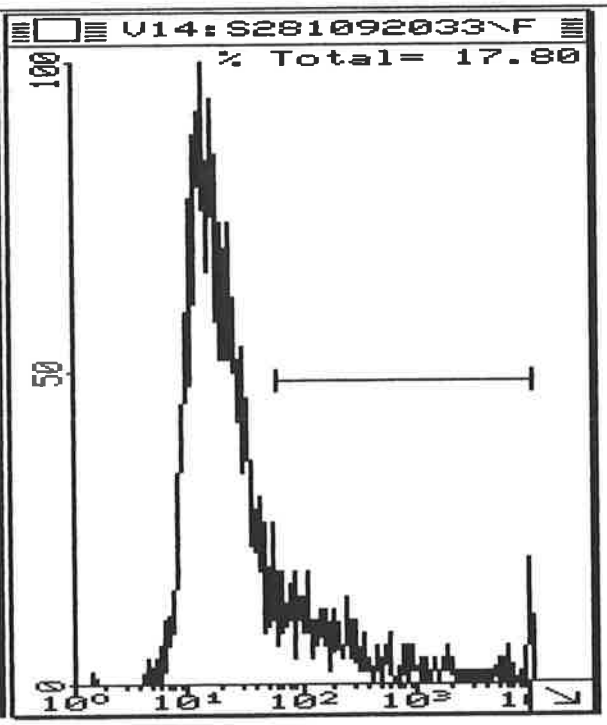
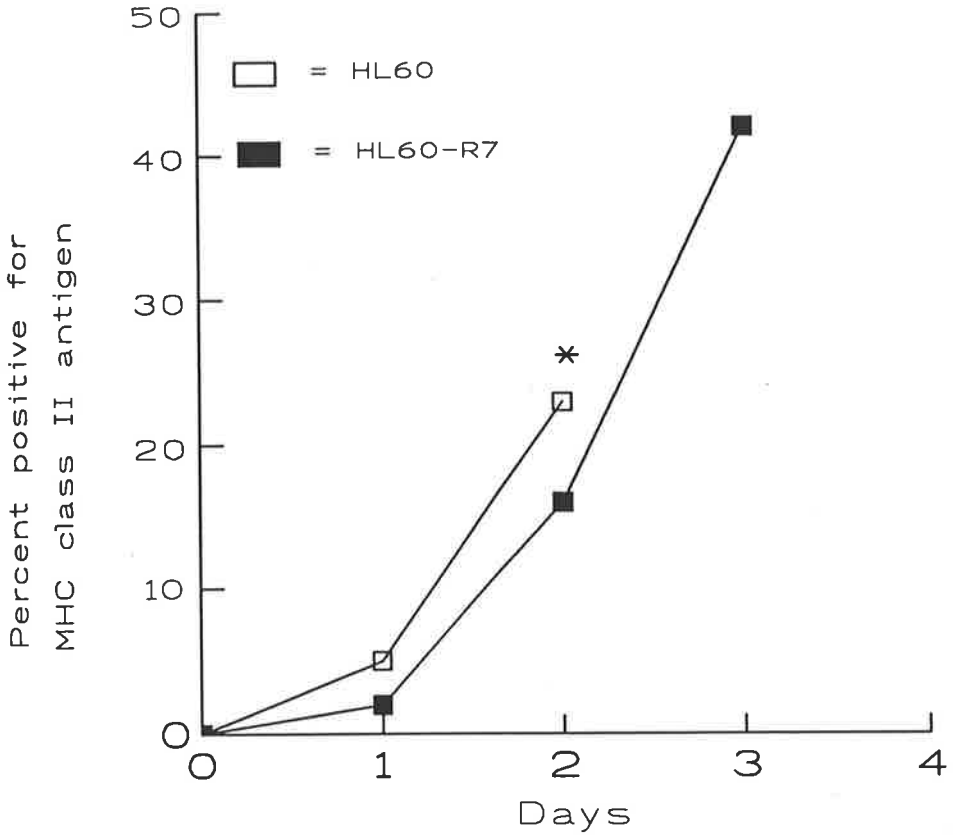
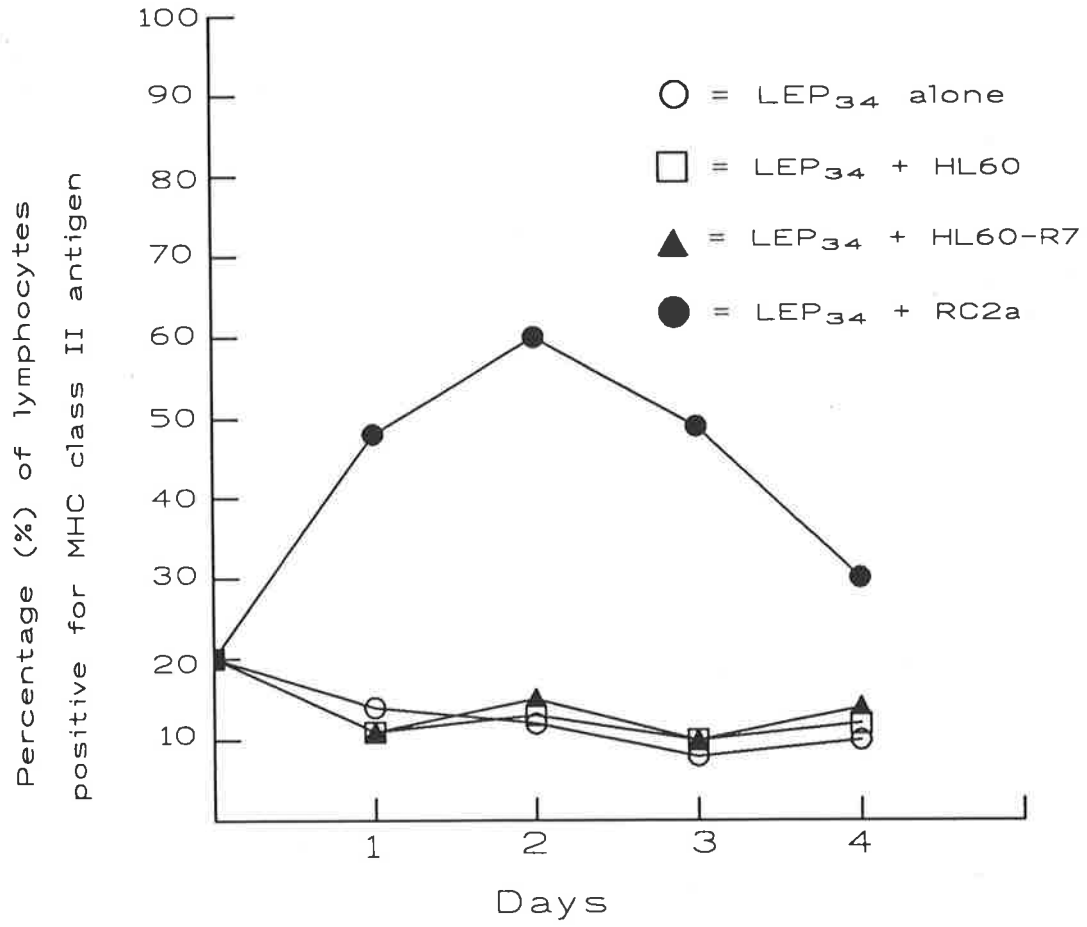

V14:S281092033\F
% Total= 17.80
100
50
0
10⁰ 10¹ 10² 10³

Figure 4.8.19: **(top) Expression of MHC class II antigen by HL-60 and HL-60-R7 cells in MLC.** Percentage of viable HL-60 and HL-60-R7 cells expressing MHC class II antigen during days 1, 2, and 3 of a MLC involving LEP_{34}

* There were too few cells to measure beyond this time-point.

Figure 4.8.20: **(bottom) Expression of MHC class II antigen by LEP_{34}.** Percentage of LEP_{34}, in MLC with designated stimulator cells, expressing MHC class II antigen.


50
40
30
20
10
0
= HL60
= HL60-R7
*
Percent positive for
MHC class II antigen
0
1
2
3
4
Days


100
90
80
70
60
50
40
30
20
10
= LEP34 alone
= LEP34 + HL60
= LEP34 + HL60-R7
= LEP34 + RC2a
Percentage (%) of lymphocytes
positive for MHC class II antigen
1
2
3
4
Days

cells. Many of these antigens were upregulated on HL-60 cells after RA treatment and were thus considered possible contributors to the enhanced immunostimulatory capacity of HL-60-R7 cells. The results of these experiments are summarized in table 4.9.1 and revealed that, of all the mAb tested, only the anti-LFA-1 mAb, TS1/22 reproducibly inhibited the response to HL-60-R7 cells, whilst none of them could reduce the proliferative response to RC-2a cells to any significant extent. An example of the effect of TS1/22, is shown in figure 4.9.1. Interestingly, TS1/22 did not inhibit the limited lymphocyte response to HL-60 cells. The mAb to MHC class I, 1B4.B12, appeared to have no effect on any of the lymphocyte responses. Such a result was also found when another mAb, 6c6b, specific for MHC class I was used (data not shown). However it is currently not known whether these two mAb are capable of functional inhibition. The mAb to CD11b, 5A4.C5, whilst ineffective at inhibiting proliferative responses here, has previously been shown to block CD11b function (Diamond *et al*, 1993)).

4.10 Discussion

4.10.1 Composition of lymphocyte-enriched populations

Somewhat surprisingly the percentage of cells expressing MHC class II antigen was far greater than would be expected, when one considers that it is often reported, or generally conceded, that approximately 10% of peripheral blood T lymphocytes are positive for this marker (Greaves *et al*, 1979). For the eight sets of lymphocyte-enriched populations analysed the percentages positive for MHC class II ranged from 12 - 54% and even taking into account that some of these cells were B lymphocytes this still leaves the $CD2^+$ population possessing a range of 11 - 52% MHC class II^+ lymphocytes.

Mean relative stimulation			
mAb	HL60	HL60-R7	RC2a
No Ab	100	100	100
3D3.3	142 ± 10	106 ± 3	103 ± 3
TS1/22	148 ± 11	34 ± 8 *1	111 ± 15
No Ab	100	100	100
1A6.11	99 ± 21	103 ± 12	102 ± 3
W-CAM-1	104 ± 21	92 ± 12	143 ± 18
No Ab	100	100	100
3D3.3	110 ± 8	98 ± 4	99 ± 2
CBR-IC2/2	174 ± 8 *2	100 ± 7	127 ± 5 *3
No Ab	100	100	100
3D3.3	112 ± 4	97 ± 7	124 ± 21
CBR-IC3/1	199 ± 11 *4	96 ± 21	107 ± 16
No Ab	100	100	100
3D3.3	142 ± 10	106 ± 3	87 ± 3
TS2/9	132 ± 13	102 ± 7	104 ± 11
No Ab	100	100	100
3D3.3	105 ± 8	102 ± 4	100 ± 4
1B4.B12	97 ± 12	89 ± 9	105 ± 5
No Ab	100	100	100
3D3.3	105 ± 11	121 ± 10	100 ± 7
5A4.C5	116 ± 10	97 ± 8 *5	118 ± 12

n = 4

Table 4.9.1: Summary of effects of various mAb on the proliferation of LEP in MLC with HL-60, HL-60-R7 or RC-2a cells. The relative stimulation of lymphocyte proliferation induced by HL-60, HL-60-R7 or RC-2a cells in the presence of a test, an isotype control or no mAb.

* Indicates that this value (for the test mAb) differed significantly from the value obtained for the isotype control treated sample, as measured by the paired t test.

*1 p = 0.002

*2 p = 0.012

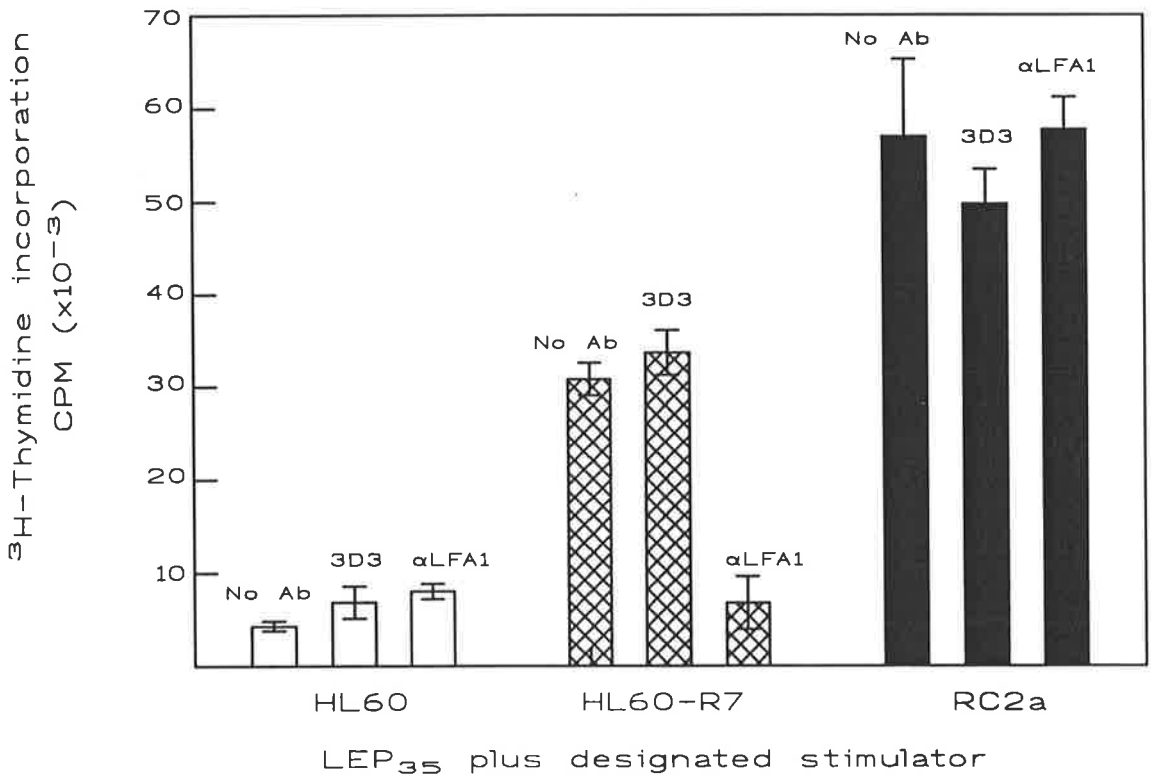
*3 p = 0.015

*4 p = 0.001

*5 p = 0.044

n = number of experiments

Figure 4.9.1: **MLC inhibition assay involving anti-LFA-1 mAb.** The inhibitory effect of TS1/22 was tested on the lymphoproliferative responses of LEP_{35} to HL-60, HL-60-R7 or RC-2a cells in a MLC. The 3D3.3 and TS1/22 hybridoma supernatants were added to the MLC at a final dilution of 1/4. Stimulator cells alone gave less than 200 cpm and LEP_{35} alone gave less than 1000 cpm.


^{3}H-Thymidine incorporation
CPM (x10^{-3})
70
60
50
40
30
20
10
No Ab
3D3
αLFA1
No Ab
3D3
αLFA1
No Ab
3D3
αLFA1
HL60
HL60-R7
RC2a
LEP$_{35}$ plus designated stimulator

An explanation for such a discrepancy, with respect to the literature, could be that the method for probing for MHC class II $^{+}$ cells in the past was not sensitive enough, resulting in slightly positive cells being designated as negative. Such an event could occur if one was using manual fluorescence to detect positive cells. Alternatively, with respect to the method used here, it may well be that some of the cells were being induced to express MHC class II antigen during the purification procedure.

The presence of MHC class II antigen on $CD2^{+}$ cells may well be indicative of currently and/or previously activated lymphocytes; an idea which receives further credence from the results obtained with anti-CD25, where 1 - 12% of the lymphocyte population proved to be positive for this marker of lymphocyte activation, albeit at very low levels of intensity.

The results obtained with a mAb specific for the monocyte marker CD14 revealed that, usually $\leq$ 1% of the LEP were $CD14^{+}$. This discovery was corroborated by the finding that $\leq$ 1% of the LEP expressed NSE (data not shown). This contrasts with results obtained with lymphocyte-rich populations that had not undergone the freeze-thawing process, where 0.5 - 3% of the population were NSE^{+}, suggesting that monocytes do not survive the freeze-thawing process particularly well. This is in accord with previous observations in this laboratory (White *et al*, 1988)

4.10.2 Mechanism of activation of lymphocytes by HL-60-R7 cells

The phenotypic analysis of the LEP responding to HL-60-R7 cells supported the notion that an alternative mode of lymphocyte activation was being employed which excluded the involvement of $CD4^{+}$ lymphocytes since it was found that the activated lymphocytes were predominantly $CD8^{+}$ and/or $CD16^{+}$ compared to LEP cultured with RC-2a cells where the majority of cells activated were $CD4^{+}$. Nevertheless, subsequent investigations revealed that $CD4^{+}$ lymphocytes played a crucial role in the response of LEP to HL-60-R7 cells as did MHC class II molecules. These two latter

findings implied that a conventional mode of lymphocyte activation was taking place. In other words $CD4^+$ lymphocytes may have bound to MHC class II^+ HL-60-R7 cells and received sufficient signals to produce IL-2 which could then aid in the proliferation of both $CD8^+$ and $CD16^+$ lymphocytes that may have been stimulated by MHC class I and/or other cell surface molecules. Such a possibility was further supported by the finding that a proportion of HL-60-R7 cells expressed MHC class II molecules within the first 2 - 3 days of the MLC. The bias toward the activation of non-$CD4^+$ lymphocytes may well reflect the limited number of HL-60-R7 cells expressing MHC class II molecules. To explain, the finding that only a minor percentage of HL-60-R7 cells expressed MHC class II antigen at low - medium levels early in the MLC (days 0 - 1), whilst the majority of cells expressed moderate levels of MHC class I may have given $CD8^+$ lymphocytes a competitive advantage over $CD4^+$ lymphocytes, with respect to binding to HL-60-R7 cells. Those $CD4^+$ lymphocytes which could bind, may have provided IL-2 for the proliferation of $CD8^+$ lymphocytes as well as the NK cells.

The expression of MHC class II molecules by HL-60-R7 cells, however, may not be responsible for their enhanced immunostimulatory nature since HL-60 cells were also found to express MHC class II antigen when placed in culture with LEP. The levels of expression and the percentage of HL-60 cells expressing this molecule were generally similar to HL-60-R7 cells. One possible explanation for the enhanced immunostimulatory ability of HL-60-R7 cells is that more of these cells survived longer in the MLC than HL-60 cells and thus they were able to signal to the lymphocytes for a longer period of time. Another possibility, not mutually exclusive to that proposed above, is that cell surface molecules expressed by HL-60-R7, but not by HL-60 cells were involved in the activation of LEP. Candidates for this role included CD11b and ICAM-1, both of which were upregulated on HL-60-R7 cells and have also been shown to be involved in the activation of lymphocytes (Dougherty *et al*, 1988). Additionally, LFA-1 and ICAM-3 were upregulated on HL-60-R7 cells and thus may have been

involved in the enhanced immunostimulatory nature of these cells. Indeed the mAb to LFA-1, TS1/22, was the only one which consistently inhibited proliferative responses to HL-60-R7 cells whilst mAb to three of the ligands to LFA-1 were generally ineffective. The finding that W-CAM-1, CBR-IC2/2 and CBR-IC3/1 were not consistently capable of even partially inhibiting the lymphoproliferative response to HL-60-R7 cells possibly suggests that LFA-1 may have interacted with more than one ligand on HL-60-R7 cells and that it may have required the addition of a combination of two or three of these mAb if substantial proliferative inhibition was to be achieved. Alternatively, it is possible that an alternative ligand, such as the recently discovered ICAM-R (Vazeux *et al*, 1992), was important in the lymphoproliferative response to HL-60-R7 cells. Finally, there is the possibility that one or more of these mAb were not added at sufficient concentrations to have an effect in this system.

It was noted that ICAM-2 expression was downregulated on HL-60-R7 cells. One could tentatively postulate that the presence of this antigen at the levels expressed by HL-60 cells may have had negative effect on the ability of lymphocytes to proliferate, however further experiments would be required to test this proposal. The downregulation of MHC class I may also have been an advantageous effect of RA, with respect to the enhanced immunostimulatory nature of HL-60-R7 cells. It may be that the ratio of MHC class I:MHC class II expression was important in determining quantitative, and possibly qualitative, lymphocyte responses to HL-60, HL-60-R7 and RC-2a cells. For instance all HL-60 cells express high levels of MHC class I and therefore may preferentially bind $CD8^+$ lymphocytes which then sterically impede the binding of $CD4^+$ lymphocytes (see figure 4.10.2(a)). HL-60-R7 cells expressed less MHC class I molecules, with some expressing no detectable levels, thereby enhancing the prospects of $CD4^+$ lymphocytes forming collaborations with the stimulator cells. As discussed above, the higher overall levels of expression of MHC class I compared to MHC class II molecules may have given the CD8+ lymphocytes a greater chance of becoming activated (see figure 4.10.2(b)). The fact that some of the HL-60-R7 cells

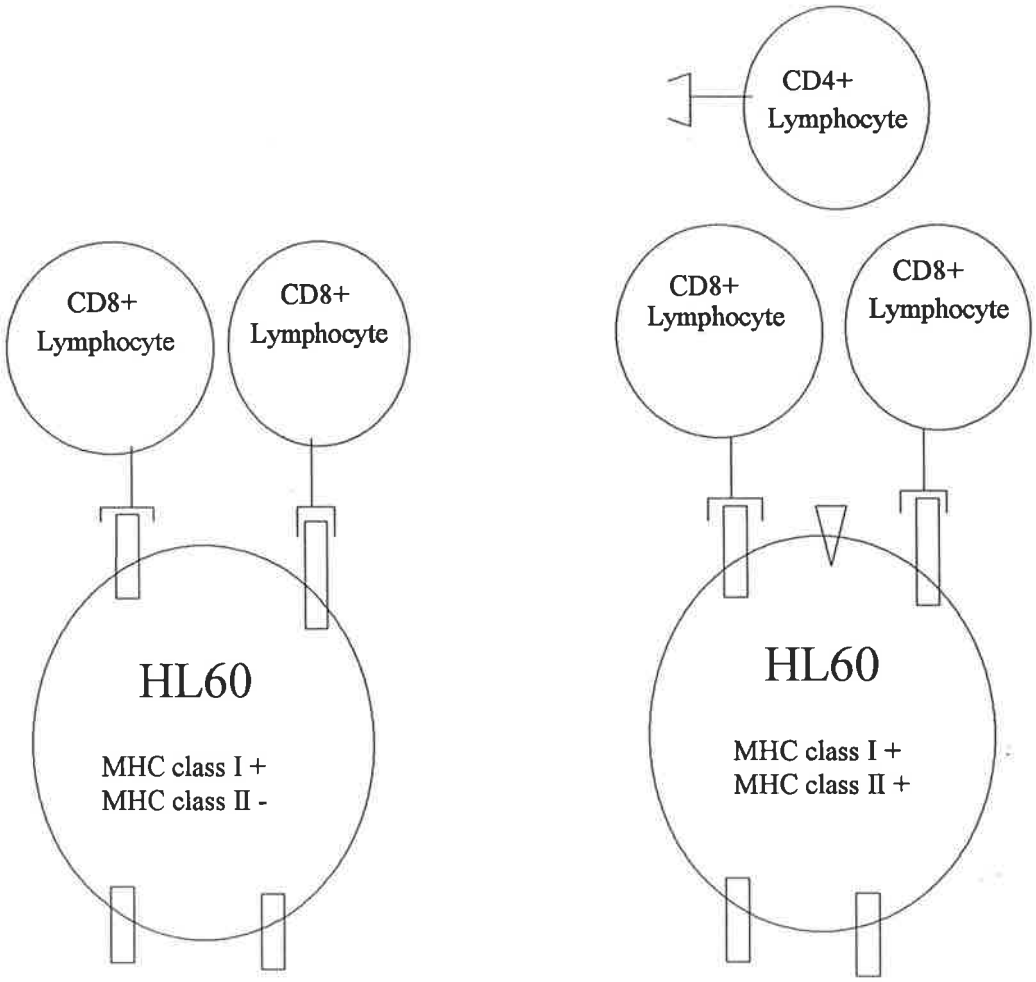
Figures 4.10.2(a) and 4.10.2(b): **HL-60, or HL-60-R7, plus LEP.** These diagrams attempt to explain how one possible contributing factor to the poor immunostimulatory nature of HL-60 cells could have been the high levels of MHC class I antigen expression relative to MHC class II expression, where the binding of $CD4^+$ lymphocytes to HL-60 cells may have been sterically hindered by the HL-60-bound $CD8^+$ lymphocytes (figure 4.10.2(a)) In the case of HL-60-R7 cells, MHC class I was downregulated, possibly enabling a small proportion of $CD4^+$ lymphocytes to bind and effect the proliferation of HL-60-R7-bound $CD8^+$ lymphocytes (figure 4.10.2(b)).


= MHC class I antigen


= MHC class II antigen

Figure 4.10.2(a): **(top) HL-60 plus LEP**

Figure 4.10.2(b): **(bottom) HL-60-R7 + LEP**


CD4+
Lymphocyte
CD8+
Lymphocyte
CD8+
Lymphocyte
HL60
MHC class I +
MHC class II -
CD8+
Lymphocyte
CD8+
Lymphocyte
HL60
MHC class I +
MHC class II +

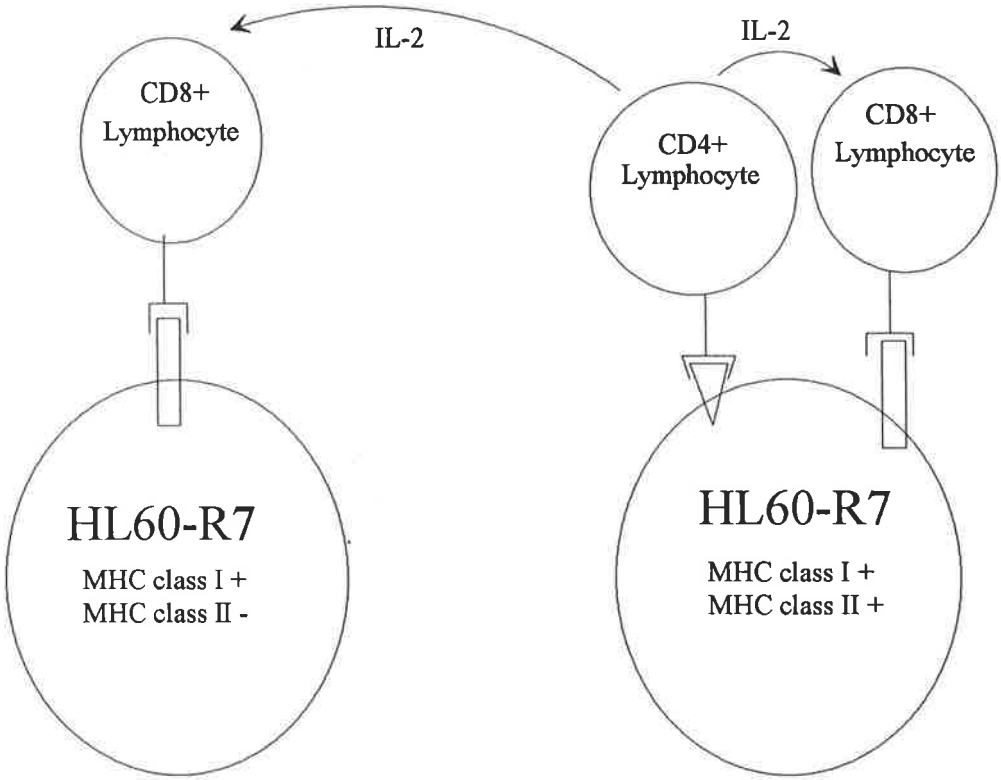
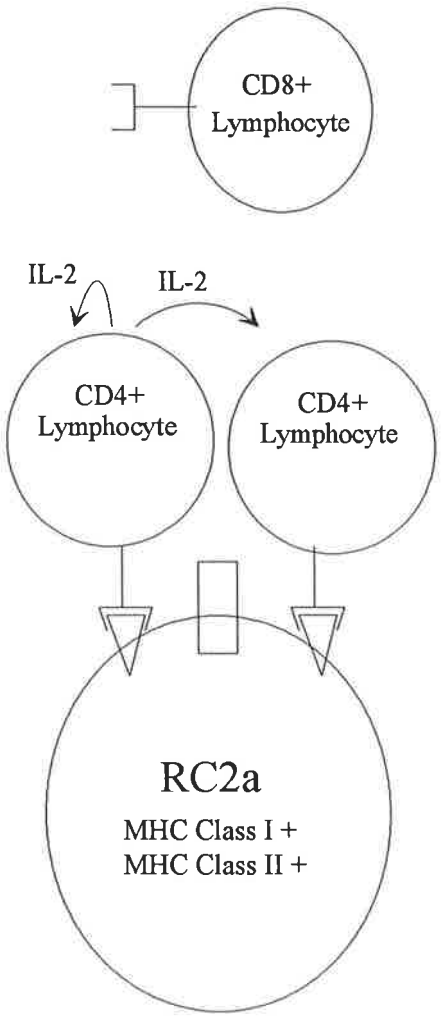

IL-2
IL-2
CD8+
Lymphocyte
CD4+
Lymphocyte
CD8+
Lymphocyte
HL60-R7
MHC class I +
MHC class II -
HL60-R7
MHC class I +
MHC class II +

Figure 4.10.2(c): RC-2a plus LEP. Diagram attempting to explain a possible reason for the predominance of activated $CD4^+$ lymphocytes in the lymphoproliferative response to RC-2a cells.

= MHC class I antigen

= MHC class II antigen


CD8+
Lymphocyte
IL-2
IL-2
CD4+
Lymphocyte
CD4+
Lymphocyte
RC2a
MHC Class I +
MHC Class II +

expressed negative to low levels of MHC class I may have enhanced the prospects of NK cell activation as it has been reported that certain MHC class I molecules, expressed by target cells, were capable of preventing NK cell activation (Ciccone *et al*, 1992b). RC-2a cells on the other hand expressed high levels of MHC class II in comparison to MHC class I and this may have given $CD4^+$ lymphocytes a selective advantage in terms of becoming activated and proliferating (see figure 4.10.2(c)).

It was found that lymphocytes cultured with HL-60-R7 cells for 6 days under MLC conditions had an enhanced capacity to lyse HL-60, HL-60-R7 and RC-2a cells compared with lymphocytes cultured with HL-60 cells or RC-2a cells. As RC-2a cells were found to activate predominantly $CD4^+$ lymphocytes this may explain the relatively weak cytolytic ability, on a cell-for-cell basis of these lymphocytes. The capacity of HL-60-R7-stimulated lymphocytes to lyse RC-2a cells would suggest that at least a portion of the cytolytic activity was non-MHC-restricted. HL-60-R7 cells themselves were found to be more vulnerable to cytolysis than HL-60 cells. The down regulation of MHC class I on HL-60-R7 cells may have been responsible for the increased susceptibility of these cells to cytolysis, especially by NK cells, being indirectly proportional to the levels of MHC class I expression by the target cell (Harel-Bellam *et al*, 1986; Storkus *et al*, 1987; Tanaka *et al*, 1988).

Chapter 5

The role of cytokines in the proliferative response of lymphocyte-enriched populations to HL-60-R7 cells

5.1 Introduction

Thus far, a number of cell surface molecules expressed by HL-60-R7 cells and/or the LEP have been investigated with regards to their involvement in the *in vitro* activation of LEP. As a result, MHC class II antigens expressed by HL-60-R7 cells have been implicated as important contributors to the stimulatory capacity of these cells. Additionally, there appeared to be a role for LFA-1 in the proliferative response. The role of cytokines, however, has not yet been defined in this system, although it was shown, indirectly, in chapter 4 that IL-2 may have been of vital importance in inducing proliferation of LEP. However, it was considered feasible that other cytokines were playing key roles in the events leading up to the production of IL-2.

The two signal hypothesis of lymphocyte activation, proposed in the 1970's (Lafferty *et al*, 1978), is still considered relevant today. The first signal is the interaction of allogeneic MHC product, or syngeneic MHC antigen in association with a foreign antigen, with the TCR of the T lymphocyte which results in expression of the high affinity IL-2R but is generally incapable of triggering lymphocyte proliferation (Palacios and Moller, 1981; Meuer and Meyer zum Buschenfelde, 1986). The second signal was proposed, and has often been found, to be delivered in the form of a cytokine of which the most commonly described are IL-1α, IL-1β, IL-6 and TNF-α (Verwilghen *et al*, 1991; Williams *et al*, 1985; Lotz *et al*, 1988). IL-1α and IL-1β are structurally dissimilar yet bind to the same receptor and share many functional traits, such that, at a functional level, they are difficult to distinguish from one another. There have been many reports that IL-1 is an important cytokine with respect to stimulating lymphocyte proliferation as antibodies to this cytokine can at least partially inhibited lymphocyte proliferation in MLC (Verwilghen *et al*, 1991). There are contrary reports,

however, which suggest that IL-1 is often not required in the activation of resting lymphocytes (Jenkins *et al*, 1991; Halvorsen *et al*, 1988).

Recently, TNF-α has been recognized as being capable of enhancing the proliferation of lymphocytes in MLC (Matthews and Sullivan, 1992). This cytokine has been reported to up-regulate expression of immunologically relevant antigens such as MHC class II molecules and CD25 (Yokoto *et al*, 1988). IL-6 has been reported to stimulate lymphocyte proliferation (Lotz *et al*, 1988), but has generally required the presence of another cytokine, such as IL-1, with which it acts in synergy to stimulate Ag-triggered lymphocytes (Houssiau *et al*, 1988)) or CD2-triggered lymphocytes (Lorre *et al*, 1990).

It was thought possible that one or more of the aforementioned cytokines were involved in the activation of LEP by HL-60-R7 cells, and that production of one or more of these cytokines may have been upregulated with respect to undifferentiated HL-60 cells. Thus, comparisons of the levels of mRNA expression for IL-1α, IL-1β, IL-6 and TNF-α by HL-60 and HL-60-R7 cells, as well as RC-2a and U937 cells, was performed by means of the RNase protection assay. RNA from U937 cells was used as a negative control as these cells have been reported not to express detectable levels of IL-1, IL-6 or TNF-α (Hass *et al*, 1991). Monkey COS cells (provided by Bronwyn Cambareri, Division of Human Immunology, I.M.V.S.) were also used as a source of negative control RNA. MLC inhibition assays were performed using functionally inhibitory antibodies specific for particular cytokines.

As NK cells figured prominently in the LEP responses to HL-60-R7 cells it was considered important to investigate those cytokines capable of playing a part in NK cell proliferation. Aside from IL-1 and, to a lesser extent, IL-6, other cytokines capable of stimulating the proliferation of NK cells include IL-12 and Stem Cell Factor (SCF) (Gately *et al*, 1991; Robertson *et al*, 1993; Matos *et al*, 1993). Matos *et al*, (1993) demonstrated that SCF could act in synergy with IL-2 to augment the proliferation of a subset of peripheral blood NK cells possessing the phenotype $CD56^{bright}$, c-kit^{+}, high

affinity IL-2R$^+$. Gately *et al*, (1991) reported that IL-12 was capable of acting as a growth factor for activated NK cells. Thus the roles of SCF and IL-12 were examined in these studies.

5.2 Expression of TNF-α mRNA by HL-60-R7 cells and other cell lines and the role of TNF-α in the MLC

For use in this and subsequently described assays, total RNA was isolated from the following cell lines; HL-60, HL60-R7, RC-2a, U937, U937 treated with TPA for 6 days, and monkey COS cells as a negative control. Additionally, total RNA was isolated from human embryonic lung cells (H.E.L.) which were provided by Dr. Greg Goodall (I.M.V.S.) and LPS-stimulated human monocytes as positive controls for IL-6 and IL-1α mRNA expression respectively (Hazuda *et al*, 1988).

The antisense TNF-α probe was synthesized using SP6 polymerase and ^{32}P-labelled UTP. The probe was gel purified, the level of labelling was measured by the Cerenkov count and then probe emanating 3 x 10^4 cpm was added independently to 1, 5, and 10 μg of total RNA derived from each of the cell lines listed above. To the same RNA samples, a probe for β-actin mRNA (3 x 10^4 cpm) was also added. The β-actin gene has been regarded as one of the "house-keeping" genes of the cell and its expression is thought to vary little from cell-type to cell-type, thus making quantitative comparisons of cytokine mRNA expression between human cell lines feasible.

Figure 5.2.1 displays the results of a RNase protection assay where probes for TNF-α mRNA and β-actin mRNA were used. HL-60 cells, it would seem, already expressed TNF-α mRNA as has been previously reported (Steffen *et al*, 1988). The level of expression was determined to be approximately 0.62 that of β-actin mRNA. This was calculated by means of analysis using a PhosphorImager (Molecular Dynamics). It should be stressed that such measurements are an approximate rather than a precise reflection of the relative levels of mRNA present. To illustrate, if one assumes the ratio of incorporated ^{32}P-labelled UTP:incorporated "cold" nucleotides

Figure 5.2.1: **Expression of TNF-α mRNA.** Autoradiograph showing the result of RNase protection assay where anti-sense ^{32}P-labelled probes were used to detect the presence of TNF-α and β-actin mRNA expressed by HL-60, HL-60-R7, RC-2a, U937 and COS cells. See section 2.3.11 for details of methods.

Legend

Lane 1 = HL-60 (1 μg RNA)

Lane 2 = HL-60 (10 μg RNA)

Lane 3 = HL-60-R7 (1 μg RNA)

Lane 4 = HL-60-R7 (10 μg RNA)

Lane 5 = COS (10 μg RNA)

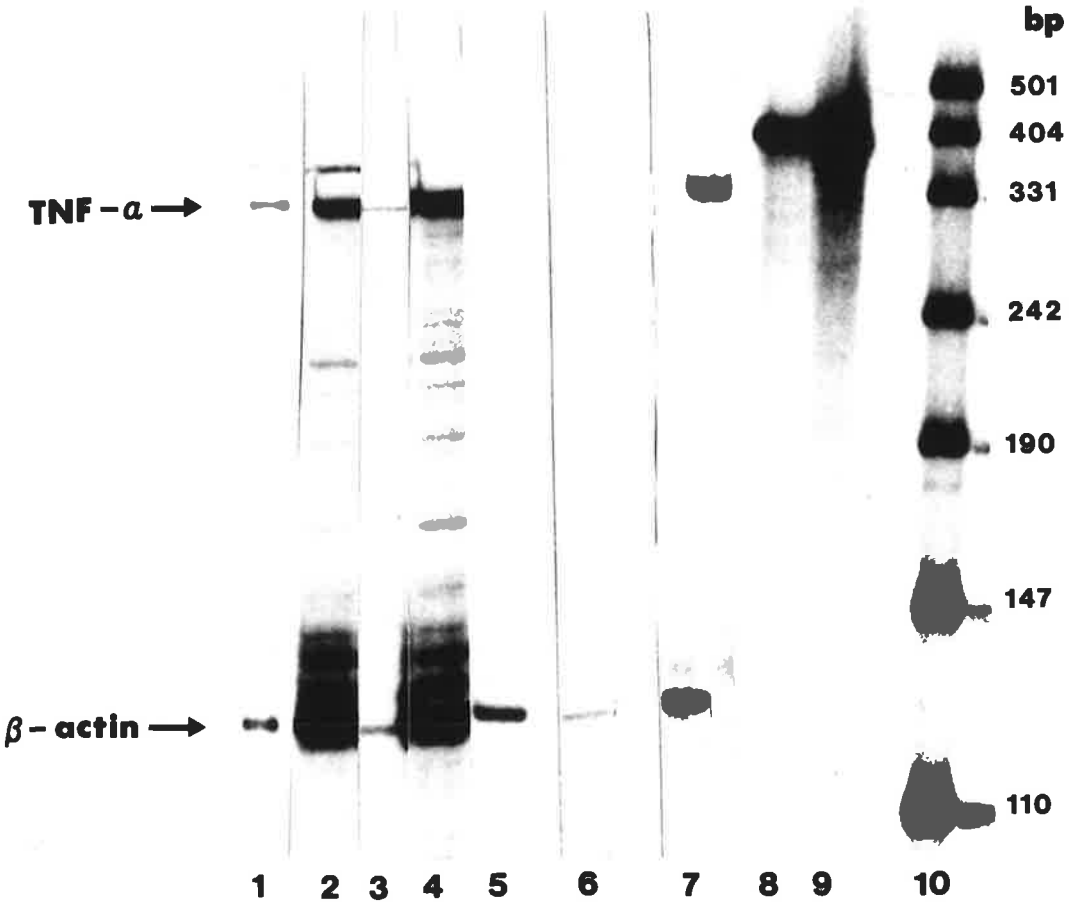
Lane 6 = U937 (10 μg RNA)

Lane 7 = RC-2a (5 μg RNA)

Lane 8 = β-actin probe (unprotected)

Lane 9 = TNF-α probe (unprotected)

Lane 10 = ^{32}P-labelled pUC19 DNA restricted with Hpa II


TNF – α
β – actin
bp
501
404
331
242
190
147
110
1 2 3 4 5 6 7 8 9 10

into the two probes was equal, then the 0.62 value could be considered an overestimate as the TNF-α probe was approximately 2.4 x larger than the β-actin probe. However, such a flaw does not interfere with making comparisons of relative cytokine mRNA expression between HL-60 and HL-60-R7 cells which is the primary concern here.

HL-60-R7 cells were also shown to express TNF-α mRNA at levels, relative to β-actin mRNA, very similar to HL-60 cells (0.63). Such comparisons can only be made if the probes added to the total RNA sample analysed, were added in excess. To ensure this occurred, incrementing amounts of total RNA (1, 5 and 10 μg) were added to a constant amount of probe. Figure 5.2.1 displays tracks representative of 1 μg and 10 μg of HL-60/HL-60-R7 cell RNA samples. The increase in intensity of the bands, indicating the presence of TNF-α and β-actin mRNA, from the 1 μg to the 10 μg samples indicates that probe was added in excess to the 1 μg total RNA samples.

Of the other cell lines tested, RC-2a cells were found to express TNF-α mRNA at higher levels than HL-60 cells. As previously reported, U937 cells did not express detectable levels (Hass *et al*, 1991), nor did the monkey COS cells. Table 5.2.1 shows the levels of TNF-α mRNA expression by the various cell lines relative to β-actin mRNA levels.

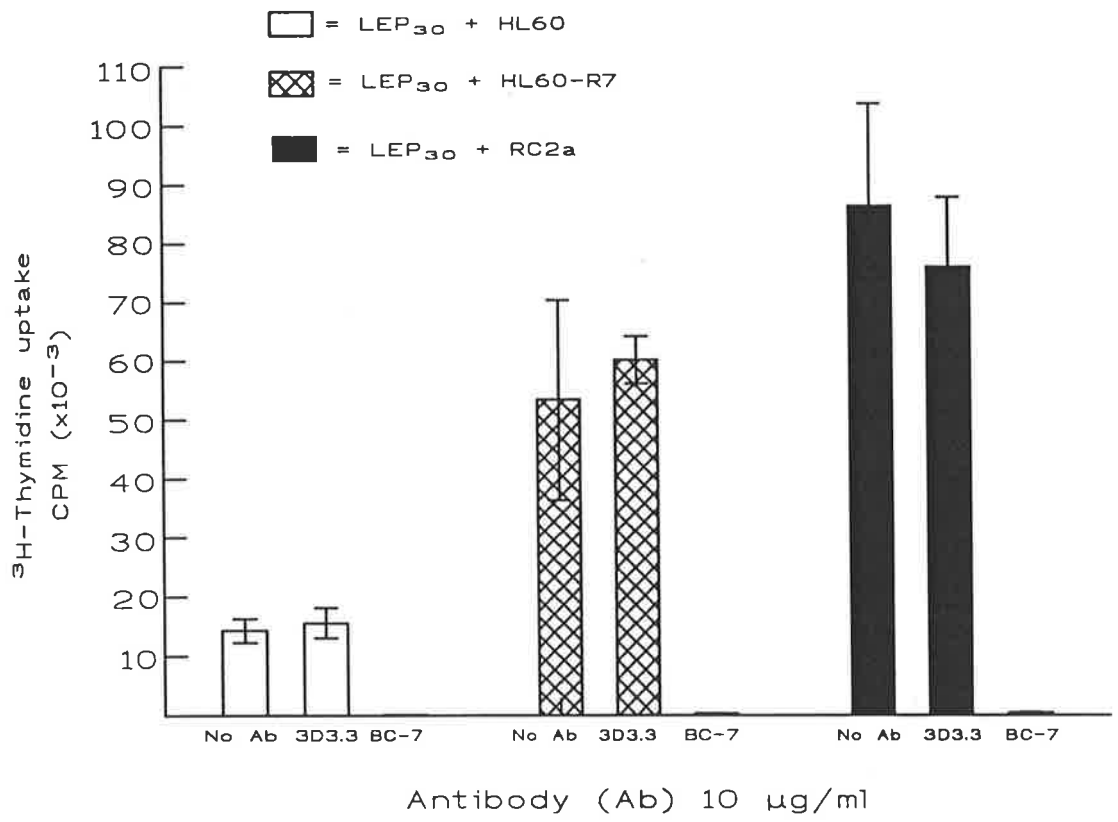
Given that TNF-α mRNA was expressed at readily detectable levels in HL-60, HL-60-R7 and RC-2a cells, it was considered possible that TNF-α could have been produced by one or more of these cell lines and subsequently effected the activation of the LEP. To ascertain the likelihood of such a proposal, a commercial mAb, BC-7 (Serotec,. U.K., cat. no. MCA 747), specific for, and capable of functionally inhibiting, human TNF-α activity was added to a MLC containing LEP and one of the three stimulator populations. Results shown in figure 5.2.2 indicate quite clearly that TNF-α played a crucial role in the proliferative responses of LEP to HL-60, HL-60-R7 and RC-2a cells. 10 μg/ml of the anti-TNF-α were capable of inhibiting all proliferative responses by > 90%. This result was shown to be reproducible for 5 other LEP. With 5 μg/ml of anti-TNF-α, inhibition of the proliferative response to RC-2a cells was

Table 5.2.1: **Relative levels of expression of TNF-α.** The levels of TNF-α mRNA expression by the indicated cell types relative to β-actin mRNA levels as determined by the PhosphorImager. Experiment was performed in duplicate (mean ± SEM)

N. T. = not tested

Cell type	Ratio of TNF-α:ß-actin
HL60	0.62 ± 0.04
HL60-R7	0.63 ± 0.02
RC-2a	1.35 ± 0.05
U937	0
U937-TPA	N.T.
H.E.L.	N.T.
COS	0

Figure 5.2.2: **Inhibition of lymphocyte proliferation in MLC by neutralizing mAb to TNF-α.** 10 μg/ml of anti-TNF-α mAb, BC-7, was added to MLC involving LEP_{17} and HL-60, HL-60-R7 or RC-2a cells. 3D3.3 (10 μg/ml) was used as an isotype class control. All stimulators alone gave < 200 cpm and LEP_{17} alone gave < 300 cpm.


= LEP30 + HL60
= LEP30 + HL60-R7
= LEP30 + RC2a
3H-Thymidine uptake
CPM (x10-3)
110
100
90
80
70
60
50
40
30
20
10
No Ab
3D3.3
BC-7
No Ab
3D3.3
BC-7
No Ab
3D3.3
BC-7
Antibody (Ab) 10 µg/ml

reduced to approximately 30% whilst responses to HL-60 and HL-60-R7 cells were still inhibited by approximately 90%.

5.3 Expression of IL-1α and IL-1β mRNA by HL-60-R7 cells and other cell lines and the role of IL-1 in the MLC

Independent RNase protection assays were performed on a variety of cell lines using IL-1α and IL-1β anti-sense RNA probes. Figure 5.3.1 reveals that all of the cell-types tested, apart from LPS-stimulated human monocytes, expressed little or no IL-1α mRNA. In the case of HL-60 cells no bands were detectable for the 1, 5 or 10 μg RNA samples even after exposing the dried gel to the PhosphorImager for 48 hours. Despite not being discernible in figure 5.3.1 it was found that HL-60-R7 cells did express very small quantities of IL-1α mRNA. The level of expression was approximately 0.005 (0.5%) that of β-actin mRNA expression. Thus there would appear to have been a marginal up-regulation of IL-1α mRNA expression by HL-60-R7 cells compared to the control HL-60 cells.

RC-2a, COS and U937 cells failed to express detectable levels of IL-1α mRNA species. Although not detectable in figure 5.3.1, analysis via the PhosphorImager showed that U937 treated with TPA for 6 days and human embryonic lung cells expressed IL-1α mRNA at levels 0.02 and 0.01 that of β-actin respectively. Probe was added in excess (5×10^4 cpm) for all cell lines tested and the relative levels of IL-1α expression are shown in table 5.3.1.

IL-1β mRNA was found to be present in all cell types tested except COS cells, although bands were often barely discernible (figure 5.3.2). The levels were so low that autoradiography (48 hours) did not lead to detection of expression for U937 cells. However exposure of the dried gel to a PhosphorImager screen for 48 hours led to detection due to the enhanced sensitivity of this system. It was calculated that HL-60 cells expressed IL-1β mRNA at a level of 0.02 of that of β-actin mRNA whilst HL-60-R7 cells expressed slightly higher levels (0.07 that of β-actin). The relative levels of

Figure 5.3.1: **Expression of IL-1α mRNA.** Autoradiograph showing the results of a RNase protection assay where anti-sense ^{32}P-labelled probes were used to detect the presence of IL-1α and β-actin mRNA in HL-60, HL-60-R7, RC-2a, U937, U937 treated with TPA, COS, human embryonic lung cells (H.E.L.) and LPS-treated monocytes. See section 2.3.11 for details of methods.

Legend

Lane 1 = HL-60 (5 μg RNA)

Lane 2 = HL-60-R7 (5 μg RNA)

Lane 3 = RC-2a (5 μg RNA)

Lane 4 = U937 (5 μg RNA)

Lane 5 = U937-PMA (5 μg RNA)

Lane 6 = COS (10 μg RNA)

Lane 7 = H.E.L. (20 μg RNA)

Lane 8 = monocytes-LPS (8 μg RNA)

Lane 9 = ^{32}P-labelled pUC19 DNA restricted with Hpa II

Lane 10 = IL-1α probe (unprotected)

Lane 11 = β-actin probe (unprotected)

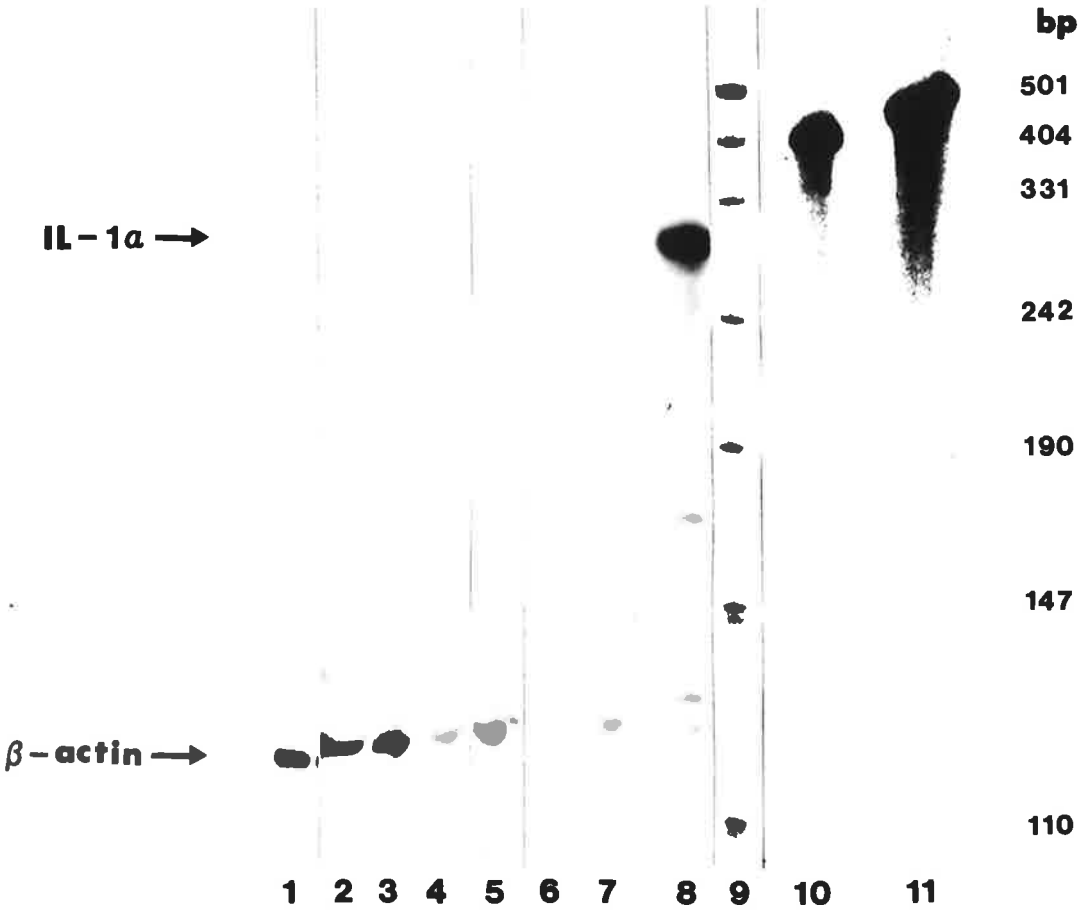

bp
501
404
331
242
190
147
110
IL-1α
β-actin
1 2 3 4 5 6 7 8 9 10 11

Table 5.3.1: **Relative levels of IL-1α mRNA expression.** The levels of IL-1α mRNA expression by the indicated cell types relative to β-actin mRNA levels as determined by the PhosphorImager. Experiment was performed in duplicate (mean ± SEM).

Table 5.3.2: **Relative levels of IL-1β mRNA expression.** The levels of IL-1β mRNA by the indicated cell types relative to β-actin mRNA levels as determined by the PhosphorImager. Experiment was performed in duplicate (mean ± SEM).

Cell type	Ratio of IL-1-α:ß-actin
HL60	0
HL60-R7	0.07 ± 0.02
RC2a	0
U937	0
U937-TPA	0.021 ± 0.001
H.E.L.	0.001
COS	0

Cell type	Ratio of IL-1-ß:ß-actin
HL60	0.019 ± 0.001
HL60-R7	0.065 ± 0.013
RC2a	0.38 ± 0.02
U937	0.035 ± 0.002
U937-TPA	4.8 ± 0.2
H.E.L.	0.614
COS	0

Figure 5.3.2: **Expression of IL-1β mRNA.** Autoradiograph showing RNase protection assay where anti-sense ^{32}P-labelled probes were used to detect the presence of IL-1β and β-actin mRNA in HL-60, HL-60-R7, RC-2a, U937, U937 treated with PMA, H.E.L. and COS cells. See section 2.3.11 for details of methods.

Legend

Lane 1 = HL-60 (5 μg RNA)

Lane 2 = HL-60 (10 μg RNA)

Lane 3 = HL-60-R7 (5 μg RNA)

Lane 4 = HL-60-R7 (10 μg RNA)

Lane 5 = RC-2a (5 μg RNA)

Lane 6 = RC-2a (10 g RNA)

Lane 7 = U937 (10 μg RNA)

Lane 8 = U937-TPA (5 μg RNA)

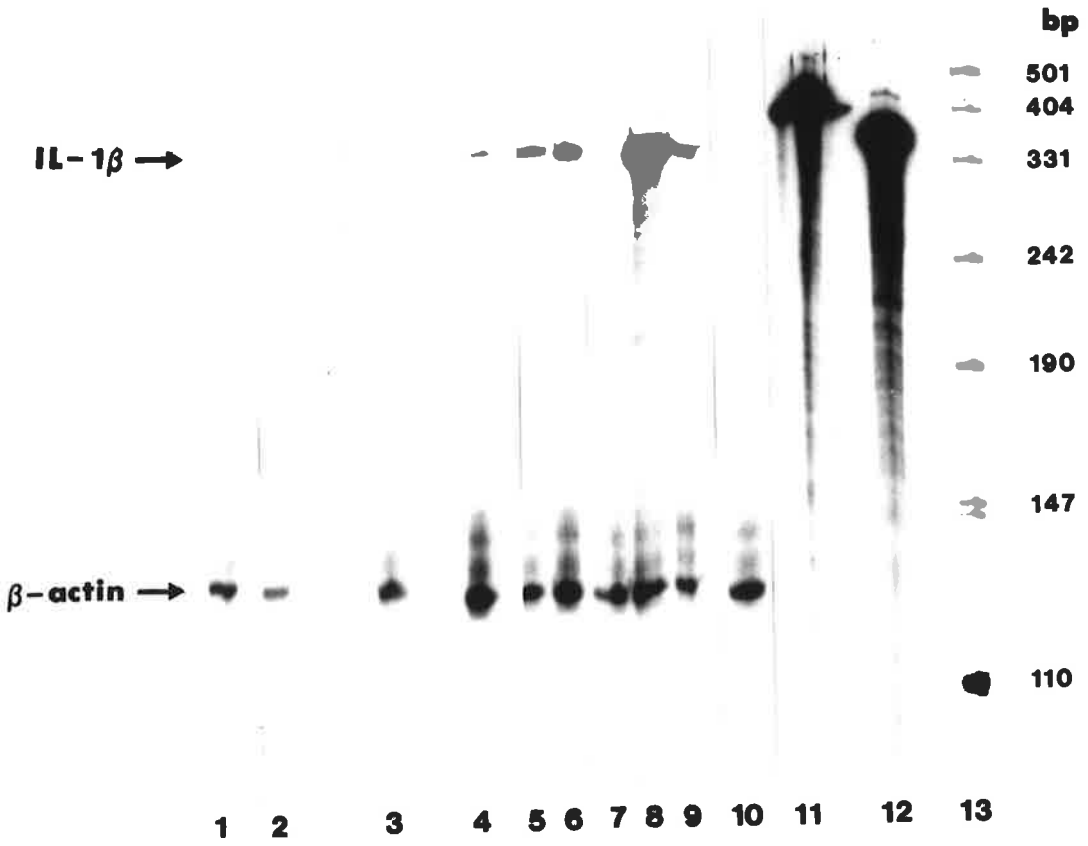
Lane 9 = H.E.L. (20 μg RNA)

Lane 10 = COS (10 μg RNA)

Lane 11 = β-actin probe (unprotected)

Lane 12 = IL-1β probe (unprotected)

Lane 13 = ^{32}P-labelled pUC19 DNA restricted with Hpa II


bp
501
404
331
242
190
147
110
IL-1β
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13

this mRNA species in the other cell types are tabulated in table 5.3.2 where it can be seen that RC-2a cells expressed abundant levels, and TPA-treated U937 cells even more so.

The role of IL-1(α and β) in the proliferative responses of LEP to HL-60, HL-60-R7 and RC-2a cells was tested using a commercial rabbit polyclonal antiserum (Cistron, USA, cat. no. 02-1100) specific for human IL-1 where the antiserum was added to a MLC of LEP plus stimulator cells. Figure 5.3.3 indicates that IL-1 does not play a significant role in the response of LEP to HL-60, HL-60-R7 or RC-2a cells. Similar results were obtained with 3 other LEP tested. The recommended dosage of the IL-1 antisera was used and was shown to be active through its capacity to inhibit the proliferation of murine thymocytes induced by 30 U/ml IL-1β, together with PHA, in a ^{3}H-thymidine incorporation assay (figure 5.3.4).

5.4 Expression of IL-6 mRNA by HL-60-R7 cells and other cell lines.

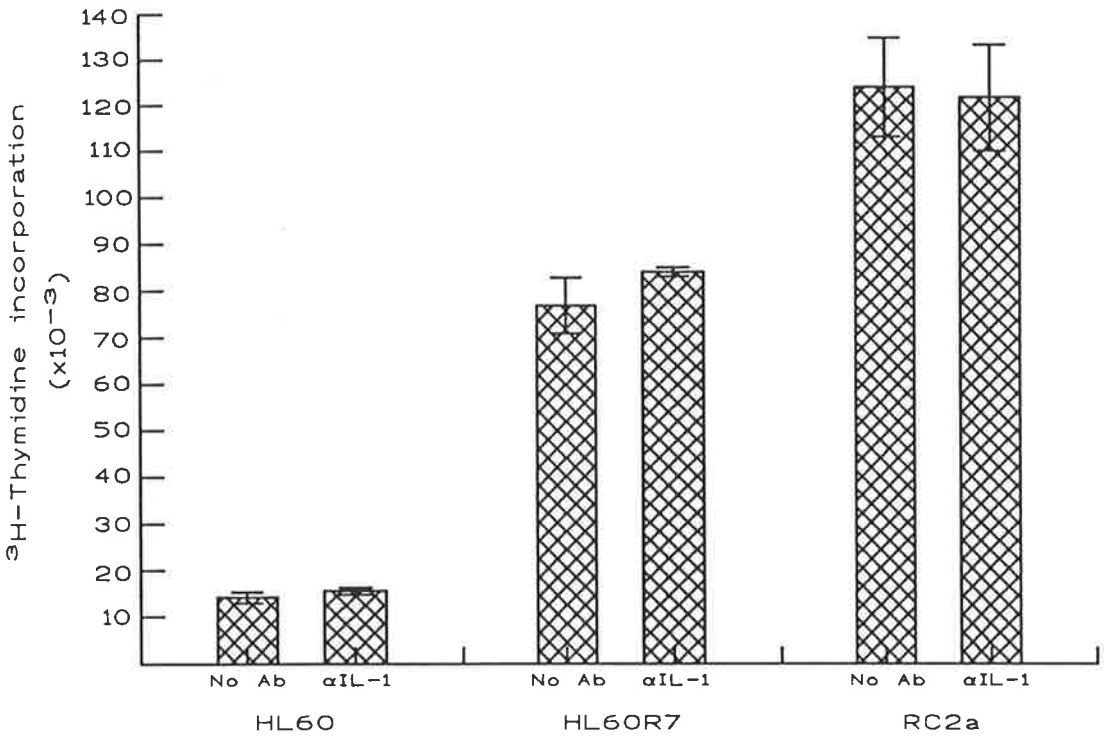
Neither HL-60-R7 nor undifferentiated HL-60 cells expressed detectable levels of IL-6 mRNA as determined by the RNase protection assay, even after exposure of the dried gel to a PhosphorImager screen for 48 hours. The only cells found to contain this mRNA species were H. E. L. cells, at levels three-fold greater than that of β-actin mRNA levels. Results of the RNase protection assay are displayed in figure 5.4.1. MLC-inhibition assays were not performed due to a lack of readily available anti-IL-6 mAb.

5.5 Expression of Stem Cell Factor mRNA by HL-60-R7 and RC-2a cells and the role of SCF in the MLC.

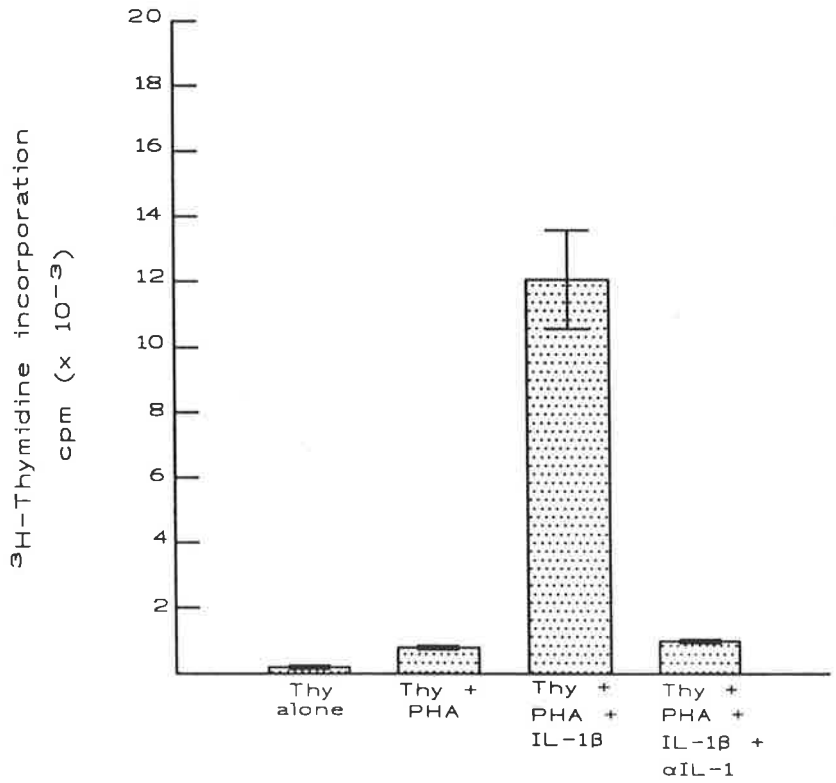
Stem Cell Factor (SCF) is a cytokine produced by a wide variety of cell types and is the ligand for the more restricted c-kit receptor tyrosine kinase (Williams *et al*, 1990). It has been recently noted that SCF can play a role in the activation of

Figure 5.3.3: **Failure of antiserum to IL-1 to block lymphocyte proliferation in MLC.** An anti-human IL-1 polyclonal rabbit antiserum (1/33 final dilution) was added to MLC containing LEP_{19} plus HL-60, HL-60-R7 or RC-2a cells. All stimulators < 200 cpm, $LEP_{19} \approx 800$ cpm.

Figure 5.3.4: **Thymocyte proliferation assay to demonstrate blocking activity of the α-IL-1 antiserum.** C3H thymocytes (1 x 10^6/ml) were cultured alone or with PHA (5 μg/ml), PHA plus rhuIL-1β (30 U/ml) or PHA plus rhuIL-1β plus anti-IL-1 (anti-human IL-1 polyclonal rabbit antiserum; 1/33 final dilution).



LEP_{19} + designated stimulator



Thymocytes (Thy) with or without PHA, IL-1β and αIL-1.

Figure 5.4.1: **Expression of IL-6 mRNA.** Autoradiograph showing RNase protection assay where antisense ^{32}P-labelled probes were used to detect the presence of IL-6 and β-actin mRNA in HL-60, HL-60-R7, RC-2a, U937, TPA-treated U937, H.E.L., and COS cells. See section 2.3.11 for details of methods.

Legend

Lane 1 = HL-60 (5 μg RNA)

Lane 2 = HL-60-R7 (5 μg RNA)

Lane 3 = RC-2a (5 μg RNA)

Lane 4 = U937 (10 μg RNA)

Lane 5 = U937-TPA (5 μg RNA)

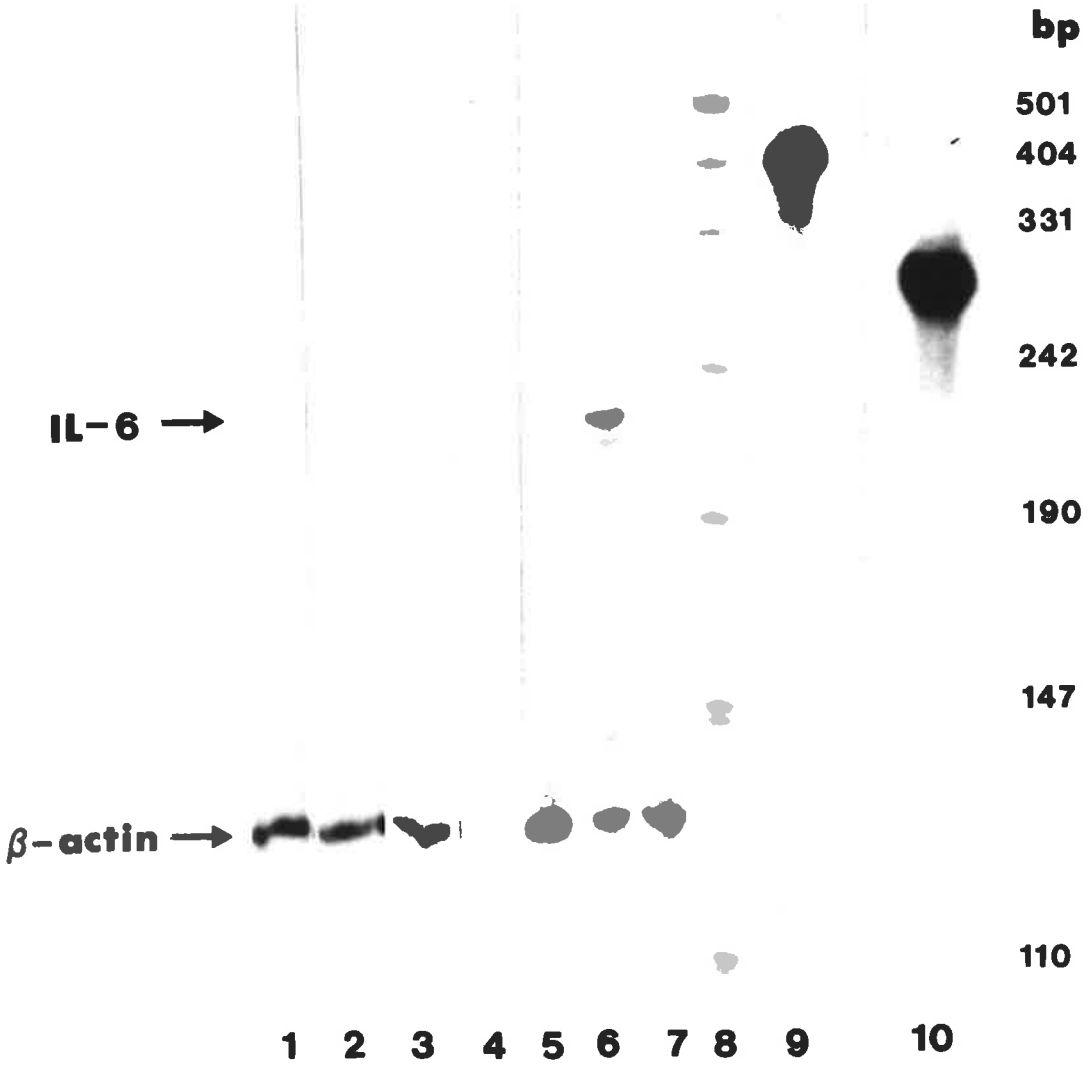
Lane 6 = H.E.L. (20 μg RNA)

Lane 7 = COS (10 μg RNA)

Lane 8 = ^{32}P-labelled pUC19 DNA restricted with Hpa II

Lane 9 = IL-6 probe (unprotected)

Lane 10 = β-actin probe (unprotected)


bp
501
404
331
242
190
147
110
IL-6
β-actin
1 2 3 4 5 6 7 8 9 10

lymphocytes, especially NK cells (Matos *et al*, 1993). Thus it was thought that SCF may be important in the activation of lymphocytes by HL-60-R7 cells.

RNase protection assays were performed to detect the presence of SCF mRNA in HL-60, HL-60-R7 or RC-2a cells. Here GAPDH was used as the control for loading variations (Siebert and Hung, 1991). It was discovered that no detectable levels were expressed by any of these cell lines after overnight exposure of the polyacrylamide gel to autoradiographic film (figure 5.5.1). HL-60 and HL-60-R7 cells expressed barely detectable levels after 2 days of exposure to a PhosphorImager screen (table 5.5.1) with little apparent quantitative differences between the two cell lines.

In order to further investigate the role of SCF in the LEP response to HL-60-R7 cells, a MLC inhibition assay was performed where three mAb, YB5.B8, 17F11 and SR-1, specific for the c-kit protein were independently added to the MLC. One of these mAb, SR-1, has been previously shown to inhibit SCF binding to c-kit (Broudy *et al*, 1992; Ashman *et al*, in press). Also, YB5.B8 has been shown to block SCF augmentation of the proliferation of an IL-2-activated NK cell subset (Matos *et al*, 1993). The results of an experiment using SR-1, displayed in figure 5.5.2, revealed there to be no significant inhibition of the proliferative responses of LEP to HL-60-R7 or RC-2a cells by any of the mAb. The concentration of SR-1 used (10 μg/ml) has previously been shown to be capable of inhibiting SCF-dependent proliferation of the cell line MO-7e (Ashman *et al*, in press). YB5.B8 and 17F11 also failed to affect the proliferative responses. These results were found to be reproducible for 3 other LEP.

5.6 The role of IL-12 in the LEP response to HL-60-R7 cells

IL-12 has been shown to stimulate the proliferation of lymphocytes, including NK cells (Gately *et al*, 1991; Perussia *et al*, 1993). Since HL-60-R7 cells were capable of stimulating NK cell proliferation within the LEP, it was thought possible that IL-12 had a role to play in the activation process. Thus, a mAb, B7, specific for IL-12 and capable of abrogating the functional activity of the cytokine (Gately *et al*, 1991), was

Figure 5.5.1: **Expression of SCF mRNA.** RNase protection assay where anti-sense ^{32}P-labelled probes were used to detect the presence of SCF and GAPDH mRNA expressed by HL-60, HL-60-R7, RC-2a and MCF-7 cells. See section 2.11.3 for further details of methods.

Legend

Lane 1 = MCF-7 (10 μg RNA)

Lane 2 = HL-60 (10 μg RNA)

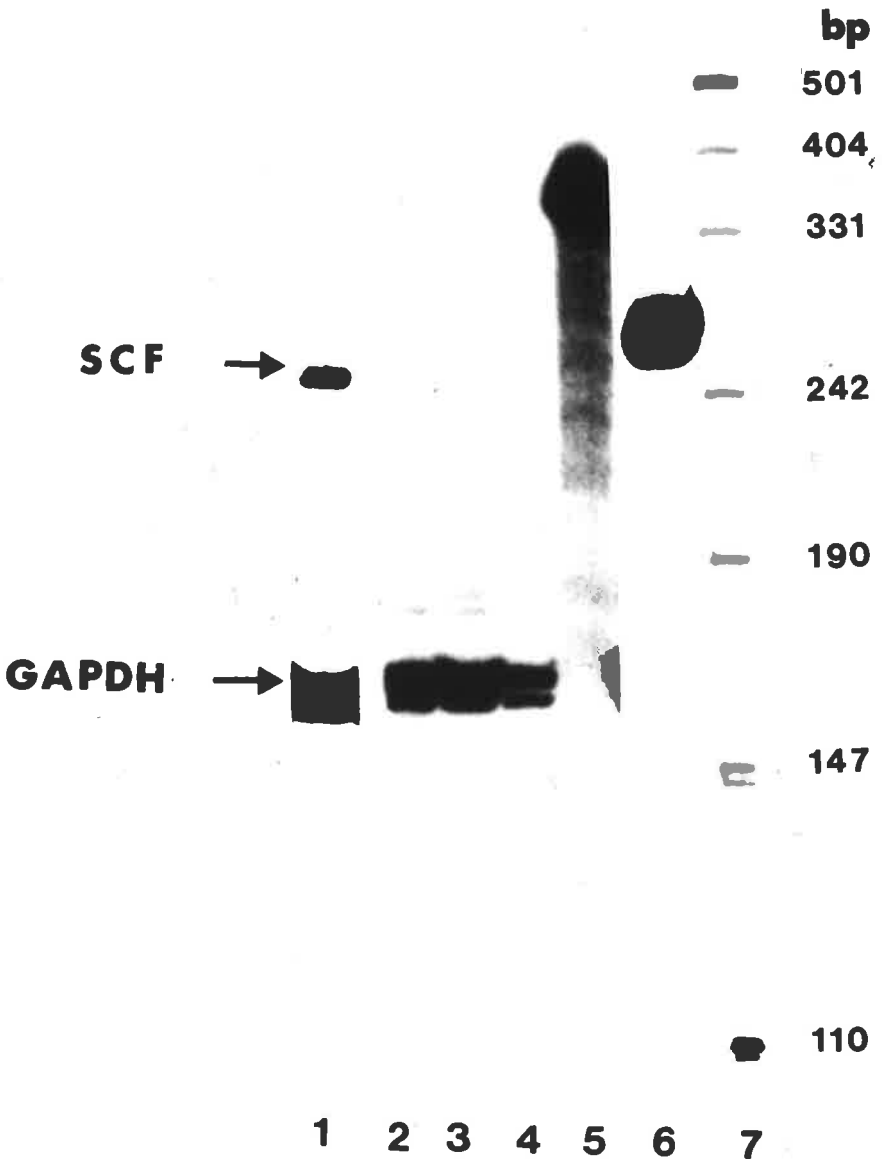
Lane 3 = HL-60-R7 (10 μg RNA)

Lane 4 = RC-2a (10 μg RNA)

Lane 5 = SCF probe (unprotected)

Lane 6 = GAPDH (unprotected)

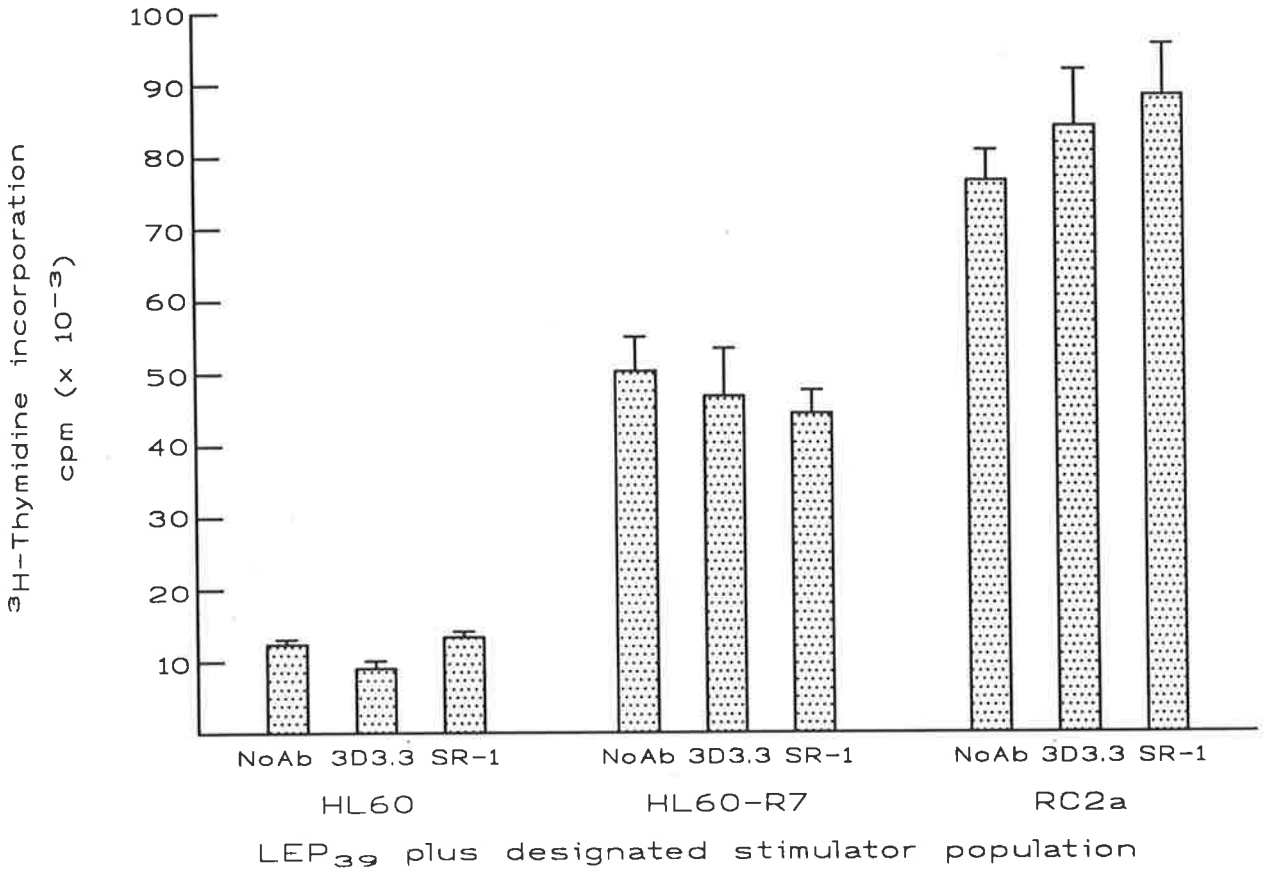
Lane 7 = ^{32}P-labelled pUC19 DNA restricted with Hpa II


bp
501
404
331
242
190
147
110
SCF
GAPDH
1
2
3
4
5
6
7

Cell type	Ratio of SCF:GAPDH
HL60	0.009
HL60-R7	0.012
RC2a	0.006
MCF-7	0.396

Table 5.5.1: **Relative levels of SCF mRNA expression.** The levels of SCF mRNA by the indicated cell types relative to GAPDH mRNA levels as determined using the PhosphorImager.

Figure 5.5.2: **Failure of blocking mAb to the SCF receptor to inhibit lymphocyte proliferation in MLC.** Anti-c-kit mAb, SR-1 (10 μg/ml), or a control mAb, 3D3.3 (10 μg/ml) was added to MLC involving LEP_{39} plus HL-60, HL-60-R7 or RC-2a cells. Stimulator cells alone gave < 500 cpm and LEP_{39} alone = 1300 ± 350 cpm.


^{3}H-Thymidine incorporation
cpm (x 10^{-3})
100
90
80
70
60
50
40
30
20
10
NoAb 3D3.3 SR-1
HL60
NoAb 3D3.3 SR-1
HL60-R7
NoAb 3D3.3 SR-1
RC2a
LEP_{39} plus designated stimulator population

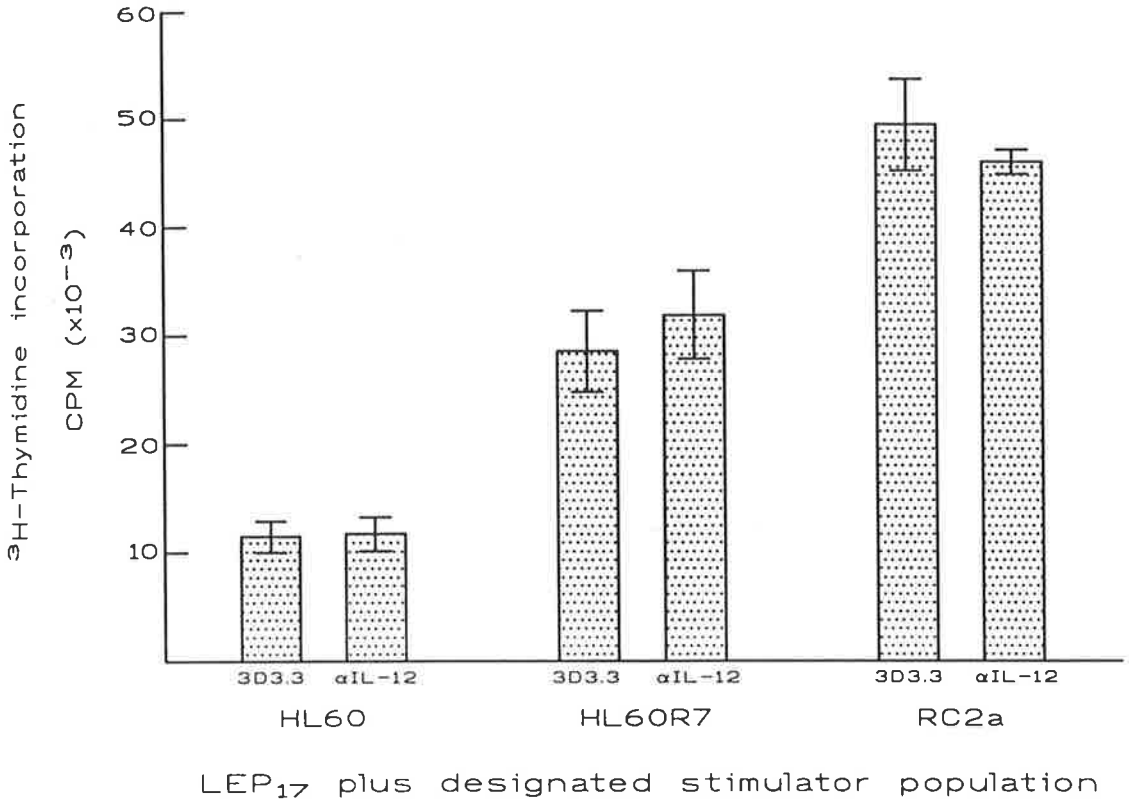
added to a MLC of LEP plus HL-60, HL-60-R7 or RC-2a cells. Figure 5.6.1 shows that there was no effect on the responses of LEP_{17} to any of the three stimulators tested upon addition of the anti-IL-12 mAb. This result was shown to be reproducible for 2 other LEP tested. The mAb to IL-12 was shown to be functional through its ability to block the recombinant human IL-12 (500 U/ml)-induced proliferation of PHA- and IL-2-stimulated lymphocytes (figure 5.6.2). A riboprobe for IL-12 mRNA was not available and so the levels of IL-2 mRNA expression by the stimulator cells was not determined.

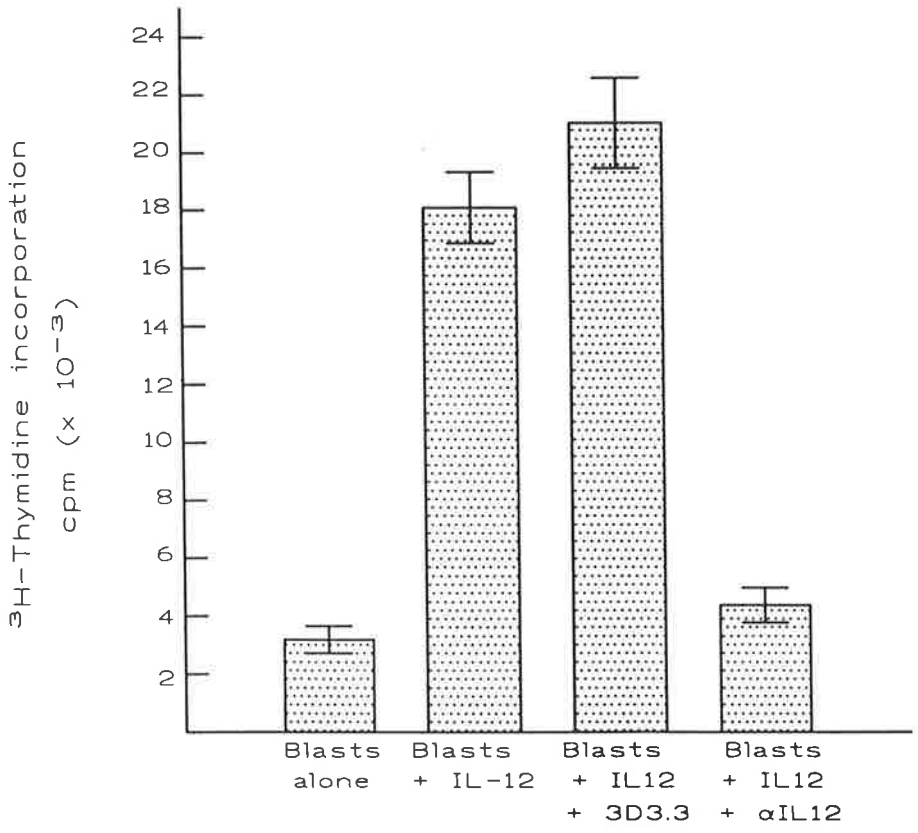
Discussion

Of all the cytokines tested, the only one found to have functional significance in the response of LEP to HL-60-R7 cells was TNF-α since 10 μg of a mAb almost completely abrogated any proliferative response to HL-60-R7 cells. This was also shown to be the case for the responses of LEP to both untreated HL-60 cells and RC-2a cells. Whether it was TNF-α produced by the stimulator cells, or by lymphocytes, that was of functional significance remains unknown. Analysis of TNF-α mRNA levels revealed that both HL-60 and HL-60-R7 cells expressed substantial and relatively equivalent levels of TNF-α mRNA suggesting that both populations were potentially capable of producing TNF-α during the MLC. The possibility that it was TNF-α production by the stimulator population that was important is supported by the recent report that monocytes are a major source of TNF-α production during *in vitro* allogeneic MLC, whilst purified T lymphocytes were poor producers of TNF-α (Lee *et al*, 1993). However, once activated, lymphocytes and NK cells are well recognized for their ability to produce TNF-α (Anegon *et al*, 1988) and have been reported to be capable of producing TNF-α during MLC (Steffen *et al*, 1985; Paya *et al*, 1988). Therefore induction of expression of this cytokine by the LEP may have been an important event in the triggering of lymphocyte proliferation. How TNF-α influenced lymphoproliferation was not investigated, however, this cytokine has been shown to

Figure 5.6.1: **Failure of neutralizing antibody to IL-12 to block lymphocyte proliferation in MLC.** Anti-IL-12 mAb, B7 (10 μg/ml), or a control mAb, 3D3.3 (10 μg/ml) was added to MLC involving LEP_{17} plus HL-60, HL-60-R7 or RC-2a cells. Stimulator cells and LEP_{17} alone gave < 500 cpm.

Figure 5.6.2: **Blocking activity of antibody to IL-12 in an IL-12-dependent lymphocyte proliferation assay.** Lymphocytes were cultured for 2 days, alone or in the presence of; rhuIL-12 (400 U/ml), rhuIL-12 plus 3D3.3 (10 μg/ml) or rhuIL-12 plus anti-IL-12 (10 μg/ml). Wells were pulsed for 18 hr with ^{3}H-thymidine.


^{3}H-Thymidine incorporation
CPM (x10^{-3})
60
50
40
30
20
10
3D3.3
αIL-12
3D3.3
αIL-12
3D3.3
αIL-12
HL60
HL60R7
RC2a
LEP$_{17}$ plus designated stimulator population


^{3}H-Thymidine incorporation
cpm (x 10^{-3})
24
22
20
18
16
14
12
10
8
6
4
2
Blasts alone
Blasts + IL-12
Blasts + IL12 + 3D3.3
Blasts + IL12 + αIL12

induce expression of IL-2 and CD25 by human T lymphocytes (Lee *et al*, 1987; Lowenthal *et al*, 1989).

With respect to the remaining cytokines that were tested, it would appear that SCF, IL-12 and IL-1 did not play a significant part in lymphoproliferative responses to HL-60-R7 or RC-2a cells as blocking mAb specific for these cytokines were incapable of abrogating these responses. The complete lack of detectable levels of IL-6 mRNA suggests that this cytokine may also be of little or no importance in these MLC.

Chapter 6

Analysis of the cell surface antigen expression and the immunostimulatory nature of a variety of cell lines.

6.1 Introduction

The cell surface antigen expression by HL-60, HL-60-R7 and RC-2a cells was analysed in some detail in chapter 3, and from these studies it was noticed that changes in antigen expression on RA-treated HL-60 cells coincided with an increased capacity to stimulate the proliferation of LEP. For example, some of the antigens, such as LFA-1, LFA-3, ICAM-1, ICAM-3 and CD11b, which were also expressed by the highly immunostimulatory RC-2a cells, were found to be up-regulated on HL-60-R7 cells. Additionally, it was discovered in chapter 4 that MHC class II molecules played an important role in the proliferative responses of LEP to HL-60-R7 and RC-2a cells, despite the fact that this molecule was not detectable on HL-60-R7 cells at the initiation of the MLC. This latter result serves as a forewarning with respect to any conclusions which may be made concerning the role of any of the cell surface molecules assayed here, as important alterations in cell surface antigen expression may have occurred during the MLC itself. Despite this reservation, a variety of myeloid, and two lymphoid, cell lines were examined for their expression of a wide range of antigens via the IIF assay and the capacity of these cell lines to stimulate or suppress the proliferation of LEP was tested. Such a study may reveal a correlation between cell surface antigen expression and the capacity to stimulate lymphocyte proliferation. Included in the study were HL-60 cells cultured with 1,25-dihydroxyvitamin D_3 (Vit D_3), IFN-γ or a combination of IFN-γ + RA, as these factors have been reported to induce HL-60 cells to become monocyte-like (Rigby *et al*, 1985; Ball *et al*, 1984; Hemmi and Breitman, 1987), and it was of interest to compare the immunostimulatory nature of these cells with that of HL-60-R7 cells.

6.2 IIF assays and MLC studies on a variety of cell lines

The results of the IIF assays performed on each cell line are shown in table 6.2.1. All cell lines tested, with the exception of K562, were found to express MHC class I molecules. As noted earlier, in chapter 3, RA-treatment of HL-60 cells resulted in a down-regulation of MHC class I expression. The treatment of HL-60 cells with IFN-γ however resulted in a 3 - 5-fold increase in expression of this molecule, whilst treatment with the combination of IFN-γ and RA resulted in levels of expression similar to those of untreated HL-60 cells. Vit D_3 treatment of HL-60 cells resulted in the down-regulation of MHC class I antigen expression.

With respect to MHC class II antigen expression, a proportion of HL-60 cells treated for 7 days with RA plus IFN-γ, or IFN-γ alone, expressed low - medium levels, whereas RA-treated and Vit D_3-treated HL-60 cells did not express this antigen. At least a proportion of each of the other cell lines expressed MHC class II molecules with the exception of U937, K562 and MOLT-4. The erythroleukaemic cell line, HEL and its subline, HEL-DR, were of interest as HEL-DR expressed significantly greater levels MHC class II molecules than HEL.

The majority of cell lines expressed both ICAM-1 and its ligand LFA-1, as well as LFA-3, albeit at varying levels. However, K562 cells did not express LFA-1 or LFA-3, and MOLT-4 and HL-60 cells did not express ICAM-1. Due to a lack of adequate quantities of anti-ICAM-2 and anti-ICAM-3 mAb only a selection of cell lines were tested for expression of ICAM-2 and ICAM-3 molecules. As previously noted, HL-60 cells down-regulated expression of ICAM-2 when treated with 10 nM RA for 7 days. Additionally, it was discovered that RA plus IFN-γ down-regulated expression of ICAM-2 by HL-60 cells, whereas IFN-γ down-regulated this antigen on approximately 30% of the population. ICAM-3 expression was not affected by IFN-γ treatment alone, however RA or the combination of RA and IFN-γ resulted in the up-regulation of

VIP-1	CD71	99# (516)	13# (318)	100 (78)	96* (18)	98# (90)
TS1/22	LFA-1	100 (30)	100 (81)	100 (45)	100 (36)	100 (45)
TS2/9	LFA-3	100 (27)	100 (87)	100 (81)	100 (51)	100 (55)
OKT3	CD3	0 (0)	0 (0)	N.T.	N.T.	0 (0)
OKT8	CD8	0 (0)	0 (0)	N.T.	N.T.	0 (0)

Table 6.2.1: Cell surface antigen expression by a range of cell lines. An overview of the IIF assays that were performed on a number of cell lines. The results are given in terms of percentage of cells positive for the relevant antigen. In brackets is given the relative mean fluorescence intensity (RMF) of the positive cells. The values obtained for the isotype control mAb ranged from 2 - 3 and were subtracted from the values obtained for the respective test mAb. Where there was a homogeneous peak shift of fluorescence compared with cells treated with the isotype control mAb, the cells were considered to be 100% positive even though the histograms may have overlapped. Where the population was heterogeneous with respect to antibody binding, cells with fluorescence intensities > the 99^{th} percentile of the fluorescence intensity of the negative control were considered positive.

* = asymmetric peak

= distinct populations

N. T. = not tested

		HL-60	HL-60-R7	HL-60 (IFN)	HL60 (IFN + RA)	HL-60 (Vit D_3) Day 4
Antibody	Antigen	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)
6c6b	Class I	100 (360)	92*(81)	100 (1666)	100 (378)	100 (93)
7b6c	Class I	100 (872)	N.T.	100 (2383)	100 (606)	100 (198)
FMC 4	Class II	0 (0)	0 (0)	39*(42)	23* (30)	0 (0)
FMC 14	Class II	0 (0)	0 (0)	N.T.	N.T.	N.T.
FMC 15	HLA-DR	0 (0)	0 (0)	N.T.	N.T.	0 (0)
W-CAM-1	ICAM-1	0 (0)	100 (21)	100 (60)	100 (264)	24*(51)
CBR-IC2/2	ICAM-2	82*(21)	11*(15)	70*(21)	100 (3)	N.T.
CBR-IC3/1	ICAM-3	100 (93)	100 (214)	100 (87)	100 (153)	N.T.
OKT11	CD2	0 (0)	0 (0)	N.T.	N.T.	0 (0)
OKT4	CD4	100 (33)	100 (30)	N.T.	N.T.	100 (6)
CD14	CD14	0 (0)	9*(9)	16*(108)	55* (45)	75*(387)
VIFcRIII	CD16	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5A4.C5	CD11b	0 (0)	89*(75)	9*(33)	43 (60)	69*(96)
B2B1.F9	CD31	37*(42)	35*(39)	N.T.	N.T.	49*(24)
VIMD5	CD15	99*(1068)	100*(291)	100 (672)	N.T.	98*(1122)
VIB-E3	CD24	0 (0)	0 (0)	N.T.	N.T.	0 (0)

VIP-1	CD71	33*(15)	100 (9)	100 (313)	100 (18)	100*(12)	100 (15)	100 (33)	100 (123)
TS1/22	LFA-1	100 (78)	100 (57)	100 (159)	100 (42)	100 (30)	0 (0)	6*(21)	100 (36)
TS2/9	LFA-3	100 (66)	100 (36)	100 (213)	100 (33)	100 (30)	0 (0)	7*(21)	100 (39)
OKT3	CD3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
OKT8	CD8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	100 (12)

Table 6.2.1: (continued)

		RC-2a	U937	KG-1	HEL	HEL-DR	K562	BALM-1	MOLT-4
Antibody	Antigen	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)	% +ve (RMF)
6c6b	Class I	100 (33)	100 (15)	100 (351)	100 (180)	100 (159)	0 (0)	100 (228)	100 (63)
7b6c	Class I	100 (153)	100 (606)	100 (678)	100 (432)	100 (330)	0 (0)	100 (369)	100 (191)
FMC 4	Class II	100 (273)	0 (0)	100 (204)	13*(15)	92*(111)	0 (0)	100 (15)	0 (0)
FMC 14	Class II	100 (897)	0 (0)	100 (609)	11*(15)	92*(138)	0 (0)	100 (117)	0 (0)
FMC 15	HLA-DR	100 (12)	0 (0)	70*(33)	N. T.	100 (6)	0 (0)	37*(96)	0 (0)
W-CAM-1	ICAM-1	100 (9)	100 (18)	100 (15)	100 (9)	12*(24)	100 (51)	100 (39)	0 (0)
CBR-IC2/2	ICAM-2	93*(33)	N.T.	100 (6)	N.T.	N.T.	N.T.	100 (39)	N.T.
CBR-IC3/1	ICAM-3	100 (420)	N.T.	100 (42)	N.T.	N.T.	N.T.	100 (63)	N.T.
OKT11	CD2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
OKT4	CD4	100 (12)	100 (36)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5*(42)
CD14	CD14	0 (0)	100 (255)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
VIFcRIII	CD16	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5A4.C5	CD11b	100 (15)	20*(9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	13*(21)
B2B1.F9	CD31	100 (117)	100 (39)	100 (42)	100 (96)	100 (78)	100 (15)	16*(6)	100 (33)
VIMD5	CD15	0 (0)	100 (39)	33*(570)	0 (0)	0 (0)	100*(126)	0 (0)	0 (0)
VIB-E3	CD24	0 (0)	9*(9)	0 (0)	67#(264)	86#(333)	100#(54)	100 (456)	0 (0)

expression of this antigen. The other cell lines tested, KG-1, BALM-4 and RC-2a, were found to express both ICAM-2 and ICAM-3.

MLC were performed with each cell line using 5 x 10^4 stimulator cells/well and 1 x 10^5 lymphocytes/well and results from selected experiments are displayed in figures 6.2.1 - 5. Figure 6.2.1 shows the immunostimulatory capacity of KG-1 and K562 relative to HL-60 and RC-2a cells using LEP_{37}. A similar experiment performed with HEL, HEL-DR or U937 cells, and using LEP_{36} is shown in figure 6.2.2. Figure 6.2.3 displays results obtained with MOLT-4 and BALM-1 cells using LEP_{17}. HL-60 cells cultured for 4 days with 5 x 10^{-8} M Vit D_3, for 7 days with IFN-γ (1000 U/ml) or for 7 days with IFN-γ (1000 U/ml) plus RA (10 nM), were also tested for their immunostimulatory capacity in MLC and examples of the results are shown in figures 6.2.4 and 6.2.5. A summary of the proliferative response of LEP to each cell line, relative to HL-60, is given in table 6.2.2. It was apparent that BALM-1 and HL-60(Vit D_3) were the only cell populations, apart from RC-2a and HL-60-R7 cells, to be consistently more immunostimulatory than HL-60 cells.

To determine whether any of the cell lines were immunosuppressive, the effect that each of them had on the proliferative response of LEP to RC-2a cells was determined by adding 5 x 10^4 mitomycin C-treated test cells to MLC containing RC-2a cells (5 x 10^4/well) and lymphocytes (1 x 10^5/well). Representative results are displayed in figures 6.2.6 - 8. An overview of the suppressive capacity of the cell lines is given in table 6.2.3 where it can be seen that a large proportion of the cell lines tested appeared to be capable of at least partially suppressing the lymphoproliferative response to RC-2a cells.

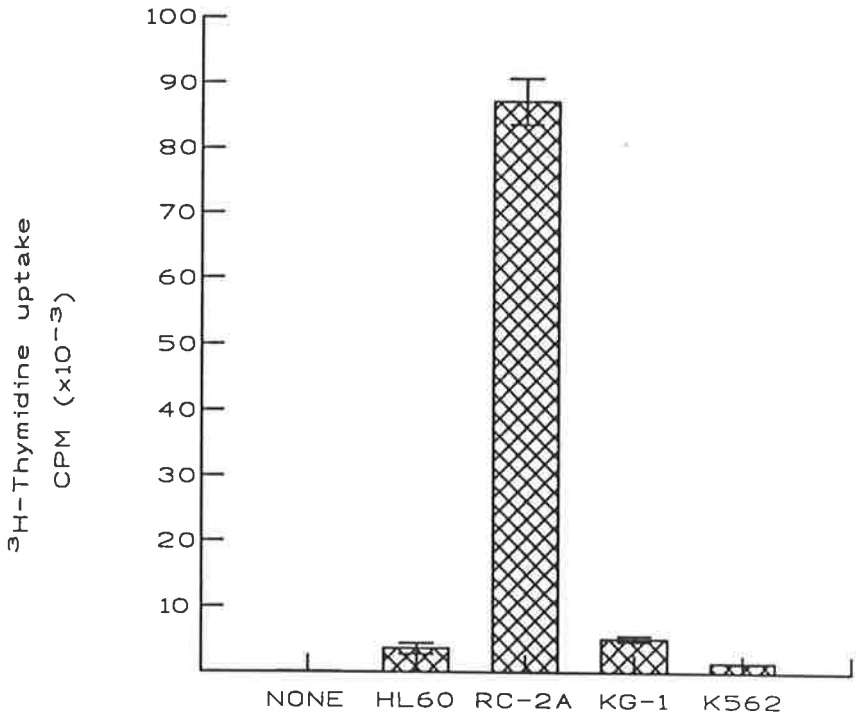
6.3 Discussion

None of the cell lines tested, other than HL-60-R7 and RC-2a, proved to be particularly immunostimulatory although BALM-1 and HL-60(Vit D_3) cells were slightly (2 - 3-fold) more stimulatory than HL-60 cells. The ability of HL-60, RC-2a,

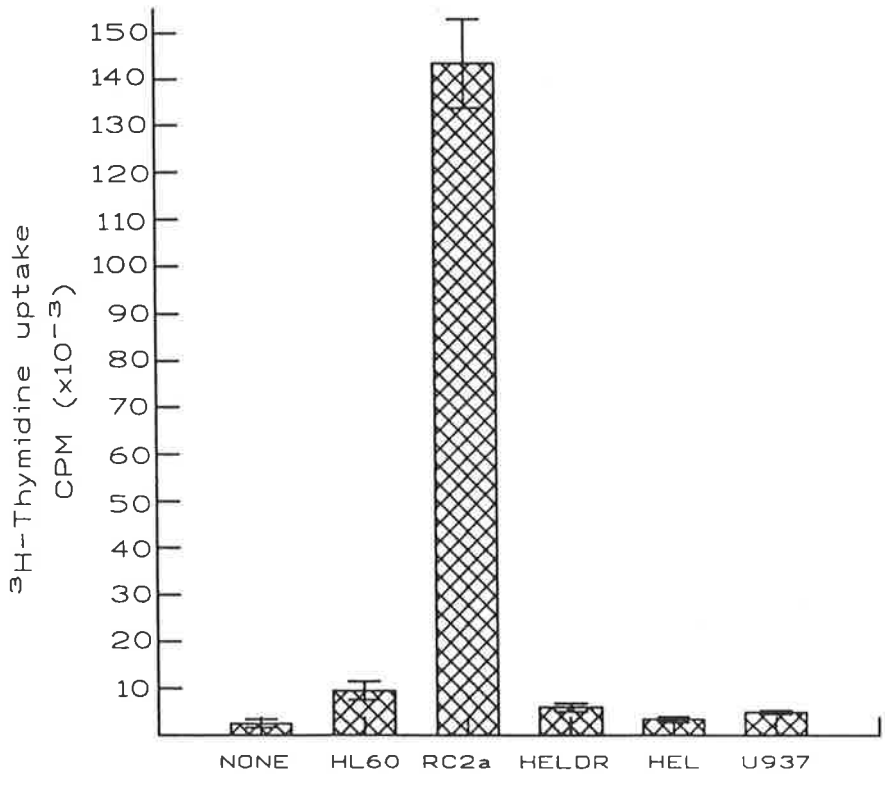
Figures 6.2.1 - 5: **Immunostimulatory capacity of leukaemic cell lines.** ^{3}H-thymidine uptake assays performed on MLC containing LEP (1 x 10^5/ml) and designated stimulator populations (5 x 10^4/ml). HL-60 (Vit D_3) = HL-60 cells cultured for 4 days in the presence of 5 x 10^{-8}M 1,25 dihydroxyvitamin D_3. HL60 (IFN) = HL60 cells cultured for 7 days with IFN-γ (1000 U/ml). HL60 (IFN + RA) = HL60 cells cultured for 7 days with IFN-γ (1000 U/ml) and RA (10 nM). Stimulators alone yielded < 300 cpm and LEP alone gave < 1500 cpm.

Figure 6.2.1: LEP_{37} plus HL-60, RC-2a, KG-1 or K562 cells.

Figure 6.2.2: LEP_{36} plus HL-60, HEL, RC-2a, U937 or HEL-DR.



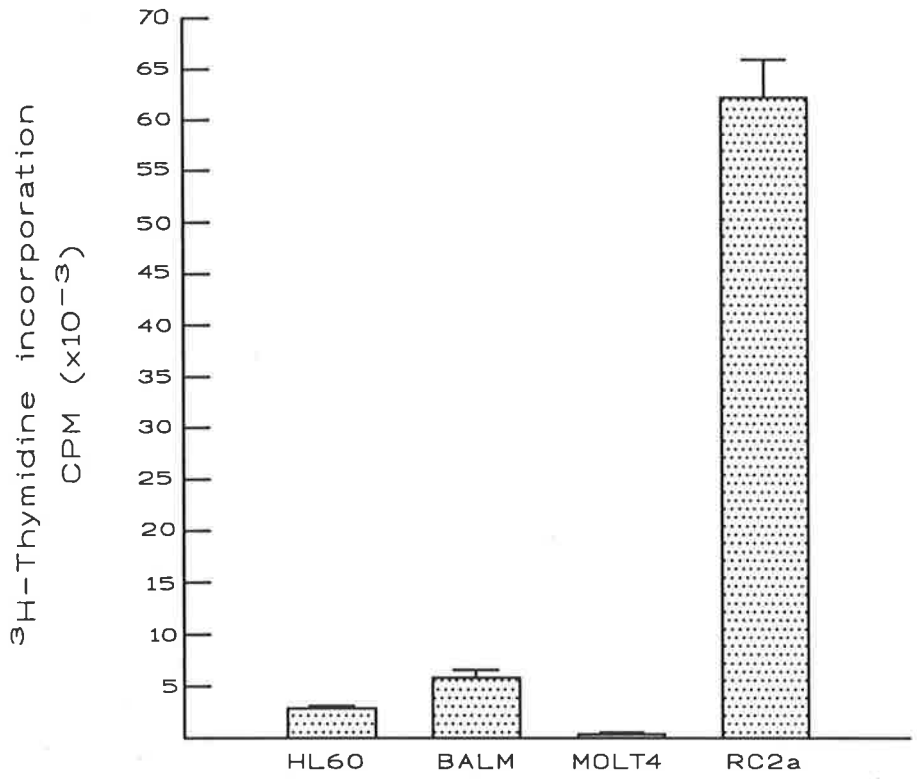
LEP_{37} plus designated stimulator



LEP_{36} plus designated stimulator

Figure 6.2.3: LEP_{17} plus HL-60, BALM-1, MOLT-4 or RC-2a cells.

Figure 6.2.4: LEP_{17} plus HL-60, HL-60-R7, HL-60 (IFN-γ), HL-60 (IFN-γ + RA) or RC-2a cells.


^{3}H-Thymidine incorporation
CPM (x10^{-3})
70
65
60
55
50
45
40
35
30
25
20
15
10
5
HL60
BALM
MOLT4
RC2a
LEP$_{17}$ plus designated stimulator

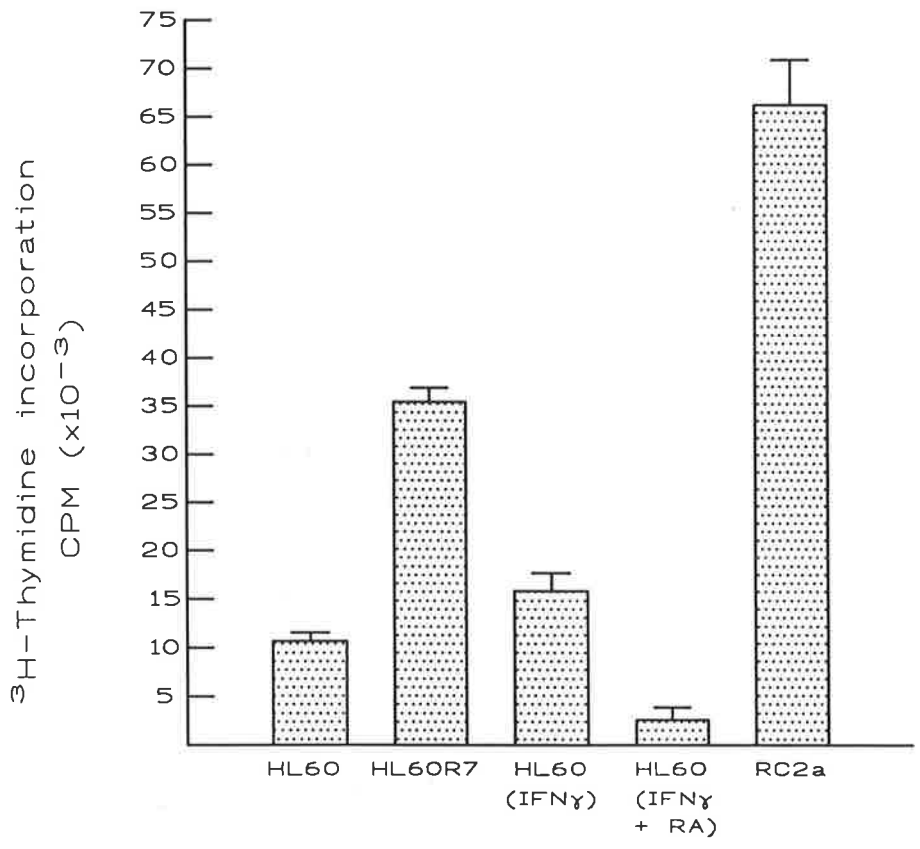
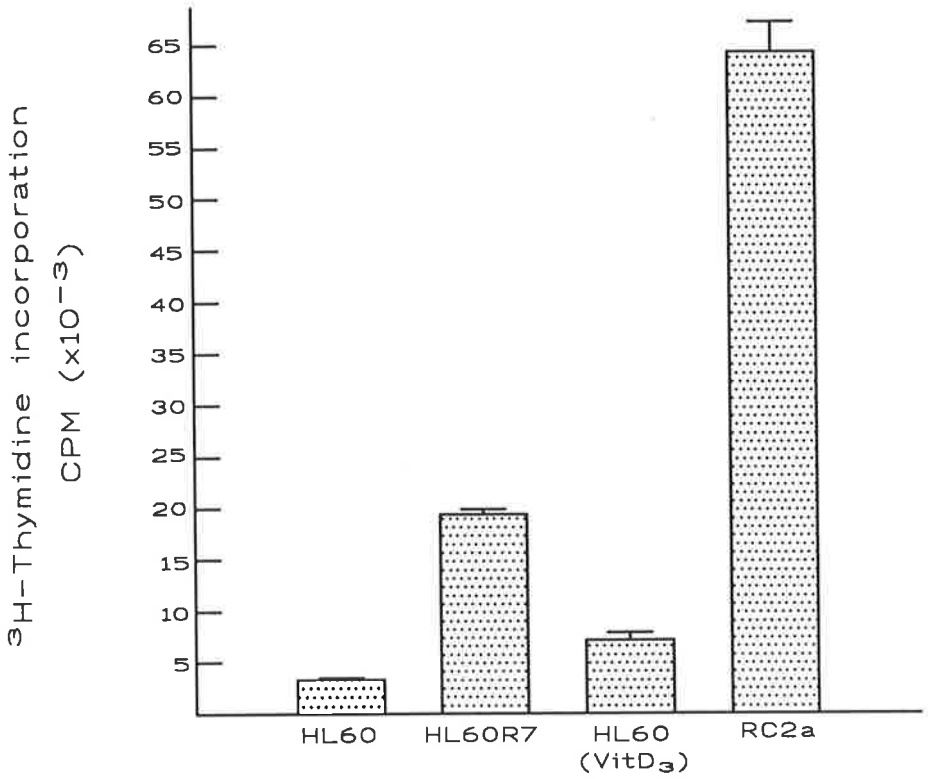

^{3}H-Thymidine incorporation
CPM (x10^{-3})
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
HL60
HL60R7
HL60 (IFNγ)
HL60 (IFNγ + RA)
RC2a
LEP$_{17}$ plus designated stimulator

Figure 6.2.5: LEP_{17} plus HL-60, HL-60-R7, HL-60 (Vit D_3) or RC-2a cells.

CELL LINE	Immunopotency relative to HL-60 cells
U937	0.24 ± 0.06 (n = 10)
MOLT-4	0.35 ± 0.18 (n = 6)
K562	0.36 ± 0.13 (n = 6)
HL60(IFN+RA)	0.42 ± 0.18 (n = 6)
HEL-DR	0.51 ± 0.08 (n = 4)
HEL-900	0.53 ± 0.23 (n = 7)
HL60(IFN)	1.02 ± 0.32 (n = 6)
KG-1	1.73 ± 0.71 (n = 4)
BALM-1	2.78 ± 0.50 (n = 6)
HL60(Vit D_3)	2.99 ± 0.54 (n = 4)
HL60-R7	11.04 ± 3.12 (n = 32)
RC-2a	24.15 ± 5.33 (n = 31)

Table 6.2.2: **Relative immunopotency of leukaemic cell lines.** Summary of the immunostimulatory nature of various cell lines relative to HL-60 cells. ^{3}H-thymidine uptake assays performed on MLC containing LEP (1 x 10^{5}/well) and designated stimulators.

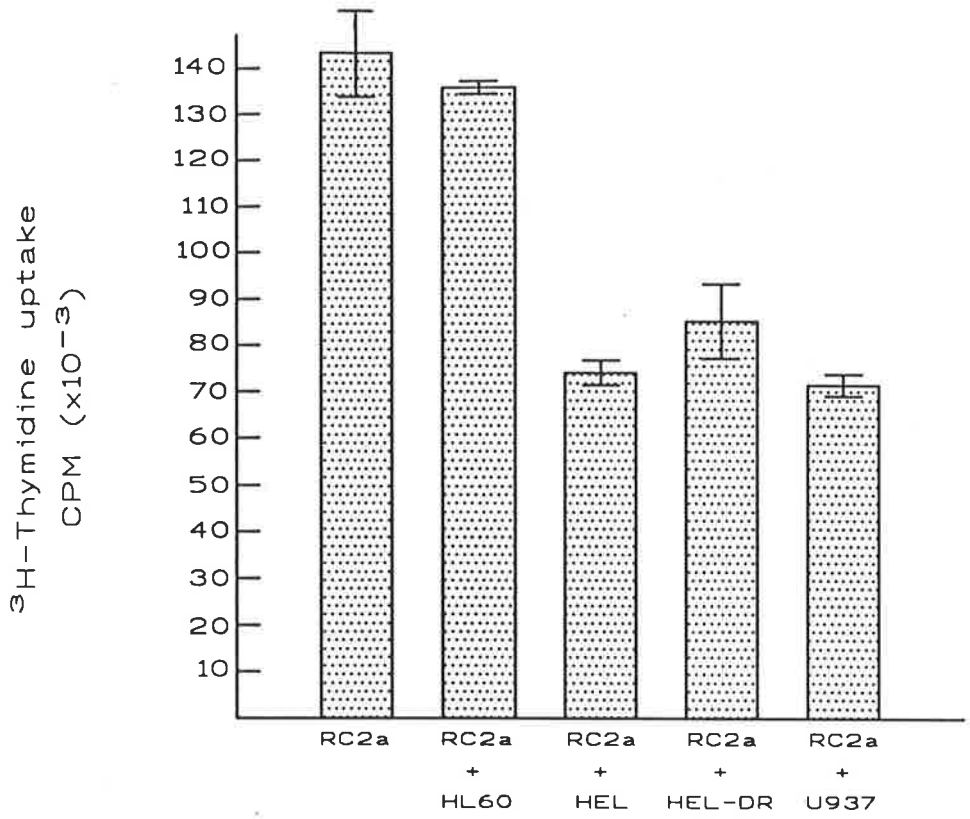
n = number of experiments


^{3}H-Thymidine incorporation
CPM (x10^{-3})
65
60
55
50
45
40
35
30
25
20
15
10
5
HL60
HL60R7
HL60 (VitD$_3$)
RC2a
LEP$_{17}$ + designated stimulator

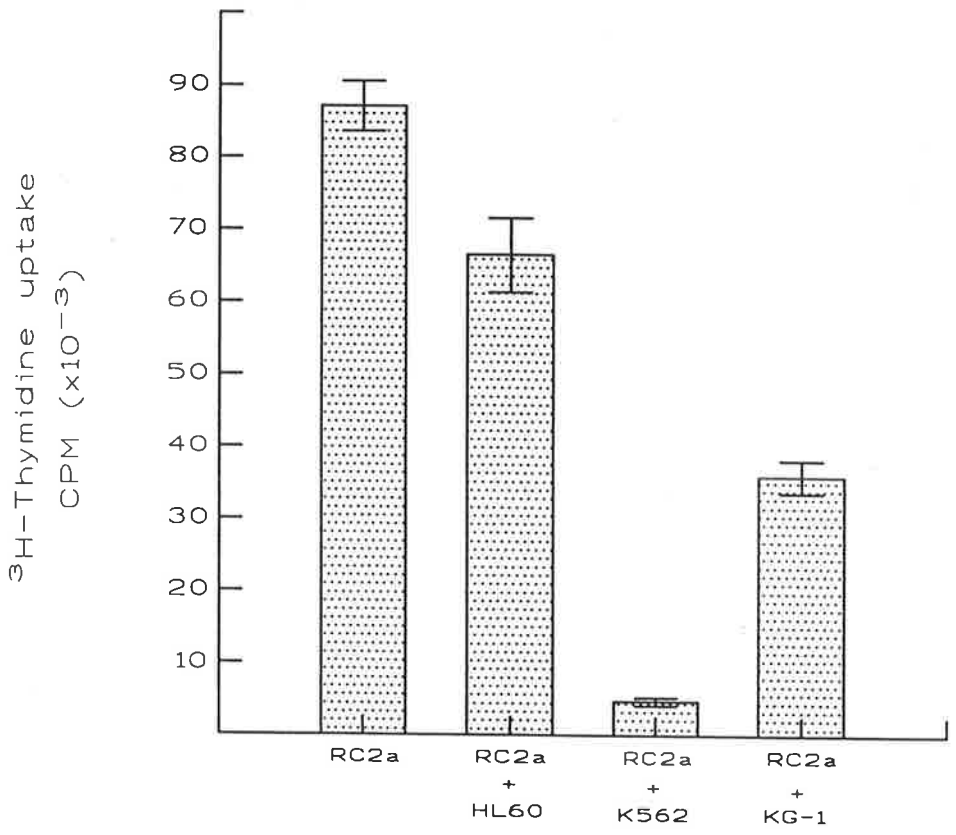
Figures 6.2.6 - 9: **Inhibitory action of leukaemic cell lines in MLC.** ^{3}H-thymidine uptake assays performed on MLC containing LEP (1 x 10^5/well) and a combination of RC-2a cells (5 x 10^4/ml) and designated stimulator cells (5 x 10^4/ml).

Figure 6.2.6: LEP_{36} and RC-2a cells plus HL-60, HEL, HEL-DR or U937 cells.

Figure 6.2.7: LEP_{37} and RC-2a cells plus HL-60, K562 or KG-1 cells.



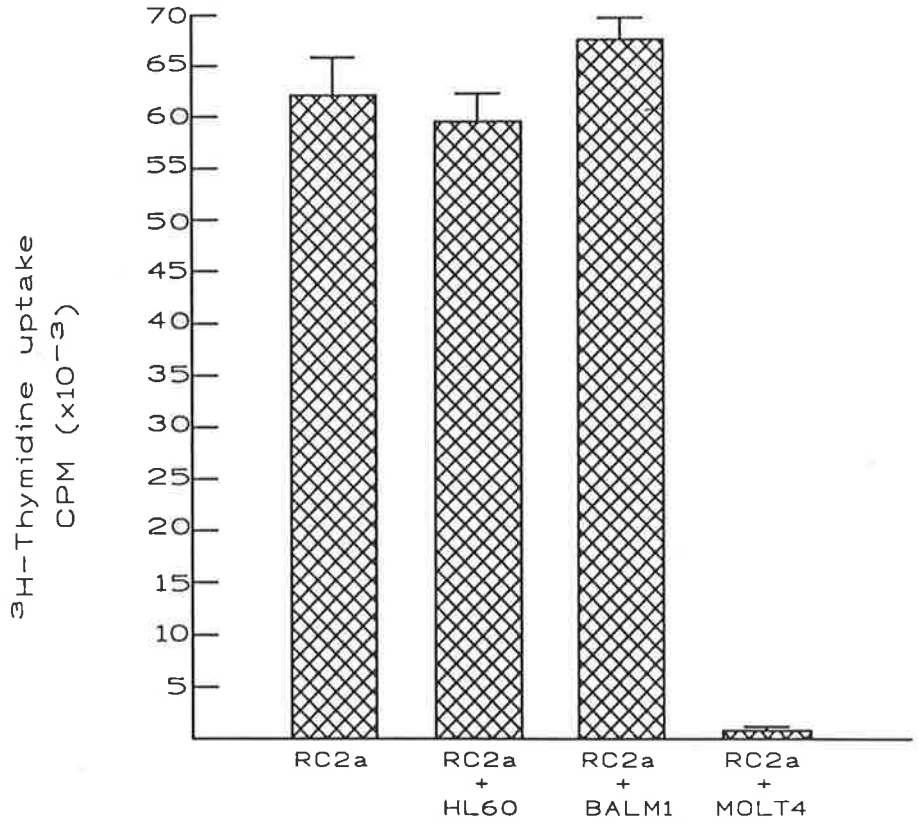
LEP_{36} plus designated stimulator(s)

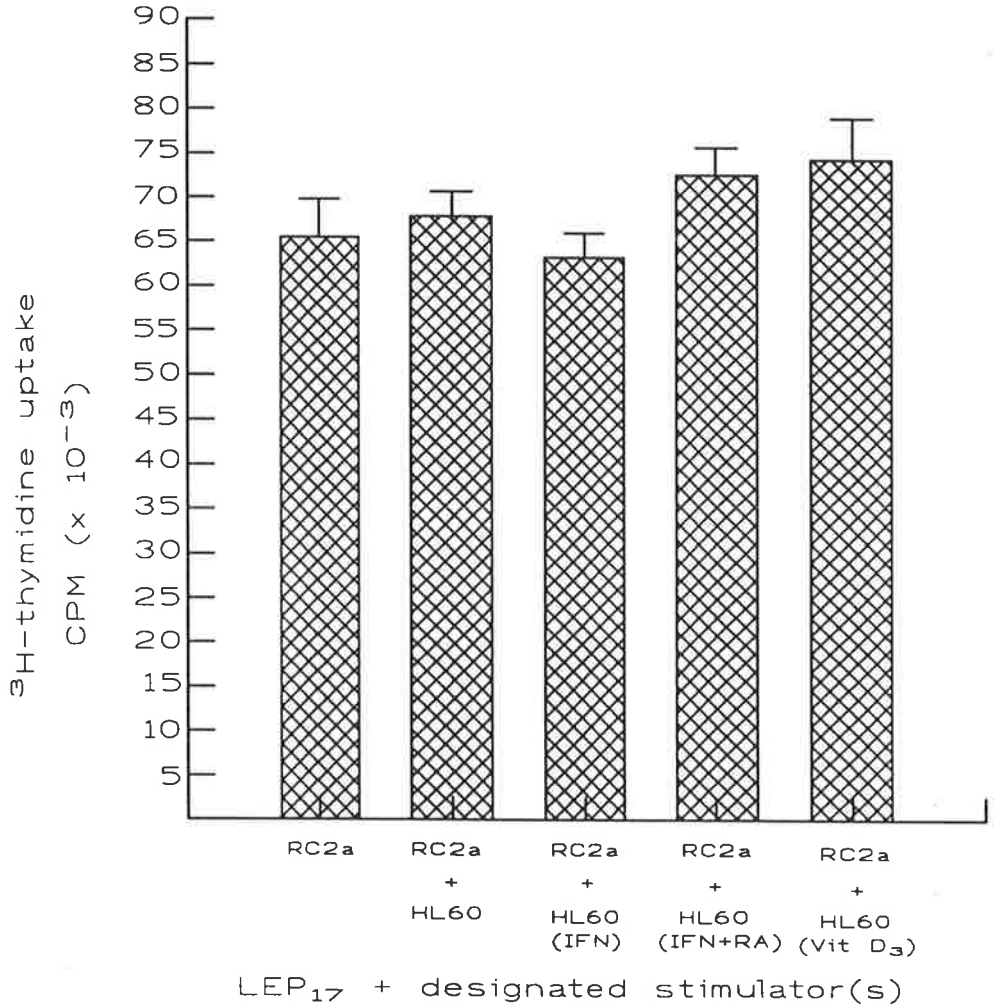


LEP_{37} plus designated stimulator(s)

Figure 6.2.8: LEP_{17} and RC-2a cells plus HL-60, BALM-1 or MOLT-4.

Figure 6.2.9: LEP_{17} and RC-2a cells plus HL-60, HL-60 (IFN-γ), HL-60 (IFN-γ + RA) or HL-60 (Vit D_3).


70
65
60
55
50
45
40
35
30
25
20
15
10
5
^{3}H-Thymidine uptake
CPM (x10^{-3})
RC2a
RC2a + HL60
RC2a + BALM1
RC2a + MOLT4
LEP$_{17}$ plus designated stimulator


90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
^{3}H-thymidine uptake
CPM (x 10^{-3})
RC2a
RC2a + HL60
RC2a + HL60 (IFN)
RC2a + HL60 (IFN+RA)
RC2a + HL60 (Vit D$_3$)
LEP$_{17}$ + designated stimulator(s)

Cell line	Mean % suppression of LEP proliferative response to RC-2a cells	
K562	93 ± 2 (n = 6)	p = 0.0001
HEL	71 ± 15 (n = 6)	p = 0.0016
HEL-DR	58 ± 4 (n = 6)	p = 0.0001
MOLT-4	57 ± 12 (n = 6)	p = 0.0032
U937	52 ± 9 (n = 10)	p = 0.0001
KG-1	31 ± 10 (n = 4)	p = 0.3424
HL60	9 ± 3 (n = 12)	p = 0.0054
HL60(IFN)	8 ± 8 (n = 6)	p = 0.2842
HL60(IFN+RA)	4 ± 10 (n = 6)	p = 0.4886
HL60(Vit D_3)	-2 ± 10 (n = 3)	p = 0.8432
BALM-1	-6 ± 13 (n = 6)	p = 0.6011

Table 6.2.3: **Suppressive nature of leukaemic cell lines in MLC.** Summary of the capacity of various cell lines to inhibit the proliferative response of LEP to RC-2a cells in MLC. The statistical significance of the suppression values were calculated for each cell line using the paired t test, and the probabilities (p) are displayed.

K562, BALM-1, MOLT-4 and U937 cells to stimulate purified $CD2^+$ lymphocytes has been examined previously and similar results were obtained to those described here, i.e. RC-2a and BALM-1 cells were found to be immunostimulatory whilst MOLT-4 and K562 cells were not (O'Keefe and Ashman, 1982). However, in contrast to the results obtained here, U937 cells were reported to be immunostimulatory. U937 cells used in these assays were shown to be mycoplasma-free and thus a simple explanation for these contradictory results appears to be unlikely.

One possible reason for the relatively non-stimulatory nature of some of the cell lines is that they were in some way suppressing the activation of LEP. In the case of K562 cells this seems almost certain to be the case, since these cells inhibited the LEP proliferative response to RC-2a cells by greater than 90% on each occasion. It should be noted that the apparent suppression caused by these cell lines may have been artefactual as a result of the differences of cell lines to deplete the medium of nutrients due to their varying metabolic activities. A means of determining whether such an event was likely would have been to use lower doses of stimulator cells. Nevertheless, other explanations for their suppressive nature include the possible release of soluble suppressive factors which may have been constitutively produced or triggered via interaction with RC-2a cells or lymphocytes. Such a possibility could be tested by adding the supernatants from cultures of K562 cells, with or without LEP, to MLC containing LEP plus RC-2a cells. Alternatively, it is conceivable that interactions between K562 cells and lymphocytes may have induced a state of non-responsiveness, or anergy. For instance the expression of high levels of ICAM-1 but no detectable levels of MHC class I or class II may result in a negative signal to the lymphocytes which interact with K562 cells, although there appears to be no precedent for such a phenomenon in the literature. Finally, K562 cells are known to be readily lysed by NK cells, and if indeed NK cytolytic activity was triggered early in the MLC as a result of the presence of K562 cells, then perhaps a subsequently released cytolytic factor could

have led to sufficient non-specific killing of neighbouring RC-2a cells and/or lymphocytes to result in very limited cellular proliferation.

With the possible exception of explanations involving NK cells and ICAM-1 molecules, very similar postulates to those given above for K562 cells can be made for the other cell lines U937, KG-1, MOLT-4, HEL and HEL-DR which partially suppressed the lymphoproliferative response to RC-2a cells. The suppressive nature of U937 cells may be due to the release of a suppressive factor which these cells have been reported to constitutively express (Onishi *et al*, 1993).

Attempting to correlate cell surface antigen expression with immunostimulatory capacity for those cell lines that were strongly, or even partially immunosuppressive is probably of little value as suppressive factor(s) possibly being released by one or more of these cell lines may have abrogated any potentially stimulatory effect due to the presence of any particular cell surface antigens. Thus those cell lines where comparisons were of possible value were the ones which possessed little or no detectable immunosuppressive activity, namely; HL-60, HL-60(Vit D_3), HL-60(IFN), HL-60(IFN + RA), RC-2a and BALM-1. From the studies performed here it would appear, superficially at least, that expression of MHC class II molecules, solely, or in combination with other adhesion molecules, such as ICAM-1 and LFA-3, was not sufficient to render cells strongly immunostimulatory, as a proportion of HL-60 cells treated with IFN-γ or IFN-γ + RA expressed MHC class II molecules, LFA-3, LFA-1 and ICAM-1. These results support the earlier finding, in chapter 4, that MHC class II antigen expression by HL-60 cells in MLC was not sufficient for these cells to become more immunostimulatory. Additionally, it has been previously reported that there is a lack of correlation between the expression of MHC class II molecules by leukaemic cell lines, or primary leukaemic cells, and the ability of these cells to stimulate lymphocyte proliferation in MLC (O'Keefe and Ashman, 1982; Ashman *et al*, 1987)

The most notable correlation of antigen expression with immunostimulatory capacity was discovered with MHC class I expression where the cell lines expressing relatively low levels of MHC class I, namely HL-60-R7 and RC-2a, were the most stimulatory. BALM-1 and HL-60(Vit D_3) expressed moderate levels of MHC class I (greater than HL-60-R7 cells, but less than HL-60 cells) and were mildly stimulatory, whilst HL-60, HL-60(IFN) and HL-60(IFN + RA) cells expressed relatively high levels of MHC class I molecules and were only weakly immunostimulatory. The correlation observed here may be coincidental, however research performed in independent laboratories suggest that this observation may well reflect the importance of MHC class I antigen expression in the stimulation of lymphocyte activation (Karre *et al*, 1986; Harel-Bellam *et al*, 1986; Eisenbach *et al*, 1986; Ljunggren *et al*, 1988; Tanaka *et al*, 1988). For instance, a murine study has revealed a situation where malignant cells of the "Lewis lung" carcinoma, expressing predominantly H-2D molecules were less immunogenic than cells expressing predominantly H-2K molecules possibly suggesting that some types of MHC class I molecules may be involved in suppressing the immune response (Eisenbach *et al*, 1986).

The enhanced immunostimulatory capacity of HL-60(Vit D_3) cells was not unexpected since it has been reported that treatment of HL-60 cells with Vit D_3 at concentrations of $> 10^{-8}$ M induces maturation into macrophage-like cells (Mangelsdorf *et al*, 1984) and that such treatment can lead to the increased expression of TNF-α mRNA (Steffen *et al*, 1988), a cytokine that was previously shown (Section 5.2) to be important in the lymphoproliferative responses to both HL-60-R7 and RC-2a cells. The relatively high levesl of expression of the transferrin receptor, CD71, by HL-60 cells treated with 5×10^{-8} M Vit D_3 suggests that these cells had not fully differentiated and therefore may have been more stimulatory if left in culture longer or treated with a higher concentration of Vit D_3. However this issue was not investigated further.

HL-60 cells treated with IFN-γ, or IFN-γ + RA, have also been reported to differentiate into monocyte-like cells (Ball *et al*, 1984; Hemmi and Breitman, 1987), however, somewhat surprisingly, these cells were no more immunostimulatory than untreated HL-60 cells. It would appear that the cells used in these experiments had become monocyte-like since a proportion of the cells expressed MHC class II antigen and CD14 and, in the case of HL-60(IFN + RA), the majority of the cells had become adherent and possessed pseudopodia by day 7. Apart from the high levels of expression of cell surface MHC class I molecules already mentioned, there were no obvious clues as to why HL-60-R7 should be immunostimulatory while HL-60(IFN) and HL-60(IFN + RA) were not.

Chapter 7: RA-resistant HL-60 cells

7.1 Emergence and characteristics of RA-resistant HL-60 cells

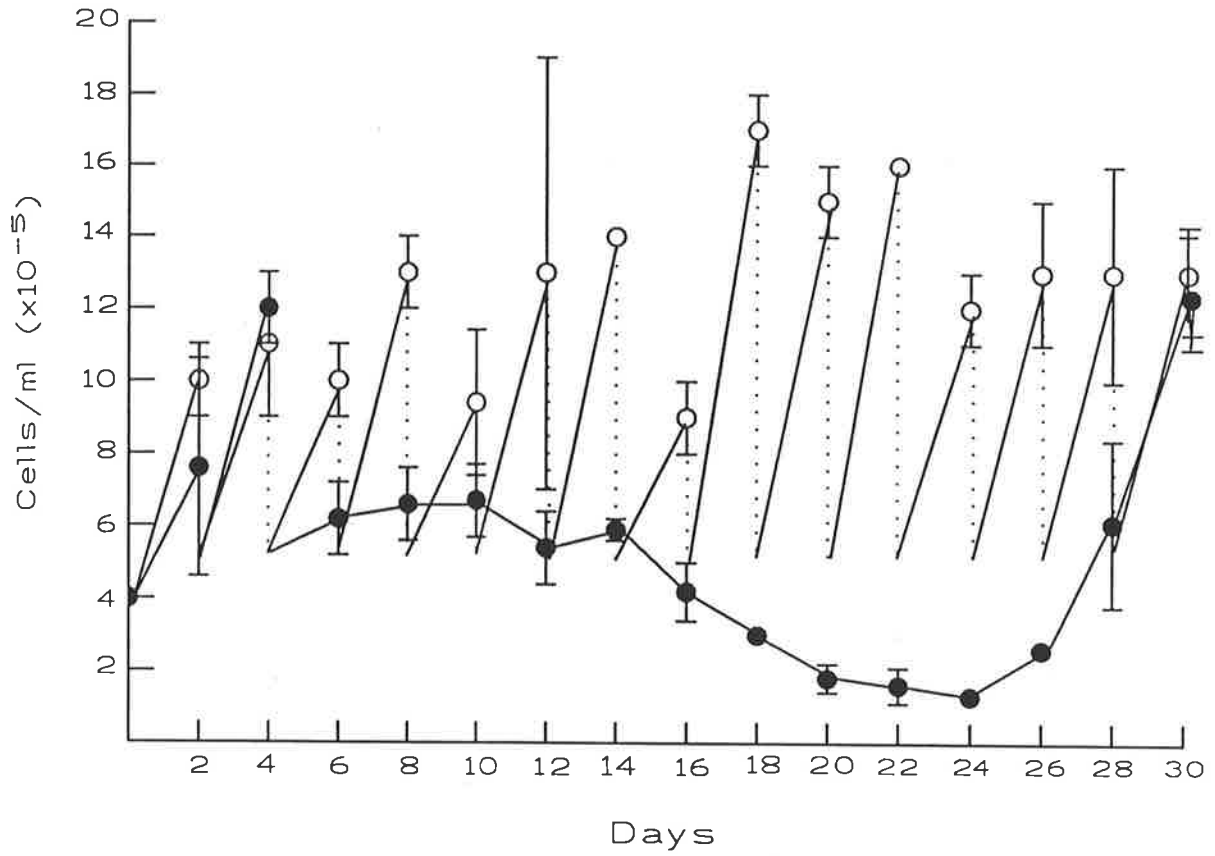
Chapter 3, section 3.2 focussed on the effect of 10 nM RA on the proliferative capacity and viability of HL-60 cells. Subsequent to those studies it was found that the extent of the effect on both these parameters could vary, depending on how the cultures were handled. For instance, in section 3.2, the cultures were initiated at 4 x 10^5 cells/ml and then subcultured at 5 x 10^5 cells/ml every alternate day with fresh medium containing 10 nM RA and this procedure was continued for 12 days. However, it was subsequently found that, from day 6 onwards, addition of only 1/5 to 1/10 of fresh culture medium (while still maintaining levels of 10 nM RA) resulted in higher viabilities for a longer period of time.

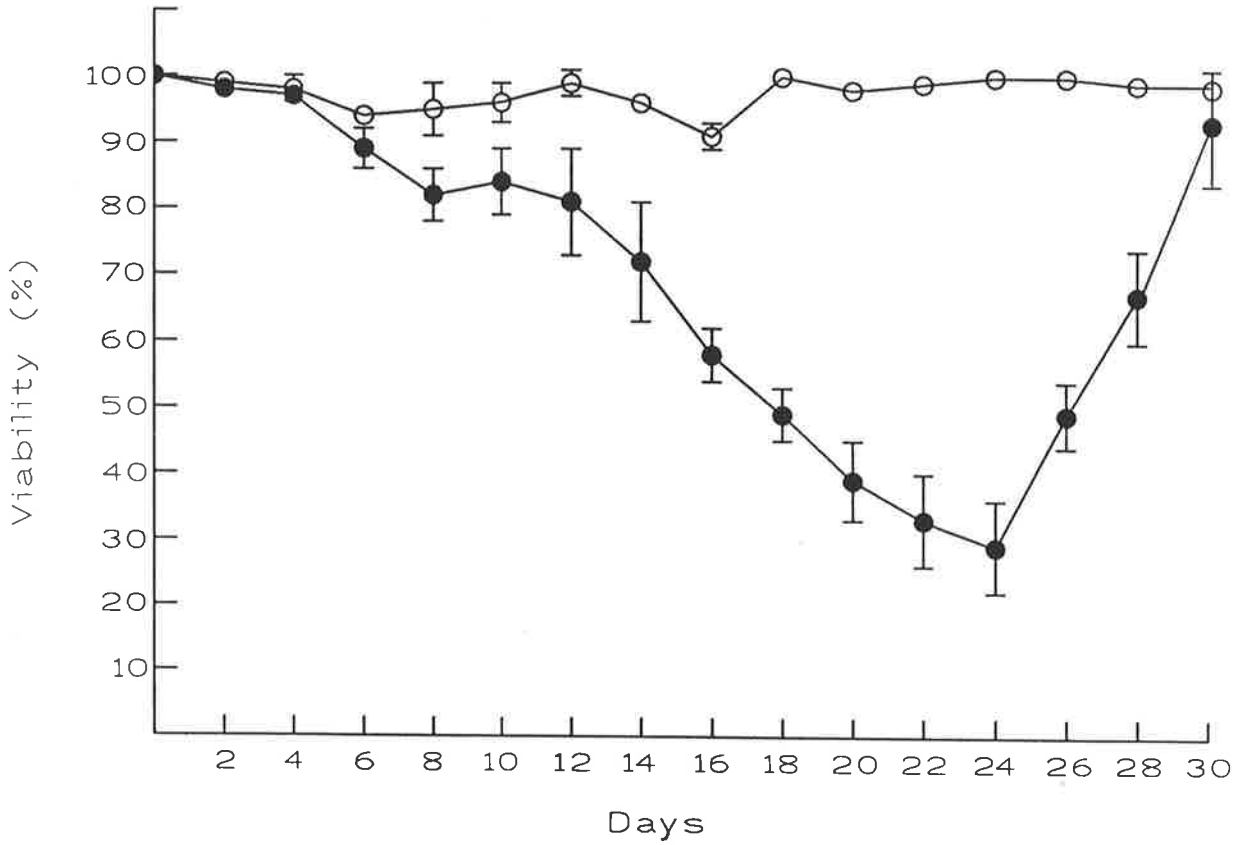
Here an experiment was performed where cultures of HL-60 cells were initiated in triplicate at 4 x 10^5/ml with or without 10 nM RA. On days 2 and 4 the cells were subcultured at 5 x 10^5/ml, however, on day 6 the RA-treated cultures were not replenished with fresh medium as the cultures did not exceed 6 x 10^6 cells/ml. On days 8 and 10 only a 1/20 supplementation of fresh medium was made whilst maintaining the levels of RA at 10 nM. No medium was added on day 12 and from days 14 through to 26 a 1/10 addition of fresh medium was made when required with the 10 nM concentration of RA being maintained. Figures 7.1.1 and 7.1.2 display the results of the viability and density studies performed. It would appear from these results that the RA-treated cultures had become resistant to the anti-proliferative effect of RA by days 28 - 30.

On day 30, two culture flasks were set up from one of the triplicate RA-treated cultures, where one flask had the RA treatment continued (HL-60-RR), whilst with the other the treatment was not continued (HL-60-BA) and thus were maintained in a similar fashion to HL-60 cells. Both cultures were sustained for greater than 4 months which corresponded to at least 40 passages. During this 4 month period a number of assays were performed with these apparently RA-resistant cells. The

Figure 7.1.1: **Proliferation of RA-treated HL-60 cells:** Analysis of the density (cells/ml) of cultures of HL-60 cells and HL-60 cells treated with 10 nM RA. The assay was performed in triplicate. The open circle = control cultures; the closed circle = RA-treated cultures.

Figure 7.1.2: **Viability of HL-60 cells maintained in RA.** Analysis of the viability HL-60 cells and HL-60 cells cultured with 10 nM RA. The assay was performed in triplicate. The open circle = control cultures; the closed circle = RA-treated cultures.


Cells/ml (x10⁻⁵)
20
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days


Viability (%)
100
90
80
70
60
50
40
30
20
10
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days

proliferative rates of HL-60, HL-60-RR and HL-60-BA cells were compared and it was found that HL-60-BA cells had the fastest doubling time, whilst HL-60-RR cells had the slowest (see figure 7.1.3.). This was ascertained through the performance of proliferation assays where cell cultures were initiated, in duplicate, at 4 x 10^5/ml and then the cell densities were recorded 2 days afterwards. This was performed on 4 occasions and the results displayed represent the mean of those 4 experiments.

MLC were performed with these three cell lines 76 and 132 days after the initial treatment of HL-60 cells with RA. The results obtained with LEP_{37} for the day 132 cultures are shown in figure 7.1.4. This, along with all other MLC performed (n = 2 for day 76 and n = 2 for day 132), indicate that neither HL-60-RR nor HL-60-BA cells could stimulate the proliferation of LEP to a greater extent than HL-60 cells.

Additionally, on days 42, 84, 132 and 178 of culture, immunofluorescence assays were performed on HL-60-BA and HL-60-RR cells to compare the expression of a variety of antigens, including many considered of interest with respect to the lymphoproliferative response to HL-60-R7 cells, such as MHC class I and class II, LFA-1 and CD11b (mAb specific for ICAM-2 and ICAM-3 were unavailable at this time). The results are shown in tables 7.1.1 and 7.1.2 respectively. MHC class I molecules and CD71 were expressed by HL-60-BA cells at levels comparable to HL-60 cells and the vast majority of the cells did not express ICAM-1 or CD11b. Interestingly, LFA-1 and LFA-3 expression on both HL-60-BA and HL-60-RR cells appeared to be maintained at levels comparable to HL-60-R7 cells. From days 42 - 178 of culture the cell surface phenotype of HL-60-BA cells did alter, although not markedly, with a small percentage of cells expressing MHC class II on days 84 and 178.

HL-60-RR cells expressed lower levels of MHC class I molecules and CD71 than HL-60-BA cells and none of the cells were found to express MHC class II molecules. On day 42 of culture ICAM-1 expression on HL-60-RR cells was comparable to that found on HL-60-R7 cells, however the majority of the population subsequently failed to express this antigen.

Figure 7.1.3: **Relative growth rates of HL-60, HL-60-RR and HL-60-BA cells.** Comparison of proliferative rates of HL-60, HL-60-RR and HL-60-BA cells. Assay was performed in duplicate and repeated 4 times and results displayed represent the mean (and SEM) of the 4 experiments. The proliferation rates of HL-60 and HL-60-BA cells were shown to be significantly different ($p = 0.048$) whilst the proliferation rates of HL-60 and HL-60-RR cells were not significantly different ($p = 0.245$), as measured by the paired t-test.

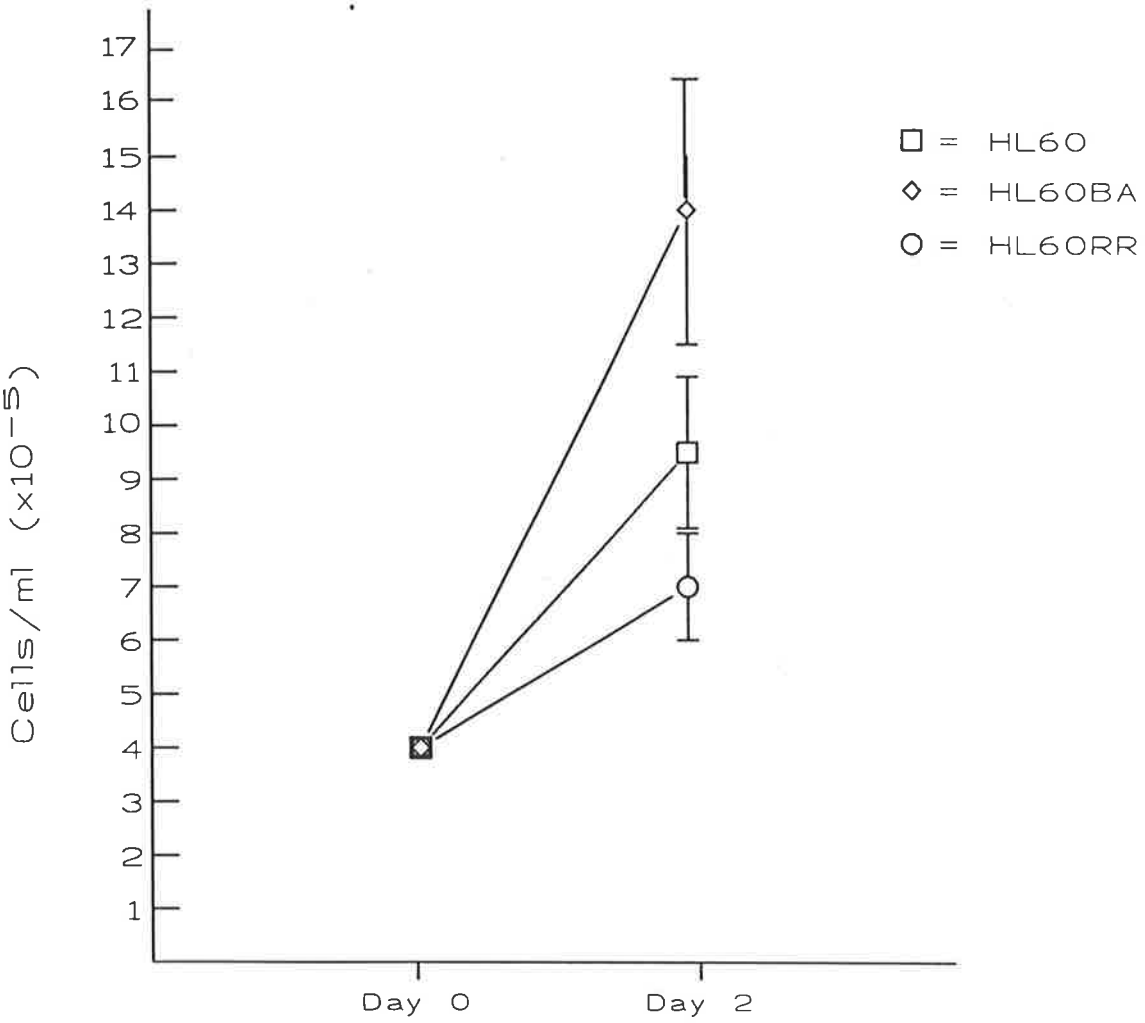
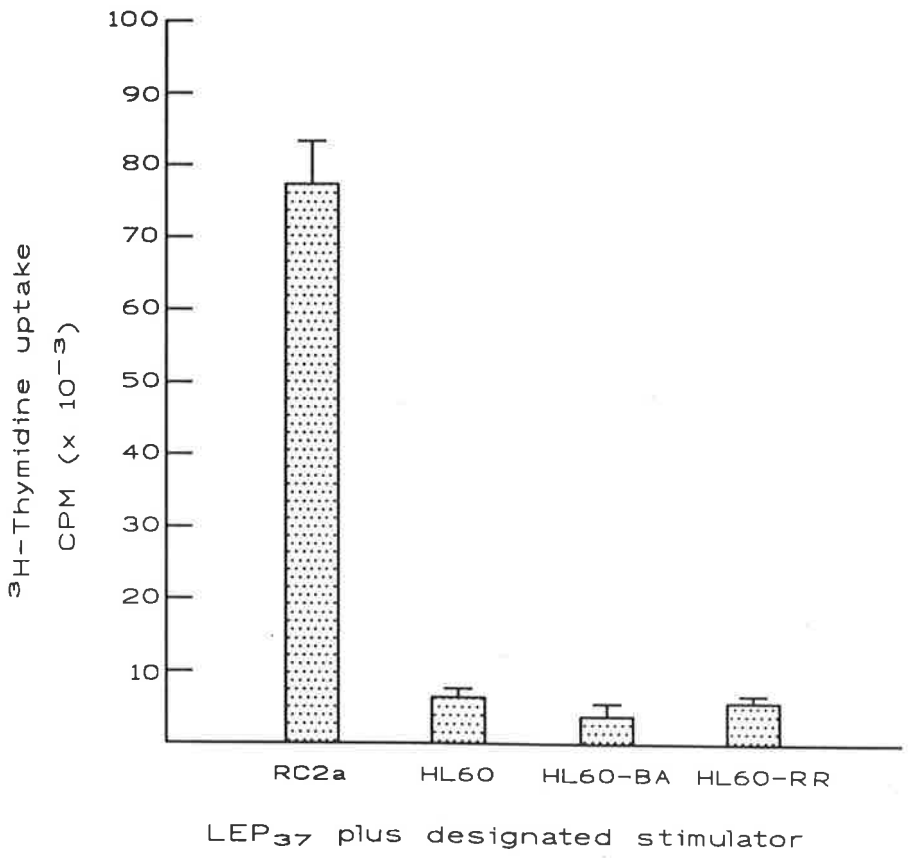

Cells/ml (x10-5)
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Day 0
Day 2
□ = HL60
◇ = HL60BA
○ = HL60RR

Figure 7.1.4: **Immunostimulatory capacity of HL-60-RR and HL-60-BA cells.** ^{3}H-thymidine uptake assay was performed on a MLC containing LEP_{37} plus designated stimulator population. Stimulators alone yielded < 300 cpm and LEP_{37} alone yielded < 200 cpm. HL-60-RR and HL-60-BA cells were used in this MLC 132 days after initiation of RA-treatment.


100
90
80
70
60
50
40
30
20
10
^{3}H-Thymidine uptake
CPM (x 10^{-3})
RC2a
HL60
HL60-BA
HL60-RR
LEP_{37} plus designated stimulator

Tables 7.1.1 and 7.1.2: Cell surface marker expression of HL-60-BA and HL-60-RR. HL-60-BA and HL-60-RR cells were assayed, by IIF, for expression of the designated cell surface markers on days 42, 84 and 178 after the initiation of the RA-treated cultures. The results are given in terms of percentage of cells positive for the relevant antigen. In brackets is given the relative mean fluorescence intensity (RMF) of the positive cells. The values obtained for the isotype control mAb ranged from 2 - 3 and were subtracted from the values obtained for the respective test mAb. Where there was a homogeneous peak shift of fluorescence compared with cells treated with the isotype control mAb, the cells were considered to be 100% positive even though the histograms may have overlapped. Where the population was heterogeneous with respect to antibody binding, cells with fluorescence intensities > the 99^{th} percentile of the fluorescence intensity of the negative control were considered positive.

* = asymmetric peak

= distinct populations

N. T. = not tested

		% Positive and (RMF)		
Antibody	Antigen	HL60-BA Day 42	HL60-BA Day 84	HL60-BA Day 178
6c6b	MHC class I	100 (252)	100 (387)	100 (558)
7b6c	MHC class I	N.T.	100 (744)	N.T.
FMC 14	MHC class I	0 (0)	3*(15)	8*(21)
TS2/9	LFA-3	100 (84)	100 (69)	100 (126)
W-CAM-1	ICAM-1	6*(30)	0 (0)	4*(24)
TS1/22	LFA-1	N.T.	100 (69)	100 (108)
VIP-1	CD71	94*(828)	100*(348)	97*(870)
5A4.C5	CD11b	N.T.	0 (0)	3*(9)

		% positive and (RMF)		
Antibody	Antigen	HL60-RR Day 42	HL60-RR Day 84	HL60-RR Day 178
6c6b	MHC class I	100#(195)	100#(150)	100#(240)
7b6c	MHC class I	N.T.	100#(384)	100#(389)
FMC 14	MHC class II	0 (0)	0 (0)	0 (0)
TS2/9	LFA-3	100 (150)	100 (63)	100 (63)
W-CAM-1	ICAM-1	100 (15)	8*(12)	4*(9)
TS1/22	LFA-1	N.T.	100 (60)	100 (57)
VIP-1	CD71	63*(132)	100*(216)	78*(156)
5A4.C5	CD11b	N.T.	3*(6)	5*(6)

7.2 Discussion

The essential finding in this section was the generation of a "RA-resistant" cell line from HL-60 cells cultured with RA. From the analysis of the cell surface markers of the RA-resistant populations produced here, it appeared that the cells, especially HL-60-BA cells, had regressed, or dedifferentiated along the maturation pathway and are close to representing the original HL-60 cells. To explain, the majority of the HL-60-BA cells did not express CD11b or ICAM-1 and also expressed similar levels of CD71 and MHC class I molecules to HL-60 cells. The idea that differentiated cells were capable of subsequent dedifferentiation is given credence through the recent finding that U937 cells treated with PMA for 2 days were apparently capable of dedifferentiation once the PMA was removed and numerous medium renewals were performed (Hosoya and Marunouchi, 1992).

Whether these sublines had become completely resistant to the effects of RA was questionable when one considers the differences between HL-60-BA and HL-60RR cells. For example, HL-60-RR cells (which had RA treatment maintained) expressed MHC class I molecules and CD71 at lower levels than HL-60-BA cells, suggesting that RA still had some influence upon these cells. Providing further support for this possibility were the results of the proliferation assays where HL-60-RR cells appeared to have a lower rate of proliferation than both HL-60 and HL-60-BA cells.

The ability of RA to induce HL-60 cells to differentiate led to the use of RA in therapy for patients with APML (Huang *et al*, 1988; Castaigne *et al*, 1990). Results were initially promising as the majority of patients went into remission, however most patients relapsed and subsequent treatment with RA alone failed to be of benefit (Sun *et al*, 1990; Chen *et al*, 1991). Thus it appeared that the leukaemic cells had become resistant to the anti-proliferative effect of the drug; an idea supported by a number of researchers who have found that HL-60 cells *in vitro* can develop a resistance to RA. It has been proposed that this resistance could be due to the loss of a functional RAR-α since transfection of the gene coding for RAR-α into RA-resistant HL-60 cells

rendered these cells sensitive to RA once again (Collins *et al*, 1990). In fact, Collins *et al* have recently shown that for one particular RA-resistant HL-60 population, the resistance to RA was due to a point mutation in the RAR-α (Robertson *et al*, 1992). A similar mechanism may apply *in vivo* and thus explain why patients become resistant to RA treatment. However this possibility has been challenged by the report that leukaemic cells from RA-resistant patients are still sensitive to RA *in vitro* (Muindi *et al*, 1992). Thus the resistance of patients to RA-treatment has been proposed to result from accelerated clearance of RA possibly due to the induction of cytochrome P-450 enzymes (Francis *et al*, 1992) or the increased expression of cellular retinoic acid-binding proteins (Cornic *et al*, 1992).

In conclusion, it is possible that RA-treated HL-60 cells became "RA-resistant" due to a mutational change in the RA receptor (RAR-α) similar to that described by Robertson *et al*, (1992). Such an event may have resulted in reduced affinity of the RAR-α for RA, which may have diminished the potency of RA without completely abrogating its effects. This would explain the finding that 10 nM RA still appeared to have an effect on the "RA-resistant cells". However, the fact that all three cultures of RA-treated HL-60 cells became "RA-resistant" at similar times makes such an explanation unlikely. Another possible explanation for the emergence of the RA-resistant cells is that HL-60 cultures contained a small population of cells that were partially resistant to the effects of RA and thus had a growth advantage over the other cells, resulting in this population dominating the culture.

Chapter 8: General discussion

The main body of this research stemmed from the initial finding that HL-60 cells, after culture with 10 nM RA, possessed an enhanced capacity to stimulate the proliferation of LEP. Subsequent studies principally involved analysis of the differentiated HL-60 cells as well as the characterization of the lymphocyte response. Based on the results obtained in chapters 3, 4 and 5, and upon what is generally known of lymphocyte activation, two models (Model 1 and Model 2) are proposed here with regards to the mechanism of lymphocyte activation by HL-60-R7 cells and are illustrated in figures 8.1 and 8.2. These models are not necessarily mutually exclusive. All the proposed biochemical signalling events were not determined from results performed here, however studies by others using a variety of different lymphocyte activation systems would suggest that the proposals here are not controversial (reviews by Rothenberg *et al*, 1992; Finkel and Kubo, 1989; Fraser *et al*, 1993).

It was apparent from the inhibitory action of mAb to MHC class II molecules in MLC that these molecules played a crucial part in the response of LEP to HL-60-R7 cells. That it was probably MHC class II antigen expression by the stimulator cells themselves, rather than the lymphocytes, that was of importance was supported by the observation that removal of MHC class II^+ lymphocytes from the LEP prior to their addition to the MLC had no effect on the lymphoproliferative response to HL-60-R7 cells. Additionally, it was discovered that these stimulator cells expressed MHC class II antigen during the MLC. The possibility that the interaction of MHC class II molecules with the TCR of $CD4^+$ lymphocytes was an important event was supported by the finding that the removal of $CD4^+$ lymphocytes from LEP prior to their addition to the MLC consistently depleted the lymphoproliferative response to HL-60-R7 cells by more than 60%. The outcome of the interaction of MHC class II antigen with the

Figure 8.1: MODEL 1: Diagrammatic representation of the possible mechanism of activation of LEP by HL-60-R7

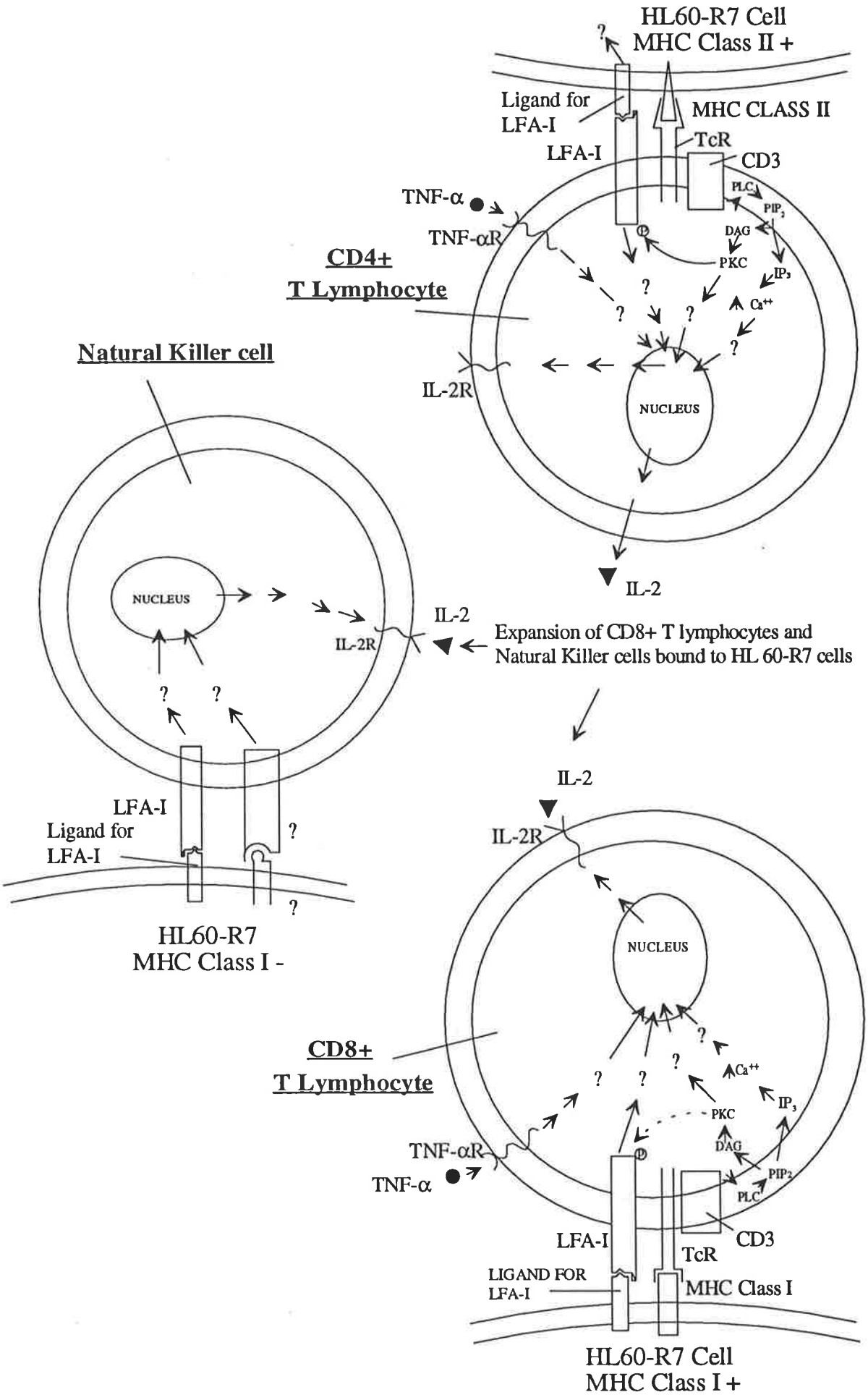
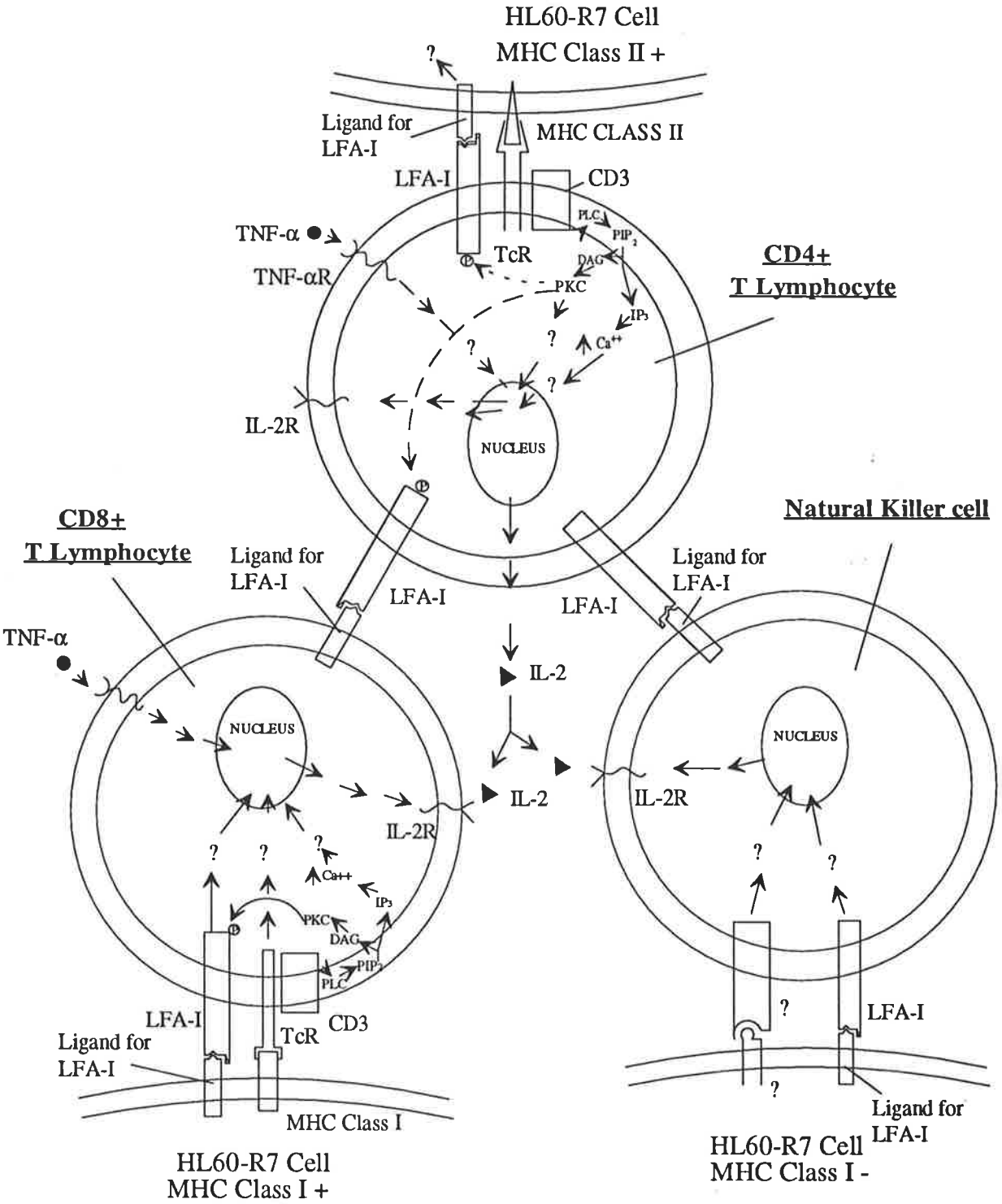

HL60-R7 Cell
MHC Class II +
Ligand for
LFA-I
MHC CLASS II
LFA-I
TcR
CD3
TNF-α
TNF-αR
PLC
PIP2
DAG
PKC
IP3
Ca++
CD4+
T Lymphocyte
IL-2R
NUCLEUS
IL-2
Natural Killer cell
NUCLEUS
IL-2
IL-2R
Expansion of CD8+ T lymphocytes and
Natural Killer cells bound to HL 60-R7 cells
IL-2
IL-2R
LFA-I
Ligand for
LFA-I
HL60-R7
MHC Class I -
NUCLEUS
CD8+
T Lymphocyte
Ca++
IP3
PKC
DAG
PIP2
PLC
TNF-αR
TNF-α
LFA-I
TcR
CD3
LIGAND FOR
LFA-I
MHC Class I
HL60-R7 Cell
MHC Class I +

Figure 8.2: MODEL 2: Diagrammatic representation of another possible mechanism of activation of LEP by HL-60-R7


HL60-R7 Cell
MHC Class II +
?
Ligand for
LFA-I
MHC CLASS II
LFA-I
CD3
PLC
PIP2
TNF-α
TcR
DAG
TNF-αR
PKC
IP3
?
Ca++
?
?
IL-2R
NUCLEUS
CD4+
T Lymphocyte
CD8+
T Lymphocyte
Natural Killer cell
Ligand for
LFA-I
Ligand for
LFA-I
LFA-I
LFA-I
TNF-α
IL-2
NUCLEUS
NUCLEUS
IL-2
IL-2R
IL-2R
?
?
?
?
?
Ca++
IP3
PKC
DAG
PIP2
PLC
LFA-I
TcR
CD3
?
LFA-I
Ligand for
LFA-I
?
Ligand for
LFA-I
MHC Class I
HL60-R7 Cell
MHC Class I +
HL60-R7 Cell
MHC Class I -

TCR was probably the expression of the IL-2R , via the activation of PKC and the triggered increase in $[Ca^{++}]_i$ (Palacios, 1982; reviewed by Finkel and Kubo, 1989)). It was likely that other stimuli, such as cytokines (e.g. TNF-α) or cell-cell interactions also contributed to the activation of PKC.

Despite the discovery that MHC class II molecules were important in the response of LEP to HL-60-R7 cells it seemed that expression of this molecule alone was unlikely to be the factor actually responsible for the enhanced lymphostimulatory capacity of HL-60-R7 cells since control HL-60 cells also expressed MHC class II molecules during the MLC at levels comparable to the those of HL-60-R7 cells. It therefore appeared that additional characteristics possessed by HL-60-R7 cells but not HL-60 cells were instrumental in enhancing their immunostimulatory nature. Cell surface marker analysis exposed a number of candidates for such a role, the most likely of which included the cell adhesion molecules, CD11b, ICAM-1, ICAM-3, LFA-1 and LFA-3 since these molecules were up-regulated on HL-60-R7 cells. The mAb TS2/9, TS1/22, W-CAM-1, CBR-IC2/2 and CBR-IC3/1 have previously been shown to inhibit the function of LFA-3, LFA-1, ICAM-1, ICAM-2 and ICAM-3 respectively (Sanchez-Madrid *et al*, 1982; Boyd *et al*, 1988; de Fougerolles *et al*, 1991; de Fougerolles *et al*, 1993) and were used here in MLC inhibition assays. It was found that only the anti-LFA-1 mAb was consistently capable of substantially inhibiting the lymphoproliferative response to HL-60-R7 cells, suggesting that the interaction between LFA-1 and one or more of its ligands was of importance in this response. The fact that the anti-LFA-1 mAb could not inhibit the limited proliferative response to HL-60 cells suggested that there was a change on HL-60-R7 cells in cell surface expression of either LFA-1 or one or more of its ligands, that contributed to their enhanced immunostimulatory ability. For instance, the upregulation of expression of LFA-1 on HL-60-R7 cells may have been of importance (not shown in model), although control HL-60 cells already expressed abundant levels of this molecule, possibly indicating that this increase may

have been inconsequential. Model 1 puts forward the possibility that LFA-1 expressed by the T lymphocyte may have interacted with one or more of its ligands (which include ICAM-1, ICAM-2, ICAM-3 and ICAM-R) on the HL-60-R7 cell, leading to the enhanced immunostimulatory capacity of these cells. This was discussed further in chapter 4. It was possible that PKC, once activated, phosphorylated the β chain LFA-1 and thus converted this molecule from a non-active to an active form, capable of binding with high affinity to its ligand(s) (Dustin and Springer, 1989). The subsequent interaction of LFA-1 on $CD4^+$ lymphocytes and ICAM-1, 2, 3 or R, or a combination of these ligands upon the HL-60-R7 cells (model 1) or other lymphocytes (model 2), could have resulted in the enhancement of the signal provided by the TCR by increasing intracellular Ca^{++} concentrations as well as increasing IL-2 production (Wacholtz *et al*, 1989). LFA-1 may have have additionally, or alternatively, played a similar role in the interaction between HL-60-R7 cells and $CD8^+$ lymphocytes or NK cells. It is also possible that the ligand for LFA-1 was responsible for signalling to the HL-60-R7 cell since ICAM-1 has already been shown to be capable of signalling (Van Seventer *et al*, 1990). Model 2 suggests that the interaction between LFA-1 and its ligand(s) may have occurred between lymphocytes. For instance, LFA-1 expressed by $CD4^+$ lymphocytes may have interacted with one or more of its ligands on NK cells or antigen-stimulated $CD8^+$ lymphocytes possibly leading to an enhanced capacity of the NK cells or $CD8^+$ lymphocytes to respond to locally produced IL-2.

There are too few clues for one to confidently postulate the most likely mode of NK cell activation. Since $CD4^+$ lymphocytes were shown to be important in the LEP response to HL-60-R7 cells, one could hypothesize that NK cells were stimulated to proliferate in response to IL-2 supplied by the activated $CD4^+$ lymphocytes. Studies by others have revealed that the majority of resting NK cells, as well as $CD8^+$ lymphocytes express cell surface p75 (β-chain of IL-2R) which, upon interaction with IL-2, is capable of signal transduction, leading to the expression of the high affinity IL-

2R (Hatakeyama *et al*, 1986; Smith *et al*, 1988; Dukovich *et al*, 1987). It has also been reported that optimal activation of NK cells required the presence of large quantities of IL-2 (≥ 100 U/ml) if IL-2 alone was used (Nagler *et al*, 1990). It was unlikely that such high levels of IL-2 were present in the MLC performed here since $CD4^-$ LEP cultured with HL-60-R7 cells were capable of proliferating in response to low levels of exogenously supplied IL-2 (≤ 10 U/ml), and the levels of proliferation were similar to those observed for MLC containing $CD4^+$ LEP plus HL-60-R7 cells that had no IL-2 added (see chapter 4). Thus it was likely that an intimate collaboration between HL-60-R7 cells and NK cells could have been necessary and since LFA-1 was shown to play some part in the stimulation of LEP by HL-60-R7 cells, it is feasible that NK cells interacted with the stimulator population (or $CD4^+$ lymphocytes) via this molecule and one or more of its ligands, such as ICAM-1, -2, -3 or -R, especially since LFA-1 has previously been shown to play a role in antigen-independent conjugate formation (Rothlein *et al*, 1986). Additionally, or alternatively, the proposed collaboration between NK cells and HL-60-R7 cells may have involved some, as yet, undefined ligand-receptor interaction, which possibly contributed to the stimulation of NK cell proliferation.

Although IL-12 and SCF can, under certain conditions, act as co-stimulators for NK cell proliferation (Gately *et al*, 1991; Matos *et al*, 1993), neither of these cytokines appeared to have been involved in NK cell proliferation stimulated by HL-60-R7 cells since functionally inhibitory mAb specific for IL-12 and SCF respectively had no effect upon the proliferative response to HL-60-R7 cells in the MLC. In fact, of the cytokines tested TNF-α appeared to be the only one that played a significant role in the lymphoproliferative response to HL-60-R7 as well as to HL-60 and RC-2a cells, as determined by the inhibitory action of neutralizing antiserum in MLC. Whether or not it was TNF-α production by the stimulator, the responder or both populations that was of importance was not ascertained. However, the possibility that the stimulator cells

could produce TNF-α was reinforced by the finding that HL-60, HL-60-R7 and RC-2a cells all expressed readily detectable levels of TNF-α mRNA (see chapter 5). Consistent with such a possibility are the *in vitro* studies have revealed monocytes to be the major source of TNF-α production during allogeneic cell interactions (Lee *et al*, 1993). Nevertheless, both NK cells and T lymphocytes have been reported to be capable of producing TNF-α under various *in vitro* conditions and thus cannot be ruled out as having provided functionally significant levels of TNF-α during the MLC involving HL-60-R7 cells (Steffen *et al*, 1985; Paya *et al*, 1988). The role of TNF-α in the generation of the response of LEP to HL-60-R7 cells could have involved indirect stimulation of lymphocyte proliferation as it has previously been shown that TNF-α can induce IL-2 and IL-2R expression by lymphocytes (Lee *et al*, 1987). Experiments performed by others with resting $CD56^{dim}$ NK cells, which comprise ≈ 90% of peripheral blood NK cells, revealed that TNF-α alone was a poor stimulator of proliferation of these cells (Robertson *et al*, 1993). However, these researchers found that when used in combination with IL-2, TNF-α could significantly increase the proliferation of these $CD56^{dim}$ NK cells. A number of other cytokines, that were not assayed for here, may have also played a role in stimulating NK cell proliferation such as IL-4 and IL-7, which have been shown to induce proliferation of human NK cell proliferation (Nagler *et al*, 1989; Naume and Espevik, 1991).

From the studies performed in chapter 6 a correlation was made between the immunostimulatory capacity of the cell lines which were not immunosuppressive (as measured by their inability to inhibit the lymphoproliferative response to RC-2a cells) and their MHC class I expression, where it was found that cell lines expressing relatively high levels of MHC class I antigen were not immunostimulatory. Of particular interest was the finding that HL-60 cells treated with IFN-γ or IFN-γ plus RA, which expressed MHC class II molecules, ICAM-1, -2 and -3 but did not possess an enhanced capacity to stimulate lymphocyte proliferation. These cells expressed

higher or equivalent levels of MHC class I molecules, respectively, compared to control HL-60 cells. Meanwhile HL-60 cells treated with RA alone (HL-60-R7) possessed an enhanced immunostimulatory capacity and had downregulated their levels of MHC class I antigen. It was possibile that the cells expressing high levels of MHC class I molecules may have bound $CD8^+$ lymphocytes to the extent that they hindered the interaction between the stimulator cells and $CD4^+$ lymphocytes, which are recognized as the predominant IL-2 producing cells (Joos *et al*, 1989). This concept was discussed further in chapter 4.

With respect to the response of LEP to RC-2a cells, the expression of high levels of MHC class II molecules by RC-2a cells and the importance of both MHC class II molecules and $CD4^+$ lymphocytes suggested that the lymphoproliferative response depended at least partially upon the interaction of MHC class II antigen expressed on RC-2a cells with the TCR expressed on $CD4^+$ lymphocytes. The signal generated within the $CD4^+$ lymphocyte by the MHC class II-TCR interactions may have been large enough such that other cell surface ligand interactions, such as LFA-1 with its receptor(s), were superfluous. This could possibly explain the inability of all mAb to cell surface markers (including anti-LFA-1), other than anti-MHC class II, to inhibit the LEP response to RC-2a cells in MLC inhibition assays. Obviously TNF-α played a crucial part in the response of LEP to RC-2a cells and this may have involved direct or indirect stimulation of proliferation as previously discussed for the response to HL-60-R7 cells.

A therapeutical perspective

Of the cell populations studied, RC-2a and HL-60-R7 cells proved to be the most immunostimulatory. Although RC-2a cells were more potent at inducing lymphoproliferative responses than HL-60-R7 cells it was apparent that the majority of the responding lymphocytes to the former were $CD4^+$ and were less cytotoxic than the

lymphocytes responding to HL-60-R7 cells. RC-2a cells expressed high levels of MHC class II molecules and this may explain the predominant $CD4^+$ lymphocyte response, whereas only a proportion of HL-60-R7 cells expressed MHC class II and this was at low to moderate levels. For there to be a substantial lymphoproliferative response by allogeneic human peripheral blood lymphocytes to either HL-60-R7 or RC-2a cells, the presence of both TNF-α and MHC class II molecules was required. Such findings may prove informative if a role for the hosts immune system is to be considered important in eradicating leukaemic cells, as there is mounting evidence to suggest that the immune system can help control leukaemia and that both MHC-restricted and MHC-unrestricted pathways of leukaemia-specific killing may be involved. To illustrate it has been found that leukaemia-free survival of patients receiving HLA-identical sibling BMT was superior in those that developed graft-vs-host disease compared to those that did not (Horowitz *et al*, 1990). This implicated a role for the immune system in eradicating the leukaemic cells possibly as a result of the graft-vs-leukaemia effect (Ringden and Horowitz, 1989). It was also reported that patients possessing decreased numbers of NK cells after ABMT had an increased relapse rate (Jacobs *et al*, 1990).

However, achieving a successful immunotherapeutic strategy will not be a simple task. For instance, one must ascertain whether the leukaemia cells are immunosuppressive as was shown to be the case for many of the leukaemic cell lines tested in these studies. However, previous reports found that most primary leukaemic cells tested were not immunosuppressive, as measured by their inability to inhibit lymphoproliferative responses to allogeneic mitomycin C-treated monocytes or RC 2a cells, suggesting that this may not be a major problem (Ashman, 1983; Ashman *et al*, 1986). Another factor to be considered is that primary leukaemic cells are a more heterogeneous population than cell lines and this may be reflected in the response to particular treatments.

One way of controlling leukaemia is through differentiation therapy, as has recently been performed with RA in APML (Huang *et al*, 1988; Castaigne *et al*, 1990). There is the potential for a wide variety of physiological factors to be used for different types of leukaemia (Ferrero *et al*, 1992) and such treatment may not only inhibit proliferation of the leukaemic cells but could also lead to the induction of cells that are more immunostimulatory or more susceptible to cell-mediated cytolysis, as was shown to occur in these *in vitro* studies with RA-treated HL-60 cells. However, further molecular manipulations of the leukaemia cells may often be required in order to induce them to become effectively immunostimulatory, as it may be that the presence (or absence) of particular molecules, such as MHC class I and class II are of vital importance in generating an effective anti-leukaemia response..

* See amendment 2 at the back of this thesis for discussion of the role of CD28 in the LEP response to HL-60-R7 cells

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Appendix 1: Supplementary results

A1.1 Fluoresceination of 3D3.3

The IgG_1 isotype control mAb, 3D3.3 was fluoresceinated as described in section 2.11.5. In brief, 40 µl of 37 µM FLUOS (in DMSO) were added to 1 ml of 1 mg/ml 3D3.3, shaken vigorously for 4 hr and then the fluoresceinated protein was isolated using a Sephadex G-25 column. The protein yield was 222 µg/ml as calculated by the equation; $(O.D._{280} - 0.25 \times O.D._{495}) \times 0.74$ = protein concentration. It was found that 0.25% of the $O.D._{495}$ would consistently contribute to the $O.D._{280}$ and, thus had to be subtracted from the initial $O.D._{280}$ reading. The $O.D._{495}$ was found to be 0.24, thus using a standard curve it was determined that the concentration of FLUOS was ≈ 1.5 µg/ml. By converting both the FLUOS and mAb concentration values to moles, it was ascertained that the ratio of FLUOS:mAb was 2:1.

A1.2 Concentration of mAb used in MLC inhibition assays

The antibody concentrations in the hybridoma supernatants that were used in the MLC inhibition assays were determined using the enzyme-linked immunosorbent assay (ELISA) as described in section 2.11.6. These mAb were derived from the supernatant of hybridoma cultures with the exception of TS1/22 and W-CAM-1, which were derived from ascites fluid and diluted 1/300 and 1/200 respectively prior to determining their concentrations. The results are shown in table A1.2(a).

mAb	Specificity	Concentration (μg/ml)
TS1/22	LFA-1	15
FMC 4	MHC class II	5
FMC 14	MHC class II	7
FMC 15	HLA-DR	14
1B4.B12	MHC class I	30
6c6b	MHC class I	16
5A4.C5	CD11b	13
W-CAM-1	ICAM-1	10
YB5.B8	c-kit	8
17F11	c-kit	18
3D3.3	IgG_1 isotype control	22
1A6.11	IgG_{2b} isotype control	14
1A6.12	IgM isotype control	18

Table A2.1(a): MAb concentrations. The concentrations (μg/ml) of various mAb used in MLC inhibition assays, as determined by the ELISA

Appendix 2: Reagents and apparati

A2.1: Reagents for non-specific and chloroacetate esterase staining

A2.1.1: Esterase fixative: Added 2.86 mM Na_2HPO_4 (BDH, Australia, cat. no. 10248), 14.7 mM KH_2PO4 (BDH, Australia, cat. no. 10203), 45% v/v acetone (Ajax Chemicals, Australia, cat. no. 6), 25% v/v formalin (BDH, S. Australia, cat. no. 10236), and 30% v/v distilled H_20.

A2.1.2: Para-rosanaline solution: Added 0.4 g of para-rosanaline hydrochloride (Sigma, USA, cat. no. P-3750) to a mixture of 8 ml of distilled H_2O and 2 ml of concentrated (10 M) HCl (BDH, Australia, cat. no. 10125) and was incubated at 56°C for 30 min in the dark. The solution was cooled and then filtered via a vacuum flask.

A2.1.3: 0.066 M Phosphate buffer (pH 7.4): Made 25.6 mM KH_2PO_4 and 110 mM Na_2HPO_4 (BDH, Australia, cat. no. 10248) in distilled H_2O.

A2.1.4: 0.066 M Phosphate buffer (pH 6.3): Made 103 mM KH_2PO_4 and 31.4 mM Na_2HPO_4 in distilled H_2O.

A2.1.5: Methyl-green counterstain: Made 2% w/v methyl-green (Sigma, USA, cat. no. M-8884) in distilled H_2O. The contaminating methyl-violet was extracted with 50 ml of chloroform.

A2.2 Preparation of $CD2^+$-lymphocyte-enriched populations.

A2.2.1: Nylon-wool column: using gloves, 0.6 g of nylon-wool (Robbins Scientific, USA, cat. no. 1078-02-0) was stretched to remove any knots, eventually creating a fine, homogenous texture. The nylon-wool was then placed into a 10 ml syringe barrel which was inserted into a plastic container, capped and autoclaved.

A2.2.2: Phosphate-buffered saline (PBS): to make 1 litre, mixed 8 g NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4. $12H_20$ and 0.2 g KH_2HPO_4 (all chemicals were analytical grade). Made up to 1 litre with Milli Q-purified H_2O, adjusted pH to 7.4. and then autoclaved.

A2.3 Indirect immunofluorescence

A2.3.1: PBS-BSA-Azide: Added 1 g of sodium azide (Ajax Chemicals, Australia, cat. no. 1222) and 1 g of BSA (Cytosystems, Australia, cat. no. 70-010-PF) to every 1 litre of PBS.

A2.3.2: 1% Paraformaldehyde: 1 g of paraformaldehyde (BDH, England, cat. no. 29447) was added to 100 ml PBS and was boiled until powder had fully dissolved. This solution was cooled to 4°C prior to the addition to cells and was stored at 4°C for up to 2 weeks.

A2.4 Purification of mouse monoclonal antibodies

A2.4.1: 0.1 M Phosphates (Na; pH 8.2): titrated 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 + .02% NaN_3 (added small amounts of 0.1 M NaH_2PO_4 to 0.1 M Na_2HPO_4 until pH is brought down to 8.2).

A2.4.2: 0.1 M Citrate, pH 5.5 or 4.5 or 3.5: titrated 0.1 M citric acid and 0.1 M sodium citrate.

A2.4.3: 0.5 M Propionic acid: 37 ml propionic acid (Ajax Chemicals, Australia, cat. no. 693) made up to 1 litre with distilled H_2O.

A2.5 Small scale preparation of plasmid DNA.

A2.5.1: Luria-broth: to 950 ml Milli Q-purified H_20 was added 10 g of bacto Tryptone (Difco, USA, cat. no. 0123-01), 5 g of bacto yeast extract (Difco, USA, cat. no. 0127-01), 10 g NaCl. The pH was adjusted to 7 with ≈ 0.2 ml 5 M NaOH and the solution was made up to 1 litre with Milli Q-purified H_2O. This solution was autoclaved before use and ampicillin was then added at 50 ng/ml.

A2.5.2: L-B agar (+ amp): Added 7.5 g of Bacto Agar (Difco, USA, cat. no. 0140-01) to 500 ml L-B, autoclaved and then incubated at 56°C and swirled

intermittently. The agar was cooled for ≈ 2 min or until agar was not too hot to touch. Then added 1 ml ampicillin (25 mg/ml).

A2.5.3: Tris-EDTA (TE): 6.1 g Tris (Sigma, USA, cat. no. T-8524), 1.86 g EDTA (Ajax Chemicals, Australia, cat. no. 181), 2 ml 10 M HCl (BDH, U.K., cat no. UN 1789) were mixed with 1 litre Milli Q-purified H_20

A2.5.4: 0.2 M NaOH + 1% SDS ("Solution 2"): 1 ml 1 M NaOH, 0.5 ml SDS (Sigma, USA, cat. no. L-4390) and 3.5 ml Milli Q-purified H_2O. This solution was made up fresh each time it was required.

A2.5.5: 3 M Potassium 5 M Acetate ("Solution 3"): for 100 ml; 60 ml 5 M potassium acetate (Aldrich, Australia, cat. no. 24038-9), 11.5 ml glacial acetic acid (BDH, Australia, cat. no. 10001) and 28.5 ml Milli Q-purified H_2O.

A2.6: RNase protection assay

A2.6.1: DEPC-treated H_2O: All water used in making up reagents was treated with diethyl pyrocarbonate (DEPC) in order to degrade RNases. This involved the addition of 0.1% DEPC (Sigma, USA, cat. no. D-5758) to Milli Q-purified H_2O and incubating for ≈ 12 hr at 37°C, then autoclaving or incubating for 15 min at 100°C.

A2.6.2: Formamide loading solution: This loading solution comprised 95% formamide (BDH, England, cat. no. 103264R), 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole FF (Sigma, USA, cat. no. X 4126).

A2.6.3: 6% Polyacrylamide gel: Added bis-acrylamide to give 6% v/v in 8 M Urea (in 1x Tris-borate-EDTA (see A2.6.4). Then added 93 µl ammonium persulphate (Sigma, USA, cat. no. A 9434) and 23.3 µl NNN'N'-tetramethylethylene-diamine (TEMED) (BDH, England, cat. no. 30385) for every 20 ml 6% bis-acrylamide and add to siliconized gel plates.

A2.6.4: Tris-borate-EDTA buffer (TBE): Made concentrated stock solution (5x) of TBE by mixing 54 g Tris base 27.5 g boric acid (Ajax Chemicals, Australia, cat. no. 101) and 20 ml 0.5M EDTA (pH 8) and making up to 1 litre with DEPC-treated Milli Q-purified H_2O.

A2.6.5: Elution Buffer: Mixed together 0.5 M NH_4Ac, 0.1 mM EDTA and SDS (0.1%) in DEPC-treated Milli Q-purified H_2O.

A2.6.6: Hybridization solution: Mixed together 40 mM PIPES pH 6.7 (Sigma, USA, F9291), 0.4 M NaCl, 1 mM EDTA and 80% v/v formamide.

A2.6.7: RNase buffer: Mixed together 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 200 mM NaCl and 100 mM LiCl in Milli Q-purified H_2O.

A2.6.8: End-labelling of Lambda restricted with Pst 1

Reaction mix: 250 µg/ml Lambda DNA (restricted with Pst 1); 1x "ONE PHOR ALL" buffer (Pharmacia); 2 mM dATP (Pharmacia, USA, cat. no. 27-1850-01), dGTP (Pharmacia, USA, cat. no. 27-1870-01); 10 µCi ^{32}P-dCTP, 1800 Ci/mmole (Bresatec, Australia, cat. no. ADC-2); 1 U/µl DNA polymerase (Pharmacia, USA, cat. no. 27-0928-01)

Stopped reaction with 0.05 M EDTA and stored at -20°C in lead-shielded container.

A2.6.9: Preparation of transfer RNA: 2000 U of tRNA (Sigma, USA, cat. no. R-8759) was dissolved in TE (pH 7.6) containing 0.1 M NaCl, to a concentration of 10 mg/ml. This solution was then extracted twice with an equal volume phenol (equilibrated with Tris-HCl (pH 8.0)), and twice with an equal volume of chloroform and then the tRNA was precipitated with 2.5 volumes of absolute ethanol at RT. The RNA was then centrifuged at 5000g for 15 min at 4°C and was redissolved at a concentration of 10 mg/ml in TE (pH 8.0).

A2.6.10: 32P-labelling of pUC 19 (digested with HPA II):

Mixed 500 ng (per 10 µl reaction vol) pUC 19 (digested with HPA II) (Bresatec, Australia, cat. no. DMW-P1), 1 mCi/ml aqueous γ ^{32}P-ATP (Bresatec, Australia, cat. no. GRA-2), 1.5 U T4 polynucleotide kinase (PNK) (Pharmacia, Australia, cat. no. 27-0736-01), 1x PNK buffer (600 mM Tris-HCl; pH 7.6, 90 mM $MgCl_2$) in milli Q H_2O. Incubated for 1 hr at 37°C, then added 9x reaction vol TE; 1x reaction vol 3M NH_4Ac and 30x reaction vol Absolute ethanol. Incubated for a further

2 hr at -20°C, washed with 70% ethanol and resuspended in 100 µl TE/500 ng pUC 19 DNA.

Appendix 3: Glossary of cytokines and cell surface markers

Glossary of cytokines

Interleukin 1

IL-1 comprises two distinct molecules, IL-1α and IL-1β, both coded for by genes on chromosome 6 (March *et al*, 1985). They are structurally dissimilar proteins, apart from a region proposed to be that which binds to the IL-1R (March *et al*, 1985). IL-1α and β bind to the same receptor which is found in two forms, high and low affinity (Kihan *et al*, 1986; Lowenthal and MacDonald, 1985). IL-1 is primarily produced by activated macrophages, keritinocytes, astrocytes and mesangial cells (Durum *et al*, 1985) and has wide range of effects on many different cell types such as the augmentation of connective tissue growth and the increase in bone resorption (reviewed by Strober and James, 1988). IL-1 stimulates transcription of genes coding for IL-2Rα (Mizel, 1990; Shirakawa *et al*, 1986) and κ immunoglobulin light chain (Mizel, 1986; Giri *et al*, 1984).

IL-2

IL-2 is a 15 kDa glycoprotein encoded by a gene on the long arm of chromosome 4 (Malkovsky and Sondel, 1987). It causes the proliferation of T cells, B cells, NK cells and thymocytes (Malkovsky and Sondel, 1987; Watson and Mochizuki, 1980) and is produced by T lymphocytes (primarily $CD4^+$) and LGL (Watson and Mochizuki, 1980). The IL-2R can be found with high, intermediate or low affinity. The high affinity receptor is a heterodimer of the low affinity α chain (p55 or Tac) and the intermediate affinity β chain (p75) (Robb *et al*, 1984; Waldmann, 1986). IL-2 can stimulate the proliferation of T lymphocytes expressing solely p75 but not T lymphocytes expressing solely p55 (Smith *et al*, 1988). Resting lymphocytes have been reported to express p75 constitutively (Dukovich *et al*, 1987; Hatakeyama *et al*, 1989).

During T cell activation p55, or IL-2Rα expression is regulated at the transcriptional level (Leonard *et al*, 1985c; reviewed by Rothenberg *et al*, 1992).

IL-6

IL-6, a 26 kDa protein coded for by a gene on chromosome 7 (Sehgal *et al*, 1986) and was originally described as a lymphokine responsible for inducing the final phase of B lymphocyte differentiation into immunoglobulin-secreting cells (Hirano *et al*, 1986) but is now known to have a wide range of effects, which include T lymphocyte differentiation (Lotz *et al*, 1988; Takai *et al*, 1988) and proliferation (Hirano *et al*, 1990), modulation of IL-2 expression (Garman *et al*, 1987) as well as playing a role in haemopoiesis (Sehgal *et al*, 1989). Sources of IL-6 include T cells, B cells, synoviocytes, chrondocytes (Hirano *et al*, 1988; Guerne *et al*, 1989; Shinmei *et al*, 1989) and monocytes (Nakajima *et al*, 1989). A possible autocrine role for IL-6 has been reported in non-Hodgkin's lymphoma, as well as CML and AML (Freeman *et al*, 1989; Yee *et al*, 1990; Biondi *et al*, 1989). The IL-6R is a 80 kD glycoprotein whose cytoplasmic domain does not appear to be required for signal transduction (Hirano *et al*, 1990).

IL-12

IL-12 is a 70 kD heterodimeric cytokine originally identified as a product of EBV-transformed human B lymphoblastoid cell lines (Kobayashi *et al*, 1989). Also known as natural killer cell stimulatory factor and cytotoxic lymphocyte maturation factor. IL-12 has a number of effects on resting and activated T lymphocytes and NK cells which include; the ability to directly induce the proliferation of activated T and NK cells (Gately *et al*, 1991); the ability to act as a comitogen with TPA or anti-CD3 mAb (Perussia *et al*, 1992; Kobayashi *et al*, 1989); the ability to induce the production of

IFN-γ, TNF and other cytokines by both resting and activated T and NK cells (Chan *et al*, 1991).

TNF-α

TNF-α is a 17 kDa protein coded by a gene on chromosome 6 (Takatsu *et al*, 1987) and is produced by activated macrophages and lymphocytes, including NK cells (Nissen-Meyer and Hammerstrom, 1982; Peters *et al*, 1986). TNF was first described through its capacity to preferentially kill tumour cells (Carswell *et al*, 1975).

Glossary of cell surface molecules

CD4

CD4 is a 55 kDa transmembrane glycoprotein that belongs to the immunoglobulin gene superfamily (Maddon *et al*, 1987) and is capable of binding to a monomorphic portion of MHC class II molecules (Doyle and Strominger, 1987). Through this interaction CD4 is thought to stabilize the physical interactions between T cells and APC (reviewed by Swain, 1983) The cytoplasmic tail of CD4 can bind to the cytoplasmic src family protein tyrosine kinase $pp56^{lck}$ (Veillette *et al*, 1988) and has thus been implicated in cell signalling (Veillette *et al*, 1989). Further details with regards to the structure and function of CD4 are reviewed by Janeway *et al*, 1989.

CD8

CD8 is a transmembrane glycoprotein, found on a subset of NK cells and T lymphocytes, that belongs to the immunoglobulin gene superfamily (Parnes, 1989). This molecule is usually a heterodimer comprising an α and a β subunit (Janeway, 1992) and is capable of binding to a monomorphic portion of the $\alpha 3$ region of MHC class I molecules (Norment *et al*, 1988; Salter *et al*, 1990). The cytoplasmic domain of CD8 can bind to the cytoplasmic src family protein tyrosine kinase $pp56^{lck}$ (Veillette *et al*, 1988) and has thus been implicated in cell signalling. Further details with respect to the structure and function of CD8 are reviewed by Rudd *et al*, 1989.

MHC class I

MHC class I molecules are coded for by the HLA-A, -B and C genes in the major histocompatibility complex on chromosome 6 and are expressed by the majority of cell types. The polymorphic 44 kDa product of these genes associates noncovalently with an invariant 12 kDa subunit known as $\beta 2$-microglobulin (reviewed by Schwartz, 1991).

These MHC class I molecules are capable of presenting portions of proteins, that have been manufactured intracellularly, to $CD8^+$ T lymphocytes (reviewed by Schwartz, 1990). Genes encoding other MHC class I molecules (HLA-E, -F and -G) have recently been identified (Reviewed by Orr, 1989).

MHC class II

MHC class II molecules are polymorphic heterodimeric glycoproteins expressed by at least 3 or 4 pairs of A and B genes and map to three regions (HLA-DR, -DP and -DQ) of the MHC class II region on chromosome 6 (Figueroa and Klein, 1986). HLA-DR are noted for being the major stimulatory molecule in the allogeneic MLC (Jaraquemada *et al*, 1986). HLA-DQ molecules however have been reported to induce suppressor activity (Jaraquemada *et al*, 1986; Nieda *et al*, 1988). The main function of MHC class II molecules is to present exogenously derived peptide to $CD4^+$ lymphocytes (reviewed by Harding and Unanue, 1990).

CD45

CD45, or leukocyte common antigen, is expressed by all haemopoietic cells and possesses a constant cytoplasmic domain (Charbonneau *et al*, 1988) and a variable extracellular domain (Thomas, 1989). The cytoplasmic domain consists of two tyrosine specific phosphatase domains (Tonks *et al*, 1989) which appear to be capable of dephosphorylating TCR-coreceptor associated tyrosine kinase (Mustelin *et al*, 1989). Many different isoforms of CD45 exist. Exons A, B and C, which partially encode the external domain of CD45, are variably expressed in different isoforms (Lefrancois *et al*, 1986). MAb binding to a subset of CD45 isoforms have their specificity designated as CD45R followed by a letter to indicate the region of the molecule they bind. CD45RA

and CD45RO are putatively expressed by naive and memory T lymphocytes respectively (reviewed by Janeway, 1992).

The T cell receptor

αβ heterodimer: This TCR comprises an acidic α chain (39 - 46 kd) and a more basic β chain (40-44 kd) (Meuer *et al*, 1983) which are linked via a disulphide bond (Samelson, 1985). The α chain gene is located on chromosome 14 (Caccia *et al*, 1985) and comprises V, J and C segments (reviewed by Moss *et al*, 1992). The β chain is located on chromosome 7 (Lai *et al*, 1988) and is comprised of V, D, J and C segments (reviewed by Moss *et al*, 1992). The diversity of TCR specificity in each host is generated by the random joining of V, J and sometimes D gene segments (Davis and Bjorkman, 1988).

CD3

CD3 is a complex of integral membrane glycoproteins of the immunoglobulin supergene family that are invariant in sequence. The complex comprises γ, δ, ε subunits and associates with a ζ:ζ homodimer or ζ:η heterodimer. TCRα and β chains cannot be expressed without coexpression of CD3 (Ashwell and Klausner, 1990). The ζ:ζ homodimer and the ζ:η hetrodimer have been shown to be involved in cell signalling (Sussman *et al*, 1988; Bauer *et al* 1991). The ζ chain is also expressed by NK cells and is involved in cell signalling (Lanier *et al*, 1989; Anderson *et al*, 1989).

LFA-1

LFA-1 is a heterodimer comprising the 180 kDa α subunit CD11a and the 95 kDa β subunit CD18, and is a member of the integrin family. LFA-1 is constitutively expressed on T lymphocytes as well as most other leukocytes and its binding capacity is transiently enhanced after TCR crosslinking (Dustin and Springer, 1989). This molecule has been shown to enhance the avidity between T cells and antigen presenting cells (Gougeon *et al*, 1985; Dougherty and Hogg, 1987; Shaw *et al*, 1986) and is

probably involved in signal transduction across the T cell membrane (van Noesel *et al*, 1988). LFA-1 has been shown to be functionally involved in cell-mediated cytotoxicity (Sanchez-Madrid *et al*, 1982). To date four receptors for LFA-1 have been discovered; ICAM-1, -2 ,-3 and -R (Marlin and Springer, 1987; de Fougerolles *et al*, 1991; Vazeux *et al*, 1992).

CD2 (LFA-2)

CD2 is a 50 kDa glycoprotein responsible for the alternative pathway of activation of T lymphocytes (Meuer *et al*, 1984; Brottier *et al*, 1985; Holter *et al*, 1986; Moretta *et al*, 1986; O'Flynn *et al*, 1985). The ligand for CD2 is LFA-3 (Selvaraj *et al*, 1987). CD2 is expressed by the vast majority of T lymphocytes and NK cells and has been shown to be functionally involved in cell-mediated cytotoxicity (Shaw and Luce, 1987).

LFA-3 (CD58)

LFA-3 is a ubiquitously expressed molecule that is the ligand for CD2 (or LFA-2) (Selvaraj *et al*, 1987) and binds via the epitopes $T11_{1a}$ and $T11_{1b}$ (Meuer *et al*, 1989). It has been shown to be functionally involved in cell-mediated cytotoxicity (Shaw and Luce, 1987).

ICAM-1 (CD54)

ICAM-1 is a 92 kDa glycoprotein and is one of at least three ligands for LFA-1 (Marlin and Springer, 1987). This molecule is expressed by vascular endothelial cells, thymic epithelial cells, fibroblasts, tissue macrophages, activated lymphocytes and germinal centre dendritic cells (Dustin *et al*, 1986). Target cell ICAM-1 expression has been shown to play a central role in T cell mediated lysis (Springer *et al*, 1987), however its presence seems to be less important for non-MHC-restricted lysis (Triozzi

et al, 1992). Also, transfection experiments have shown ICAM-1 to be important in antigen presentation (Dougherty *et al*, 1988; Schulz *et al*, 1988). ICAM-1 is also a ligand for MAC-1 (CD11b/CD18) (Diamond *et al*, 1990).

AMENDMENTS

Amendment 1 (for Introduction 1.4.5(d))

CD28

CD28, a homodimer (44 KD) and a member of the immunoglobulin supergene family (Aruffo and Seed, 1987, Williams and Barclay, 1988), is expressed by most T lymphocytes (Hansen *et al*, 1980). The exceptions include a suppressive population of $CD28^{-}CD11b^{+}$ T cells (Damle *et al*, 1983, Morishito *et al*, 1989). T lymphocyte proliferation assays involving the anti-CD28 mAb 9.3 (cross-linked) revealed CD28 to have an important stimulatory role when used in combination with anti-CD3 or anti-CD2 mAbs (Weiss *et al*, 1986, Pierres *et al*, 1988). It has subsequently been shown that CD28, in its role as a costimulator, enhanced the expression of CD25, as well as IL-2, γ-IFN, TNF-α and IL-1 (Martin *et al*, 1986, Thompson *et al*, 1989, Ledbetter *et al*, 1990, van Kooten *et al*, 1988).

The first counter-receptor for CD28 to be discovered was B7 (B7-1) (Freeman *et al*, 1989, Linsley *et al*, 1991). This molecule is also a member of the immunoglobulin supergene family and is expressed on activated B lymphocytes, macrophages, activated monocytes, dendritic cells and activated T cells (Hart *et al*, 1993; Sansom and Hall, 1993). It has also been found to be a counter-receptor for CTLA-4, a molecule related to CD28 and only expressed on activated T cells (Linsley *et al*, 1992). Subsequently another counter-receptor to CD28 and CTLA-4 has been cloned and found to play a role in the costimulation of T lymphocytes (B7-2) (Freeman *et al*, 1993).

B7 has been shown to deliver a strong costimulatory signal for the activation of T cells by binding to one of its ligands CD28 or CTLA-4 (Ohnishi *et al*, 1993; Ding and Shevach, 1994). That CD28 may be important for providing secondary activation signals to T cells was supported by the finding that MHC class I+ and MHC class II+ human cardiac myocytes were not capable of stimulating allogeneic peripheral blood mononuclear cells

unless immobilized anti-CD28 mAb were present (Ansari *et al*, 1993). Further support for such a notion came with the finding that NIH 3T3 cells transfected with HLA-DR and B7 were capable of stimulating the proliferation of human PBL whilst those cells transfected with HLA-DR alone caused anergy (Gimmi *et al*, 1993). Additionally, it has been found that, whilst not expressed on resting T cells, B7 expression could be induced upon activation and was found to be functionally involved in T-T cell interactions (Wyss-Coray *et al*, 1993). It was independently observed that soluble anti-CD28 mAb and soluble anti-B7 mAb could inhibit ag-specific T cell proliferation, implicating a role for the CD28-B7 interaction in accessory-cell mediated T lymphocyte activation (Lesslauer *et al*, 1986, Kuolova *et al*, 1991). Interestingly, it has been recently shown that B7 costimulates the TCR-dependent proliferation of human ag-primed $CD4^+$ T cells more efficiently than that of resting non-activated $CD4^+$ T cells and that the proliferation of resting $CD4^+$ T cells can be efficiently costimulated by either ICAM-1 or VCAM-1 via interactions with their respective ligands, LFA-1 and VLA-4 (Damle *et al*, 1993). It was further shown, by the same researchers, that responsiveness of resting T cells to B7 was augmented by ICAM-1 or VCAM-1 costimulation. Reciprocally it has been discovered that cross-linking of CD28 can enhance integrin-mediated adhesion in T lymphocytes (Shimizu *et al*, 1992).

Separate studies indicated that when PMA and the non-cross-linking form of the anti-CD28 mAb 9.3 were used in combination to activate T lymphocytes, CD28 was capable of signalling in a calcium and PKC-independent fashion (June *et al*, 1989). However, the cross-linking of CD28 on T lymphocytes provoked an increase in intercellular calcium (Ledbetter *et al*, 1987). It was subsequently shown that this increase in calcium was due to the triggering of a PTK, which in turn triggered $PLC\gamma_1$ (Ledbetter *et al*, 1992). Thus it would appear that CD28 is capable of providing two types of

signals. T lymphocyte proliferation inhibition assays suggested that B7 is capable of triggering both arms of the CD28 signal (Ledbetter *et al*, 1992). It was further suggested by Linsley and Ledbetter (1993) that both arms of the CD28 signal are necessary for ag-stimulated T lymphocyte activation since soluble mAb 9.3 actually inhibits T cell responses to antigen, as mentioned previously.

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Amendment 2 (for General Discussion)

The role of CD28 in the LEP response to HL-60-R7 cells

It is feasible that, along with MHC class II, LFA-1 and TNF-α, CD28 played an important part in the response of LEP to HL-60-R7 cells. To illustrate, if B7-1 and/or B7-2 were expressed by HL-60-R7 cells then these molecules could interact with CD28 on T lymphocytes, possibly assisting in the initiation of the immune response. That CD28 could be involved in costimulatory activity has been previously shown (Ansari *et al*, 1993). Additionally, or alternatively, CD28-B7(-1 and/or -2) interactions may have been important in T-T cell (see Wyss-Coray *et al*, 1993) or T-NK cell collaboration. Resting NK cells do not express CD28, however IL-2 or IL-12-activated NK cells or NK cell lines have been reported to express this molecule (Shaw *et al*, 1994: Gong *et al*, 1994). Additionally, the CD28-B7 interaction was shown to be responsible for MHC-unresticted cytotoxicity mediated by a human NK leukaemia cell line (Azuma *et al*, 1992). It is therefore possible that such an interaction was important in the cytotoxicity of HL60-R7-activated lymphocytes towards the HL60-R7 targets. In order to ascertain whether the above , or any other, proposals are tenable, one would be required to perform immunofluorescence assays to determine whether HL-60 and HL-60-R7 cells express B7-1 or B7-2. Additionally, one would need to perform MLC-inhibition assays using functionally inhibitory mAb specific for CD28 (and possibly CTLA-4) and /or its ligands. Unfortunately, neither of these assays could be performed here due to a lack of access to the relevant mAb.

* See amendment 1 for references.

Amendment 3

Synonyms for some cell surface molecules

It was discovered that, in some instances, the same molecules were referred to by different names in different parts of this thesis. Here is a list of those antigens and the alternative names that were used.

Antigen	Alternative name
CD2	LFA-2, T11
CD16	FcγRIII
CD25	IL2Rα, p55
CD54	ICAM-1
CD58	LFA-3
CD64	FcγR1
HLA-D	MHC class II
IL-2Rβ	p75