



HUMAN MYELOID DIFFERENTIATION ANTIGENS

Alan Bruce Lyons, B.Sc. Hons (Adelaide),
Department of Microbiology and Immunology,
The University of Adelaide.

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ABSTRACT

This thesis is concerned with the identification and characterization of antigens defined by monoclonal antibodies (MoAb's) that can be used in the investigation of human myeloid haemopoiesis and differentiation.

A model system for the study of human monocytic differentiation was investigated. It was found that cells of the human myelomonocytic cell line RC-2A would differentiate towards macrophages when treated with a source of lymphocyte derived factors obtained from mitogen stimulated human peripheral blood mononuclear cells.

A panel of MoAb's was used to investigate the surface marker changes involved in this process, and in addition, new MoAb's were produced which were myeloid specific. A colorimetric assay for screening MoAb's to cell surface antigens was modified to enhance its sensitivity, in an attempt to maximise the chances of identifying MoAb's binding to antigens of low copy number.

Cells of the human myelomonocytic line RC-2A were induced to differentiate toward macrophages by culturing for up to twelve days in the presence of supernatant from phytohaemagglutinin stimulated human peripheral blood mononuclear cells (PHA-LCM). The process of differentiation was monitored by changes in expression of two macrophage related enzymes (α -Naphthol Butyrate Esterase and Acid Phosphatase), the changes in expression of the monocyte/macrophage cell surface markers detected by the monoclonal antibodies anti-Mo1 and anti-Mo2, Ia antigen detected by the monoclonal antibody FMC-14, and alteration in cell morphology.

RC-2A cells over a period of 8 days culture in the presence of PHA-LCM expressed α -Naphthol Butyrate Esterase very strongly compared to untreated cells, and *de novo* Acid Phosphatase activity was found within lysosomal granules. Such a pattern of enzymic expression is typical of macrophages. Investigation of cell surface markers detected by MoAb's showed changes consistent with differentiation towards macrophages, with the binding of α -Mo1 and α -Mo2 increasing. Induced RC-2A cells were able to stimulate in one-way Mixed Leukocyte Culture more effectively than control cells. Maturation induced by PHA-LCM was accompanied by a marked decrease in the proliferative potential of the cell population, and a reduced ability to form colonies in semi-solid medium. After 9 days in culture with PHA-LCM, these RC-2A cells had only 3% of the clonogenic potential of control untreated cells, suggesting that differentiation was accompanied by an irreversible loss of proliferative ability.

Investigations into the effects of the recombinant human biological factors tumour necrosis factor (TNF), γ -Interferon, Granulocyte-Macrophage colony stimulating factor (GM-CSF) and Granulocyte colony stimulating factor (G-CSF) revealed that none of these alone could induce the full complement of differentiation effects mediated by PHA-LCM, suggesting full differentiation is likely to be a multifactorial process. For example, of the four factors investigated, only γ -Interferon and G-CSF were able to reduce the clonogenic potential of RC-2A cells, and only γ -Interferon was able to increase the expression of α -Naphthol Butyrate Esterase. The alteration of antigen expression on RC-2A cells mediated by γ -Interferon and TNF was quite different to the pattern of expression induced by PHA-LCM.

A polyclonal antiserum against RC-2A cells raised in mice and absorbed with the autologous B lymphocyte cell line Cess B, was shown to increase the proliferation of RC-2A cells at appropriate dilutions, demonstrated by a 3 fold increase in ^3H -thymidine uptake. The antiserum was also able to stimulate the formation of exclusively macrophage colonies in semi-solid agar culture of human bone marrow mononuclear cells. These properties were not shared by normal mouse serum, suggesting the feasibility of raising monoclonal antibodies with functional effects on haemopoietic cells.

In an effort to isolate MoAb's which exerted functional effects on RC-2A cells, in addition to those recognising human myeloid differentiation antigens, a screening assay was required which would identify antibodies binding antigens of low copy number. A previously described assay for detecting MoAb's binding to cell surface antigens, called the Rose Bengal Assay (RBA), was modified to give increased sensitivity. This assay involved coating the wells of 96-well microtitre trays with an anti-murine immunoglobulin reagent, allowing any immunoglobulin in test hybridoma supernatants to bind. Then target cells were added, which would be bound to wells that contained specific antibody from the hybridoma supernatant. Bound cells were stained with Rose Bengal dye, and optical density at the relevant wavelength correlated with presence of antibody specific to the target cells. During the course of these investigations, a number of antibodies reproducibly demonstrated binding to target cells in the RBA, but gave negative or very weak binding in indirect immunofluorescence assay, and would probably have been overlooked had such an assay been used for the initial screening process.

Hybridomas were produced by fusion of spleen cells from mice immunised with RC-2A cells with cells of the murine myeloma line x63Ag8.653, and selection in HAT medium. Hybrids were screened using the RBA, and those which produced antibody binding to RC-2A, but not to the autologous B lymphocyte cell line Cess B, were chosen for cloning by limit dilution and further investigation. Eventually, five differing MoAb's were selected to be characterised more fully. These MoAb's were screened for functional effects on RC-2A cells and haemopoietic cells, but were found to have no activity in the systems studied. The binding pattern of these five MoAb's to human haemopoietic cell lines, normal blood cells and tissue sections was determined, and their specificity for human haemopoietic progenitors was studied using negative selection by complement mediated cell lysis. The molecular weights of antigens detected by the MoAb's were determined for four of the five antibodies. Comparisons between these antibodies and those described in the literature suggest that the specificities of two of the MoAb's obtained may be different to those of antibodies previously published.

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.

Alan Bruce Lyons.

June, 1987

To Ildikó

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ABBREVIATIONS

CFU-S	Colony forming unit spleen
CFU-C	Colony forming unit culture
CFU-M	Macrophage Colony forming unit
CFU-G	Neutrophil " " "
CFU-Eo	Eosinophil " " "
CFU-GM	Macrophage/Neutrophil "
CFU-Mix/GEMM	Mixed " " "
CFU-e	Erythroid " " "
BFU-e	Erythroid burst forming unit
CFU-Leuk	Leukaemic colony forming unit
CSF	Colony stimulating factor
M-CSF	Macrophage " " "
G-CSF	Granulocyte " " "
GM-CSF	Macrophage/Granulocyte " "
Multi-CSF /IL-3	Pluripotential " "
EDF	Eosinophil differentiation factor
DMSO	Dimethylsulphoxide
TPA	12-0-tetradecanoyl phorbol 13-acetate
f-MLP	methionine-leucine-phenylalanine tripeptide
PHA-LCM	Phytohaemagglutinin lymphocyte conditioned medium
γ -IFN	Interferon gamma
TNF- α	Tumour necrosis factor alpha
LT	Lymphotoxin
DIF	Differentiation inducing factor
M ϕ	Macrophage

Nø	Neutrophil
Eø	Eosinophil
Pl	Platelet
T cell	Thymus derived lymphocyte
B cell	Bursa or marrow derived lymphocyte
MNC	Mononuclear cells
PMN	Polymorphonuclear cells
AML	Acute myeloid leukaemia
AMML	Acute myelomonocytic leukaemia
AMoL	Acute monocytic leukaemia
FAB M1-M7	French-American-British classification of myeloid leukaemias
ALL	Acute lymphocytic leukaemia
CLL	Chronic lymphocytic leukaemia
HLA-A,B,C	Human class I major histocompatibility antigen
HLA-DR	Human class II major histocompatibility antigen
Ia	Class II " " "
MoAb	monoclonal antibody
LFA-1	Lymphocyte functional antibody type 1
C3biR	Receptor for third complement fragment
FAL	3- α -fucosyl-N-acetyl-lactosamine
FcR	Receptor for the Fc region of immunoglobulin
ADCC	Antibody dependant cellular cytotoxicity
ABMT	Autologous bone marrow transplantation
CPM	Counts per minute
kd	Kilodaltons
FCS	Foetal calf serum
PEG	Polyethylene glycol

HAT	Hypoxanthine-aminopterin-thymidine
HT	Hypoxanthine-thymidine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
TRIS	Tris(hydroxymethyl)amino-methane
PBS	Phosphate buffered saline
BSA	Bovine serum albumen
IMDM	Iscoves modification of Dulbecos medium
RPMI	Roswell Park Memorial Institute medium
Az	Sodium azide
IgA	Immunoglobulin class A
IgG1	" " G1
IgG2a	" " G2a
IgG2b	" " G2b
IgM	" " M
FITC	Fluorescein isothiocyanate
RBA	Rose Bengal Assay
RIA	Radioimmunoassay
IF	Indirect immunofluorescence assay
FACS	Fluorescence activated cell sorter
HA	Haemagglutination assay
ELISA	Enzyme-linked immunosorbent assay
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
EBV	Epstein-Barr virus

CONTENTS.

ABSTRACT	-	-	-	-	i
STATEMENT	-	-	-	-	v
ACKNOWLEDGEMENTS	-	-	-	-	vi
ABBREVIATIONS	-	-	-	-	vii

CHAPTER 1: Introduction

1.1.1 Differentiation	-	-	-	1
1.1.2 Haemopoietic differentiation	-	-	-	1
1.1.3 Models of commitment to differentiation	-	-	-	3
1.1.4 Clonal culture of haemopoietic progenitor cells	-	-	-	7
1.1.5 Properties of the colony stimulating factors-	-	-	-	9
1.1.5.1 Murine CSF's-	-	-	-	9
1.1.5.2 Human CSF's -	-	-	-	11
1.1.5.3 Other haemopoietic growth factors	-	-	-	14
1.1.5.4 Functional effects of CSF's on mature cells	-	-	-	15
1.1.5.5 <i>In vivo</i> activity of the CSF's	-	-	-	15
1.2.1 Myeloid cell lines: Suitability and usefulness as models of haemopoietic differentiation	-	-	-	16
1.2.2 Induction of differentiation of myeloid cell lines	-	-	-	19
1.2.3 Induction of differentiation of HL-60	-	-	-	20
1.2.3.1 Granulocytic differentiation of HL-60	-	-	-	21
1.2.3.2 Monocyte/macrophage differentiation of HL-60	-	-	-	22
1.2.4 Induction of differentiation in other human myeloid cell lines	-	-	-	24
1.2.5 Identity of biologically-derived agents which induce differentiation in myeloid cell lines	-	-	-	28
1.2.6 Myeloid cell lines: Summary	-	-	-	32
1.3.1 Monoclonal antibodies and haemopoietic differentiation	-	-	-	33
1.3.2 Monoclonal antibodies to human myeloid antigens	-	-	-	34
1.3.3 Less well-defined monoclonal antibodies	-	-	-	38

1.3.3.1 MoAb's with relative affinity for myeloid progenitor cells	-	-	-	38
1.3.3.2 MoAb's with functional effects on myeloid cells				40
1.3.3.3 Miscellaneous MoAb's to myeloid cells	-			41
1.3.4 Classification of myeloid leukaemia using MoAb's				43
1.3.4.1 Prognostic significance of anti-myeloid MoAb's				49
1.3.4.2 Lineage fidelity	-	-	-	50
1.3.4.3 Clonogenic cells in AML		-	-	51
1.3.4.4 MoAb's with potential therapeutic uses.		-		52
1.4 Aims of the project	-	-	-	53

CHAPTER 2: Materials and methods.

2.1 Buffers and general reagents	-	-		55
2.2 Antisera	-	-	-	56
2.2.1 Preparation of rabbit antiserum to mouse immunoglobulin-		-	-	56
2.2.2 Preparation of goat antiserum to mouse immunoglobulin-		-	-	57
2.2.3 Affinity purification of goat antibodies to mouse immunoglobulin	-	-	-	58
2.2.4 Labelling of antibodies with I ¹²⁵	-		-	59
2.2.5 Labelling of antibodies with FITC	-		-	60
2.3 Tissue culture	-	-	-	60
2.3.1 General tissue culture medium		-	-	61
2.3.2 IMDM; Single strength	-	-	-	61
2.3.3 IMDM; Double strength	-	-	-	62
2.3.4 <i>In vitro</i> culture of cell lines		-	-	62
2.3.5 Human haemopoietic cell lines		-	-	63
2.3.6 Cryopreservation of cells		-	-	63
2.3.7 Thawing of frozen cells	-	-	-	64

2.4 Isolation of human haemopoietic cells	-	64
2.4.1 Preparation of peripheral blood mononuclear cell fraction	- - - -	64
2.4.2 Preparation of peripheral blood polymorphonuclear cell fraction	- - - -	65
2.4.3 Preparation of peripheral blood erythrocytes-		65
2.4.4 Preparation of bone marrow mononuclear cells-		65
2.4.5 Preparation of bone marrow nucleated cells	-	66
2.4.6 Preparation of cord blood mononuclear cells	-	66
2.4.7 Determination of cell morphology	- -	66
2.5 Immunoassay of MoAb binding to haemopoietic cells		67
2.5.1 Indirect immunofluorescence assay of MoAb binding to haemopoietic cells	- - -	67
2.5.2 Manual scoring of FITC-Sheep/Goat anti-mouse labelled cells-	- - -	68
2.5.3 Flow cytometric scoring of FITC-Sheep/Goat anti-mouse labelled cells	- -	68
2.5.4 Haemagglutination assay	- - -	68
2.6 Culture of myeloid progenitor cells	-	69
2.6.1 Production of lymphocyte conditioned medium (PHA-LCM)	- - - -	69
2.6.2 Culture of myeloid progenitor cells in semi-solid medium-	- - - -	69
2.6.3 Triple stain of myeloid colonies	- -	70
2.7 Determination of molecular weights of proteins detected by MoAb's to cell surface antigens	-	72
2.7.1 Immunoabsorbtion of biotinylated cell surface proteins	- - -	72
2.7.2 Polyacrylamide gel electrophoresis of immunoabsorbed cell surface proteins	- - -	74
2.7.3 Polyacrylamide gel electrophoresis; reagents and method	- - - -	74
2.7.4 Western transfer onto nitrocellulose of proteins separated by electrophoresis	- -	76
2.7.5 Detection of biotin-labelled protein on nitrocellulose paper	- - -	77

2.8 Immunocytochemistry	-	-	-	78
2.8.1 Immunoperoxidase detection of MoAb binding	-			79
2.8.2 Alkaline phosphatase anti-alkaline phosphatase (APAAP) detection of MoAb binding	-		-	79

CHAPTER 3: Studies on the differentiation of the human myelomonocytic cell line RC-2A in response to lymphocyte derived factors.

3.1 Introduction	-	-	-	82
3.2 Materials and methods	-	-	-	83
3.2.1 Cell line	-	-	-	83
3.2.2 Preparation of PHA-LCM	-	-	-	83
3.2.3 Induction of differentiation of RC-2A cells with PHA-LCM	-	-	-	84
3.2.4 Culture of RC-2A cells in the presence of purified/cloned human growth factors			-	84
3.2.5 Cytochemical stains	-	-	-	85
3.2.6 Indirect immunofluorescence assay of surface marker expression	-	-	-	86
3.2.7 Optimization of growth of RC-2A cells in semi-solid agar	-	-	-	87
3.2.8 Clonal assay of cellular proliferative potential				88
3.2.9 Ability of purified/cloned growth factors to replace the requirement of RC-2A cells for RC-2A conditioned medium for the formation of colonies in semi-solid agar	-	-	-	88
3.2.10 One-way mixed leukocyte culture	-	-	-	89
3.2.11 Production of a polyclonal murine antiserum to RC-2A cells	-	-	-	89
3.2.12 Effect of adsorbed anti-RC-2A serum on ³ H-Thymidine uptake by RC-2A cells	-	-	-	90
3.2.13 Effect of adsorbed anti-RC-2A serum on bone marrow myeloid progenitor cells		-	-	90
3.3 Results	-	-	-	91
3.3.1 Growth characteristics of RC-2A cells			-	91
3.3.2 Effect of differentiation inducers on α -Naphthol-Butyrate esterase expression by RC-2A cells	-			91

3.3.3	Effect of differentiation inducers on morphology of RC-2A cells-	-	-	-	92
3.3.4	RC-2A surface marker expression changes caused by inducers of differentiation	-	-	-	93
3.3.5	The effect of differentiation inducing agents on the clonogenic potential of RC-2A cells	-	-	-	95
3.3.6	Ability of cloned/purified growth factors and PHA-LCM to replace the requirement for RC-2A conditioned medium for culture of RC-2A cells in semi-solid agar	-	-	-	96
3.3.7	Effect of culture of RC-2A cells in the presence of PHA-LCM on their ability to stimulate in one-way mixed leukocyte culture	-	-	-	97
3.3.8	Effect of anti-RC-2A serum on uptake of ³ H-Thymidine by RC-2A cells-	-	-	-	97
3.3.9	Effect of anti-RC-2A serum on bone marrow myeloid progenitor cell growth	-	-	-	97

3.4 Discussion

CHAPTER 4: Development of an improved screening method for monoclonal antibodies binding to cell surface antigens.

4.1	Introduction	-	-	-	-	105
4.2	Materials and methods	-	-	-	-	106
4.2.1	Monoclonal antibodies and sera	-	-	-	-	106
4.2.2	Cell lines	-	-	-	-	106
4.2.3	The rose bengal assay	-	-	-	-	107
4.2.4	Indirect immunofluorescence	-	-	-	-	108
4.2.5	Whole cell radioimmunoassay	-	-	-	-	108
4.3	Results	-	-	-	-	109
4.3.1	Optimisation of the RBA	-	-	-	-	109
4.3.2	Comparison with whole cell RIA	-	-	-	-	109
4.3.3	Comparison with manual indirect immunofluorescence	-	-	-	-	110
4.3.4	Comparison with indirect immunofluorescence scored by flow cytometry	-	-	-	-	110
4.3.5	Screening of an anti-AML fusion: Comparison with RIA	-	-	-	-	111

4.3.6 Results with other human cell lines and cell types				112
4.3.7 Detection of MoAb's specific for minor cell populations	-	-	-	113
4.4 Discussion	-	-	-	114

CHAPTER 5: Production of monoclonal antibodies binding to human myeloid cells.

5.1 Introduction	-	-	-	116
5.2 Materials and methods	-	-	-	117
5.2.1 Immunization of mice	-	-	-	117
5.2.2 Preparation of single cell suspensions from mouse spleen and thymus	-	-	-	118
5.2.3 Production of monoclonal antibody secreting murine hybridomas	-	-	-	118
5.2.4 Screening by RBA	-	-	-	120
5.2.5 Screening by ³ H-Thymidine uptake assay	-	-	-	120
5.2.6 Limit dilution cloning of antibody producing murine hybridomas	-	-	-	121
5.2.7 Expansion of hybrids	-	-	-	122
5.2.8 Determination of immunoglobulin sub-class by radial immunodiffusion	-	-	-	122
5.2.9 Determination of immunoglobulin concentration				123
5.3 Results	-	-	-	125
5.3.1 Screening by RBA	-	-	-	125
5.3.2 Screening for ability to alter ³ H-Thymidine uptake by RC-2A cells	-	-	-	125
5.3.3 Limit dilution cloning of antibody secreting hybridoma cells	-	-	-	126
5.3.4 Immunoglobulin sub-classes of monoclonal antibodies				128
5.3.5 Establishment of frozen stocks of hybridoma cells, and of supernatants	-	-	-	128
5.3.6 Concentrations of immunoglobulin in MoAb stocks				129
5.4 Discussion	-	-	-	129

CHAPTER 6: Characterization of monoclonal antibodies binding to human myeloid cells.

6.1 Introduction	-	-	-	130
6.2 Materials and methods	-	-	-	131
6.2.1 Estimation of molecular weights of antigens detected by anti-myeloid MoAb's	-	-	-	132
6.2.2 Binding to human haemopoietic cell lines	-	-	-	132
6.2.3 Binding to normal peripheral blood leukocytes and erythrocytes, and bone marrow nucleated cells	-	-	-	132
6.2.4 Complement mediated cell lysis of cord blood progenitor cells	-	-	-	133
6.2.5 Immunohistochemistry	-	-	-	134
6.2.6 The effects of purified growth factors and PHA-LCM on the surface marker expression of RC-2A cells	-	-	-	135
6.2.7 Effects of antibodies on differentiation of RC-2A cells and cord blood myeloid progenitor cells	-	-	-	135
6.2.8 Binding of antibodies to peripheral blood leukocytes of AML patients	-	-	-	136
6.3 Results	-	-	-	137
6.3.1 Molecular weights of antigens detected by anti-myeloid MoAb's	-	-	-	137
6.3.2 Binding to human haemopoietic cell lines	-	-	-	138
6.3.3 Binding to human peripheral blood leukocytes and erythrocytes, and bone marrow nucleated cells	-	-	-	138
6.3.4 Complement mediated cell lysis of myeloid progenitors from cord blood.	-	-	-	139
6.3.5 Immunohistochemistry	-	-	-	140
6.3.6 Binding to RC-2A cells treated with inducers of differentiation	-	-	-	141
6.3.7 Functional effects on RC-2A cells and myeloid progenitor cells	-	-	-	141
6.3.8 Binding of antibodies to myeloid leukaemic cells	-	-	-	142
6.4 Discussion	-	-	-	
<hr/>				
CHAPTER 7: General discussion.	-	-	-	147
BIBLIOGRAPHY	-	-	-	154

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CHAPTER 1

Introduction.



1.1.1 Differentiation

Cellular differentiation is the process by which cells become increasingly more specialised to fulfil their role as functional, mature elements in a tissue or organ. Metazoans, such as mammals, are derived from an omnipotent, single celled embryo, which has the capacity to give rise to all the diverse cell types in the organism. How this process is orchestrated is still largely mysterious, but rapid advances in cell biological techniques and monoclonal antibody technology are providing much information enabling the clarification of some fundamental steps in the process of differentiation. It is apparent that the central process in cell differentiation involves the sequential activation and suppression of genes in an orderly manner. It is also clear that for such genetic programs to manifest themselves, the cellular environment must be permissive for their expression.

One of the key steps in differentiation seems to be the concomitant loss of proliferative or self-renewal capacity as specialized morphological and functional traits are acquired. In this way differentiation and proliferation are thought to be coupled but independent events (e.g. Sachs, 1980; Greaves, 1982). This may of course be a little simplistic, as there is conflicting evidence in at least one system, namely the proliferation of mature lymphocytes.

1.1.2 Haemopoietic Differentiation

Because of the accessibility of cells at different levels of differentiation, the haemopoietic system has been widely studied as a model of differentiation. The process of haemopoietic differentiation gives rise to all the mature formed elements of the blood and lymphoid system, including the tissue macrophages and mast cells. These cells are

ultimately derived from pluripotential stem cells, common to the myeloid and lymphoid series [Wu *et al*, 1968; Abramson *et al*, 1977; Keller *et al*, 1985]. The term stem cell in this context refers to cells which are able to undergo extensive self renewal and also able to give rise to cells that are committed to differentiation.

The haemopoietic system is accessible to investigation by several methods, both *in vivo* and *in vitro*. Avenues of investigation of haemopoietic differentiation include *in vivo* experiments in which animals, usually rats or mice, have been treated with radiation or cytotoxic drugs to ablate bone marrow and thus cease all haemopoietic activity, followed by infusion with autologous or syngeneic bone marrow cells to reconstitute haemopoiesis [Till *et al*, 1961]. After haemopoietic 'rescue', colonies comprising of mixtures of haemopoietic cell types in various combinations can be seen in the spleen. The cells giving rise to these colonies were operationally designated 'colony forming unit - spleen' (CFU-S). Infusion of cells from primary spleen colonies, resulting in secondary colonies, demonstrates the ability of CFU-S to self renew [Till and McCulloch, 1961]. The number of CFU-S assayable within individual primary spleen colonies varies considerably, indicating that the CFU-S comprise a heterogeneous population with respect to self renewal ability [Siminovitch *et al*, 1963].

The *in vitro* systems employed include bulk liquid culture [Dexter *et al*, 1977] and culture in semi-solid media, originally described by Bradley and Metcalf, 1966 and Pluznik and Sachs, 1965. Both experimental systems have limitations on the type of information that can be obtained, and require care in the interpretation of results. Nevertheless, much valuable information on the progeny of individual cells and their response to regulatory growth factors has been obtained.

1.1.3 Models of Commitment to Differentiation

A number of models have been proposed which attempt to explain the mechanism by which stem cells 'choose' between division resulting in daughter cells identical to themselves, or progeny which are committed to differentiation. As most of the information gained on this subject is based on the study and enumeration of cells which are several cell divisions 'downstream' of this event, interpretation of such data needs to be carried out with caution. Environmental effects on cell maturation may alter the frequencies of the various haemopoietic cell types without affecting commitment of stem cells.

The models of differentiation fall into two main groups, the deterministic and stochastic models [Trentin, 1970; Till *et al*, 1964]. Deterministic models suggest that commitment of stem cells to differentiation is influenced by the environment within which the stem cells reside. One such proposal, called the haemopoietic inductive microenvironment (HIM) model, proposes that the stem cell decision to renew or commit is instructed by the immediate environment of the stem cell [Trentin, 1970]. This model was based on a time course study of the spleen colonies of haemopoietically reconstituted mice, in which cytochemical staining of spleen colonies was performed at various times after infusion of bone marrow [Curry and Trentin, 1967]. However, the evidence for this model may require further investigation, as Magli *et al*, 1982 have shown that two distinct types of colonies are identifiable in the spleen colony assay system. Colonies which appeared by day 7 were transient and are thought to be derived from unipotent progenitors, whereas day 14 colonies appear to be derived from more primitive multipotential cells. There did not seem to be any direct connection between the two colony types, i.e. day 7 colonies did not develop into day

14 type colonies, thus sequential study of colonies may give misleading results.

Stochastic models of differentiation differ from deterministic models in that the decision to renew or differentiate is proposed to be inherently determined by events within the stem cell itself. That is, random genetic events determine the probability of self renewal or commitment to differentiation as the outcome of a given division of a stem cell. Till *et al*, 1964 proposed a stochastic model of stem cell differentiation based on the statistical analysis of CFU-S distribution in spleen colonies. They found that primary spleen colonies of haemopoietically reconstituted mice varied in their content of cells able to give rise to secondary colonies when infused into irradiated mice. The frequency distribution of the number of spleen colonies derived from individual primary spleen colonies approximated a gamma distribution, which suggested the decision of a stem cell to self-renew was governed by a stochastic rule. The value of p , the distributional parameter, signifying the probability of self renewal, was found to be approximately 0.6, i.e. the probability of self renewal was greater than the probability of commitment to differentiation, thus ensuring the perpetuation of the stem cell clone. Some recent experimental data lend support to a stochastic model of differentiation. Nakahata *et al*, 1982 analysed the frequency of self renewing cells within blast cell-like colonies from murine spleen cells cultured in semi-solid medium. Such blast colonies are thought to arise from cells analogous to CFU-S. The frequency of self renewing cells in these colonies also fitted a gamma distribution, with a p value of 0.589, very close to the value obtained by Till *et al*, 1964, and within the range proposed by Schofield *et al*, 1980, which would enable maintenance of the stem cell pool. This model has been elaborated by Korn *et al*, 1973, who proposed that progressive and stochastic restriction of the range of

end cell types a progenitor cell could give rise to occurred as stem cells divided to give increasingly specialised progeny. Support for such a model is provided in the identification of progenitors that are able to give rise to two or three end cell types in various combinations when cultured in semi-solid agar under the same conditions [Suda *et al*, 1983; Suda *et al*, 1984]. It has been demonstrated that the injection of bacterial endotoxin into mice, known to increase serum G-CSF, increased numbers of stem cells and progenitor cells by up to 50-100 fold, but did not change the proportions of progenitor types [Apte *et al*, 1976, Staber and Metcalf, 1980]. In this way it can be seen that haemopoietic growth factors may regulate the composition and quantity of end cells without affecting the commitment of stem cells.

Even though evidence suggests that the commitment step which determines that the progeny of a stem cell will give rise to more mature cells is a stochastic process, it is also apparent that local microenvironmental conditions govern the type of mature end cells that will appear [Trentin, 1971; Lord *et al*, 1975; Frassoni *et al*, 1982]. That is, the local microenvironment of stromal and other supportive cells has a permissive role in the haemopoietic process. For example, it has been shown using the murine spleen colony assay of Till *et al*, 1961 that reconstitution of irradiated mice using cells from primary single lineage colonies can result in the appearance of multi-lineage colonies in the spleens of recipient mice, suggesting unexpressed differentiation potentials of stem or progenitor cells in the colonies due to environmental 'incompatibility' of the primary colony site [Trentin, 1971]. It has been noted in both murine marrow and spleen colonies that stromal cells with recognisable characteristics seem to be associated with a given lineage [Western & Bainton, 1979, LaPushin & Trentin, 1977], and putative

haemopoietic stem cells appear to associate closely with bone marrow macrophages [Lambertson, 1984].

There exists a strain of mice, $S1/S1^d$, that has a genetic abnormality resulting in a form of macrocytic anaemia which can be demonstrated to be due to an inability of the bone marrow stroma to support haemopoiesis, suggesting a microenvironmental defect. Bone marrow from these mice are able to reconstitute irradiated histocompatible mice [McCulloch *et al*, 1964], thus the genetic abnormality does not impair the ability of the stem cells to give rise to progeny. Adherent cell layers of stromal cells from $S1/S1^d$ mice in Dexter type cultures cannot support long term proliferation of CFU-S, providing additional evidence for a microenvironmental defect at the level of the stroma [Dexter and Moore, 1977]. A similar situation exists in humans. Juneja and Gardner [1985] have shown that some human patients with aplastic anaemia also show functionally abnormal haemopoietic stromal cells, which are less able to support *in vitro* CFU-C growth.

The murine strain W/W^v is also characterised by anaemia, but in this case the abnormality resides in the stem cell compartment, as infusion of compatible normal bone marrow is able to cure the anaemia [McCulloch *et al*, 1964]. The degree of cytopenia differs between lineages in this mutant, with the most affected cell types being macrophages and mast cells, and the neutrophils and megakaryocytes being affected to a lesser extent. These two murine mutants serve as models of the dual importance of stroma and stem cells in haemopoiesis, and are essentially complementary in their haemopoietic defects. Transplantation of bone marrow from $S1/S1^d$ to W/W^v strain mice can cure the W/W^v anaemia [McCulloch *et al*, 1965, Tavassoli *et al*, 1973], and the $S1/S1^d$ stromal defect can be cured using transplanted normal spleen fragments [Russel and Bernstein, 1968] or W/W^v bone marrow stromal cells. These experiments have been repeated *in vitro* and show the

same ability to cross correct [Dexter & Moore, 1977]. Recently Suda *et al* [1985] reported that W/W^v mice, which show a 100 fold decrease in dermal mast cells, are able to give normal numbers of mast cell colonies in semi-solid culture. This suggests that the abnormality may be due to the inability of the mast cell progenitors to migrate to the skin, or the failure of progenitors in the skin to give rise to mature mast cells. This difference between *in vivo* and *in vitro* behavior could mean that for these defects to manifest themselves, interaction with the tissue environment is required. Thus the W/W^v defect appears to be due to the inability of the progenitor cells to respond to the environment necessary for mast cell growth, whereas the Sl/Sl^d defect is presumably due to the inability of the stromal cells to provide a permissive environment. This type of experimental system must be interpreted with caution, as these mutant strains have other abnormalities, such as pigmentation defects. These defects in pigmentation also seem to involve the dermal environment of Sl/Sl^d mice [Mayer and Green, 1968], suggesting a close relationship between stem cell systems.

1.1.4 Clonal culture of haemopoietic progenitor cells.

The development of clonal assay methods for haemopoietic progenitors [Bradley and Metcalf 1966; Pluznik and Sachs, 1965] has paved the way for the dissection of the process of myeloid differentiation. Committed progenitors of all myeloid lineages can now be cultured in semi-solid media [Table 1.1]. Suspensions of bone marrow or other haemopoietic progenitor-containing cell sources are immobilized in cell culture medium using a gelling agent such as agar or methyl cellulose. The cultures are supplemented with a source of stimulatory molecules which are specific for distinct lineages. The progenitor cells are able to form colonies of progeny cells, the composition of which depends on the progenitor as well

Table 1.1

Haemopoietic Progenitors Culturable in Semi-solid Medium

CFU-GM	Neutrophil/Macrophage)	Murine - Bradley and Metcalf, 1966
)	Pluznik and Sachs, 1965
CFU-G	Neutrophil)	
)	Human - Pike and Robinson, 1970
CFU-M	Macrophage)	
BFU-e	Erythroid- 'Burst'		Murine - Axelrad <i>et al</i> , 1974
CFU-e	Erythroid		Murine - Stephenson <i>et al</i> , 1971
			Human - Tepperman <i>et al</i> , 1974
CFU-Meg	Megakaryocyte		Murine - Metcalf <i>et al</i> , 1975
			Human - Vainchenker <i>et al</i> , 1978
CFU-Eo	Eosinophil		Murine - Johnson and Metcalf, 1980
			Human - Chervenick and Boggs, 1971
CFU-Baso	Basophil		Murine - Leary and Ogawa, 1984
CFU-Mast	Mast Cells		Murine - Schrader, 1981
			Human - McCarthy <i>et al</i> , 1980
CFU-Mix (CFU-GEMM)	Mixed Erythroid		Murine - Johnson and Metcalf, 1977
	Erythroid + Neutrophil)	
	Macrophage)	(in multiple combinations)
	Eosinophil)	Suda <i>et al</i> , 1983
	Megakaryocyte)	
			Human - Fauser and Messner, 1978

as the type of stimulus supplied. The range of differentiation potentials a given progenitor is able to express is inherent, but will only be expressed if the relevant stimuli are supplied [Reviewed in Metcalf, 1984]. The progenitor cell which gives rise to progeny of multiple lineages (CFU-GEMM or CFU-mix) and CFU-S are thought to be overlapping populations, as individual spleen colonies assayed for both CFU-mix and CFU-S demonstrate significant correlation between the numbers of these progenitors obtained.

At present there is controversy over evidence for the successful culture of a common lymphoid-myeloid progenitor [Messner *et al*, 1981], as it is difficult to demonstrate that lymphoid cells in culture have arisen from primitive progenitors and not from activation of resting T lymphocytes. It has been demonstrated using time lapse cinematography that T lymphocytes in semi-solid agar are very migratory and are commonly seen to 'home in' to other colonies [Donnenberg and Cameron, 1985]. Until experiments involving transfer of single cells, such as by Metcalf, 1980b are performed, this area will remain unclear. Given the expected rarity of such progenitor cell types, this will constitute a daunting task.

The growth of myeloid progenitor cells in culture is absolutely dependant on the addition of specific growth factors, operationally defined as colony stimulating factors (CSF's). They are required *in vitro* for survival, stimulation of division and differentiation of myeloid progenitor cells [Metcalf, 1970; Metcalf, 1977]. CSF's have been demonstrated to be glycoproteins which range in molecular weight from approximately 20 to 70 kd and, in contrast to many regulatory molecules and hormones, are produced by a variety of tissue types [Metcalf, 1984; Metcalf, 1986]. The *in vitro* activities of the CSF's demonstrate that extremely small amounts of these growth factors are required to exert stimulatory effects on progenitor cells, with concentrations in the vicinity of 10^{-12} able to stimulate at half maximal activity [Reviewed in

Metcalf, 1986]. In some cases CSF's are able to exert functional effects on mature end cells produced as a result of stimulation of progenitor cells by the same CSF's [Reviewed in Metcalf, 1987]. Many of these CSF's have been purified and genes for a number of them, of both human and murine origin, have been cloned. Recombinant CSF's expressed in bacterial systems have a similar activity to native factors, indicating that carbohydrate moieties are not required for activity, but internal disulphide bridges have been shown to be essential for activity [Burgess *et al*, 1987; Reviewed in Metcalf, 1986; Metcalf, 1987]. Table 1.2 gives a brief description of the known CSF's.

1.1.5 Properties of the Colony Stimulating Factors.

1.1.5.1 Murine CSF's.

Four murine colony stimulating factors have been well characterised in terms of biological activity and have been purified to homogeneity.

Multi-CSF: This CSF, also commonly known as IL-3, as well as by other operationally defined terms [Table 1.2], is a glycoprotein of approximately 23-28 kd which is able to stimulate colony formation by granulocytic, monocytic, eosinophilic, erythroid, megakaryocytic and multipotential progenitor cell types [Burgess *et al*, 1980; Iscove *et al*, 1982; Bazill *et al*, 1983; Williams *et al*, 1985]. Other factors defined by their activities on a variety of cell types are thought to be the same molecule, for example, BPA (Burst promoting activity) [Iscove *et al*, 1982] and PSF (Persisting cell stimulating factor) [Clark-Lewis and Schrader, 1981]. Thus far, T lymphocytes stimulated with mitogen or antigen are the only normal cell type demonstrated to produce IL-3 [Metcalf, 1984], however the WEHI-3B murine myelomonocytic cell line produces IL-3 constitutively [Ihle *et*

Table 1.2

The Colony Stimulating Factors.

	Synonyms	Mr(kd)	Cloning	Purification
<u>MURINE</u>				
M-CSF	CSF-1	70 (dimer)	-	Stanley and Heard, 1977
G-CSF	MGI-2	25	-	Nicola <i>et al</i> , 1982
GM-CSF	CSF-2	23	Gough <i>et al</i> , 1984	Burgess <i>et al</i> , 1977
IL-3	Multi-CSF BPA PSF	23-28	Fung <i>et al</i> , 1984	Ihle <i>et al</i> , 1982
<u>HUMAN</u>				
huM-CSF	Urinary CSF	45 (dimer)	Kawasaki <i>et al</i> , 1985	Das <i>et al</i> , 1981
huG-CSF	CSF- β	30	Nagata <i>et al</i> , 1985	Nicola <i>et al</i> , 1979
huGM-CSF	CSF- α	22	Wong <i>et al</i> , 1985	Lusis <i>et al</i> , 1981
huIL-3	Multi-CSF	16 f	Yang <i>et al</i> , 1986	-

al, 1982], due to a retroviral insertion [Ymer *et al*, 1985]. The gene for IL-3 has been cloned, and comparisons between different clones suggests that an extra exon is coded for which may possibly allow the production of a membrane bound form of the CSF [Fung *et al*, 1984; Yokota *et al*, 1984; Metcalf, 1986]. The activity of IL-3 is half maximal between 10^{-12} and 10^{-13} molar, depending on the target cell type, and this molecule is able to stimulate CFU-s generation, allow proliferation of IL-3 dependant cell lines, but does not induce differentiation of murine myeloid cell lines [reviewed in Metcalf, 1986]

GM-CSF: This molecule is a glycoprotein of about 23 kd [Burgess *et al*, 1977] which is able to stimulate colony formation by granulocytic, monocytic and bipotential granulocytic/monocytic progenitor cells [Burgess and Metcalf, 1977]. The concentration of this CSF effects the relative frequency of granulocyte and monocyte colonies obtained in culture. Low concentrations of GM-CSF favours monocytic colony formation and at high concentrations mainly granulocytic colony formation occurs [Burgess and Metcalf, 1977b]. In addition, it has been shown that the concentration of GM-CSF is able to influence the type of colonies that paired daughter cells from a single progenitor are able to form. Using a micro-manipulator, Metcalf, 1980 showed that daughter cells replated in low GM-CSF concentrations preferentially gave rise to colonies consisting mainly of monocytes, whereas 'sister' cells replated into high concentrations of GM-CSF gave rise to colonies of predominantly granulocytic type. The gene encoding GM-CSF has been cloned, and like the IL-3 gene, it appears to be able to give rise to an extra exon [Stanley *et al*, 1985, Gough *et al*, 1984]. Such a mechanism would allow the membrane bound form of the growth factor to exert its effects in direct cell-cell interactions. GM-CSF requires a concentration of about 10^{-12} for half maximal activity, and has a minor

differentiation inducing effect on myeloid cell lines [Reviewed in Burgess and Nicola, 1983].

G-CSF: This CSF is a glycoprotein of approximately 25 kd which, at low concentrations, stimulates the formation of granulocytic colonies consisting of small numbers of mature neutrophils, but at higher concentrations also stimulates monocytic colony formation [Nicola *et al*, 1983; Metcalf *et al*, 1983]. The serum levels of CSF are dramatically increased by injection with bacterial endotoxin, and subsequently *G-CSF* was shown to be a major component of this colony stimulating activity [Metcalf, 1971; Nicola *et al*, 1983]. *G-CSF* has half maximal activity of about 5×10^{-12} molar and is a potent inducer of differentiation of myeloid cell lines [Nicola *et al*, 1983; Metcalf and Nicola, 1982].

M-CSF: The activity of this 70 kd dimeric glycoprotein is predominantly for the monocytic lineage, but it is able to stimulate a limited number of granulocyte-containing colonies in cultures with serum-containing medium [Stanley and Guilbert, 1980; Metcalf, 1984]. It has a half maximal activity between 5×10^{-13} - 10^{-12} molar and requires intact subunit structure for its function [reviewed in Metcalf, 1986].

1.1.5.2 Human CSF's

The human colony stimulating factors are not quite as well characterized as those of the murine system, but four CSF's which appear to be analogous have been described.

huIL-3: Recently Yang *et al*, 1986 cloned the gene for a human IL-3-like growth factor. Initially, the gene for a gibbon IL-3 was cloned, and using

this the human gene was isolated from a library of human DNA by hybridization. The human gene showed a high degree of homology with the gibbon gene, with only 11 different amino acids from an estimated total of 152. The predicted molecular weight of the non-glycosylated polypeptide was approximately 16 kd. *In vitro* activity of the gibbon activity included the ability to stimulate mixed colony formation, and by inference the human gene would therefore code for a true multilineage IL-3-like growth factor. The human IL-3 gene shares 45% homology at the nucleotide level, and a 29% homology at the amino acid level with the murine IL-3 gene.

huGM-CSF: This 22 kd glycoprotein has activity for granulocyte and/or monocyte progenitors, as well as eosinophil precursors. It stimulates highest numbers of colonies when scoring is carried out at day 14. These colonies consist mainly of monocyte or eosinophil type [Metcalf *et al*, 1986]. The gene for *huGM-CSF* has been cloned, and it has been demonstrated that this growth factor is the same as the previously described *CSF α* [Wong *et al*, 1985; Lee *et al*, 1985; Metcalf *et al*, 1986; Tomonaga *et al*, 1986]. The sequence data of the cloned growth factor shows a 70% homology with the murine *GM-CSF* gene in the protein coding region, but these factors are not cross reactive [Metcalf, 1986].

huG-CSF: This glycoprotein of approximately 18 kd has preferential stimulatory activity for relatively mature granulocytic progenitors [Begley *et al*, 1985], and shares complete functional homology with murine *G-CSF*, being able to induce differentiation of myeloid cell lines and to stimulate formation of colonies by murine granulocytic precursors [Nicola *et al*, 1985]. The gene coding for *huG-CSF* has been cloned [Nagata *et al*, 1986].

huM-CSF: This dimeric 45 kd glycoprotein shares N-terminal homology with its murine counterpart, and has been cloned [Kawasaki *et al*, 1985]. Unlike murine M-CSF, it does not act directly on monocytic progenitors, rather it acts indirectly, probably on adherent cells, which then produce a colony stimulating activity. However, it is able to stimulate monocytic colony formation by murine progenitors [Motoyoshi *et al*, 1978; Motoyoshi *et al*, 1982].

The granulocyte/monocyte colony stimulating factors from a single species demonstrate quite a large amount of functional overlap, in that the same progenitor populations are able to respond to stimulation by a number of growth factors. Within a species, structural homology between the CSF's is not demonstrable, but considerable homology exists between analogous human and murine CSF's [Reviewed in Metcalf, 1986; Metcalf, 1987]. Even though such functional redundancy exists, the murine CSF's are able to down regulate the expression of receptors for CSF's in a hierarchical fashion, such that IL-3 down-regulates the other three CSF receptors, GM-CSF the receptors for G- and M-CSF, and G-CSF the receptor for M-CSF [Walker *et al*, 1985]. This may indicate that a complex 'fine tuning' mechanism exists for regulation of growth factor effects. In addition, it has been suggested that co-ordinate production of haemopoietic growth factors by T lymphocytes may occur. A common decanucleotide sequence is found in the 5' flanking regions of a number of genes encoding growth factors and may function as a regulatory element. [Kelso and Metcalf, 1985; Metcalf, 1986].

1.1.5.3 Other haemopoietic growth factors.

Several recent reports in the literature suggest that other haemopoietic growth factors distinct from the ones mentioned above may exist.

Stanley *et al*, 1986 reported that a factor they previously described (Hemopoietin 1) was able to stimulate the growth and differentiation of more primitive cells than those stimulated by IL-3.

Enokihara *et al*, 1985 reported that the T cells of a patient with allergic eosinophilia, when challenged with the allergen, were able to produce a factor which stimulated eosinophil colony formation but had no effect on neutrophil colonies.

Warren and Sanderson, 1985 reported that a murine T cell hybrid (NIMP-TH1) produced a factor they named Eosinophil Differentiating Factor (EDF) which was able to induce eosinophil differentiation, and this was distinct from IL-3 and GM-CSF. Lopez *et al*, 1986 reported that purified EDF selectively supported the proliferation and differentiation of murine eosinophil progenitors, and was active on human eosinophil progenitors.

Tanno *et al*, 1987 reported that liquid cultures of human cord blood mononuclear cells stimulated with conditioned medium from the human Mo T cell leukaemic cell line showed a population of basophil cells arising by week 2, followed by a peak of eosinophils at week 4. A proportion of cells from these cultures were found to have mixed eosinophilic/basophilic granulation, like which the authors previously reported to be found within human eosinophil-like colonies [Denburg *et al*, 1985]. Growth stimulating activities for the two cell types were partially purified from Mo conditioned medium. The two fractions were able to selectively stimulate more colonies of apparently pure basophils or eosinophils respectively, when compared with unfractionated Mo conditioned medium. The numbers of monocytic/neutrophilic colonies was unaffected. The authors suggest a

common progenitor for eosinophils and basophils, and the existence of specific factors for the two lineages. The relationships between these reported eosinophil growth factors are not as yet known.

1.1.5.4 Functional effects of CSF's on mature cells.

Apart from the ability of the CSF's to stimulate the production of mature myeloid cells, the functional activities of the mature end cells of the macrophage, neutrophil and eosinophil lineages can be substantially modified by the exposure to CSF's in a dose dependant and reversible manner [Reviewed in Metcalf, 1987]. These functional activities include survival, chemotactic stimulation/inhibition, phagocytic ability, antibody dependant cell cytotoxicity, parasite killing and elaboration of biologically active molecules involved in inflammation [Begley *et al*, 1986; Gasson *et al*, 1984; Lopez *et al*, 1983; Vadas *et al*, 1983; Handman and Burgess, 1979; Ziboh *et al*, 1982; Weisbart *et al*, 1985]. The production of high concentrations of colony stimulating factors after injection of mice with endotoxin suggests a dual function of this response to bacterial products in combatting infection. The high levels of CSF stimulate production of new cells and also augment their functional capacity.

1.1.5.5 *In Vivo* activity of the CSF's.

Until recently, the evidence for an *in vivo* role for CSF's was circumstantial. Transplantation of tumours which constitutively produce colony stimulating factors into mice were able to increase concentrations of serum CSF, induce granulocytosis and expand pools of bone marrow granulocytic cells in a way which correlated with tumour size [e.g. Yunis *et al*, 1984]. Injection of partially purified huM-CSF into mice was able to give a modest increase in monocyte/granulocyte formation [Metcalf and Stanley, 1971]. Recently, the availability of recombinant growth factors

has enabled the demonstration of *in vivo* activities of GM-CSF and IL-3 which correlate well with their previously demonstrated *in vitro* activities. Injection of GM-CSF resulted in substantial increase in spleen cellularity and circulating neutrophils, and in monocyte and neutrophil infiltration of the lungs and the peritoneal cavity. A decrease in the cellularity of the bone marrow was noted [Metcalf *et al*, 1987]. Similar effects were found for IL-3 injected into mice, except that increases in splenic mast cells were demonstrated, and the numbers of non-erythroid progenitor cells in the spleen increased more dramatically than with GM-CSF treatment, whereas the numbers of bone marrow progenitors remained constant [Metcalf *et al*, 1986].

1.2.1 Myeloid cell lines: Suitability and usefulness as models of haemopoietic differentiation.

A number of human myeloid cell lines have been derived from leukaemic cell specimens from peripheral blood, marrow or other tissues of patients with Acute non-lymphocytic or Chronic myelogenous leukaemias [Table 1.3, Figure 1.1]. Cell lines differ from fresh leukaemic cells in some fundamental ways. They tend to be less heterogenous in phenotype than fresh leukaemic cells [Rovera, 1985], and exhibit a much larger proportion of cells able to undergo cell division. Generally, less than one percent of leukaemic blasts from acute myelogenous leukaemias are able to form colonies *in vitro* [Reviewed in Sabbath and Griffin, 1985], whereas myeloid cell lines can range from 10 up to greater than 80% of cells able to form colonies in semi-solid medium [eg. Brennan *et al*, 1981; Ruscetti *et al*, 1981; Bradley *et al*, 1982; Fibach *et al*, 1982; Alder *et al*, 1984].

Freshly isolated leukemic cells, like haemopoietic progenitor cells, are in most cases absolutely dependent on added growth factors for

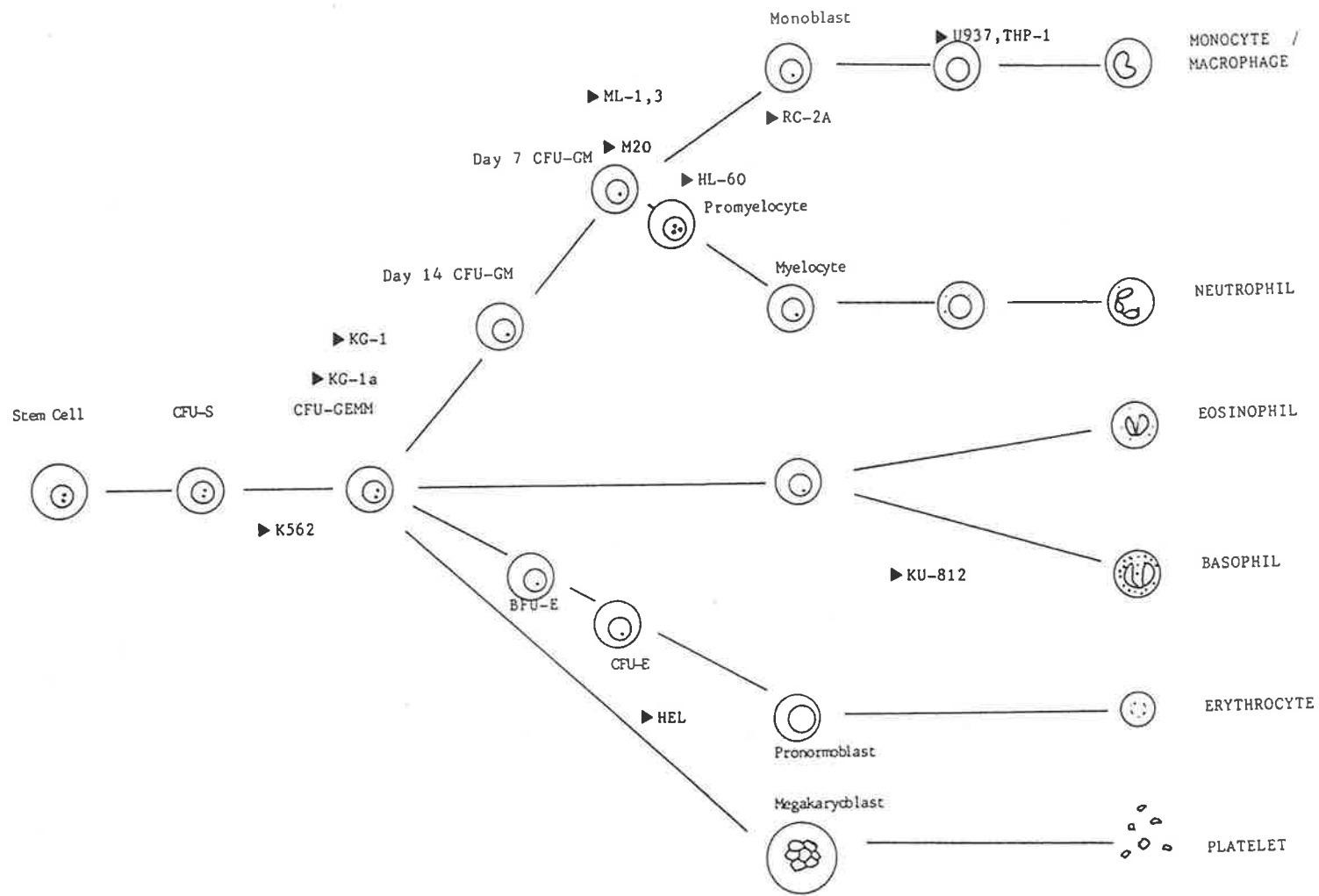
Table 1.3

Human Myeloid Cell Lines

Name	Predominant Cell Type	Reference
K562	Early myeloid blast/ erythroblast	Lozzio and Lozzio, 1975
HEL	Erythroblast	Martin <i>et al</i> , 1982
KG-1a	Early myeloblast	Koeffler <i>et al</i> , 1980
KG-1	Myeloblast	Koeffler and Golde, 1978
HL-60	Promyelocyte	Collins <i>et al</i> , 1977
M-20	Myelomonoblast	Treves <i>et al</i> , 1985
ML-1, ML-3	Myelomonoblast	Takeda <i>et al</i> , 1981
RC-2A	Myelomonocyte	Bradley <i>et al</i> , 1982
THP-1	Monocytoid	Tsuchiya <i>et al</i> , 1980
U937	Monocytoid	Sundstrom and Nilsson, 1976
KU812	Basophil	Kishi, 1985

Figure 1.1

Human myeloid cell lines. Likely relationship between cellular composition of the lines and a schematic representation of normal haemopoietic differentiation.



survival and division in culture [reviewed in Metcalf, 1984], whereas in the process of adaptation to tissue culture conditions, cell lines become autonomous with respect to growth factor requirements [reviewed in Markham *et al*, 1984; Schrader *et al*, 1985], however exceptions do exist [Oliff *et al*, 1984; Ford *et al*, 1985; Dexter *et al*, 1980]. One possibility is that in some cases cell lines constitutively produce autostimulatory growth factors [Brennan *et al*, 1981; reviewed Schrader, 1986; Metcalf, 1984].

Schrader *et al*, 1983 reported that transformation of the IL-3 dependant but non-leukaemic murine cell line R6-X to produce tumourigenic IL-3 independant variants was due to autocrine production of IL-3. The murine myelomonocytic cell line WEHI-3B constitutively produces IL-3 due to a retroviral insert near the gene coding for IL-3 [Ymer *et al*, 1985], indicating that mechanisms for the autocrine production of growth factors do exist.

Conversely, the murine haemopoietic cell line FDC-P1 [Dexter *et al*, 1980], like RX-6, is dependant on exogenous sources of IL-3 or GM-CSF for proliferation and survival *in vitro* [Hapel *et al*, 1984; Metcalf, 1985], but recently it has been shown that Abelson virus infection of a variant of the FDC-P1 cell line can result in growth factor autonomy and acquisition of tumourigenicity, with no evidence for the autocrine production of IL-3 or GM-CSF by the transformed cells [Cook *et al*, 1985].

The role for autocrine production or growth factor autonomy as causative agents in leukaemogenesis in humans has not been convincingly demonstrated [reviewed in Metcalf *et al*, 1985; Metcalf, 1986]. Freshly isolated myeloid leukaemic cells, or monocytes of leukaemic origin, do not produce growth factors at levels higher than their normal counterparts [Griffin *et al*, 1982; Metcalf, 1984]. Given the clonal nature of leukaemias, it has been suggested that the amount of growth factor produced by a

newly emerging leukaemic clone would be inconsequential in an environment already containing physiologically active concentrations of such growth factors [Metcalf, 1986]. As previously stated, *in vitro* proliferation of leukaemic blast and colony forming cells requires exogenous growth factors, indicating that growth factor autonomy is unlikely to be responsible for *in vivo* growth advantage of the leukaemic clone. The experimental manipulation of cell lines demonstrates that growth factor autonomy or autocrine production may confer tumourigenicity on previously non-tumourigenic cell lines, indicating that unregulated growth factor production may potentially be involved in leukaemia, however it seems that such mechanisms are unlikely to be involved in the early pathogenesis of the disease.

Even though human haemopoietic cell lines may in time drift from their original characteristics, most seem to represent to some extent the leukaemic populations from which they were derived, which in turn appear to represent their normal progenitor cell counterparts [reviewed in Greaves, 1982; Greaves,^{etal} 1986]. Thus in most cases myeloid cell lines represent, albeit in an abnormal fashion, a segment of the continuum of normal myeloid haemopoietic differentiation. Cell lines have been isolated which appear to be derived from pluripotent myeloid progenitors (K562) [Lozzio and Lozzio, 1975] through to quite differentiated monocytoid cells (U937) [Sundstrom and Nilsson, 1976], with others representing intermediate phenotypes [Table 1.3, Figure 1.1]. If leukaemia is thought of as an arrest in normal differentiation, then these cell lines may reflect the 'block' in differentiation exhibited by the leukaemia from which they were derived [Sachs, 1982; Greaves, 1982].

Using due care in the interpretation of experimental data, haemopoietic cell lines are a useful adjunct to *in vivo* and other *in vitro* studies of

normal and leukaemic haemopoiesis. Such investigations fall into several categories;

1. Studies of *in vitro* induction of differentiation.
2. Studies of genetic abnormalities involved with leukaemogenesis.
3. Preparation of haemopoietic growth factors for purification/cloning.
4. Provision of renewable sources of haemopoietic differentiation antigens defined by monoclonal antibodies, for structure/function analysis and cloning.

This section will concentrate on the first category.

1.2.2 Induction of differentiation of myeloid cell lines.

Many myeloid cell lines retain some ability to undergo differentiation to more mature phenotypes in response to wide range of substances of a chemical nature or of biological derivation [reviewed in Abrahm and Rovera, 1981; Koeffler, 1983; Harris and Ralph 1985], as do freshly isolated leukaemic cells [eg. Fibach and Rachmilewitz, 1981; Breitman *et al*, 1981; Perussia *et al*, 1983b; Tuow and Löwenberg, 1985]. Such behavior suggests that the putative differentiation block in leukaemia is not necessarily absolute, and that some responsiveness to normal biological and other regulators is retained. It is also clear that *in vivo*, leukaemia is characterized by a limited amount of differentiation, with the renewal of the leukaemic clone being due to a small fraction of more primitive cells comprising the leukaemic 'stem' cell compartment [Minden *et al*, 1978; Wouters and Löwenberg, 1984]. It has been demonstrated, using a murine model, that leukaemic cell lines retain ability to differentiate *in vivo* in response to normal regulators. Cells of the murine M-1 myeloid leukaemia line, when injected into mouse embryos, contribute in part to a normal granulocytic population in adult mice [Gootwine *et al* 1982],

demonstrating responsiveness to normal embryonic controls. Similarly, the leukaemic clones of patients with ANLL in remission also demonstrate responsiveness to haemopoietic controls (eg. Gunz and Henderson, 1983).

1.2.3 Induction of differentiation of HL-60

The myeloid cell line studied in the most detail for its ability to undergo differentiation *in vitro* is HL-60, a cell line derived from the peripheral blood of a patient with promyelocytic leukaemia [Collins *et al*, 1977]. This cell line consists predominantly of promyelocytes, but has a small number of more mature cell types in the culture population, including some bands and granulocytes. When treated with certain substances, this cell line can differentiate towards granulocytes (DMSO, Butyric acid [Collins *et al*, 1978], Retinoic acid [Breitman *et al*, 1980] or toward macrophages (TPA [Rovera *et al*, 1979], Lymphokines [Dayton *et al*, 1980], Vitamin D metabolites [McCarthy *et al*, 1983]), indicating that it retains at least a bi-lineage potential. Fibach *et al*, 1985 demonstrated that exogenous serine proteases were able to induce the differentiation of HL-60 cells towards neutrophils after about 3-6 days, and upon further incubation a population of monocytic cells appeared and the neutrophilic cells declined, resulting in a predominantly macrophage cell-like population remaining by day 12. Thus, unlike other inducers, serine proteases brought about differentiation along two pathways, but with dissimilar kinetics of appearance of the two types of cells.

Culture of HL-60 cells in alkaline conditions has been reported to give rise to cells with atypical eosinophilic granules [Metcalf, 1983; Fischkoff *et al*, 1984], suggesting that they have further capacity to give rise to a third lineage of differentiation. However, cells treated in this way show a very abnormal phenotype. Acute leukaemias with eosinophilic

differentiation also show similar atypical granules and, like HL-60, usually have a genetic abnormality which involves chromosome 16 [Berger *et al*, 1985].

1.2.3.1 Granulocytic differentiation of HL-60 cells.

A number of compounds have been shown to induce HL-60 cells to differentiate towards neutrophils, including the physiologic compound retinoic acid, the polar compound DMSO and butyric acid [reviewed in Abraham and Rovera, 1981].

Morphological changes to HL-60 cells treated with inducers of granulocytic differentiation are very characteristic. Nuclear morphology became distinctively neutrophil-like in appearance, with the majority of cells exhibiting band forms or segmented nuclei [Collins *et al*, 1980; Breitman *et al*, 1980]. However, these cells did not develop secondary granules typical of normal mature neutrophils [Newberger *et al*, 1980], which may be attributable to incomplete rather than aberrant maturation.

HL-60 cells showed a decrease in proliferative ability when induced with DMSO, butyrate or retinoic acid [Collins *et al*, 1978; Breitman *et al*, 1980; Todd and Malech, 1986], but due to the nature of these compounds, it is difficult to assess whether this reflects a true differentiation related decrease in self renewal or cytotoxicity of the inducing agents.

Histochemically, a number of changes occurred in HL-60 cells treated with inducers of granulocytic differentiation. Increase in expression of the enzyme chloroacetate esterase occurred [Harris and Ralph, 1985], and cells became able to reduce nitro-blue tetrazolium [Newberger *et al*, 1979], due to production of reactive oxygen radicals. Myeloperoxidase, a marker of immature myeloid cells, remained constitutively produced [Koeffler and Golde, 1980] in contrast to monocyte/macrophage differentiation (see below). The granulocytic markers of alkaline phosphatase activity and

lactoferrin synthesis were absent from DMSO induced HL-60 cells [Gallagher *et al*, 1979].

Functionally, HL-60 derived granulocytic cells shared properties with normal granulocytes, such as phagocytosis and chemotaxis [Fontana *et al*, 1980], and DMSO treated cells were able to kill bacteria [Collins *et al*, 1979].

Several groups have reported changes in cell surface antigen expression by HL-60 cells undergoing granulocytic differentiation. The mature granulocyte/monocyte marker detected by the MoAb OKM1 (anti-C3bi receptor) showed a marked increase in expression when cells were treated with retinoic acid or DMSO, but in contrast, the immature myeloid antigen detected by the MoAb S5.7 underwent a decrease in expression [Ferrero *et al*, 1983]. The MoAb MY3, an antibody which binds predominantly to monocytic cells and to a minor population of granulocytes, showed an augmentation of binding after DMSO treatment [Todd and Schlossman, 1984]. The receptor for the chemotactic attractant/activating peptide f-mlp demonstrated increased expression on granulocytic induction [Niedl *et al*, 1980]. Comparisons of two dimensional peptide mapping of DMSO or retinoic acid induced HL-60 surface membrane proteins with those of normal human granulocytes demonstrated a number of peptides whose expression was altered consistent with differentiation towards neutrophils [Felsted *et al*, 1983].

1.2.3.2 Monocyte/Macrophage differentiation of HL-60 cells.

The inducers of monocytic differentiation of HL-60 cells include both chemical compounds and biologically derived proteins.

TPA treated HL-60 cells acquired macrophage morphology, such as cerebriform nuclei lacking nucleoli, pseudopodia and became more adherent

to plastic [Rovera *et al*, 1979] as did cells after incubation with lymphocyte derived factors and Vitamin D3 derivatives [Elias *et al*, 1980; Chiao *et al*, 1981; Camussi *et al*, 1982; Fibach *et al*, 1982; McCarthy *et al*, 1983]. TPA exerted its effects more quickly than did lymphocyte derived factors, producing maximal differentiation within 2-3 days [Fibach *et al*, 1982b], whereas lymphocyte derived factors required about 7-12 days to induce a similar degree of differentiation [Chiao *et al*, 1981].

The proliferation of HL-60 decreased when cells underwent differentiation towards macrophages in response to TPA [Rovera *et al*, 1980], lymphocyte derived factors [eg, Elias *et al*, 1980] and Vitamin D3 derivatives [McCarthy *et al*, 1983]. As TPA is toxic to HL-60 cells [Todd *et al*, 1981], the investigation of differentiation linked clonogenic suppression is not possible, however, Gullberg *et al*, 1986 demonstrated clonogenic suppression of HL-60 cells by lymphocyte derived factors.

Histochemical changes to HL-60 cells treated with inducers of monocytic differentiation have been shown to be consistent with differentiation towards macrophages [reviewed in Harris and Ralph, 1985]. TPA has been shown to induce expression of α -Naphthol Acetate Esterase [Rovera *et al*, 1979], Acid Phosphatase and increased lysozyme [Rovera *et al*, 1977], weak NBT reducing activity [Newberger *et al*, 1981], β -Glucuronidase [Koeffler *et al*, 1981], and to decrease myeloperoxidase activity [Rovera *et al*, 1979]. Lymphocyte derived factors have also been shown to induce α -Naphthol Acetate Esterase expression [Olsson *et al*, 1981], NBT reduction [Olsson *et al*, 1982] and to decrease myeloperoxidase activity [Chiao *et al*, 1981]. Vitamin D3 derivatives induced α -Naphthol Acetate Esterase activity in HL-60 cells [McCarthy *et al*, 1983].

HL-60 cells induced to differentiate along the monocytic pathway by lymphokines were able to phagocytose latex or *Candida* particles [Chiao *et al*, 1981] and to mediate antibody dependant cell cytotoxicity [Dayton *et*

al, 1983], as were TPA treated cells [Rovera *et al*, 1979; Weinberg *et al*, 1981]. TPA treated cells were able to kill bacteria [Koeffler *et al*, 1981].

TPA and lymphocyte derived factors have been shown to induce expression of monocyte/macrophage associated surface antigens. The C3b1 receptor bound by α -Mo1 and OKM1 monoclonal antibodies underwent increased expression in response to TPA and lymphokines [Todd *et al*, 1981; Dayton *et al*, 1983]. The expression of the monocyte antigen Mo2 and HLA-DR antigens were induced by lymphocyte derived factors, but not by TPA [Todd *et al*, 1981; Dayton *et al*, 1983; Perussia *et al*, 1982]. TPA and lymphokine preparations induced expression of Mo3 antigen [Todd and Schlossman, 1982] and lymphokines increased expression of the mature myeloid marker MY3 [Todd and Schlossman, 1984]. Receptors for the Fc portion of human IgG1 were induced by TPA and lymphokines [Koeffler *et al*, 1981; Takeda *et al*, 1983], and the expression of the early myeloid marker detected by the monoclonal antibody S5.7 decreased in response to both agents [Ferrero *et al*, 1983]. In addition, in common with maturing normal monocytes, HL-60 cells treated with TPA elaborated colony stimulating factor into their culture medium [Ascensao and Mickman, 1984].

1.2.4 Induction of differentiation in other human myeloid cell lines.

A number of human cell lines of myeloid origin other than HL-60 have been demonstrated to have the ability to undergo *in vitro* differentiation [reviewed in Koeffler, 1983; Harris and Ralph, 1985]. K562, a cell line derived from a patient with Chronic Myelogenous Leukaemia in blast crisis [Lozzio and Lozzio, 1975] represents early myeloid blast cells, and some sublines also demonstrate erythroid characteristics, such as glycophorin expression [Horton *et al*, 1981]. Such sublines also co-expresses granulocytic markers, which suggests that they display lineage infidelity [Marie *et al*, 1981], or represent a leukaemic counterpart of a normal

erythroid/myeloid progenitor [Rimmer and Horton, 1984]. When treated with haemin or butyrate, these cells differentiate along the erythroid lineage, and exhibit haemoglobin synthesis [Andersson *et al*, 1979; Rutherford *et al*, 1979]. K562 has been shown to differentiate towards more mature phagocytic cells in response to TPA [Huberman and Callahan, 1979], but others reported that TPA has no effect [Koeffler *et al*, 1979]. This may be due to subline variations between laboratories. Megakaryocytic differentiation of K562 cells treated with TPA has recently been reported [Tetteroo *et al*, 1984]. Induction of differentiation was assessed by the loss of glycoprotein A and the acquisition of platelet glycoprotein IIIa.

The HEL cell line was isolated from a patient with Hodgkin's lymphoma who subsequently developed erythroleukaemia. This erythroid line expresses the erythroid proteins spectrin and glycoprotein A, and synthesizes low levels of haemoglobin, which can be greatly enhanced by treatment with haemin [Martin and Papayannopoulou, 1982; Rimmer and Horton, 1984]. It also expresses granulocyte/monocyte markers, and may, like K562, be of erythroid/myeloid progenitor origin [Rimmer and Horton, 1984].

The cell line KG-1 was derived from a patient initially with erythroleukaemia, who subsequently developed undifferentiated AML [Koeffler and Golde, 1978]. This line has cells of myeloblastic phenotype as the main constituents of the culture population, appears to represent a progenitor which is more primitive than HL-60. KG-1 was induced to differentiate towards monocytes by exposure to TPA or lymphokines, but in keeping with HL-60 results, TPA was unable to increase the expression of HLA-DR antigens, whereas lymphocyte derived factors did [Dayton *et al*, 1983]. Even though KG-1 appears to be of earlier origin than HL-60, attempts to induce differentiation along other lineages have been unsuccessful [Koeffler and Golde, 1978; reviewed in Koeffler, 1983]. KG-1a, a subline derived from KG-1, was reported to represent a less

differentiated form of the parent line [Koeffler *et al*, 1980]. It was subsequently shown to be resistant to the differentiating effects of TPA [Koeffler *et al*, 1981]. Recently, it has been reported that KG-1a expresses three T lymphocyte markers (TCR β and T3 α mRNA's, CD3 surface glycoprotein), as well as the myeloid marker detected by MY7 [Furley *et al*, 1987]. This cell line may represent an aberrant phenotype due to dysregulated gene expression, or represent a common myeloid-T lymphocyte progenitor from which KG-1 was originally derived.

Recently a myelomonoblastic cell line, named M-20, was isolated from the peripheral blood of a patient with myeloblastic leukaemia. The cell line consists of a mixed population of myeloblasts and promyelocytes, and could be induced to differentiate towards macrophage-like cells in response to TPA. Even though the cells of this line appear to represent a similar stage of differentiation to HL-60, attempts to induce differentiation towards neutrophils with DMSO or Retinoic acid were unsuccessful [Treves *et al*, 1985].

ML-1 and ML-3 are cell lines of promonocytic phenotype, and are also able to differentiate to a more mature monocyte/macrophage phenotype in response to TPA and lymphokines [Takeda *et al*, 1981; Ferrero *et al*, 1983; Dayton *et al*, 1983]. Expression of antigens associated with mature monocytes was induced or augmented, but again lymphokines, but not TPA, were able to induce expression of HLA-DR antigens [Dayton *et al*, 1983].

The cell line RC-2A was derived from the peripheral blood of a patient with acute myelomonocytic leukaemia, and consists of predominantly myelomonocytic cells. As the line is relatively mature, the cells constitutively express many monocyte markers, such as Mo1, HLA-DR antigens, and weak α -Naphthyl Acetate Esterase activity [Bradley *et al*, 1982; Ralph *et al*, 1982]. This line can be induced to differentiate towards macrophages in response to TPA. Such differentiated cells demonstrated

antibody dependant cell cytotoxicity and increased α -Naphthyl Acetate Esterase activity [Ralph *et al*, 1982].

U937, derived from the pleural effusion cells of a patient with diffuse histiocytic lymphoma [Sundstrom and Nilsson, 1976], consists of fairly mature monocytoïd cells, and seems to be more mature than RC-2A. The cells of this line express many mature markers of macrophage differentiation, such as complement receptor detected by OKM1, Fc receptors, α -Naphthyl Acetate Esterase, peroxidase and lysozyme [Sundstrom and Nilsson, 1976; Ralph *et al*, 1976; Moscicki *et al*, 1983], however do not constitutively express HLA-DR antigens [Koren *et al*, 1979]. Exposure of U937 cells to TPA or lymphokines induced macrophage differentiation [Larrick *et al*, 1980; Ralph *et al*, 1983; Moscicki *et al*, 1983], as did vitamin D derivatives [Olsson *et al*, 1983; Rigby *et al*, 1984]. Such changes were usually of a quantitative rather than qualitative nature, involving increases in expression of constitutively expressed enzymes and receptors [Reviewed in Harris and Ralph, 1985]. TPA again did not induce HLA-DR antigens on U937 cells [Santoli *et al*, 1983], nor did vitamin D3 derivatives [Rigby *et al*, 1984], or a preparation of lymphocyte derived factors which induced them on HL-60 [Elias *et al* 1980]. This may of course relate to differences in sensitivity of the two cell lines to the agent in the preparation causing HL-DR expression, however γ -interferon has been reported to induce HLA-DR antigens on U937 cells by some workers [Vizilier *et al*, 1984; Santoli *et al*, 1983], but to not induce them by others [Kelley *et al*, 1984]. Such disparities may be due to inadequate controls for Fc binding, as most monoclonal antibodies directed against HLA-DR antigens used in these studies were of IgG2a subclass, which bind strongly to Fc receptors of monocytic cells [Gadd and Ashman, 1983]. Alternatively, this may be a result of sub-line variation.

1.2.5 Identity of biologically derived agents which induce monocyte/macrophage differentiation in myeloid cell lines.

The identity of factors derived from biological sources which exert monocytic differentiation inducing effects on human myeloid lines has been investigated by a number of research groups. Olsson *et al*, 1981 described two protein factors which they termed differentiation inducing factors (DIF's) purified from mitogen stimulated human peripheral blood mononuclear cell supernatants. One had a molecular weight of about 40 kd, and had no colony stimulating activity, while the other was about 25 kd and co-purified with colony stimulating activity. Both these DIF's were able to induce differentiation of HL-60 cells toward macrophages. The DIF of \approx 25 kd may be the same as G-CSF, with which it shares similar molecular weight and ability to induce differentiation in cell lines [Burgess and Metcalf, 1980].

Subsequent papers by this group described a DIF purified from the conditioned medium of the T lymphocyte cell line HUT-102. This factor had a molecular weight of between 46 and 55 kd and was able to induce differentiation in HL-60 cells but had no colony stimulating activity, which suggests similarity to the 40K DIF described above. This factor also acted synergistically with retinoic acid to induce monocytic differentiation of U937, a property it lacked on its own [Olsson *et al*, 1984]. Gullberg *et al*, 1986, from the same group, further characterized the DIF from HUT-102 conditioned medium, and demonstrated that it was distinct from γ -interferon by showing that an anti- γ -interferon monoclonal antibody capable of blocking the effects of γ -interferon had no effect on DIF activity. At concentrations about ten-fold lower than that required for differentiation inducing effects, the DIF was able to inhibit clonogenicity of normal myeloid progenitor cells (CFU-GM), freshly isolated AML blasts and sublines of HL-60 and U937. Apart from its effects on myeloid

progenitors, the DIF's relationship to the known colony stimulating factors is not clear.

A molecule, named D factor, derived from a fibroblast cell line, was shown to exert differentiative effects on the murine M1 myeloid cell line [Tomida *et al*, 1984]. This molecule has no colony stimulating capacity for normal myeloid progenitors and is also known as MGI-2 [Lotem *et al*, 1980; Lotem and Sachs, 1984]

Of the four known murine colony factors, two have been shown to exert differentiation inducing effects on myeloid cell lines. G-CSF is a powerful inducer of differentiation of the murine myelomonocytic cell line WEHI-3B [Moore, 1982; Nicola *et al*, 1983] and is able to suppress self renewal in these cells [Metcalf, 1980; Metcalf, 1982]. Murine G-CSF is also able to induce differentiation in the human cell line HL-60 [Metcalf, 1983]. Murine GM-CSF has weak differentiating effects on WEHI-3B cells [Metcalf, 1979] and the two other murine colony stimulating factors M-CSF and IL-3 have not been shown to induce differentiation in myeloid cell lines [Metcalf, 1984].

Human G-CSF, which is analogous to the murine G-CSF, was shown to induce differentiation in murine WEHI-3B cells [Nicola *et al*, 1985], and the human HL-60 cell line was reported to differentiate in response to partially purified human GM-CSF and G-CSF from human placental conditioned medium [Metcalf, 1983]. Recombinant human GM-CSF [Wong *et al*, 1985] has been reported to be active in inducing HL-60 but not KG-1 to differentiate towards monocytes and eosinophils [Tomonaga *et al*, 1986], but not to have an effect on murine WEHI-3B cells [Metcalf *et al*, 1986]. A human pluripoiectin isolated by Welte *et al*, 1985, was reported to be able to induce differentiation in WEHI-3B and HL-60 cells. Subsequently, this factor was reported to probably be identical to G-CSF [Metcalf, 1987].

Takeda *et al*, 1986 reported that a 17 kd DIF isolated from PHA-LCM was able to induce differentiation of the human ML-1, HL-60 and THP-1 myeloid cell lines towards monocytes. They showed that this molecule shared 20 amino acids of N-terminal homology with recombinant human tumour necrosis factor (TNF) [Pennica *et al*, 1984; Wang *et al*, 1985] and its activity was blocked by an anti-TNF monoclonal antibody, suggesting that it was in fact TNF. TNF and lymphotoxin (LT) are two biologically derived peptides which have a cytotoxic or cytostatic effect on tumour cell lines. TNF is contained in post-endotoxin serum [Carswell *et al*, 1975] and LT is contained in supernatants of cultured lymphocytes after antigenic or mitogenic stimulation [Ruddle and Waksman, 1968; Williams and Granger, 1968]. The two factors have recently been purified and cloned [Pennica *et al*, 1984; Wang *et al*, 1985; Aggarwal *et al*, 1984; Gray *et al*, 1984], and show some homology, and some workers now call TNF 'TNF- α ' and lymphotoxin 'TNF- β '. Trinchieri *et al*, 1986 recently demonstrated that these two factors were able to induce monocytic differentiation of HL-60, and to a lesser extent, ML-3 and U937, and that γ -interferon was able to act synergistically, resulting in potentiation of differentiative ability of TNF and LT, as determined by enhanced induction of OKM1, Fc receptors, ADCC and α -Naphthol Acetate Esterase activity.

The ability of γ -interferon to induce differentiation in human myeloid cell lines is well documented [Reviewed in Harris and Ralph, 1985; Trinchieri *et al*, 1986]. It has been shown to be a potent inducer of Fc receptors [eg. Perussia *et al*, 1983], HLA-DR antigen [eg. Virelizier *et al*, 1984; Koeffler *et al*, 1984], and an inhibitor of proliferation [Ralph *et al*, 1983] of human myeloid cell lines. The differentiation inducing effects of γ -interferon appear to be only partial, and effects are not as pronounced as those obtained with other inducers, as demonstrated by only minimal increases in OKM1 antigen and α -Naphthol Acetate Esterase expression by

HL-60 cells [Trinchieri *et al*, 1986]. Gamma interferon has been reported to act synergistically with Vitamin D3 to induce differentiation of HL-60 cells. Either substance alone induced only partial differentiation, with end cells exhibiting different properties, suggesting different modes of action, but together they resulted in more complete monocytic differentiation and augmented clonogenic suppression [Ball *et al*, 1986]

The use of purified and recombinant growth factors has been essential in associating factors with defined *in vitro* activities. A major problem using conditioned media from various cell types is that they differ widely in the type and relative concentrations of growth factors, and in the case of PHA-LCM, the time of harvest could alter such relative concentrations. Discrepancies in the literature where conditioned media have been used can probably be accounted for in this way. For example, PHA-LCM harvested at 48 hours contains γ -interferon but no colony stimulating factor, that harvested at 72 hours contains γ -interferon, colony stimulating factor, LT and sometimes TNF, whereas conditioned medium from U5637 cells contains colony stimulating factor but lack γ -interferon, LT and TNF, yet all three media induce differentiation in human myeloid cell lines [Ralph *et al*, 1983; Trinchieri *et al*, 1986]. The preceding examples stress the need for defined growth factors to elucidate the agents responsible for the induction of differentiation in human myeloid cell lines. Obviously, the majority of cell line work was performed with crude growth factor preparations before the general availability of such defined molecules, but recent work seems to point to the following;

1. A number of discrete biologically derived molecules are able to induce differentiation in human myeloid cell lines, some less effectively than others.

2. Some of the above act synergistically with each other.

3. Different factors can give rise to different "subsets" of differentiation associated attributes, suggesting different modes of action.

From the above, it seems that differentiation in these cell line model systems is a multifactorial process. Even though purified and recombinant growth factors eliminate many of the uncertainties associated with using undefined preparations, properties of the cells being investigated may also pose problems. For example, HL-60 cells treated with TPA and LPS produce TNF [Wang *et al*, 1985] and γ -interferon can cause normal mononuclear cells to release TNF [Nedwin *et al*, 1985], suggesting that it is possible for defined factors to act indirectly on a target cell population.

1.2.6 Myeloid cell lines: Summary.

Human myeloid cell lines are a useful adjunct to the study of normal and leukaemic haemopoietic differentiation, but have some features which necessitate the use of caution in the interpretation of experimental results. Cell lines obviously are very far removed from their normal haemopoietic counterparts, as well as from fresh leukaemic cells. Induction of differentiation of these lines does not result in the acquisition of all the traits of normal mature end cells [eg. Ferrero *et al*, 1983; Dayton *et al*, 1983; reviewed in Abrahm and Rovera, 1981; Koeffler, 1983; Harris and Ralph, 1985]. The extent of differentiation achieved appears to be a property of the cell line used, as well as the inducing agent employed. For example, both KG-1 and HL-60 undergo differentiation towards monocytes when treated with TPA or lymphokines, but KG-1 does not express as many mature monocytic markers detected by MoAb's as HL-60 does [Ferrero *et al*, 1983; Dayton *et al*, 1983]. TPA, obviously a non-physiological inducer, acts independantly of receptor(s) for biologically derived reagents by

interacting directly with Protein Kinase C, due to its structural analogy to diacylglycerol [Niedl *et al*, 1983; Nishizuka *et al*, 1984]. This limits the ability of TPA to induce the full complement of monocytic markers, an example being the induction of HLA-DR antigens on myeloid cell lines by lymphokines and γ -IFN, but not by TPA [Perussia *et al*, 1982; Santoli *et al*, 1983; Dayton *et al*, 1983; Vizilier *et al*, 1984].

The cell line K562 can co-express antigens associated with the granulocytic and erythroid lineages [Marie *et al*, 1981]. A subclone of this line was studied in terms of clonal analysis of heterogeneity of surface marker expression, and it was demonstrated that rather than being a result of an ordered program, expression was at random [Adler *et al*, 1984]. Even though the authors chose a subcloned variant of the cell line which clearly exhibited lineage infidelity and may be atypical, it is still apparent that interpretation of cell line work is not straight-forward.

Nevertheless, cell lines have contributed much knowledge to the understanding of haemopoiesis. They have the advantage of being a renewable source of cells of haemopoietic origin which exhibit features that have allowed the elucidation of some of the mechanisms involved in differentiation, and the identification of agents which may have therapeutic uses [Koeffler, 1983].

1.3.1 Monoclonal Antibodies and Haemopoietic Differentiation.

The introduction of monoclonal antibody technology [Köhler and Milstein, 1975] has aided the study of haemopoietic differentiation. Monoclonal antibodies (MoAb) are very useful because of their single defined specificity, which allows the analysis of differentiation in terms of markers defining lineage and stage of differentiation, as well as in some cases allowing functional attributes of structures defined by them to be determined. One of the most important attributes of monoclonal

antibodies, since they are derived from 'immortalized' cell lines, is that large scale production of antibody with defined specificity is possible. This enables direct comparison of data obtained from different laboratories using the same reagents, unlike non-standard individual polyclonal antisera which are available only in limited supply.

MoAb's do have some disadvantages compared with conventional heteroantisera, in that they may not be able to fix complement or agglutinate cells or may not be able to effectively cross-link antigen to enable immunoprecipitation. These disadvantages are in the main of a technical nature, and as such can usually be overcome by alternative technology. For example, a second complement fixing polyclonal antiserum to mouse immunoglobulin can be used in a 'piggy-back' system to bring about complement mediated cell lysis. MoAb bound to Sepharose beads or erythrocytes can be employed for immunoprecipitation of antigen and rosetting of cells. Even though MoAb's generally show very high specificity, their binding to different cell types is not in itself sufficient as the sole indicator that an identical molecule is present in all cases. [reviewed in Lane and Kaprowski, 1982]. A number of cross reactivities have been described and can be due to common haptenic structures on different molecules (e.g. CD15 MoAb's, which are described later), similar but non-identical epitopes, or artifactual binding caused by Fc receptors [Gadd and Ashman, 1983].

1.3.2 Monoclonal antibodies to human myeloid antigens.

Since the development of monoclonal antibody technology, a number of MoAb's exhibiting specificity for antigens found on human myeloid cells have been produced and reported in the literature. These antibodies have been produced after immunization of mice with a variety of normal myeloid cell types, myeloid leukaemic cells or cell lines [reviewed in Robak and

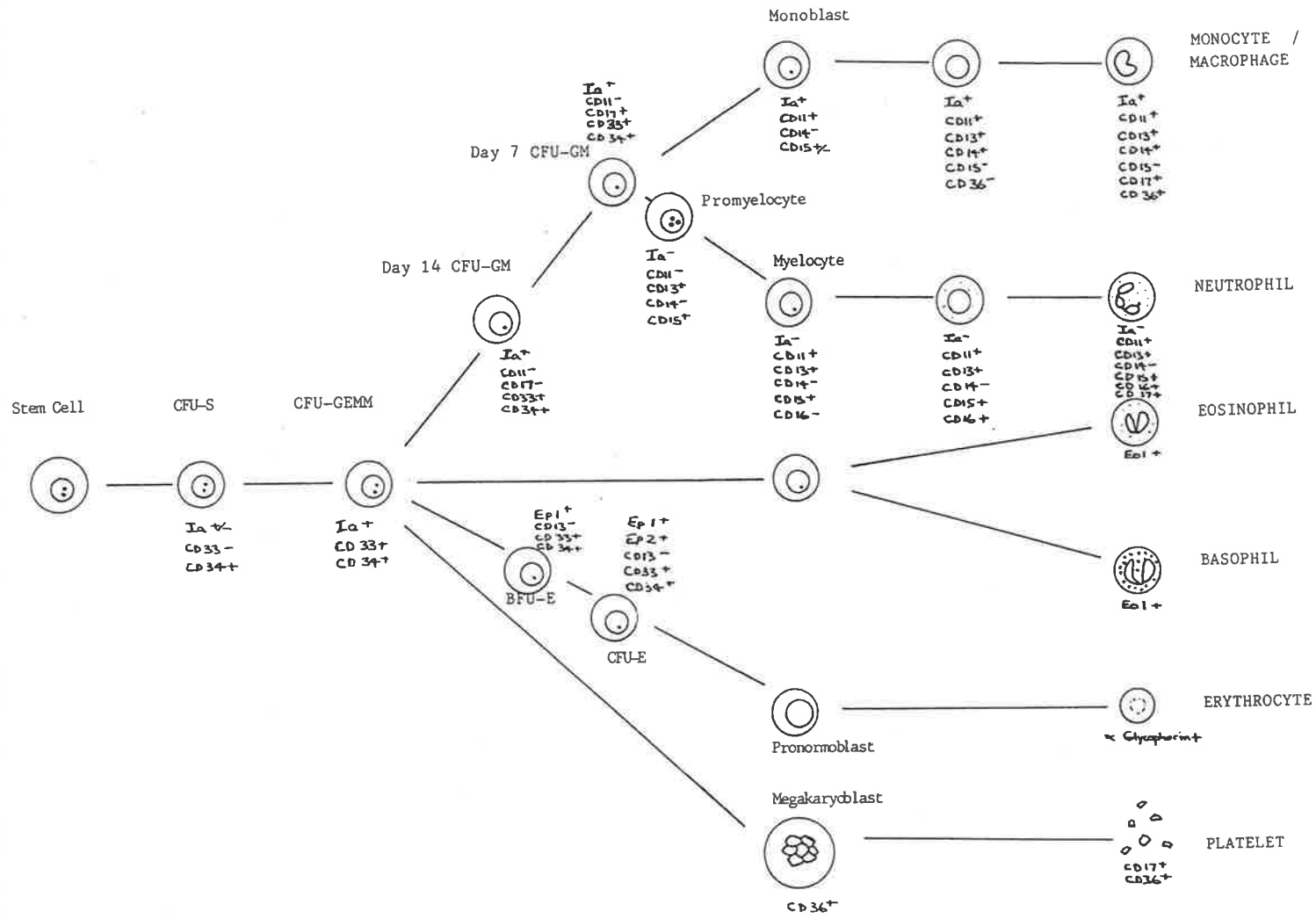
Goldman, 1985; Reinherz *et al*, 1986]. These antibodies have been essentially restricted to cells of the myeloid series on the basis of their binding patterns to a variety of cell types, but some also show a limited amount of binding to cells of lymphoid origin. To obtain myeloid specific MoAb, it is necessary to use a strategy to maximise the chances of obtaining such MoAb, as it is apparent that the murine immune system responds most strongly to common species specific antigens, such as human major histocompatibility antigens (MHC), whereas antigens of special interest may be rare or only on a proportion of the immunising cells, or may only be weakly immunogenic, especially if they are structures that show evolutionary conservation. Probably for this reason, many of the antibodies produced in different laboratories have been demonstrated to bind to the same structures on human cells [reviewed in Reinherz *et al*, 1986].

The process of normal myeloid differentiation results in the loss or decrease in expression of certain antigens detected by MoAb's, and in the acquisition of new antigens. Presumably these antigens relate to the functional capacities of the cells. Therefore MoAb's to myeloid antigens can be used to investigate lineage relationships between myeloid cells, either by directly identifying cells binding MoAb's or by culturing progenitor cells after positive or negative selection. Such studies have enabled the construction of 'lineage maps', which provide an immunophenotypic view of lineage interrelationships [Figure 1.2].

Due to an ever increasing number of monoclonal antibodies directed at surface structures on haemopoietic cells reported in the literature, three international workshops have been held since 1982 with the purpose of classifying MoAb's into defined groups which bind to the same antigens and/or have very similar characteristics, or bind unique antigens. To date,

Figure 1.2

Immunophenotypic schematic representation of normal haemopoietic differentiation, based on binding of MoAb's of various CD groups.



the reports of the first two workshops have been published [Bernhard *et al*, 1984; Reinherz *et al*, 1986].

This section will describe the main clusters of MoAb's with predominantly myeloid specificity. Table 1.4 summarises the specificities of these clusters, some of which are discussed in more detail below.

CD11

This cluster is further subdivided into three groups of antibodies which recognise three related heterodimeric glycoproteins found on leukocytes. These heterodimers share a common 95 kd β chain, which is recognised by antibodies of the CD18 group.

CD11a antibodies bind to the 180 kd α chain of the leukocyte functional antigen (LFA-1) complex found on most leukocytes. This structure is thought to be an adhesion protein involved in a variety of interactions of leukocytes with other cells [Springer *et al*, 1982; Sanchez-Madrid *et al*, 1983; Springer *et al*, 1985]. Antibodies binding to LFA-1 have been reported to block T lymphocyte-mediated killing and T helper cell activities [Krensky *et al*, 1983; Hildreth *et al*, 1983].

CD11b antibodies bind to the 160 kd α chain of the Mo1/OKM1/HuMAC-1 heterodimer, which is found on monocyte/macrophages, neutrophils, natural killer (NK) cells but not on lymphocytes [Sanchez-Madrid *et al*, 1983]. This structure has been shown to be the receptor for the C3bi fragment of complement which is involved in adherence and phagocytosis [Wright *et al*, 1983; Arnaut *et al*, 1983].

CD11c antibodies detect the 150 kd α chain of the third member of this group. This structure appears to be relatively macrophage specific, shows variable expression on neutrophils, and also appears to have an adhesive function [Hogg *et al*, 1986; Malhotra *et al*, 1986].

Table 1.4

Summary of the properties of the myeloid CD group antibodies.

f - positive after de-sialation.

- positive on some lymphoblastoid leukaemias.

¶ - positive on KG-1a, the less differentiated subtype of KG-1.

Table 1.4a

CD Group	Mr(kd)	Struct.	Peripheral blood elements					Myeloid Leukaemic cells	
			T	B	N ϕ	M ϕ	Pl.	M1/M2	M4/M5
CD11a	180	LFA-1 α	+	+/-	+	+	-		
CD11b	160	Mol α	-	-	+	+	-	+/-	+
CD11c	150	p150 α	-	-	+	+	+		
CD12			-	-	+	+	+	+	+
CD13	150-160		-	-	+	+	-	+	+
CD14	55		-	-	+/-	+	-	+/-	+
CD15	180(+)	FAL	-f	-	+	+/-	+/-	+/-	+/-
CD16	50-60	FcR _{1a}	-	-	+	-	-		
CD17	-	Lactosyl- ceramide	-	-	+	+	+		
CD18	95	LFA- β	+	+	+	+	-	+/-	+#
CD31	130-140	?gpIIa	+/-	-	+	+	+		
CDw32	40	?FcR ₁₁	-	+	+	+	+		
CD33	67		Little binding to normal cells					+	+
CD34	115		"	"	"	"	"	+/-	+/-#
CD35	220	CR1	-	+	+	+	-	-	+
CD36	85	gpIV	-	-	-	+	+	+/-	+

Table 1.4b

CD Group	CFU-Mix	CFU-GM		BFU-e	CFU-e	KG1	Human Cell Lines			Lymphoid	
		d 14	d 7				Myeloid			T	B
							HL-60	U937	K562		
CD11a	-	-	-	-	-		+	+	+/-		
CD11b	-	-	-	-	-	+/-	+w	+/-	-	-	-
CD11c	-	-	-	-	-			+		-	-
CD12											
CD13			+	-	-	+	+	+	+/-		
CD14	-	-	-	-	-	-	-	+/-	-		
CD15	+/-	+/-	+/-	+/-		+/-	+/-	+/-	+/-	+/-	-
CD16											
CD17		-	+/-	-	-	+	+	+	-	+	+
CD18						+	+	+	-	+	+/-
CD31						+		+	-	+	
CDw32											
CD33	+	+	+	+	+	+	+	+	+	-	-
CD34	+	+	+	+		+ ¶		-			
CD35											
CD36	-	-	-	-	+/-			+			

Table 1.4c

CD Group	Example	Reference
CD11a	MHM 24	Hildreth <i>et al</i> , 1983
CD11b	Mo1	Todd <i>et al</i> , 1981b
CD11c	3.9	Malhotra <i>et al</i> , 1986
CD12	20.2	Morishima <i>et al</i> , 1986
CD13	MY7	Griffin <i>et al</i> , 1981
CD14	Mo2	Todd <i>et al</i> , 1981b
CD15	VIM D5	Knapp <i>et al</i> , 1984b
CD16	CBL FcR gran 1	Werner <i>et al</i> , 1986
CD17	T5A7	Andrews <i>et al</i> , 1983
CD18	TS1/18	Sanchez-Madrid <i>et al</i> , 1983b
CD31	SG134	Goyert, in McMichael <i>et al</i> , 1987
CDw32	CIKM5	Pilkinton, in Bernard <i>et al</i> , 1984
CD33	L4F3	Andrews <i>et al</i> , 1983
CD34	MY-10	Civin <i>et al</i> , 1984
CD35	E11	Hogg <i>et al</i> , 1984
CD36	5F1	Bernstein <i>et al</i> , 1982

This family of glycoproteins has important *in vivo* function, as it has been shown that patients with a genetic deficiency of these structures are susceptible to recurrent bacterial infections [Springer *et al*, 1984; Springer *et al*, 1985].

CD13

This group recognises a 150-160 kd glycoprotein found on monocytes and neutrophils and their precursors. This 'pan myeloid' antigen has been found to be on over 85-95% of acute myeloid leukaemias [Drexler and Minowada, 1986; Neame *et al*, 1986].

CD14

Antibodies of this cluster bind to a 50-55 kd glycoprotein found almost exclusively on monocytes, however this group does include some antibodies which bind neutrophils [Bernstein and Self, 1986].

CD15

This group of antibodies recognise the 3- α -fucosyl-N-acetyl-lactosamine (FAL) carbohydrate moiety found on certain myeloid cell proteins of 180, 110, 68 and 50 kd molecular weight. It has been demonstrated that antibodies recognising this structure may bind to different epitopes [Majdic *et al*, 1984] and in addition, the level of sialylation of glycoproteins influences the binding of CD15 antibodies, as desialylation using neuraminidase enabled CD15 antibodies to bind to cALL cells and increased binding to M2 myeloid leukaemic cells [Tetteroo *et al*, 1984; Tetteroo *et al*, 1984b; Tetteroo *et al*, 1986]. These observations may explain the fact that antibodies in this group show some differences in binding. One group of MoAb's bind only to neutrophils and precipitate a

single 180 kd glycoprotein, whereas another group also bind weakly to monocytes and precipitate some other bands.

CD16

This group of antibodies bind to the 50-60 kd low affinity Fc receptor (FcR_{1b}) of granulocytes [Bernstein and Self, 1986].

CD17

Antibodies of this cluster recognise the lactosyl-ceramide glycolipid moiety found on monocytes, neutrophils and platelets. Contained within this structure is the FAL unit recognised by CD15 antibodies [Symington *et al*, 1986].

1.3.3 Less well-defined MoAb's.

A number of antibodies detecting myeloid antigens have been reported in the literature, but have not been assigned to any cluster group. This may be due to their uniqueness, or that the results of the third Leukocyte Typing workshop have not as yet become available. In addition, many of these antibodies were not submitted to the workshops. The examples given below are by no means an exhaustive review, but serve to demonstrate the diversity of antibodies that have been characterized. Some of these examples are from less well defined cluster groups.

1.3.3.1 MoAb's with relative specificity for myeloid progenitors.

This group of antibodies is rather small, as the majority of MoAb's that have been produced to date bind to mature end cells or at least to morphologically recognisable cells such as promyelocytes, with a proportion of such antibodies binding to myeloid progenitors [reviewed in Foon and Todd, 1986, Reinherz *et al*, 1986].

Two antibodies, L4F3 and MY9, in the tentative grouping CD33, bind to a 67 kd glycoprotein found on immature myeloid cells of the bone marrow, but virtually undetectable on mature monocytes or granulocytes. These antibodies also bind to the majority of myeloid colony forming cells of the bone marrow (CFU-Mix, CFU-GM, BFU-e, CFU-Meg), but not to the precursors of these cells [Andrews *et al*, 1983; Griffin *et al*, 1984; Andrews *et al*, 1986]. They also bind to blast cells in most AML cases [Neame *et al*, 1986; Drexler and Minowada, 1986].

Three antibodies, two of which are in the tentative grouping CD34 (BI-3C5, MY-10) and one which is a non-workshop antibody (12.8), appear to recognise the same 115 kd glycoprotein which is found on a small (1-5%) subset of bone marrow mononuclear cells. The majority of the myeloid colony forming cells were found within this population [Katz *et al*, 1985; Civin *et al*, 1984; Civin *et al*, 1987; Andrews *et al*, 1986]. The cells detected by MY-10 have been shown to be intermediate in size and lack antigens associated with any mature lineages, indicating they represent primitive blast cells [Civin *et al*, 1987]. Antibody 12.8 appeared to bind to cells able to give rise to colony forming cells in long term bone marrow culture [Andrews *et al*, 1986]. CD34 antibodies also bind to tonsil endothelium [McMichael *et al*, 1987].

It is interesting to speculate that antibodies from the CD33 and CD34 groups may be able to define a subpopulation of cells (CD33 (-)ve, CD34 (+)ve) that contains progenitor cells more primitive than those already defined by *in vitro* colony culture methods.

Peng *et al*, 1984 reported two antibodies associated with immature myeloid cells and progenitors. Pro-Im1 detected an 85 kd glycoprotein found on 25 to 40% of bone marrow cells, including the majority of CFU-GM, BFU-e and CFU-Mix, but not on any mature peripheral blood cells. Pro-Im2 detected a heat stable glycolipid determinant which had a similar

distribution, but was also found on mature granulocytes. HL-60 cells expressed both the antigens detected by these antibodies, but expression was lost when the cells were induced to differentiate towards monocytes with TPA.

A number of MoAb's which bind to erythroid but not other types of progenitors have also been described. The antibodies 5F1 [Bernstein *et al*, 1982] and FA6-152 [Edelman *et al*, 1986] appear to recognise the same 85 kd glycoprotein which is found on CFU-e, BFU-e and immature erythroid cells, including foetal erythrocytes. These antibodies demonstrated binding to monocytes and platelets, and have been tentatively named CD36. Yokochi *et al*, 1984 reported two antibodies which appear to be distinct from the above examples. Ep-2 binds to immature erythroid precursors but not to erythroid progenitors (CFU-e, BFU-e) and does not bind to other cell types. Ep-1 binds to erythroid progenitors and precursors, as well as to some myeloid cell lines. Neither binds to any mature peripheral blood elements.

1.3.3.2 MoAb's with functional effects on myeloid cells.

Several antibodies have been reported to exert functional effects on myeloid cells. These effects can involve the activation of functions, or more usually the blocking of functions.

WEM-G1, a granulocyte specific antibody, binds to a 110 kd cell surface protein and stimulates neutrophil and eosinophil function as measured by antibody dependant cell cytotoxicity (ADCC). This ability is also shared by GM-CSF, and WEM-G1 and GM-CSF were shown to stimulate in an additive fashion [Lopez and Vadas, 1984]. Similarly, another antibody from the same group, WEM-G11, was able to enhance both ADCC and phagocytosis by granulocytes, as well as enhancing the cytotoxic effect of eosinophils on schistosomulae. This antibody recognised the 95 kd chain of

a gp 95,43 which may be related to the LFA-1 group of heterodimers [Lopez *et al*, 1985].

The monoclonal antibody NCD1 inhibited degranulation and chemotaxis of granulocytes [Cotter *et al*, 1981]. VIM-D5, a granulocyte specific CD15 group antibody inhibited phagocytosis of zymosan particles and *S. Aureus* bacteria [Bjerknes *et al*, 1984]. The antibody Mo3e detects a p50,80 kd on monocytes, and is able to block the response of monocytes to migration inhibition factor (MIF) [Liu *et al*, 1986]. This structure appears to be involved in the Protein Kinase C transmembrane signal system [Todd *et al*, 1986]. As previously described, antibodies of the CD11 group are also able to block some functions of myeloid and lymphoid cells.

1.3.3.3 Miscellaneous MoAb's to myeloid cells.

Todd and Malech, 1986 reported a MoAb which was raised against HL-60 cells and identified an antigen which was associated with cell proliferation. This antibody, Y201, identified the subset of cells in a population of HL-60 which was able to give rise to colonies in semi-solid medium. Differentiation of HL-60 cells with a number of agents (TPA, DMSO, Retinoic acid) resulted in marked decrease in Y201 binding, which correlated with the decrease in clonogenic potential of the treated populations. The binding pattern of this antibody was inconsistent with it recognising the transferrin receptor, as it did not bind to other cell lines tested, and only bound to about 3% of bone marrow mononuclear cells. The authors were unable to obtain a molecular weight for the antigen detected, and did not investigate the binding of this antibody to haemopoietic progenitor cells.

Another monoclonal antibody, AGF2.3, raised on HL-60 cells was shown to bind to a 215 kd protein found on the nuclear envelope membrane [Brown *et al*, 1985]. This antigen appears also to be associated with proliferation

as TPA or DMSO treatment resulted in an almost complete loss of expression by HL-60 cells. This structure was detected on all haemopoietic cell lines, and on the majority of non-haemopoietic cell lines which were tested, however it was not expressed by any of the primary non-transformed lines tested.

De Jong *et al*, 1986 reported two MoAb's which bound to the same 70-78 kd glycoprotein found on granulocytes and band cells. 38D2 and 41D2 did not bind to monocytes, platelets, leukaemic lymphoblasts or myeloblasts, myeloid cell lines or immature bone marrow myeloid cells. The antibodies did not effect phagocytic or chemotactic ability, and appear to bind almost exclusively with relatively mature granulocytes.

Eosinophilic leukaemia cells were used by Saito *et al*, 1986 to raise a monoclonal antibody designated EO-1, which detected a 23 kd protein found essentially only on eosinophils, basophils and platelets, and not on neutrophils. This antibody did not bind to myeloid leukaemic cells or cell lines, but did bind to a number of B cell leukaemias.

Avnstrom *et al* 1985 reported SA-1, a MoAb raised on megakaryoblastic leukaemia cells which bound to a 15 kd polypeptide on neutrophils, immature myeloid cells, and a subpopulation of activated T lymphocytes. This antibody bound to 65% of AML specimens studied, but did not bind to myeloid cell lines, CFU-GM, platelets or normal or leukaemic lymphoid cells.

Andreesson *et al*, 1986 reported a series of MoAb's binding to late differentiation antigens of macrophages. MAX.1, MAX.2 and MAX.3 showed similar patterns of binding, but recognised distinct molecules of 64, 200 and 68 kd respectively. All three antibodies bound strongly to monocyte derived macrophages but not to day 1 adherent monocytes, and in addition MAX.3 bound weakly to platelets. These antibodies did not bind to any other lymphoid or myeloid cell types tested. A fourth antibody described,

MAX.11, had similar binding characteristics, but also bound variably to day 1 adherent monocytes, to a subpopulation of peripheral blood monocytes, and weakly to U937 cells.

Similarly, Zwadlo *et al*, 1985 reported an antibody which recognised an 86 kd antigen present on day 3 cultured monocytes and tissue macrophages, but not on freshly isolated peripheral blood monocytes, granulocytes, platelets, lymphocytes, and most cell lines tested, apart from two melanoma cell lines.

Gadd and Ashman, 1985 reported a MoAb named YB5 which was raised on M1 AML cells, and bound to a proportion of myeloid leukaemias but not to any other cell type or cell line tested. Subsequently, this antibody was shown to bind to mast cells in sections of tonsil, skin and gut, and to detect a protein of 150 kd [Ashman *et al*, 1987; Mayrhofer *et al*, 1987].

The above examples illustrate the diversity of MoAb's to myeloid antigens reported in the literature. It is apparent that antibodies are rarely absolutely specific for a particular cell type or lineage, and a number of antibodies show specificity for other tissue types besides the haemopoietic system, for example cells of neural, endothelial or melanocyte origin [Hogg *et al*, 1981; Zwadlo *et al*, 1985; reviewed in Reinherz *et al*, 1986; McMichael *et al*, 1987].

1.3.4 Classification of myeloid leukaemia using MoAb's.

Myeloid leukaemias appear to represent an uncontrolled clonal expansion of abnormal myeloid progenitor cells, which fail to give rise to mature, non-proliferating progeny. Comparison of leukaemic cells with normal cell types has enabled the identification of the cell lineage and cell type the leukaemic cells represent, and has allowed speculation regarding the identity of the cell target of the events responsible for the

establishment of the leukaemic state [e.g. Greaves, 1982; Messner and Griffin, 1986]. Such comparisons have enabled classification of the leukaemias into groups based on their lineage origins. It is well recognised that different types of leukaemia respond differently to therapy, thus accurate diagnosis is important for correct treatment [reviewed in Gunz and Henderson, 1983]. Acute Myeloid Leukaemia (AML) has been classified into seven groups, based on criteria defined by the French-American-British (FAB) cooperative workshops, to enable standardization of diagnosis of AML, and to allow comparisons between treatment protocols [Bennett *et al*, 1976; Bennett *et al*, 1985; Bennett *et al*, 1985b]. This classification system was based on a combination of morphology in Romanowsky stained smears and cytochemistry (Myeloperoxidase, Sudan Black B, Chloroacetate esterase for granulocytic differentiation; Non-specific esterase (NSE) for monocytic differentiation), which classified AML according to the degree and type of differentiation of their blast cells in bone marrow biopsies.;

M1- predominantly myeloblasts with <10% granulocytic differentiation.

M2- more than 30% myeloblasts with >10% granulocytic differentiation,
<20% NSE (+)ve.

M3- a) Promyelocytic, Chloroacetate esterase, Auer rods.

b) Variant with kidney shaped nuclei but NSE (-)ve.

M4- myelomonocytic, >20% and <80% NSE (+)ve.

M5- monocytic, >80% NSE (+)ve.

a) Monoblastic, poorly differentiated.

b) Monocytic, differentiated.

M6- erythroid, more than 50% erythroblasts.

M7- megakaryoblastic.

Even though the FAB classification system has standardized diagnosis of leukaemia, and allowed statistical evaluation and assessment of treatment regimes between centres, a number of deficiencies exist. These are mainly due to the subjective nature of the system, and resulting differences in the interpretation of the criteria for classification. For example, it is sometimes difficult to distinguish poorly differentiated M1 AML from lymphoid L2 subtypes when cytochemical staining is not present [e.g. Herrmann *et al*, 1983; Avnstrom *et al*, 1985]. Within the myeloid AML classification system, M1 and M2 subtypes are often difficult to assign accurately, and the subjective nature of the FAB system has resulted in considerable observer variation [Whittaker *et al*, 1979; Head *et al*, 1985; Head *et al*, 1985b].

A number of recent studies suggest that immunophenotypic subtyping of AML with monoclonal antibodies may provide a useful adjunct to FAB classification [Ball and Fanger, 1983; Griffin *et al*, 1983; van Reijden *et al*, 1983; Linch *et al*, 1984; Neame *et al*, 1986; reviewed Griffin, 1985; Foon and Todd, 1986; Drexler and Minowada, 1986]. These studies also demonstrate the need to carefully choose MoAb's for inclusion into screening protocols, as considerable variation in the expression of some markers occurs both within well defined subtypes and within the blast population of a given patient [Drexler and Minowada, 1986; Pessano *et al*, 1983].

Griffin *et al*, 1983 used a panel of MoAb's to immunophenotype 70 cases of AML. The antibodies used were MY4 (CD14), MY7 (CD13), MY8, α -Mo1 (CD11b) and anti-I2 (Ia binding). The authors used these antibodies to group the AML blasts into four phenotypic subtypes, whose patterns of antigen expression resembled their normal counterparts;

Group I-(CFU-C-like);	MY4(-),MY8(-),	Mo1(-),	Ia(+)
Group II-(myeloblast-like);	MY4(-),MY8(weak+),	Mo1(weak+),	Ia(+)
Group III-(promyelocyte-like);	MY4(-),MY8(+),	Mo1(+),	Ia(-)
Group IV-(promonocyte-like);	MY4(+),MY8(+),	Mo1(+),	Ia(+)

Generally, all the AML blasts they tested bound MY7. Comparison of classification by FAB subtype with this scheme revealed that M3 and M5 subtypes were found within Group III and Group IV respectively. M1,M2 and M4 subtypes were dispersed through all groups, with M4 showing marked monocytic involvement having a tendency to be in Group IV.

Studies by van Reijden *et al*, 1983 on 55 cases of AML showed that two antibodies which bound to normal granulocytes but not monocytes (M1/N1 (CD15) and UJ308) generally did not bind to monocytic M4/M5 AML cells. Promyelocytic M3 AML cells were found to be negative for Ia antigens and OKM1 (CD11b) binding was found to be non-discriminatory between FAB subtypes.

Ball and Fanger, 1983 used the neutrophil specific antibodies PMN-6 and PMN-29, and the monocyte/granulocyte binding antibody AML-2-23 to type 28 cases of AML. These antibodies which do not bind to CFU-GM failed to bind to cells of the M1/M2 subtypes, confirming the immature phenotype of these leukaemias. Six out of the 8 M4 subtypes tested bound all three antibodies, whereas 4 out of the 5 M5 subtypes tested bound only AML-2-23, correlating with the binding of the three antibodies to normal cell types.

Pessano *et al*, 1984, using a panel of 16 antibodies to immunophenotype 63 cases of AML, reported that they found no correlation between the pattern of antibody binding and FAB classification, with the possible exception that M3 subtypes tended to be negative for anti-Ia antibody binding. The binding patterns of the antibodies emphasised the considerable antigenic heterogeneity of leukaemic cells from a given

patient. The results obtained by the authors may relate more to the choice of the antibodies in the panel, rather than a general inability of MoAb's to classify leukaemias.

Linch *et al*, 1984 used a panel of antibodies including UCHM1 (CD14) and E11 (CD35) to subtype 70 AML cases. It was found that both MoAb's bound almost exclusively to leukaemic cells of monocytic M4 and M5 subtypes.

Recently, Drexler *et al*, 1986 subtyped 724 cases of leukaemia or lymphoma with a panel of 15 antibodies. MCS-2 (CD13) was found to bind to 96% of 195 AML cases, but only 2.4% of 421 cases of lymphocytic leukaemia or lymphoma. There was wide variability of binding of other monoclonal antibodies to myeloid leukaemic cells, however three antibodies of the CD14 group (Leu M3, MY4, anti-Mo2) showed a clear tendency to bind to cells of monocytic variants AML (M4 and M5).

Drexler and Minowada, 1986 reviewed the literature on immunophenotypic subtyping, and grouped the antibodies used into four categories. Group 1 were pan myeloid and included MY7, MCS-2 (both CD13), MY9 (CD33) and VIM-2. Group 2 antibodies were predominantly monocytic in specificity, with some granulocyte binding, and included anti-Mo1, OKM1 (both CD11b). Group 3 antibodies were specific for monocytes, and included CD14 MoAb's, anti-Mo2, Leu-M3, MY4 and UCHM1 as well as AML-2-23. Group 4 comprised those antibodies which bound to a proportion of monocytic and granulocytic cell types. The MoAb's in groups 1 and 3 were quite discriminatory. Group 1 antibodies were able to accurately distinguish between myeloid and lymphocytic leukaemias, and group 3 antibodies were reliably able to identify monocytic M4 and M5 subtypes. Group 2 and 4 antibodies bound quite variably to AML FAB subtypes, and it remains to be seen if such binding patterns have any prognostic significance.

Neame *et al*, 1986 used a panel of commercially available antibodies to immunophenotype 72 cases of AML. The antibodies used were MY9 (CD33), MY7 (CD13), Leu M1 (CD15), PMN-6/PMN-29 (anti-neutrophil), AML-2-23 (anti-monocyte/granulocyte) and HLA-DR (anti-Ia). This panel enabled immunophenotypic classification which concurred with FAB subtyping in over 80% of cases. Their data showed that MY7 and MY9 bound to almost all cases of AML, with MY4 binding to mainly M4/M5 subtypes. Leu M1 bound to most AML cases except those classified as M1, and PMN-6/29 bound mainly to AML of M2,M3,M4 subtypes. AML-2-23 bound infrequently to M1 and M2 subtypes, and Ia tended not to be expressed by M3 AML.

Andreasen *et al*, 1985 reported a MoAb (NAT-9 II:3F-6F) which bound to 20 of 20 M2 and 12 of 12 M4 AML cases tested, but did not bind to any M1 or M5 subtypes (12 of each tested).

From the above examples, a number of clear associations are apparent;

1. Pan-myeloid antibodies such as MY7, MCS-2 (both CD13), VIM-2 and MY9 (CD33) bind to the majority of Acute Myeloid Leukaemias, and are useful in distinguishing between myeloid and lymphoid leukaemias, especially in undifferentiated cases [Neame *et al*, 1986; Drexler *et al*, 1986; Drexler and Minowada, 1986]
2. Monocyte specific CD14 antibodies such as MY4, anti-Mo2, Leu M3 and UCHM1 have marked specificity for leukaemias with monocytic involvement, such as M4/M5 subtypes [Griffin *et al*, 1983; Linch *et al*, 1984; Drexler *et al*, 1986; Drexler and Minowada, 1986; Neame *et al*, 1986].
3. MoAb's which bind to granulocytes, such as PMN-6/29, are able to distinguish myelomonocytic (M4) leukaemias from the 'pure' monocytic (M5)

subtype [Ball and Fanger, 1983; van der Reijden *et al*, 1983; Neame *et al*, 1986].

4. Anti-Ia MoAb's usually do not bind to promyelocytic M3 leukaemias [Griffin *et al*, 1983; van der Reijden *et al*, 1983; Pessano *et al*, 1984; Neame *et al*, 1986].

5. M1 subtypes may be distinguishable from M2 by the tendency of M1 cells not to bind the Leu M1 CD15 MoAb [Neame *et al*, 1986]. In addition, the antibody NAT-9 II:3F-6F appears to bind M2 but not M1 cells [Andreasen *et al*, 1985].

1.3.4.1 Prognostic significance of anti-myeloid MoAb's.

Antibodies to myeloid differentiation antigens may in time be of prognostic value, as are MoAb's which distinguish distinct subgroups of ALL [Greaves, 1981]. The outcome in response to chemotherapy of AML is extremely variable, ranging from no remission to apparent cure in 10-15% of cases, with FAB classification having little prognostic value [e.g Gunz and Henderson, 1983]. It has been shown in some studies that AML with monocytic involvement have a slightly worse prognosis than non-monocytic types, with AML's with blasts that bind MY4 or MY7 antibody having a poorer prognosis than their counterparts not binding MY4 or MY7 [Griffin *et al*, 1983; reviewed in Foon and Todd, 1986]. Civin *et al*, 1983 reported that MY-1 (+)ve AML's were more likely to enter remission than those which were MY-1 (-)ve, conversely, MY-10 (-)ve AML's were more likely to enter remission than those which bind MY-10 antibody. The subgroup of AML's expressing the antigen detected by YB5 were reported to have a poor prognosis [Gadd and Ashman, 1985]. Subsequently, a larger series confirmed this result, and this antibody appears to identify a subgroup of AML's

which responds poorly to standard daunorubicin/cytosine arabinosine/6-thioguanine (DAT) chemotherapy [Ashman, unpublished results].

1.3.4.2 Lineage fidelity

There have been several reports in the literature of leukaemic cells displaying 'lineage infidelity' i.e. co-expression of normally mutually exclusive lineage markers on individual cells [reviewed in Greaves, 1986]. This may be due to aberrant gene expression by the leukaemic cells, or perhaps may be interpreted as representing the immortalization of cells which are too rare to be detected in normal individuals. Therefore, such leukaemias may be subclasses of acute leukaemia that were previously unrecognised [Knapp *et al*, 1984]. Leukaemias of mixed myeloid/lymphoid immunophenotype are in the main very undifferentiated [Neame *et al*, 1986], and therefore it may be speculated that they could represent myeloid/lymphoid stem cells. In some cases, it is possible that aberrant glycosylation which unmasks determinants that are associated with another lineage may be responsible for some of the reported examples of marker co-expression e.g. CD15 anti FAL antibody binding to ALL blasts [Bettelheim *et al*, 1982]. Generally, acute myeloid leukaemias tend to exhibit lineage fidelity, and those co-expressing mixed lineage markers are quite rare, comprising of the order of 1-5% of the total, depending on the study [reviewed in Messner and Griffin, 1985]. As previously discussed, few antibodies show absolute specificity for a given lineage or cell type, and the state of knowledge of antigen expression on normal haemopoietic progenitor cells is insufficient at present to exclude the possibility of 'mixed' immunophenotypes occurring normally.

1.3.4.3 Clonogenic cells in AML.

Blast cell populations of some AML patients have been shown to contain a small proportion of cells, known as CFU-Leuk, which are able to form colonies in semi-solid medium in analogous way to normal haemopoietic progenitor cells [reviewed in Sabbath and Griffin, 1985; Griffin and Löwenberg, 1986]. If these cells represent leukaemic 'stem' cells which are responsible for the maintenance of the leukaemic population *in vivo*, then it is conceivable that the immunophenotype of these clonogenic cells is of more relevance in terms of potential therapy involving MoAb's than the immunophenotype of the bulk blast population.

There is some indirect evidence that the *in vitro* CFU-Leuk are related to, or comprise an as yet unknown proportion of the *in vivo* growth fraction of AML cells. A large proportion of the CFU-Leuk have been shown by thymidine suicide experiments to be synthesizing DNA in S-phase of the cell cycle [Minden *et al*, 1983]. Clonogenic cells have been shown to undergo self-replicative divisions *in vitro*, resulting in the ability of colonies to form secondary colonies upon replating, however the efficiency of replating was usually under 10% [McCulloch *et al*, 1982].

The immunophenotype of AML CFU-Leuk has been reported to be less mature than the bulk population from which they were derived, and their progeny appear to exhibit a more mature antigenic makeup than the CFU-Leuk from which they were derived [reviewed in Griffin and Löwenberg, 1986]. In some cases it has been reported that the immunophenotype of the colony cells demonstrates a greater degree of maturity than the original blast population [Tuow and Löwenberg, 1985]. This may be due to the PHA-feeder cell culture system used, which produces biologically active molecules able to induce differentiation in these cells. Lange *et al*, 1984 noted that the immunophenotypes of AML CFU-Leuk's were similar to those of normal myeloid colony forming cells.

Hast *et al*, 1986 demonstrated that the immunophenotype of AML CFU-Leuk's correlated to some degree with FAB classification. Tuow and Löwenberg, 1985 suggested that immunophenotyping the progeny of clonogenic cells from undifferentiated or unclassifiable leukaemias may be useful in identifying the lineage(s) to which they are related.

1.3.4.4 MoAb's with potential therapeutic uses.

Monoclonal antibodies have potential for purging remission AML bone marrow of residual leukaemic cells by complement mediated cell lysis prior to autologous bone marrow transplant (ABMT). Such procedures have been successfully employed for ABMT in cases of acute lymphocytic leukaemia [Ritz *et al*, 1982; Jansen *et al*, 1984]. The main criterion for judging the suitability of a MoAb for use in purging marrow is that the antibody must bind to the cells which are responsible for the *in vivo* perpetuation of the leukaemic clone (leukaemic 'stem' cells), but not bind to the stem cells responsible for the repopulation of the haemopoietic system after chemotherapeutic/radiotherapeutic ablation of leukaemic bone marrow.

Ball *et al*, 1986 used two monoclonal antibodies together to purge remission bone marrow of 10 AML ABMT cases. The antibodies PM-81 and AML-2-23 were chosen because they fixed complement, bound to AML blasts in 77 to 91% of cases, bound to the majority of CFU-Leuk in two-thirds of the AML's tested, but only to a proportion of CFU-GM. They did not bind to erythroid or multipotential haemopoietic progenitors. Two antibodies were used together to maximise the probability of removing all clonogenic cells by compensating for the heterogeneity of antigen expression on blast and clonogenic cells [Pessano *et al*, 1984; Sabbath *et al*, 1985]. At the time of submission of the paper, seven of the ten patients were surviving disease-free at 2 to 21 months post-transplantation. The pan-myeloid CD33 MY9 antibody is currently being used for purging of marrow [Ritz, 1987]. As

time and the state of knowledge of myeloid specific antibodies progresses, trials of more antibodies will be undertaken to assess their utility for ABMT.

1.4 Aims of the Project.

Monoclonal antibodies which bind to surface structures of human myeloid cells are potentially very useful in the study of normal and leukaemic differentiation. They allow molecules which are unique to a lineage to be defined, and this information, combined with that gained about structures common to different lineages may allow the formulation of models of haemopoiesis. Such models serve to explain the interrelationships between the different haemopoietic lineages. When this project commenced, there were only a small number of MoAb's which were myeloid specific described in the literature, and very few bound to structures with known functional activities [e.g. Bernard et al, 1984].

One aim of this project was to set up a cell line model of differentiation using a human myeloid cell line which could be induced to differentiate in response to biologically derived growth factors. Such an approach would provide a reproducible, renewable source of cells which would allow monitoring of changes in antigen and enzyme expression as differentiation occurred. In addition, this type of model would facilitate the study of the molecular identity of structures detected by monoclonal antibodies.

A major difficulty in the production in the production of Murine MoAb's to human antigens is that the mouse immune system responds mainly to strongly immunogenic species-specific determinants such as MHC antigens. Monoclonal antibodies to potentially interesting weakly immunogenic antigens may therefore represent only a small fraction of the

total number of hybrids produced in a given fusion. To overcome this problem, the immune system of the mouse may be diverted away from strongly immunogenic structures [e.g. Gadd *et al*, 1985], or a screening procedure employed which is both rapid and sensitive. A further aim was to set up a screening procedure which would enable a large number of hybridoma supernatants to be assayed for specificity rapidly and accurately, allowing myeloid specific antibodies to be identified as early as possible after the fusion.

The final aim of the project was to raise monoclonal antibodies against cells of the human myeloid cell line used as a model of differentiation. These MoAb's could then be used to investigate changes in antigen expression as differentiation of the cell line occurred. In addition, these monoclonal antibodies could be used to 'immunophenotype' myeloid cells from a variety of sources.

CHAPTER 2

Materials and Methods.

2.1 Buffers and general reagents.

Non-tissue culture reagents and solutions were prepared using deionized and filtered water that had been processed by the milli-RO60 system [Millipore Corporation, USA]. Unless otherwise stated, solutions were stored at 4° C prior to use. General chemicals were of analytical grade.

Saline: 0.9% w/v (0.154 M) NaCl in water.

PBS pH 7.4: Phosphate Buffered Saline was prepared by dissolving NaCl (8g), KCl (0.2g), Na₂HPO₄ (1.115g) and KH₂PO₄ (0.2g) in 1 litre of water and adjusting the pH to 7.4 with 1M HCl.

PBS/Az: 1 ml of 10% w/v NaN₃ in saline was added to 500 ml PBS pH 7.4.

PBS/BSA/Az: 0.5g of Bovine Serum Albumen [Flow, Australia] was added to 500 ml PBS/Az.

Isotonic Phosphate, pH 7.4: NaH₂PO₄ (1.964g) and Na₂HPO₄ (19.21g) were dissolved in 500 ml of water.

Iso-PBS, pH 7.4: 4 volumes of saline was buffered with 1 volume of Isotonic Phosphate pH 7.4.

Red blood cell lysis solutions: Solution A; Tris base (2.0954g) was dissolved in 90 ml of water, the pH adjusted to 7.65 with 1 M HCl and made up to a final volume of 100 ml. Solution B; NH₄Cl (8.3g) was dissolved in one litre of water. Both solutions were sterile filtered through a 0.22µ pore size Millex filter (Millipore Corp., USA). Immediately before use, 1 volume of solution A was mixed with 9 volumes of solution B.

Alkaline phosphate enzyme diluent: Diluent for alkaline phosphatase conjugated antibodies was made by mixing 40 ml of 0.5 M Tris-HCl pH

7.5, 10 ml of 100x Mg/Zn (5 ml of 2 M $MgCl_2$, 0.25 ml of 0.1 M $ZnCl_2$ made up to 100 ml with water), 200 mg BSA, 5 ml of 10% v/v NaN_3 and 940 ml of saline.

Giemsa's Buffer: $NaHPO_4$ (2.724g) and KH_2PO_4 (2.376) were ground together using a mortar and pestle, then dissolved in 900 ml of water. The pH was adjusted to 7 with 1M HCl and the volume made up to 1 litre.

Giemsa's stain: A working strength solution was made by diluting Giemsa's stain (BDH) 1/25 in Giemsa's buffer. Cell smears were stained for 10 min, and washed in Giemsa's buffer until the colour of the smears changed from blue to salmon pink.

2.2 Antisera.

2.2.1 Preparation of Rabbit antiserum to Mouse Immunoglobulin.

Pooled murine immunoglobulin of IgG1, IgG2a, IgG2b and IgM subclasses was used to immunize rabbits. IgM and IgG2b were produced from hybridomas grown as ascites in mice. IgG2a and IgG1 were purified from normal mouse serum using Protein A conjugated Sepharose 4B (Pharmacia) columns (kindly provided by Dr. P Ey in this department). For each immunization, 1 ml of saline containing 2-3 mg of pooled immunoglobulins was emulsified with 1 ml of Span 85 (Sorbitan trioleate, Koch-Light)/2 drops of colloidal alumina (C-γ gel, CalBiochem). A rabbit was injected subcutaneously with 0.5 ml of the emulsion in four sites. The rabbit was immunized a further three times at monthly intervals and was bled out by cardiac puncture 14 days after the final immunization. The blood was allowed to clot and the

serum was collected and freed of residual erythrocytes by centrifugation for 10 min at 200g. The serum was made up to 40% ammonium sulphate using a cold saturated solution of ammonium sulphate, and allowed to stand on ice for 30 min. The precipitated immunoglobulin fraction was collected by centrifugation at 1000g for 10 min. The pellet of precipitated protein was dissolved in PBS and dialysed against two changes of PBS over two days at 4°C. Each change of PBS was 100 times the volume of the sample being dialysed. The immunoglobulin fraction was diluted to 1 mg/ml, sterilized by filtration and stored at -20°C. At each stage of the process, Ouchterlony double diffusion was used to ensure that the reagent had activity against IgG1,2a,2b and IgM (see section 5.2.7).

2.2.2 Preparation of Goat antiserum to Mouse Immunoglobulin.

Hybridomas secreting IgG1, IgG2a, IgG2b and IgM immunoglobulins to *Salmonella enteritidis* cytoplasmic antigens [O'Connor and Ashman, 1982] were grown as ascites in pristane primed mice, and the immunoglobulins purified by the method of Ey *et al*, 1978 by S Gadd in this laboratory. A total of 1 mg of an equimolar solution of these antibodies in saline was emulsified in Freund's complete adjuvant, and injected subcutaneously into four sites of the goat. After 4 weeks, the goat was injected with a further 0.5 mg of the same immunoglobulin mixture in Freund's incomplete adjuvant. Eighteen days later the goat was bled from the jugular vein and the serum obtained was shown to contain antibodies to the four immunoglobulin subclasses used as the immunogens, by Ouchterlony immunodiffusion (see section 5.2.7).

2.2.3 Affinity purification of goat antibodies to mouse immunoglobulin.

A column of mouse IgG-conjugated Sepharose 4B (kindly provided by Dr. P L Ey) was equilibrated with PBS pH 8.2, and goat anti-mouse immunoglobulin serum (see section 2.2.2) was adjusted to pH 8 with 1 M NaOH and loaded onto the column. The serum was washed through the column with PBS pH 8 at a flow rate of 0.3 ml/min using a peristaltic pump and 2 ml fractions were collected. The fractions were monitored for their protein content by absorbance at 280nm, and when the peak of protein not binding to the column had passed (i.e. $OD_{280} < 0.05$), 0.1 M citrate buffer pH 3.3 was applied to the column to elute antibodies binding to mouse IgG. Fractions were collected into an equal volume of PBS/Az to neutralize the acid, and the fractions containing the peak of protein were pooled. The eluate of unbound serum proteins was then re-run through the column to remove any remaining antibodies binding to mouse IgG and fractions containing antibodies were pooled with those previously obtained.

A column of mouse IgM-conjugated Sepharose 4B (kindly provided by Dr. P Ey) was used to isolate any antibodies remaining in the eluent from the IgG column which bound to IgM, using the same procedure as above.

The fractions (anti-IgG and anti-IgM) were pooled and passed over a column of human immunoglobulin-conjugated Sepharose 4B (kindly provided by Dr. P L Ey) to remove any human immunoglobulin cross-reactivity, using the same procedure as above, except that the unbound material from the column was pooled and retained. The pooled affinity purified goat anti-mouse Ig(G+M) was dialysed against 100 volumes of

PBS/Az, adjusted to 1 mg/ml and stored as 100 μ l aliquots at -20°C for use in the Rose Bengal assay, or conjugated with I¹²⁵ (see section 2.4) or FITC (see section 2.5) for use in RIA or indirect immunofluorescence assays respectively.

2.2.4 Labelling of antibodies with I¹²⁵.

The method of Salacinski *et al*, 1981 was essentially used. An Iodogen bead (Pierce Chemicals) was placed in the bottom of a polypropylene vial (Eppendorf, W.Germany). Ten μ l of carrier-free Na¹²⁵I (Amersham, England) at 100 mCi/ml and 20 μ l of 0.5 mg/ml affinity purified goat antibody to mouse immunoglobulin (see section 2.2.3) in saline was added to the vial. After 15 minutes incubation at room temperature, the reaction was quenched with 70 μ l of 0.2 mg/ml tyrosine (Sigma) in distilled water, followed by 100 μ l of PBS/BSA/Az. To separate the labelled antibodies from the free ¹²⁵I, the reaction mixture was layered onto a Sephadex G-25 column (Pharmacia) of approximately 2 ml volume and eluted at 0.3 ml/minute with PBS/BSA/Az. Fractions of 0.5 ml were collected and 10 μ l samples of each were counted in a Packard Auto Gamma model 5110 spectrometer. The ¹²⁵I eluted in two peaks, the first of which contained the labelled antibodies. Typically, 60 to 90% of the recovered radioactivity was found to be associated with the labelled antibodies. The labelled antibody was stored at 4°C in PBS containing 30% BSA as a carrier, and 0.01% Azide as a preservative.

2.2.5 Labelling of antibodies with FITC.

This method is essentially that described by Rinderknecht, 1962. Affinity purified goat antibodies to mouse immunoglobulin (see section 2.2.3) were dialysed overnight against 100 volumes of azide-free 0.1 M NaHCO₃ pH 9. The immunoglobulin concentration was adjusted to 2 mg/ml in the same buffer, and an equivalent amount (w/w) of FITC-celite (CalBiochem) was added and the mixture incubated for 2 hours in the dark at room temperature. The solution was centrifuged at 200g for 5 mins and the supernatant was run through a Sephadex G-25 column (Pharmacia) of approximately 3 ml volume at a flow rate of 0.3 ml/min, with 0.5 fractions collected. The appearance of green fluorescence in the void volume eluate corresponded to the labelled antibodies. Fractions having visible green fluorescence were pooled, and the degree of substitution was determined with the method described in Hudson and Hay, 1980;

$$\text{FITC:Protein} = \frac{2.87 \times \text{OD}_{495}}{\text{OD}_{280} - 3.5 \times \text{OD}_{495}}$$

Generally, the FITC:Protein ratio obtained was greater than 4:1.

2.3 Tissue Culture.

Water used for tissue culture solutions was de-ionised using a Milli RO60 system (Millipore Corp., USA) and further purified by passing through two beds of ion exchange resins, a carbon filter and an organic filter using a Milli Q system (Millipore).

2.3.1 General Tissue culture medium.

The contents of two sachets of RPMI 1640 powder containing added glutamine (GIBCO, USA) were dissolved in 1.5 litres of water, to which NaHCO_3 (4g) was added and the pH was adjusted to 7.4 with 1 M HCl. Sterile stock solutions of HEPES pH 7.2 (CalBiochem), Penicillin/Streptomycin (GLAXO, Australia) were added to give final concentrations of 15 mM, 500 IU/ml and 100 $\mu\text{g}/\text{ml}$ respectively and the final volume adjusted with water to 2 litres. Ninety ml aliquots were placed in sterile glass bottles after passing through a Sterivex GS filter unit with filling bell (0.22 μ pore size) (Millipore). The tissue culture medium was supplemented with 10% v/v foetal calf serum which had been heat inactivated by incubation at 56°C for 30 min. The foetal calf serum used had been batch tested for its ability to support the growth of x63 Ag8.653 myeloma cells at both high and low densities. This solution will be hereafter referred to as 'medium'. Medium stored for longer than one week was supplemented with glutamine to a final concentration of 2 mM before using.

2.3.2 IMDM: Single strength.

Iscoe's modification of Dulbecco's medium (IMDM) was made by dissolving the contents of one sachet of IMDM powder with HEPES (GIBCO, USA) in 900 ml of water, with NaHCO_3 (2g). Penicillin and Streptomycin were added as in 2.3.1, the volume adjusted to one litre, and the medium was sterile filtered and aliquotted as described above.

2.3.3 IMDM; Double strength.

Double strength IMDM (2xIMDM) was made by dissolving the contents of one sachet of IMDM powder with HEPES (GIBCO, USA) in 390 ml of water and adding 5.9 μ l 2-mercapto-ethanol, 0.2g L-asparagine (Sigma) and Penicillin/Streptomycin at double the final concentration used in section 2.3.1. The medium was sterile filtered as before, but aliquotted into 49ml volumes. Immediately before use, 0.57g NaHCO₃ was dissolved in the 49 ml aliquot, which was then sterilised using a push-through 0.22 μ filter (Millipore). Bicarbonate was not added to 2xIMDM until immediately before use, due to problems with precipitation during storage.

2.3.4 In Vitro culture of cell lines.

Cell lines were generally cultured in RPMI 1640 + 10% FCS in sterile plastic tissue culture flasks of 25 and 75 cm² growth area (Falcon, Becton Dickinson, USA. or Lux, Miles Scientific, USA). The flasks were incubated at 37° C in a fully humidified atmosphere containing 5% CO₂ in air. Cultures were maintained in a logarithmic phase of growth by subculturing, and cell densities were maintained between 10⁵ and 10⁶ cells/ml, depending on the idiosyncrasies of the individual cell lines. Exceptions to the above conditions were the lines KG-1, which was cultured in single-strength IMDM + 20% FCS, and Daudi, which was cultured in RPMI 1640 + 10% FCS without bicarbonate/carbon dioxide buffering. The pH of densely growing Daudi cultures was adjusted using sterile filtered 1 M NaOH solution.

2.3.5 Human haemopoietic cell lines.

B lymphoblastoid cell lines employed for screening of MoAb's were BALM-1 (Minowada *et al*, 1977) and the EBV transformed lines Daudi (Klein *et al*, 1968) and Cess B (Bradley *et al*, 1982). T-ALL derived lines used were Molt-4 (Minowada *et al*, 1975) and CCRF-HSB2 (Adams *et al*, 1970). A null cell line Nalm-6 (Minowada *et al*, 1978) derived from a patient with non-T non-B ALL was also used.

Myeloid cell lines used were K562, derived from a patient with CML in blast crisis (Lozzio and Lozzio, 1975), KG-1 derived from a patient with erythroleukaemia who subsequently developed AML (Koeffler and Golde, 1978), HL-60 derived from a patient with acute promyelocytic leukaemia (Collins *et al*, 1977), RC-2A derived from an individual with AMML (Bradley *et al*, 1982) and U937, a monocytoid cell line derived from a patient with histiocytic lymphoma (Sundstrom and Nilsson, 1976).

The cell lines Molt-4, CCRF-HSB2, BALM-1, Nalm-6 and Daudi were obtained from Dr. H Zola, Flinders Medical Centre, Adelaide; RC-2A, Cess B and K562 from Dr G Pilkington, The Cancer Institute, Melbourne; KG-1 from T Radloff, The Kanematsu Institute, Sydney; HL-60 from Prof. D Metcalf, The Walter and Eliza Hall Institute, Melbourne.

2.3.6 Cryopreservation of cells.

Using sterile technique, cells to be cryopreserved were suspended at between $1-2 \times 10^7$ /ml in tissue culture medium, to which an equal volume of cryoprotectant (30% FCS, 20% Dimethylsulphoxide, 50% RPMI 1640 v/v) was added dropwise while shaking. The cell suspension was placed into 1 or 2 ml plastic screw top vials (Nunc, Denmark) and

frozen in a controlled rate freezer (Paton Industries, Adelaide, South Australia). The rate of cooling was 5°/min down to 0°, 1°/min to -25° and 5°/min to -100°C. After freezing, the vials were stored in liquid nitrogen cannisters (Union Carbide).

2.3.7 Thawing of frozen cells.

The vials of frozen cells were rapidly thawed in a 37°C water bath, and the cell suspension was diluted with an equal volume of medium added dropwise over 5 min, then allowed to stand for 15 min. An equal volume of medium was again added dropwise over 5 min, and the suspension was allowed to stand for a further 15 min. The cells were pelleted by centrifugation at 200g for 5 min, resuspended in medium and likewise washed two more times. Cell counts were determined using a haemocytometer and the viability by the ability to exclude 0.2% trypan blue dye.

2.4 Isolation of human haemopoietic cells.

2.4.1 Preparation of peripheral blood mononuclear cell fraction.

Samples of normal human peripheral blood were kindly supplied by the Red Cross Blood Transfusion Service, Adelaide.

Mononuclear cells were isolated from human peripheral blood by bouyant density gradient separation based on the method of Boyum, 1968. Five ml of blood collected into lithium heparin anti-coagulant containing tubes was layered onto 4 ml of ficoll-hypaque density 1.076 (Ficoll-Paque, Pharmacia) and centrifuged for 25 minutes at 400g at room temperature. The band of cells located at the interface was

aspirated with a pasteur pipette and washed three times in medium by centrifugation at 200g for 5 min followed by resuspension in 5 ml of medium. Cytospin smears stained with Giemsa's stain (see section 2.4.7) generally revealed less than 5% contamination by polymorphonuclear cells.

2.4.2 Preparation of peripheral blood polymorphonuclear cell fraction.

The method of Ferrante and Thong, 1980 was used to separate polymorphonuclear cells from lithium-heparinized whole blood by density gradient centrifugation. Five ml of blood was layered onto 4 ml of Ficoll/Angiograffin/Urovisin density 1.114 (prepared in this laboratory) and centrifuged for 25 min at 400g. Two bands of cells resulted, the top layer containing mononuclear cells and the lower containing polymorphonuclear cells. After aspiration cells were washed as previously described in 2.4.1. To assess the purity of the preparations, cytospin/giemsa staining as described in 2.4.7 was carried out and purity of both fractions was usually greater than 90%.

2.4.3 Preparation of peripheral blood erythrocytes.

Suspensions of 0.5% erythrocytes were prepared by adding 50 μ l of the erythrocyte pellet from a sample of peripheral blood centrifuged on a 1.114 gradient (see section 2.10.2) to 10ml of PBS/BSA/Az.

2.4.4 Preparation of bone marrow mononuclear cells.

Bone marrow aspirated from the iliac crest of patients with either non-haemopoietic disease, leukaemia in remission or from normal volunteers into citrate anti-coagulant was provided by Drs. C Juttner

and L B To of the Institute of Medical and Veterinary Science, Adelaide.

Bone marrow was mixed with sterile 0.6 % Dextran 4000 in saline (Pharmacia) in a ratio of 5:1 in 50 ml conical plastic tubes (Falcon) and allowed to stand undisturbed at 37°C for 1 hr. The mononuclear cell-rich layer of serum was aspirated using a sterile pasteur pipette and washed in medium by centrifugation and resuspension.

2.4.5 Preparation of bone marrow nucleated cells.

Nucleated cells were isolated by centrifugation of bone marrow on a 1.114 gradient as described in section 2.4.2, except that the two bands were pooled.

2.4.6 Preparation of cord blood mononuclear cells.

Cord blood was obtained from Dr I Kowanko (The Adelaide Childrens Hospital) and Dr J Gamble (Institute of Medical and Veterinary Science, Adelaide) and the mononuclear fraction isolated by gradient centrifugation (see section 2.4.1) and samples were cryopreserved and stored in liquid nitrogen (see section 2.3.6).

2.4.7 Determination of cell morphology.

Cell smears were made using a cyto-centrifuge (Shandon Southern) by centrifuging 5×10^4 of the haemopoietic cells under investigation in neat FCS onto a glass microscope slide. The cell smears were air dried, fixed for 30 sec in ethanol, air dried, then stained with Giemsa's stain (BDH, England) diluted 1/25 in Giemsa buffer (see section 2.1) for 10 minutes. Excess stain was washed from smears with

Giemsa buffer. The smears were mounted in D.P.X (Gurr) and overlaid with a coverslip. An Olympus microscope (Model BH2) was used to examine cells and identify them according to their morphology.

2.5 Immunoassay of MoAb binding to haemopoietic cells.

2.5.1 Indirect Immunofluorescence assay of MoAb binding to haemopoietic cells.

This assay is based on the RIA of Williams *et al*, 1977, except that FITC-labelled anti-mouse immunoglobulin detecting reagent is substituted for a radiolabelled reagent. Target cells obtained from frozen stocks (see section 2.17, 2.18), direct from culture (see section 2.8) or purified from peripheral blood (see section 2.10) were washed by centrifugation and resuspension in PBS/BSA/Az, and finally resuspended at a density of 5×10^6 cells per ml in PBS/BSA/Az supplemented with 10% heat inactivated normal rabbit serum. Azide was included to prevent capping and endocytosis or shedding of any antibody-bound antigens, and normal rabbit serum was included to prevent any non-specific binding of antibodies via Fc receptors on target cells. One hundred μ l of target cell suspension was pipetted into plastic V bottomed tubes (Sarsdet No. 57.477), followed by an equal volume of hybridoma supernatant or antibody solution, and incubated on ice for 60 min. The cells were washed by the addition of 1 ml of PBS/BSA/Az, centrifuged for 5 min at 200g, and all but approximately 25 μ l of the supernatant aspirated. The cells were resuspended, and a further 1 ml of PBS/BSA/Az was added. This washing procedure was repeated twice.

The cells were resuspended in about 25 μ l of PBS/BSA/Az and 25 μ l of a 1/10 dilution of FITC-labelled F(ab')₂ fragments of sheep anti-mouse immunoglobulin (Silenus, Australia) or an appropriate dilution of the FITC-labelled goat anti-mouse immunoglobulin reagent described in section 2.5 was added followed by incubation on ice for 45 min. The cells were washed twice as previously described and scored manually (see section 2.5.2) or by flow cytometry (see section 2.5.3).

2.5.2 Manual scoring of FITC-Sheep/Goat anti-mouse labelled cells.

Labelled cells (see section 2.5.1) were resuspended in approximately 50 μ l of PBS/BSA/Az and scored manually using an Olympus microscope (model BH2/BH-RFL-W) with epi-illumination. A minimum of 200 cells were scored per sample.

2.5.3 Flow cytometric scoring of FITC-Goat/Sheep anti-mouse labelled cells.

Labelled cells (see section 2.5.1) were fixed with 1% paraformaldehyde in PBS using the method of Lanier and Warner, 1981, and stored at 4°C in the dark for up to a week before analysis. The scoring was carried out by Mr. J Webster in the Department of Clinical Immunology at Flinders Medical Centre, Adelaide using a Becton Dickinson FACS IV. Between ten and fifty thousand cells per sample were analysed, depending on the experiment.

2.5.4 Haemagglutination assay.

Suspensions of human erythrocytes (see section 2.10.3) were incubated with 50 μ l aliquots of serial 1/2 dilutions of hybridoma

supernatants for 45 min at room temperature in flexible round bottomed 96 well plates (Linbro, No. 76-364-05). The diluent used was PBS/BSA/Az. An erythrocyte binding antibody (DA1.F9) raised in this laboratory, and Sal-2 (IgG1) were used as positive and negative controls respectively. Haemagglutination titre was determined by visual inspection.

2.6 Culture of myeloid progenitor cells.

2.6.1 Production of Lymphocyte conditioned medium (PHA-LCM).

Blood from consenting volunteers was separated on Ficoll-Hypaque Gradients (Pharmacia) and the mononuclear cells were collected, washed in medium as described in section 2.4.1. The cells were cultured at a density of 10^6 /ml in 1xIMDM (see section 2.3.2) with 10% FCS and 1% PHA-M (DIFCO) in 75 cm² tissue culture flasks (Lux, Miles Scientific, IL., USA.) for 7 days in a humidified atmosphere of 5% CO₂ in air. The supernatant was harvested and filtered through a 0.22µm membrane (Millipore), and stored at 4°C or -20°C for up to twelve months before use.

2.6.2 Culture of myeloid progenitor cells in semi-solid medium.

Bone marrow or cord blood myeloid progenitor cells (CFU-C) were cultured in semi-solid agar essentially using the method of Nakahata and Ogawa, 1982. Five $\times 10^4$ or 1×10^5 mononuclear cells/ml were cultured in 1 ml of semi-solid medium in 35 mm plastic tissue culture dishes (Kayline, Adelaide) as follows. A variety of FCS samples were batch tested for their ability to grow the largest number of colonies under

identical conditions, and a supply of the best batch was set aside and used for all assays. A solution of 0.66 % agar (Difco bacto-agar) was prepared by boiling 0.66g of agar in 100 ml of water for 5 min, after which the solution was held at approximately 40°C. This was mixed with 2xIMDM (see section 2.4.3) and FCS at a ratio of 2:1:1 (v/v/v) and the required number of bone marrow or cord blood mononuclear cells (see sections 2.4.4 and 2.4.6) added. The suspension was quickly mixed to ensure an even dispersion of cells and dispensed in 1 ml aliquots into tissue culture dishes containing PHA-LCM (see section 2.6.1) as a source of haemopoietic growth factors. The amount of PHA-LCM added was determined by titration, and the final amount chosen was based on a combination of the number and ratio of types of colonies obtained. Typically, 50-100µl per dish was used, as this level gave near to maximum numbers of colonies (50 to 100 per 5×10^4 cells plated) and also gave the best spread of colony types. The ratio of GM:G:M:Ec colonies was typically about 20:15:55:10. Colonies were defined as aggregates of greater than 40 cells, and were counted using an Olympus SZ dissecting microscope *in situ* before staining, and using an Olympus BH-2 microscope after staining (see section 2.6.3).

2.6.3 Triple stain of myeloid colonies.

Agar discs were fixed *in situ* with 1 ml of a 2.5% v/v solution of glutaraldehyde (50% stock solution, BDH) in PBS for 5 min. The fixed agar discs were washed briefly in distilled water, picked up onto a 75 x 50 mm glass slide and covered with a circle of Whatman No.1 filter paper. After drying overnight at room temperature, the filter paper was floated off by immersing slides in distilled water for 1 min.

The dried down agar slides were then stained with a three stage protocol to identify the cell types within the colonies.

α-Naphthol-acetate esterase; monocyte/macrophage specific.

The method of Lojda *et al*, 1979 was used to identify monocytes/macrophages. The substrate solution was made by adding 40 mg of Fast Garnet GBC salt (Sigma) to 50 ml of 2.8% Na_2PO_4 solution, adjusting the pH to 6.5 and then adding 1 ml of 1% w/v α -Naphthyl-acetate (Sigma) in acetone. The solution was filtered through Whatman No.1 filter paper and the dried down slides incubated in the solution at room temperature for 10 min. The slides were rinsed briefly in water and the next staining procedure was followed.

Chloroacetate esterase; neutrophil specific.

The method of Kubota *et al*, 1979 was used to identify neutrophils. The substrate solution was made by adding 20 mg of Fast Blue BB salt (Sigma), 4 mg naphthol AS-D chloroacetate (Sigma) dissolved in 2 ml of N-N'-dimethyl-formamide (Univar) to 38 ml of PBS M-15 (0.174% w/v KH_2PO_4 , 0.768% Na_2HPO_4 , pH 7.4). The solution was filtered through Whatman No. 1 filter paper and the slides were incubated for 1 hr at room temperature. The slides were rinsed in water in preparation for the next stage.

Luxol Fast Blue stain; eosinophil specific.

The method of Metcalf, 1984 was used to identify eosinophils. Slides were stained for 2 hrs in a solution of 0.15% Luxol Fast Blue MBS (Gurr) in 70% ethanol saturated with urea, then washed in running tap water for 5 min to remove urea. The slides were mounted in D.P.X (Gurr), a coverslip was applied, and colony types were assessed using an Olympus microscope model BH-2. Monocyte/macrophage cytoplasm

stained reddish-brown, neutrophil cytoplasm stained with blue granules and eosinophils exhibited yellowish-green cytoplasmic granules (see Chapter 6).

2.7 Determination of molecular weights of proteins detected by MoAb's to cell surface antigens.

The technique of Cole *et al*, 1987 was used to determine the molecular weights of protein cell surface antigens detected by MoAb's.

2.7.1 Immunoabsorption of biotinylated cell surface proteins.

Target cells (RC-2A and Cess B) were cultured as described in section 2.3.4 and harvested by centrifugation at 200g for 5 min. The cells were washed by three times by repeated centrifugation and resuspension in PBS at 4°C, and finally resuspended at 10⁸ cells/ml in PBS in a 50ml conical plastic tube (Falcon). To each 10⁸ cells, 50µl of freshly prepared 0.1 M NHSS-Biotin (BRESA, Adelaide) in PBS was added to give a final concentration of 5 mM Biotin. The reaction was allowed to proceed for 30 min at 4°C with occasional mixing.

Solubilization of cell surface membranes was achieved by the addition of 1 ml of Nonidet P40 buffer (1% NP40 (BDH), 1 mM EDTA, 50 mM Tris-HCl, 150 mM NaCl pH 8.0) and 10µl of protease inhibitor cocktail (2 mg/ml each of phenyl-methyl-sulphonyl-fluoride (PMSF), L-(-1-tosylamido-2-phenyl)-ethyl-chloromethyl-ketone (TPCK), 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) and p-nitrophenyl-p'-guanidino-benzoate-HCl (NPGb) (all Sigma) in N-N'-dimethyl-formamide (Univar)) per 10⁸ cells. The cell suspension was gently mixed and incubated on ice for 30 min. The lysed cell suspension was was

centrifuged for 10 min at 200g to remove nuclei and large cell debris. The supernatant was aspirated and centrifuged for 30 min at 100,000 and the resulting supernatant (NP40 lysate) was retained for immunoadsorption.

Protein A-Sepharose 4B (Pharmacia) was swollen in PBS for 15 min, then equilibrated in 1% NP40 buffer. The Protein A-Sepharose was 'armed' with anti-mouse immunoglobulin by addition of a 50% slurry of beads in 1% NP 40 buffer to an equal volume of affinity purified rabbit anti-mouse IgG1 (2 mg/ml, kindly provided by Dr P By in this department), and incubation for 2 hrs at 4°C. The beads were washed three times by centrifugation at 200g for 5 min and resuspension of the pellet in 1% NP 40 buffer as a 50% slurry ready for use.

Four hundred μ l of hybridoma culture supernatant was added to 1 ml of NP40 lysate in a 1.5 ml Eppendorf tube, and this mixture was incubated for 16 hrs at 4°C on a rotating mixer. After incubation, the mixture was transferred to a fresh Eppendorf tube containing 125 μ l of a 50% v/v suspension of anti-mouse immunoglobulin armed Protein A-Sepharose 4B. The tubes were incubated 1 hr at 4°C on a rotating mixer and then centrifuged for 10 sec in an Eppendorf microfuge. The supernatants were aspirated, and the pellets of Protein A-Sepharose were quickly washed 4 times by alternate resuspension of the pellets, aspiration of the supernatants and centrifugation; twice in 1% NP 40 buffer, once in 0.1% NP 40 buffer, and once in 50 mM Tris-HCl, 150 mM NaCl pH 8.0. The pellets were stored at -20°C prior to electrophoresis.

2.7.2 Polyacrylamide gel electrophoresis of immunoadsorbed cell surface proteins.

For electrophoresis, 50 μ l of SDS sample buffer (reducing, see section 2.7.3) was added to each Eppendorf tube containing Protein A-Sepharose-adsorbed MoAb/antigen complex and the tubes incubated for 3 min at 100°C. In addition, 20 μ l of NP40 lysate was incubated with 20 μ l of SDS sample buffer (reducing) to visualize total biotinylated protein. A cocktail of molecular weight markers (see section 2.7.3) was also electrophoresed on each gel to enable molecular weight estimations of bands in test tracks to be estimated (see section 2.7.4). After incubation, the Eppendorf tubes were centrifuged for 10 sec and the entire supernatant loaded onto a single track of a 7.5% polyacrylamide gel and electrophoresis carried out according to the method of Lugtenberg *et al*, 1975.

2.7.3 Polyacrylamide gel electrophoresis; reagents and method.

Acrylamide/bis-acrylamide stock solution.

A solution of total concentration of 30% acrylamide was made by dissolving 29.2 g of acrylamide (BDH) and 0.8 g of bis-acrylamide (BDH) in water to 100 ml. To remove free acrylic acid, the solution was mixed with 2 g Amberlite resin beads (BDH) and allowed to stir overnight at 4°C using a magnetic stirrer. The solution was filtered through sintered glass and stored in a the dark at room temperature.

Buffer A (separating gel).

1.5 M Tris-HCl pH 8.8 was made by adding 18.5 g of Tris-HCl to 100 ml of water and adjusting the pH to 8.8 using 1 M HCl.

Buffer B (stacking gel).

0.5 M Tris-HCl pH 6.8 was made by adding 6.6 g of Tris-HCl to 100 ml of water and adjusting the pH to 6.8 with 1 M HCl.

Electrode Buffer, pH 8.3.

3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS were dissolved in 900 ml water, the pH adjusted with 1 M HCl, and water added to a final volume of 1 litre.

Sample Buffer.

Reducing sample buffer was made by mixing 1 ml of buffer B, 0.8 ml glycerol, 1.6 ml of 10% w/v SDS, 0.4 ml β -mercaptoethanol, 0.2 ml of 0.05% w/v Bromophenol blue and 4 ml of water.

Protein standards.

A cocktail of proteins was made using equivalent weights of myosin (205 kd), β -galactosidase (116 kd), phosphorylase B (97 kd), bovine albumen (66 kd), egg albumen (45 kd) and carbonic anhydrase (29 kd) in sample buffer. Approximately 20 μ g of protein in total was applied to wells.

Separating Gel.

The main resolving gel (7.5 %) was made by mixing 12.2 ml of water, 6.25 ml of buffer A and 6.25 ml of acrylamide/bis-acrylamide solution, de-gassing using a Buchner flask connected to a vacuum line, and addition of 0.25 ml of a 10% w/v SDS solution, 100 μ l of a 10% w/v ammonium persulphate solution and 20 μ l of TEMED. This solution was placed in a gel former of 150 mm x 150 mm x 1.5 mm and then overlaid carefully with 2 ml of water to exclude air and then allowed to set for 1 hr.

Stacking Gel.

The stacking gel (4%) was made by mixing 6.1 ml of water, 2.5 ml of buffer B and 1.3 ml of acrylamide/bis-acrylamide, which was degassed, and 0.1 ml of 10% w/v SDS, 50 μ l of 10% w/v ammonium persulphate and 8 μ l TEMED was then added. This solution was pipetted into the gel former after aspirating the overlay, and a 20 well comb was inserted into the top of the stacking gel.

Running of samples.

After 30 min, the comb was removed and the gel placed in a vertical gel box. Electrode buffer was placed in both upper and lower reservoirs. Samples were placed in wells using a micro-syringe (Hamilton, USA). A potential difference of 60 V was applied until the bromophenol blue dye front entered the main gel, at which time the voltage was increased to 90 V until the dye front reached about 3-5 mm from the end of the main gel.

2.7.4 Western transfer onto nitrocellulose of proteins separated by electrophoresis.

Separated proteins were transferred electrophoretically onto nitrocellulose paper (Hibond C, Amersham) using the method of Towbin *et al*, 1979. The main separating polyacrylamide gel was carefully removed and placed on a Scotch Brite scouring pad. A sheet of nitrocellulose which had been pre-soaked in distilled water was overlaid carefully on top of the gel taking care to exclude air bubbles. One corner of the sheet was marked for registration purposes. A second Scotch Brite pad was placed over the nitrocellulose, and the 'sandwich' was placed between two perforated plastic supports and

secured with rubber bands. This assembly was placed into an electrophoresis transfer box, which was filled with transfer buffer consisting of 25 mM Tris-HCl pH 8.3, 192 mM glycine and 20% methanol. The protein in the gel was transferred electrophoretically to the nitrocellulose paper by applying a potential difference of 90 V for 16 hr at 4°C.

After transfer, the section of nitrocellulose paper containing molecular weight standards and one track of total biotinylated protein was stained with 0.1% amido black in 5:5:1 methanol/water/acetic acid for 5 min and destained in several changes of the same solvent. The remainder of the gel containing the test antibody tracks and a second total biotinylated total protein track was blocked in 100 ml of 3% w/v BSA in PBS/Az for one hour at room temperature with gentle mixing.

2.7.5 Detection of biotin-labelled protein on nitrocellulose paper.

The biotin labelled proteins bound to the nitrocellulose paper were detected enzymically as follows. The blocked sheet of nitrocellulose was washed four times for 5 min in PBS/0.1% Tween 20, and then once in PBS for 5 min. The sheet was then incubated for 30 min in 10 ml of a 1/400 dilution of Streptavidin-biotinylated horseradish peroxidase complex (Amersham, No. RPN 1051) in PBS. The sheet was washed with 4 x with PBS/0.1% Tween 20 and once with PBS as previously described. The sheet was incubated for approximately 10 min in a solution of 7.5 mg diaminobenzidine (Sigma) and 9µl H₂O₂ (30% v/v solution) in 15 ml of PBS to detect peroxidase activity, which appeared as brown staining.

2.8 Immunocytochemistry.

2.8.1 Immunoperoxidase detection of MoAb binding.

The binding of monoclonal antibodies to smears of RC-2A cells prepared by cytocentrifugation of cells onto glass slides (see section 2.4.7), and to cryostat sections of normal human tonsil and small intestine (kindly supplied by Mr. L Spargo) was assayed as described by Mayrhofer *et al*, 1987.

Blocking of endogenous peroxidase;

The blocking methods used were derived from the methods described by Streefkerk, 1972 and Weir *et al*, 1974 respectively.

A. Acid ethanol.

Slides were incubated in 96% v/v ethanol containing 0.074% HCl for 10 minutes at 4°C before fixation.

B. Methanol/hydrogen peroxide.

Slides were incubated in absolute methanol containing 0.5% v/v H₂O₂ for 10 min at 4°C prior to fixation.

Primary antibody.

Hybridoma supernatants to be assayed were ultra-centrifuged at 100,000g for 10 min to remove aggregates and supplemented with 10% heat inactivated normal human serum.

Peroxidase conjugated anti-mouse immunoglobulin.

Affinity purified sheep anti-mouse immunoglobulin conjugated with horseradish peroxidase was kindly supplied by Dr. G Mayrhofer and used undiluted, supplemented with 10% normal human serum.

Peroxidase substrate/staining solution.

Five mg of diaminobenzidine (Sigma) was dissolved in 10 ml of 0.05 M Tris-HCl pH 7.6, then 200 μ l of 1%v/v H₂O₂ was added. This solution was made up immediately before use and filtered onto the slides through a push-through 0.22 μ filter (Millipore, USA).

Blocked or unblocked slides were fixed in 96% v/v ethanol at 4°C for 10 min and then washed in three changes of PBS over 5 min. Fifty μ l of test supernatant was applied to smears/sections and slides were incubated in a humid chamber at 4°C for 1 hr. Slides were washed in PBS as previously, and 20 μ l of peroxidase conjugate was applied, followed by a further 1 hr incubation. After washing as before, substrate/staining solution was filtered onto the slides and they were incubated at room temperature for 10 min. Slides were washed in water and counterstained with haematoxylin for 2 min, followed by another wash in water. Smears were air dried, mounted in D.P.X. (Gurr) and coverslips applied. Sections were dehydrated by sequential 5 min baths in 25%, 50% and 96% v/v ethanol, followed by a bath in HistoClear and immediately mounted with D.P.X. and coverslips applied. Antibody binding was demonstrated by brown staining.

2.8.2 Alkaline phosphatase anti-alkaline phosphatase (APAAP) detection of MoAb binding.

The method of Erber *et al*, was used to investigate the binding of monoclonal antibodies to smears of bone marrow and peripheral blood nucleated cells harvested as in sections 2.4.1, 2.4.2 and 2.4.5, and cytocentrifuged onto glass slides as described in section 2.4.7.

Buffered formol acetone fixative.

The fixative was made by dissolving 10 mg of Na_2HPO_4 , 100 mg KH_2PO_4 in 30 ml of water, to which was added 45 ml acetone and 25 ml formalin.

Tris-buffered saline (TBS).

Normal saline (see section 2.1) was buffered using 0.05 M Tris-HCl and the pH adjusted to 7.6 using 1 M HCl.

Sheep anti-mouse immunoglobulin.

Silenus sheep anti-mouse immunoglobulin (F(ab')₂ fragments, affinity purified, Silenus) reagent was diluted 1/50 in TBS before use.

Alkaline phosphatase developer.

To a solution of 49 ml of 0.1 M Tris-HCl pH 8.2 was added 25 mg of naphthol AS-MX phosphate (Sigma) dissolved in N-N'-dimethylformamide (Univar). Into this solution was dissolved 12 mg of levamisole (Sigma) and 50 mg of Fast Red TR salt (Sigma). This solution was used immediately after preparation.

Preparation of APAAP complex.

An aliquot of 10 μ l of an anti-alkaline phosphatase monoclonal antibody (Ascites, kindly provided by Mr. F Stomski, Institute of Medical and Veterinary Science, Adelaide.) was diluted 1/500 in 5 ml TBS and mixed with 21.5 μ l of alkaline phosphatase (type VII-N, Sigma No. P2276). Before use, this stock solution was diluted 1/4 with TBS.

Smears on slides were circled with a diamond pencil to stop solutions from running. Slides were fixed in buffered formol acetone for 30 sec, rinsed once in distilled water and washed twice in TBS.

Hybridoma supernatants, which had been ultracentrifuged for 10 min at 100,000g to remove aggregates, were diluted 1/2 in PBS/BSA/Az and aliquots of 50 μ l placed on smears, followed by incubation at room temperature for 30 min in a humid chamber to ensure that the smears did not dry at any stage. The slides were washed in three changes of TBS over 3 min then anti-mouse immunoglobulin was applied and they were incubated for a further 30 min as before, followed by three washes as previously described. The slides were then incubated with APAAP complex for 30 min and washed as before. The slides were incubated with two further cycles of anti-mouse immunoglobulin/wash/APAAP complex/wash, with incubations of 10 min duration. After the final wash, the slides were incubated for 15 min in the alkaline phosphatase developer, washed in running water, counterstained with haematoxylin for 2 min and mounted in glycerol/glycine mountant. Cells exhibiting antibody binding were stained bright reddish-pink.

CHAPTER 3

Studies on the differentiation of the human myelomonocytic cell
line RC-2A in response to lymphocyte derived factors.

3.1 Introduction.

Acute myelomonocytic leukaemia, in common with other acute non-lymphocytic leukaemias, is usually thought of as a disease arising from the inability of clonally derived myeloid progenitors to differentiate to mature progeny, while retaining the ability to proliferate, resulting in the accumulation of immature cells and suppression of normal myelopoiesis. Nevertheless, the differentiation arrest is not absolute, as samples of blasts from patients with acute non-lymphocytic leukaemia can be induced to differentiate *in vitro* (see section 1.2).

Similarly, cell lines derived from human myeloid leukaemias, such as HL-60 [Gallagher *et al*, 1979] KG-1 [Koeffler and Golde, 1978] U937 [Sundstrom and Nilsson, 1976] and K562 [Lozzio and Lozzio, 1975] can be induced to differentiate *in vitro* and have been useful in the study of the differentiation process, and in elucidating cell/factor interactions. Such investigations have involved the use of chemical inducers or biologically derived materials [Gallagher *et al*, 1979; Koeffler and Golde, 1978; Sundstrom and Nilsson, 1976; Koren *et al*, 1979; Lozzio and Lozzio, 1975; Lozzio and Lozzio, 1979; Hoffman *et al*, 1979] (See section 1.2).

The human myelomonocytic cell line RC-2A was isolated from a sample of peripheral blood from a patient with acute myelomonocytic leukaemia in relapse [Bradley *et al*, 1982]. The original cell line, RC-2, was initially dependant on colony stimulating factor (CSF), but after further culture became autonomous (RC-2A) and no longer required a source of CSF for growth, while still resembling the original line in all other respects. The predominant cell type in the culture population is the myelomonocyte, with some more mature forms arising from limited spontaneous differentiation.

Few studies of induction of differentiation of this line have been carried out [eg. Ralph *et al*, 1982].

Many biologically active molecules, such as colony stimulating factors and interleukins, are contained in the supernatant of human peripheral blood mononuclear cells which have been stimulated by mitogens, for example, phytohaemagglutinin [Price *et al*, 1975]. PHA-LCM has been used by a number of groups to induce differentiation in myeloid cell lines such as HL-60 [eg. Dayton *et al*, 1980]. Unlike other inducers of differentiation, such as Phorbol Esters and Dimethyl Sulphoxide, PHA-LCM is not toxic to cells. In this chapter, the effect of PHA-LCM and some recombinant human growth factors on differentiation, proliferation clonogenic potential of RC-2A cells is examined.

3.2 Materials and Methods.

3.2.1 Cell line.

The cell line RC-2A was obtained from Glenn Pilkington, The Cancer Institute, Melbourne, Australia. The cells were seeded at 2×10^5 cells/ml and were maintained in suspension culture as described in section 2.3.4 with subculture every 3-4 days to maintain a cell density between 2×10^5 /ml.

3.2.2 Preparation of PHA-LCM.

PHA-LCM was prepared as described in section 2.6.1. Except where otherwise stated, the same batch of PHA-LCM was used for the following experiments. This batch was chosen because it induced more complete differentiation of RC-2A cells than other batches, based on cytochemistry and morphology. This batch also stimulated the formation of myeloid

colonies from bone marrow cells cultured in semi-solid agar (see section 2.6), indicating that this preparation contained colony stimulating factors.

3.2.3 Induction of differentiation of RC-2A cells with PHA-LCM.

RC-2A cells were seeded at 2×10^5 cells/ml in RPMI 1640 medium with 10% FCS containing 20% PHA-LCM into 250ml tissue culture flasks (LUX, Miles Scientific, or Falcon). Cultures were re-fed on day 4 and day 6-8, depending on the growth rate. Cells were harvested at various times up to day 12, depending on the experiment, taking care to dislodge adherent cells by vigorous tapping of the culture flasks.

3.2.4 Culture of RC-2A cells in the presence of purified/cloned human growth factors.

The following human growth factors, all kind gifts of Prof. MA Vadas, Department of Human Immunology, Institute of Medical and Veterinary Science, were used;

GM-CSF (recombinant, purified)

G-CSF (recombinant, Cos cell supernatant)

TNF- α (recombinant, purified)

γ -IFN (recombinant, purified)

Unless otherwise stated, the four growth factors were used at a final concentration of 40 ng/ml, 100 μ l/ml, 200 U/ml and 200 U/ml respectively. These values have been shown by other workers to be optimal for *in vitro* effects [eg. Koefler *et al*, 1984; Trinchieri *et al*, 1986; A. Lopez, pers. comm.].

One ml cultures of RC-2A cells were seeded at 2×10^5 cells/ml in 24 well plates (Costar). Cultures were supplemented with GM-CSF, G-CSF, TNF- α and γ -IFN as detailed above. In addition, control cultures containing no

growth factors, or containing 20% v/v PHA-LCM (see section 2.6.1) were initiated. Cultures were re-fed on day 4 using one ml of medium supplemented with the relevant growth factors to maintain a constant concentration. Cells were harvested using a sterile pasteur pipette on day 7, taking care to dislodge adherent cells, and assayed for their α -Naphthol-Butyrate esterase activity and their morphology (see section 3.2.5) and their ability to form colonies in semi-solid medium (see sections 3.2.7, 3.2.8.). RC-2A cultures of a larger size were treated with γ -IFN and TNF- α to assess surface marker effects, and were set up as described in section 3.2.6.

3.2.5 Cytochemical stains.

Cell smears were made by cytocentrifuging 10^4 cells onto a glass slide using a Shandon Cytospin as described in section 2.16. Smears were subsequently fixed for 30 seconds with methanol, stained for morphological characterisation with Geimsa's stain (see section 2.1), or fixed in esterase fixative ($1.5 \times 10^{-3} M$ Na_2HPO_4 , $7.5 \times 10^{-3} M$ KH_2PO_4 , 45% Acetone, 7.5% Formaldehyde in water) for cytochemical staining.

Smears were stained for α -Naphthol Butyrate Esterase activity using a modification of the method of Li *et al*, 1973. Three hundred μ l of 1% pararosaniline dye (Sigma) in 2N HCL, was hexadiazotized by adding an equal volume of freshly made 4% w/v sodium sulphite and allowing the reaction to proceed at room temperature for 1 min. The hexadiazotized pararosaniline dye was placed in 38 ml of 0.15 M phosphate buffer, pH 6.3, to which 50 μ l of α -Naphthol Butyrate (Sigma) dissolved in 2' Ethoxy-Ethanol (BDH) had been added. Fixed smears were incubated for one hour at room temperature, washed in water, and counterstained with 0.1% w/v methyl green (BDH) in PBS. Alpha-Naphthol Butyrate Esterase activity was indicated by diffuse deposition of red/brown dye in the cytoplasm.

Acid Phosphatase activity was determined using a modification of the method of Burstone, 1962. Ten μg of Naphthol AS-MX Phosphate (SIGMA) was dissolved in 500 μl of N-N'-Dimethyl Formamide (Univar) and added to 38 ml of 0.1 M Acetate buffer, pH 5.2, into which 50 μg of Fast Blue BB dye (Sigma) was added. Smears were incubated for 90 minutes at 37°C, and counterstained with 0.1% eosin in PBS. Lysosomal Acid Phosphatase activity was detected by the appearance of blue granules in the cytoplasm.

3.2.6 Indirect immunofluorescence assay of surface marker expression.

The monoclonal antibodies FMC-14 (IgG1) (binding to Ia antigen) [Zola *et al*, 1983] (a kind gift of Dr H Zola, Flinders Medical Centre, Adelaide), anti-Mo1 (IgM) (CD11b) (Australian Monoclonal Developments, or a kind gift of Dr G Burns, Institute of Medical and Veterinary Science, Adelaide) and anti-Mo2 (IgM) (CD14) (Coulter Clone, Florida) [Todd *et al*, 1981] were tested for their ability to bind to RC-2A cells which had been cultured with PHA-LCM for zero, 3, 6 and 8 days, by indirect immunofluorescence with analysis by flow cytometry, as described in section 2.5. Culture were set up in a staggered fashion, such that cells from all time points were assayed at the same time.

Large scale cultures of RC-2A cells were set up to investigate the effects of TNF- α and γ -IFN on surface marker expression by RC-2A cells. Two of three flasks (75 cm² Lux, Miles Scientific) containing 30 ml of RC-2A cells at 2×10^5 cells/ml were supplemented with 200 U/ml of TNF- α or γ -IFN, and one left as a control. Cultures were re-fed on day 4 with 30 ml of medium containing the same concentration of the relevant growth factor, and then harvested on day 7. The cells were assayed for their binding of anti-Mo1, anti-Mo2 and FMC-14 by indirect immunofluorescence with scoring by flow cytometry.

Briefly, approximately 2-5 μg of each monoclonal antibody was allowed to bind to 5×10^5 RC-2A cells in RPMI 1640, in a total volume of 200 μl , containing 10% FCS, and 10% normal rabbit serum to block F_c binding, for 1 hour on ice. The normal rabbit serum was omitted in some experiments to investigate F_c binding changes. The antibodies Sal-2 (IgG1), Sal-3 (IgM) and Sal-5 (IgG2a) were used as negative controls and to investigate F_c binding. Cells were washed three times with phosphate buffered normal saline, pH 7.4 containing 0.1% BSA and 0.01% azide. Cells labeled with primary antibody were incubated for 1 hour with affinity purified sheep $F(ab')_2$ anti-mouse immunoglobulin conjugated with FITC (Silenus, Australia), and washed twice as before. The cells were then analysed using a Becton Dickinson FACS IV. Ten or 25 thousand cells were analysed per sample.

3.2.7 Optimization of growth of RC-2A cells in semi-solid agar.

RC-2A cells were seeded at a density of 3×10^3 cells per 1 ml tissue culture dish (Kayline, Adelaide) in semi-solid medium. The medium was made by mixing 2 volumes of 0.8% agar (w/v Difco Bacto agar in water, dissolved at 100°C and held at 45°C), 1 volume of FCS (Flow) and 1 volume of 2xIMDM (see section 2.7.3). The final concentration of agar of 0.4% was chosen as RC-2A colonies in the more usual agar concentration of 0.33% tended to be very disperse and difficult to count. The dishes containing RC-2A cells were supplemented with zero, 25, 50, 100, and 200 μl of conditioned medium from exponentially growing cultures of RC-2A cells. This conditioned medium was harvested, sterile filtered using a 0.22M push through filter (Millipore) and used on the same day. Dishes were cultured for 14 days in a humidified atmosphere of 5% CO_2 in air, fixed for 10 minutes with 1% glutaraldehyde in water, dried onto 76 x 50 mm slides and stained with haematoxylin. Colonies of greater than 40 cells were

enumerated under 5X magnification using a back-lit bacterial colony counter. Table 3.1 shows the titration of RC-2A conditioned medium. Henceforth, 100 μ l of the conditioned medium was used to ensure optimum growth of RC-2A colonies.

3.2.8 Clonal Assay of Cellular Proliferative Potential.

RC-2A cells were cultured in the presence of PHA-LCM in a staggered fashion, such that cells harvested on the final day had been exposed to PHA-LCM for zero, 5 or 9 days. During this culture period, cells were re-fed to ensure cultures did not overgrow. RC-2A cells were cultured with recombinant growth factors for 7 days as described in section 3.2.4.

Harvested cells were seeded at densities of 5×10^3 and/or 2×10^4 viable cells/ml in IMDM/0.4% agar (see section 3.2.6), then dispensed into plates immediately to prevent premature gelling. Agar was used at a final concentration of 0.4% instead of the more usual 0.3% to prevent dispersal of RC-2A colonies. Five dishes were plated for each time sample and supplemented with 100 μ l of sterile filtered RC-2A culture supernatant, which was found to be necessary for RC-2A to grow at such low cell densities, and plates were incubated and scored as previously described (see section 3.2.7).

3.2.9 Ability of purified/cloned growth factors to replace the requirement of RC-2A cells for RC-2A-Conditioned Medium for the formation of colonies in semi-solid agar.

Triplicate cultures of 2×10^3 RC-2A cells/ml in semi-solid agar were set up as described in section 3.2.6. Cultures were supplemented with PHA-LCM (100 μ l/ml), GM-CSF (100 μ l/ml), G-CSF (20ng/ml), TNF- α (100 U/ml), γ -IFN (100 U/ml) or left unsupplemented. Plates were cultured and scored as described in section 3.2.7.

Table 3.1

Ability of RC-2A conditioned medium to support the growth of RC-2A colonies (>40 cells) in semi-solid agar.

Dilution of RC-2A-CM	Colonies/3 x 10 ⁵ cells
none	some clusters of 4-8 cells
1/40	18±7.2
1/20	23±1.5
1/10	27±3.2
1/5	15±2.5

Results are averages ± Standard Error of the Mean of triplicate assays.

3.2.10 One-way mixed leukocyte culture.

Peripheral blood mononuclear cells were obtained from healthy donors as described in section 2.4.1. Cultures were set up essentially as previously described [O'Keefe and Ashman, 1982]. Triplicate cultures of responder mononuclear cells and RC-2A cells that had been induced to differentiate for 8 days, as well as control RC-2A cells, were set up as follows. RC-2A cells were washed three times by centrifugation (200g for 5 min) to ensure that no carry-over of residual PHA occurred. Cells used as stimulators had previously been treated with 20 µg of Mitomycin C (Sigma) per 2×10^5 cells/ml at 37°C for 1 hour, washed, and resuspended at the appropriate density in RPMI 1640/10% FCS. Cultures of 2×10^5 responder cells with 2×10^3 to 2×10^5 stimulator (RC-2A) cells in 0.2 ml of RPMI 1640/10% FCS with or without the addition of 1 µg/ml of Indomethacin (Merck), in 96 well tissue culture plates (Linbro, VA, USA Cat. No.76-032-05) were incubated at 37°C in humidified 7.5% CO₂, 7.5% O₂ in N₂ for 5 days. The cultures were pulsed with 1 µCi per well of ³H-Thymidine (Amersham, England. TRK.120, 25 Ci/mmol) 18 hours before harvesting with a Titertek cell harvester. Filter disks were placed in 2 ml of Beckman Ready Solve EP scintillation fluid and counted using a Beckman LS 7500 scintillation counter.

3.2.11 Production of a polyclonal murine antiserum to RC-2A cells

Two BALB/c mice were immunized twice by the intra-peritoneal route with 10^7 RC-2A cells in PBS, with a 3 week interval between injections. A final injection of 10^7 RC-2A cells was given via the tail vein, and the mice were bled from the retro-orbital complex under ether anaesthesia. The blood was allowed to clot and the serum was carefully removed with a pasteur pipette and centrifuged at 200g for 5 min to remove red blood cells. Two ml of serum was adsorbed to the autologous B cell line Cess B

[Bradley *et al*, 1982] by sequential incubation with 10^7 Cess B cells until all activity to the cells had been removed, as judged by indirect immunofluorescence assay scored manually (see section 2.5). The adsorbed serum retained activity to RC-2A cells, as assessed by indirect immunofluorescence.

3.2.12 Effect of adsorbed anti-RC-2A serum on ^3H -Thymidine uptake by RC-2A cells.

Fifty μl serial 1/2 dilutions, starting at 1/2.5, of anti-RC-2A serum in medium were placed in triplicate wells of a 96 well tissue culture tray (Linbro, round-bottomed), followed by 50 μl of a 5×10^5 cells/ml suspension of RC-2A cells in medium per well, such that there were 2.5×10^5 cells per well containing triplicate serial 1/2 dilutions of anti-serum from 1/5 down to 1/320. As a control, normal mouse serum was serially diluted and seeded with RC-2A cells in triplicate in the same way. The cultures were incubated in a fully humidified atmosphere of 5% CO_2 in air at 37°C for 3 days. The wells were pulsed for 18 hr with 1 μCi of ^3H -Thymidine then harvested and counted as described in section 3.2.10.

3.2.13 Effect of adsorbed anti-RC-2A serum on bone marrow myeloid progenitor cells.

The ability of adsorbed anti-RC-2A serum to stimulate the formation of colonies in cultures of bone marrow mononuclear cells was carried out as follows. Quadruplicate plates containing 5×10^4 bone marrow mononuclear cells in 1 ml of semi-solid agar were prepared as described in section 2.14, and supplemented with either 50 μl of a 1/4 dilution of anti-RC-2A (see section 3.2.10), 50 μl of a 1/4 dilution of normal mouse serum, 50 μl of PHA-LCM (see section 2.6.1) or 50 μl of medium as a negative control. Cultures were incubated for 14 days, fixed and mounted on slides as

described in section 2.6. The slides were stained for Acid phosphatase activity as described in section 3.2.5 to enable the visualization of macrophage colonies, then counterstained lightly with haemotoxylin.

3.3 Results.

3.3.1 Growth characteristics of RC-2A cells.

The growth of RC-2A cells with and without 20% PHA-LCM was investigated over a 12 day period by sampling at regular intervals and performing viable cell counts using trypan blue exclusion. At appropriate intervals, cultures were split with RPMI 1640/10% FCS ± PHA-LCM to maintain a cell density between $2-10 \times 10^5$. It was found that control cultures grew exponentially with a doubling time of 48 hours and viability of over 90%, as determined by trypan blue exclusion. Initially, cultures containing PHA-LCM grew exponentially with a similar doubling time, but from day 6 onwards showed a progressive decline in growth rate, culminating in a slow decrease in cell number (Figure 3.1). The viability of these cultures was comparable with control cultures until day 10-12, at which time it dropped slightly to 80-85%.

3.3.2 Effect of differentiation inducers on α -Naphthol-Butyrate esterase and Acid phosphatase expression by RC-2A cells.

Alpha-Naphthol Butyrate Esterase expression by PHA-LCM induced RC-2A cells progressively increased in intensity over time. Figure 3.2 shows a time course experiment over five days, in which esterase activity was scored as negative or very weak, moderate, and strong. Intensity of staining was assessed subjectively, however scoring was performed blind on smears of duplicate cultures. After about 4 days induction, esterase expression rose from 20% to 100% of cells. By day 8, virtually all cells

Figure 3.1

Growth curves of RC-2A cells cultured with and without PHA-LCM. Cultures of RC-2A cells were seeded at 2×10^5 /ml into medium with 20% PHA-LCM (○) and without PHA-LCM (●). Cultures were split as appropriate (dashed vertical lines) and viable counts performed using trypan blue exclusion, over a twelve day period.

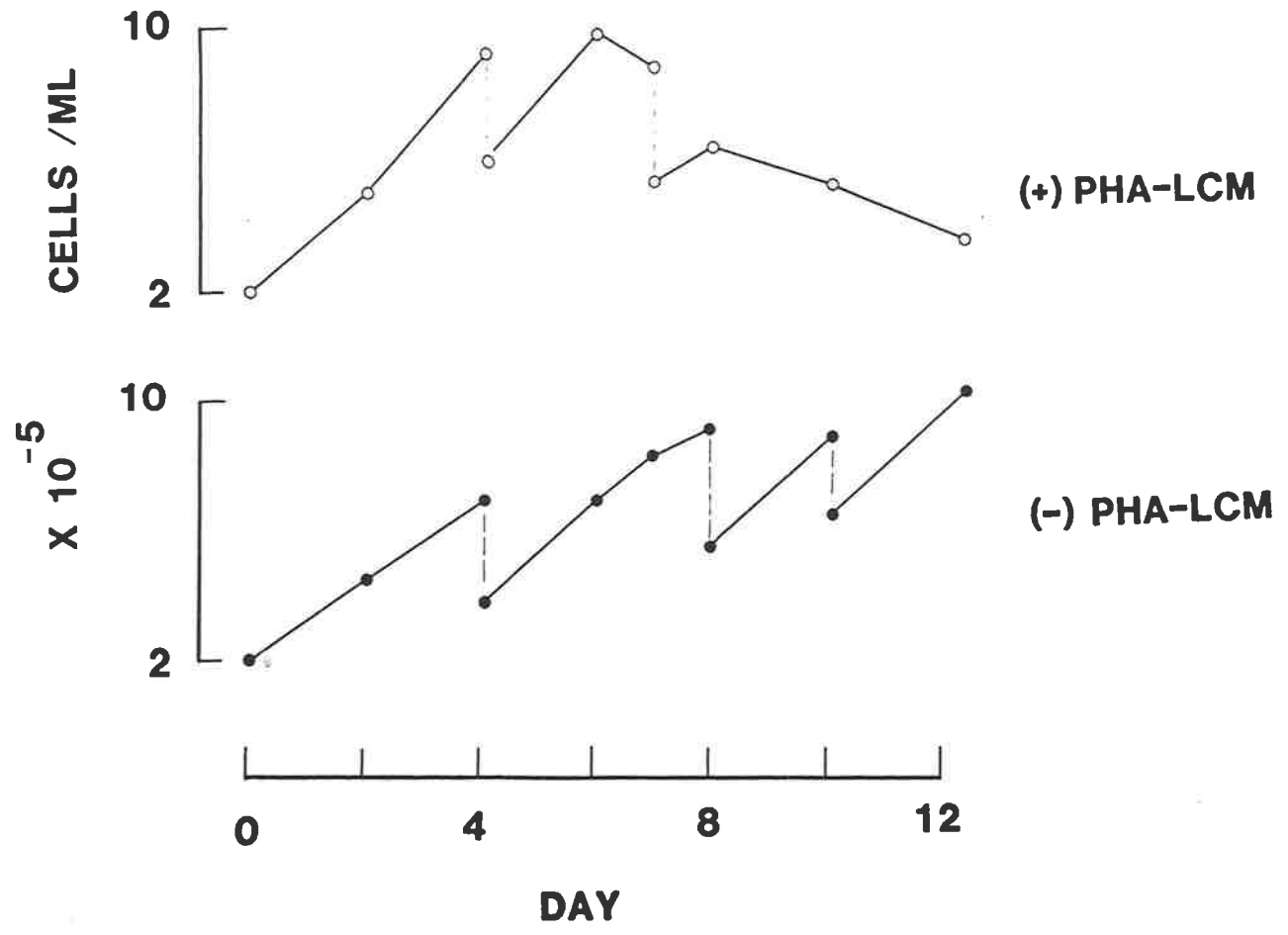
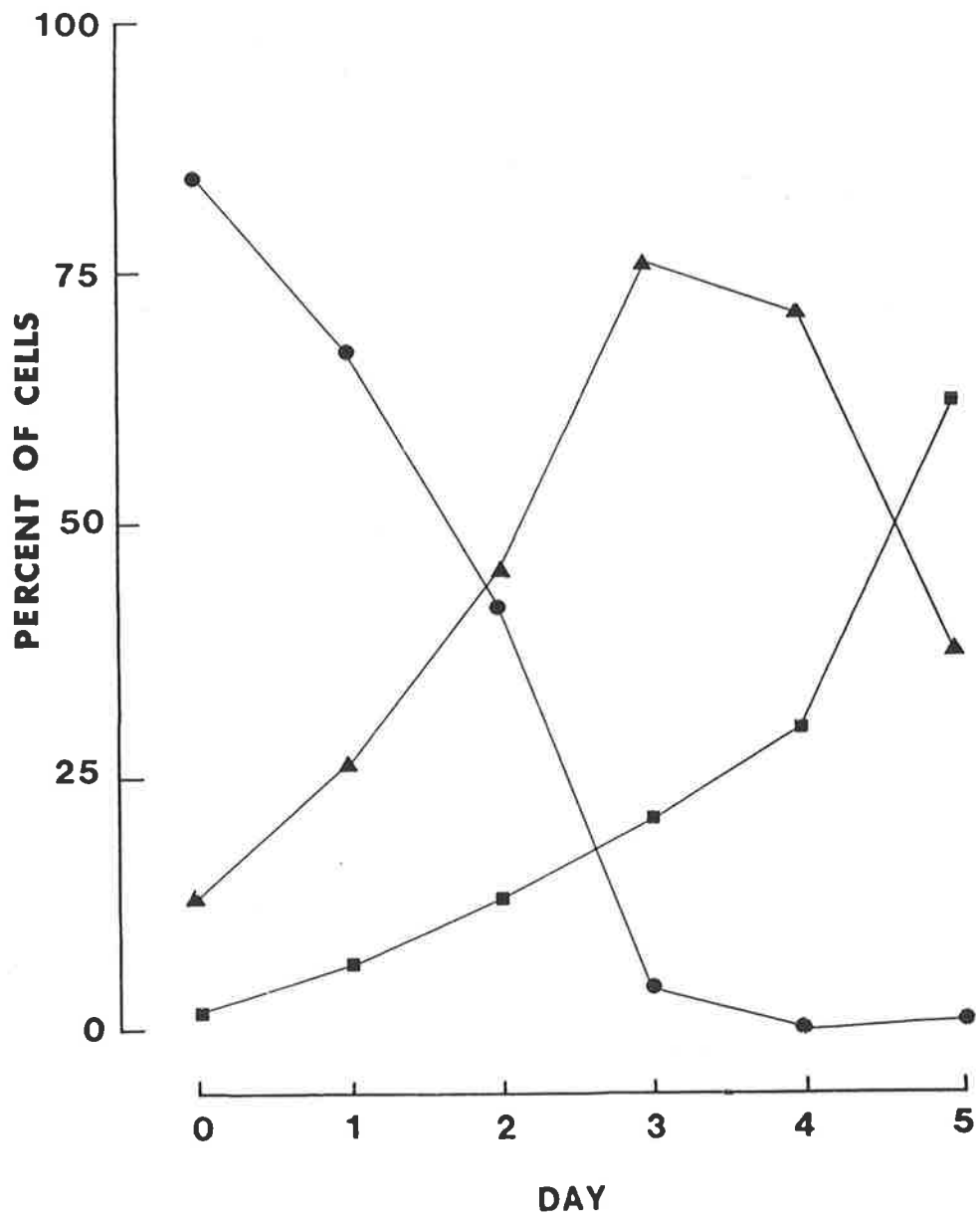


Figure 3.2

Duplicate cultures of RC-2A cells were seeded at 2×10^5 /ml into medium containing 20% PHA-LCM. Samples were removed daily and stained for α -Naphthol Butyrate Esterase activity. Cell viabilities were above 90% by trypan blue exclusion. Cells were scored negative or low (●), moderate (▲) or strong expression (■). Three hundred cells were counted for each time period.



were expressing the enzyme strongly (Figure 3.3A). Expression of the lysosomal marker Acid Phosphatase increased from very light scattered staining to multiple intensely stained granules after 8 days of induction (Figure 3.3C). This enzyme activity was slower to appear than the esterase.

None of the purified/cloned growth factors altered the expression of α -Naphthol-Butyrate esterase expression by RC-2A cells except γ -IFN, which caused a marked increase in expression of the enzyme to at least the level induced by PHA-LCM over the same period of time (data not shown).

3.3.3 Effect of differentiation inducers on morphology of RC-2A cells.

After induction of differentiation, RC-2A cells became more adherent to plastic and were larger in size (subsequently confirmed by flow cytometry, see below). Figure 3.4 shows RC-2A cells cultured in the presence of PHA-LCM for 7 days compared with control cells. Photographs were taken of cells growing in plastic flasks through an Olympus inverted microscope. Metabolic activity appeared to be increased in PHA-LCM treated cells, as the phenol red indicator demonstrated a slight change to orange in these cultures. In separate experiments prolonged, culture in the presence of PHA-LCM resulted in marked acidification compared with control cultures set up at the same time. Smears of PHA-LCM treated cells stained with Geimsa's stain demonstrated increased vacuolation and some multinucleation (Figure 3.3B).

RC-2A cells cultured with γ -IFN also exhibited greater adherence to plastic, and in addition caused accelerated yellowing of tissue culture medium when the culture time without re-feeding was prolonged. None of the other growth factors tested appeared to affect the morphology of RC-2A cells.

Figure 3.3 A

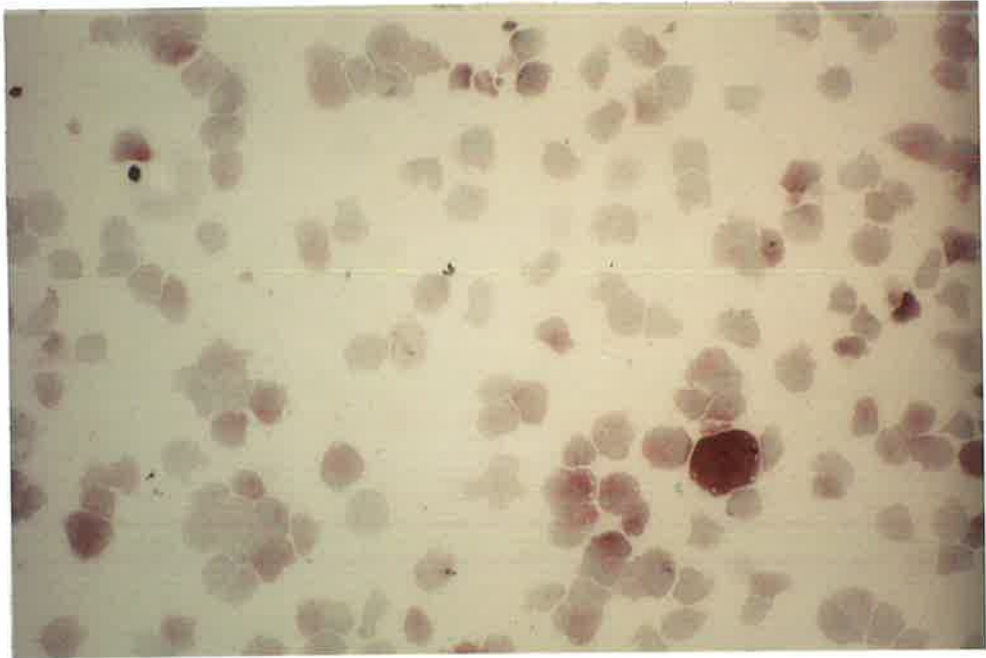
Photographs of RC-2A cells cultured with or without 20% PHA-
LCM for 8 days. Cell viabilities ranged between 85-95%.

α -Naphthol Butyrate Esterase stain.

A: no LCM x500

B: + LCM x500

A



B

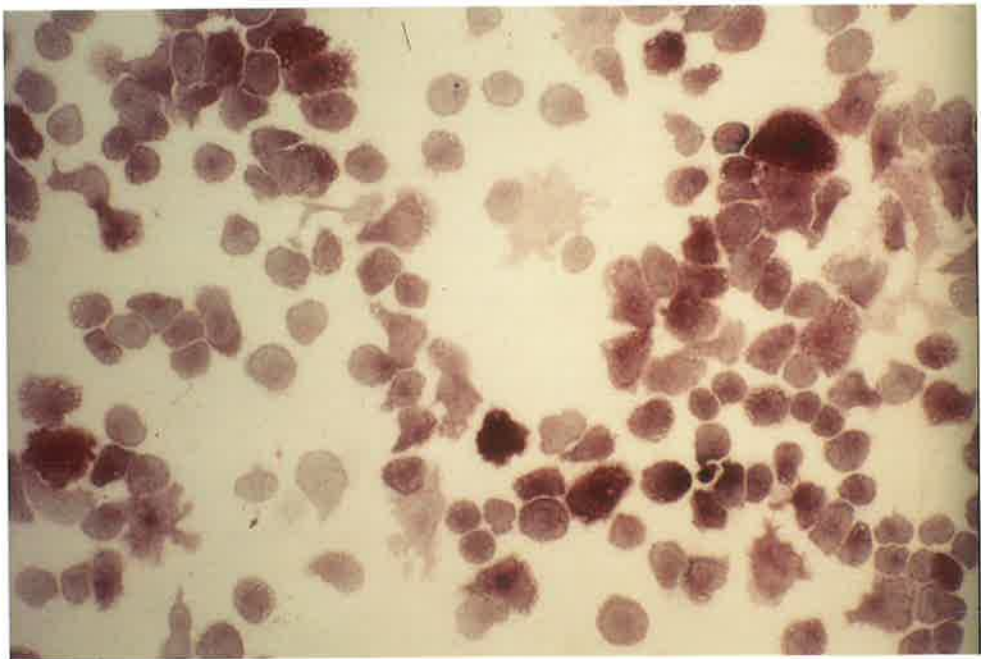


Figure 3.3 B

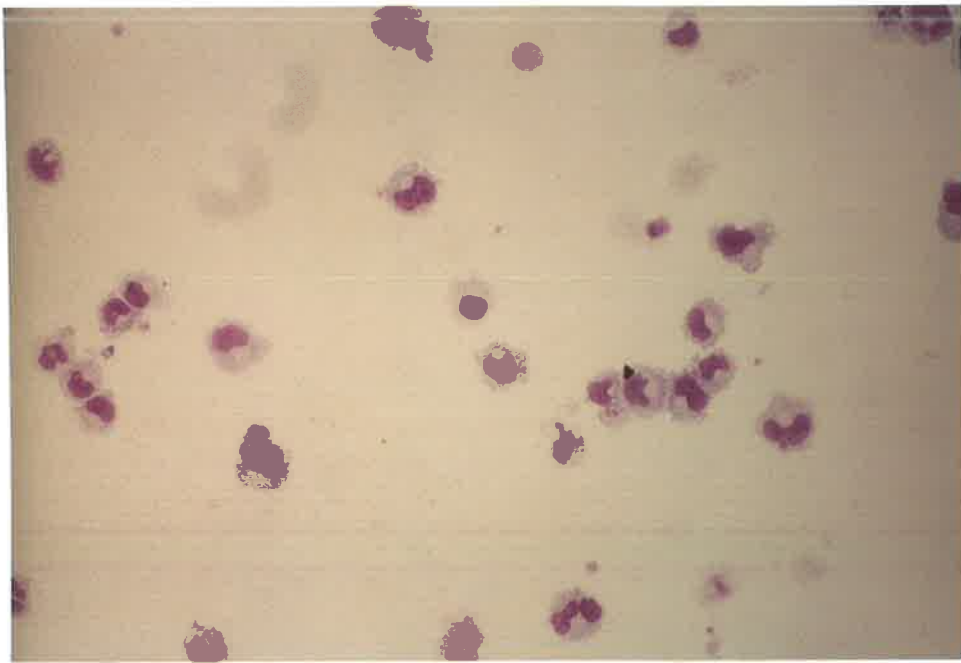
Photographs of RC-2A cells cultured with or without 20% PHA-
LCM for 8 days. Cell viabilities ranged between 85-95%.

Giemsa's stain.

A: no LCM x500.

B: + LCM x500.

A



B

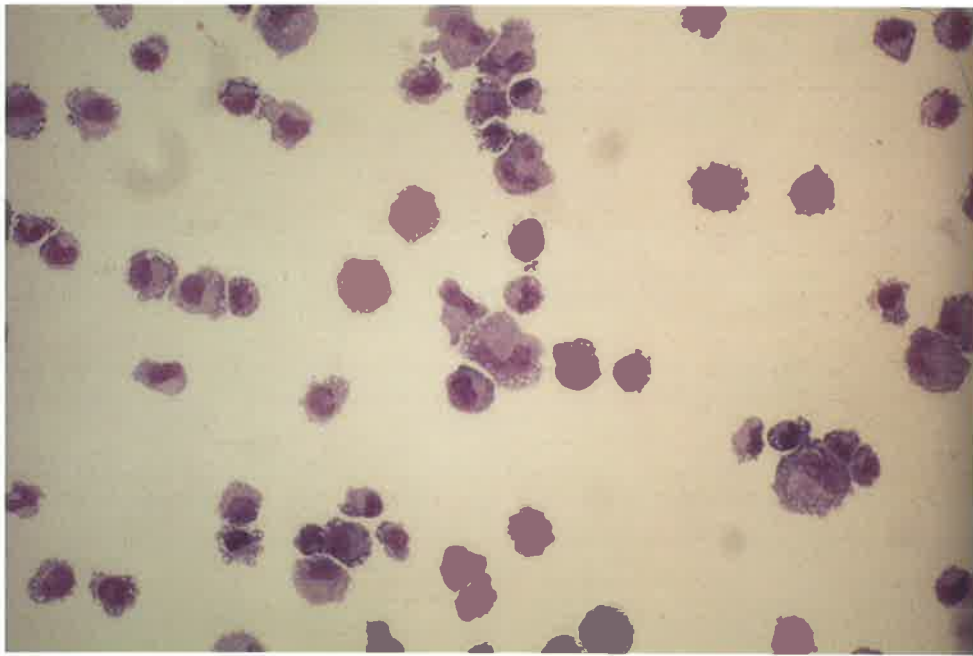


Figure 3.3 C:

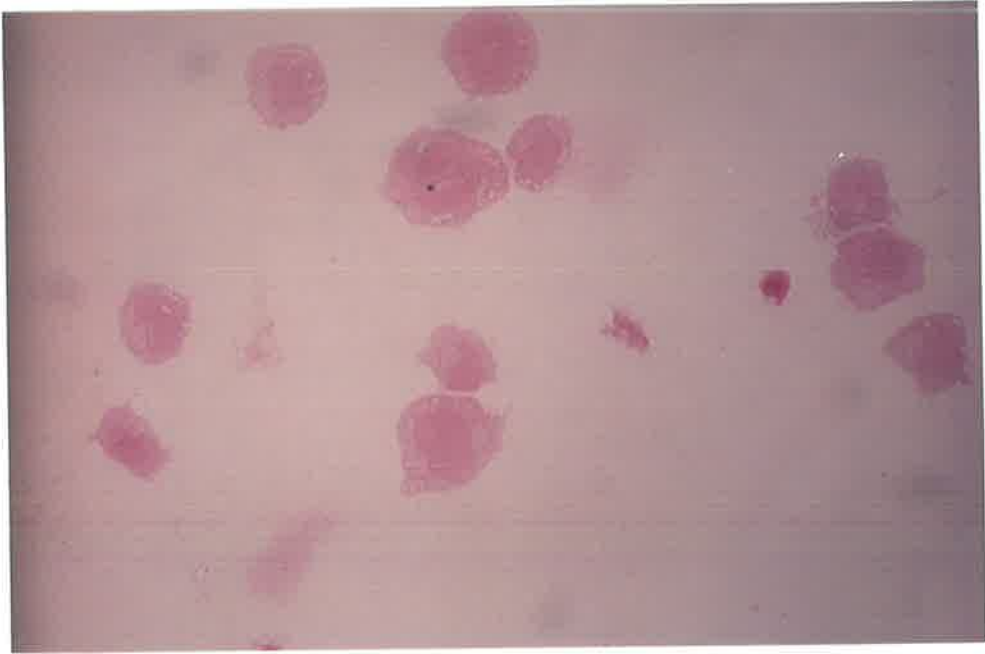
Photographs of RC-2A cells cultured with or without 20% PHA-
LCM for 8 days. Cell viabilities ranged between 85-95%.

Acid Phosphatase stain

A: no LCM x800

B: + LCM x800

A



B

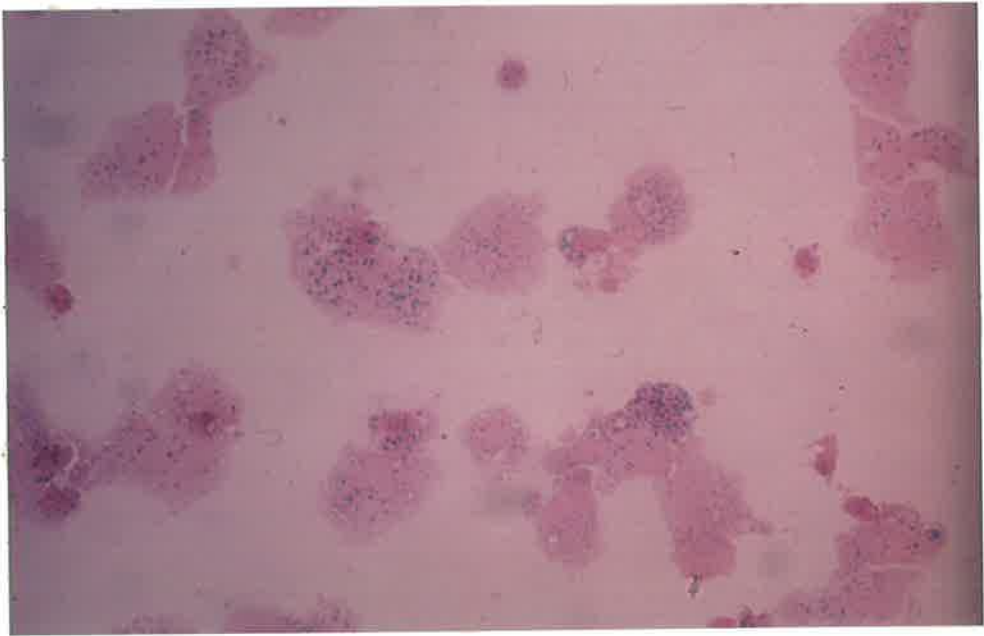


Figure 3.4

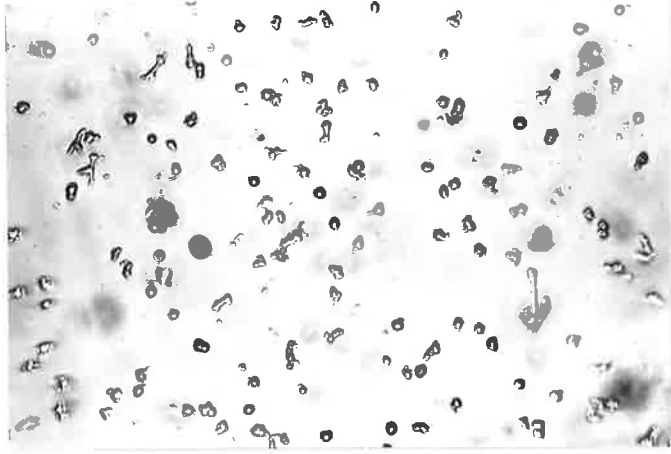
Photograph taken through an inverting microscope of RC-2A cells seeded into culture initially at 2×10^5 /ml, and refed on day 4.

A. No PHA-LCM, 7 days.

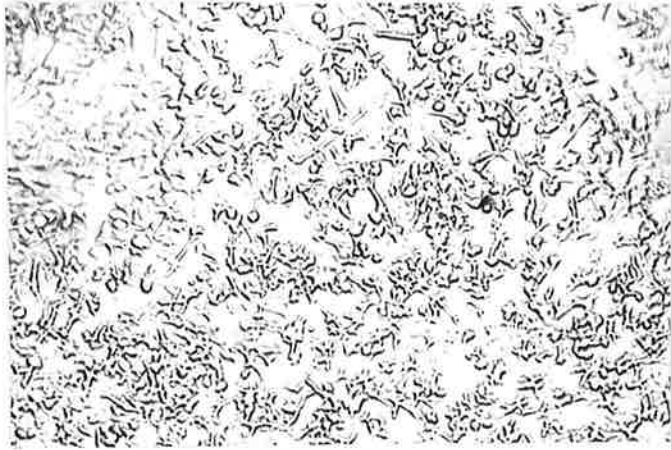
B. With 20% PHA-LCM, 7 days.

Magnification approximately x250

A



B



3.3.4 RC-2A cell surface marker expression changes caused by inducers of differentiation.

The expression of Ia antigen detected by the monoclonal antibody FMC-14 and of the leukocyte functional antigen (C3bi receptor) detected by anti-Mo1 were found to undergo significant changes as RC-2A cells differentiated in response to PHA-LCM. The expression of Ia showed a bimodal distribution, showing peaks at channels 160 and 220 on a logarithmic scale, with the proportion of cells binding less FMC-14 increasing in size as the process of differentiation occurred (Figure 3.5). Over the period of induction, the proportion of cells in the lower fluorescence population rose from approximately 30% at day 0 up to about 90% by day 8. The expression of Mo1 antigen showed an increase as differentiation occurred. Initially, cells showed a broad spectrum of binding, but the range of fluorescence intensity narrowed, with the peak shifting from channel 60 at day 0 to 130 at day 8, on a logarithmic scale, as cell differentiation progressed (Figure 3.5).

In a separate experiment on the binding of FMC-14 and anti-Mo1, the analysis of fluorescence was carried out using linear gain. This allowed the negative control median fluorescence intensity to be subtracted from the test antibody median fluorescence intensity (assuming that fluorescence intensity was normally distributed in the cell population) to give an estimate of the amount of fluorescence attributable to test antibody binding. Over the 8 days of culture with PHA-LCM, the total fluorescence due to FMC-14 binding decreased by four-fold. As the fluorescence histograms for FMC-14 binding were not normally distributed, this can only be considered as an approximate value. Similarly, the total fluorescence due anti-Mo1 binding was found to increase by four-fold. The binding of the negative control antibodies Sal-2 (IgG1), Sal-3 (IgM) and Sal-5 (IgG2a) underwent slight increases that were not of a sufficient

Figure 3.5

Changes in the expression of antigens on RC-2A cells detected by monoclonal antibodies FMC-14 (anti-Ia, IgG1) and anti-Mo1 (anti-C3B1 receptor, IgM) (solid lines) during zero, three, six and eight days culture in the presence of 20% PHA-LCM. Cultures were re-fed every 48 hours. Negative control antibodies Sal-2 (IgG1) and Sal-3 (IgM) (dotted lines) show little alteration in binding. Bound antibody was detected by indirect immunofluorescence and flow cytometry on a FACS IV. Fluorescence intensity was measured on a logarithmic scale. Peak fluorescence channels are given in the text.

DAY
0

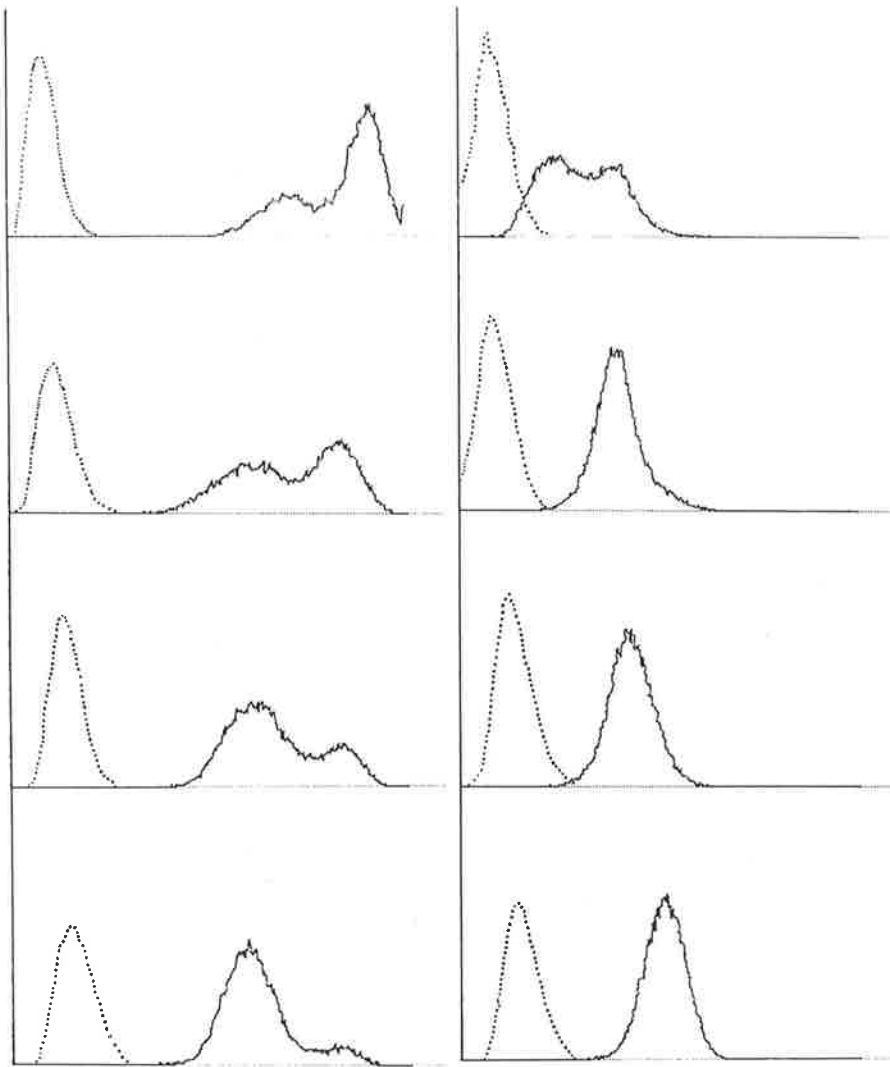
3

6

8

FREQUENCY ↑

FLUORESCENCE INTENSITY →
FMC 14 Mo1



magnitude to account for changes in binding of test antibodies. Typically, peak channel fluorescence rose from 20 to 30 on a logarithmic scale over 8 days induction with PHA-LCM, for the three sub-classes of negative control antibody used. These slight increases were most probably due to the increased size of induced RC-2A cells, shown by forward scatter characteristics in Figure 3.6.

The binding of anti-Mo2 was essentially little different to the negative control until day 8, when there was a moderate peak shift of 30 channels on a linear scale over the negative control binding, and the appearance of a small number of cells (comprising about 5-10% of the cell total) with a peak fluorescence intensity of about 180 channels on a linear scale. Peak fluorescence due to negative control antibody (Sal-3, IgM) binding rose from channel 28 to 60 on a linear scale over the induction period, whereas fluorescence due to anti-Mo2 binding rose from channel 30 to 90. (Figure 3.6).

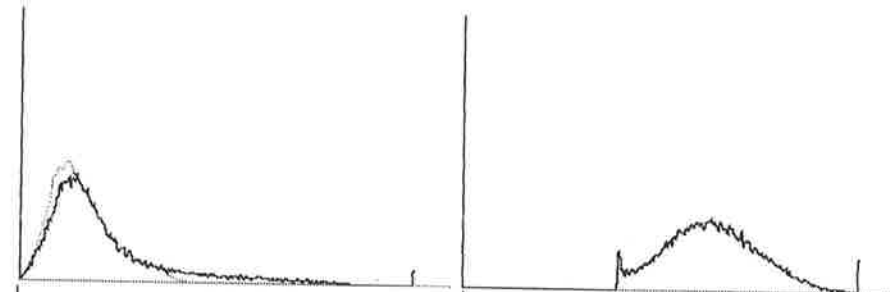
The binding of antibodies by F_c receptors on RC-2A cells was investigated by allowing cells cultured for up to 8 days with PHA-LCM to incubate with negative control antibodies of IgG1, IgG2a, and IgM subclass with and without 10% normal rabbit serum. It was found that an IgG2a subclass monoclonal antibody (Sal-5) showed increased binding to RC-2A cells after culture with PHA-LCM. This binding was blockable by the addition of 10% normal rabbit serum. Typically, over an 8 day culture with PHA-LCM, indirect immunofluorescence due to Sal-5 binding showed an increase in peak channel fluorescence from 20 to 30 on a logarithmic scale. Without normal rabbit serum, the peak channel rose from 30 up to 90, indicating increased F_c binding. Peripheral blood blasts from patients with acute leukaemias, especially those with monocytic differentiation, are sometimes able to bind irrelevant antibodies of IgG2a subclass via the F_c receptor [Gadd and Ashman, 1983; White *et al*, 1987]. The high affinity F_c

Figure 3.6

Change in the expression of the antigen on RC-2A cells detected by the monoclonal antibody anti-Mo2 (IgM) (solid lines) compared with negative control antibody Sal-3 (IgM) binding (dotted lines), during zero, three, six and eight days culture in the presence of 20% PHA-LCM. Cultures were re-fed every 48 hours. Bound antibody was detected by indirect immunofluorescence and flow cytometry on a FACS IV. Fluorescence intensity was measured on a linear scale. Peak fluorescence channels are given in the text.

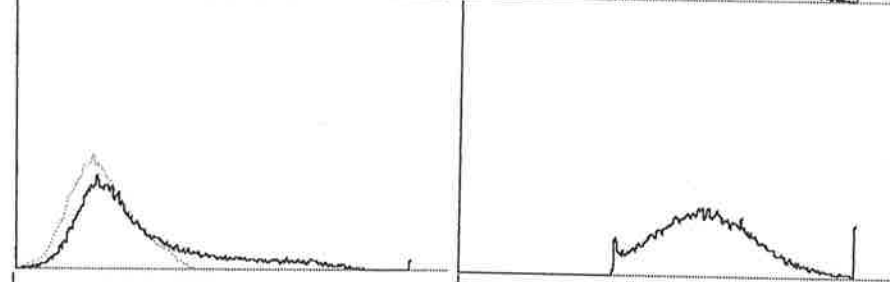
Forward scatter characteristics of the same cell populations are shown in the right half of the diagram, demonstrating a size increase in the cells as induction occurred.

DAY
0

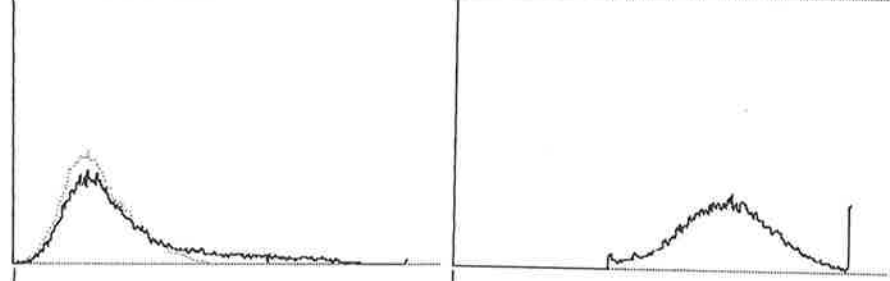


3

FREQUENCY ↑



6



8



FLUORESCENCE
INTENSITY →
Mo2

FORWARD
SCATTER →

receptor, which is able to bind monomeric murine IgG2a, is restricted to cells of the monocytic lineage [Anderson and Looney, 1986].

Figure 3.7 shows fluorescence histograms of binding of FMC-14, anti-Mo1 and anti-Mo2 to RC-2A cells treated with TNF- α and γ -IFN. Binding to control untreated RC-2A cells is also shown, and all histograms include binding by negative control anti-Salmonella antibodies Sal-2 (IgG1) and Sal-5 (IgM). It can be seen that the expression of Ia antigen, as determined by the binding of FMC-14, was reduced by both factors. The expression of Mo1 was increased moderately by TNF- α and strongly by γ -IFN, whereas the expression of Mo2 was not changed by either.

Further studies of surface marker expression alterations by TNF- α and γ -IFN are presented in Chapter 6 and summarized in Table 6.3.

3.3.5 The effect of differentiation inducing agents on the clonogenic potential of RC-2A cells.

The cloning efficiency of RC-2A cells in semi-solid agar showed a progressive decrease with increasing periods of culture in PHA-LCM (Table 3.2). The numbers of colonies which grew in the control cultures of uninduced cells were significantly greater than in the cultures from 5 day induced cells, which in turn yielded more colonies than 9 day induced cells, at both plating densities. (Students T test, $p < 0.001$ in all cases.) The size and appearance of colonies derived from control and PHA-LCM treated cultures were very similar, however histochemical studies of the colonies were not performed.

The clonogenic potential of RC-2A cells cultured in the presence of GM-CSF, G-CSF, TNF- α and γ -IFN was assayed by the ability to form colonies in semi-solid agar, the results of which are summarized in table 3.3. As a positive control, cells incubated with PHA-LCM were also cultured. The PHA-LCM used was from two different batches, distinct from

Figure 3.7 A

Histograms showing binding of MoAb FMC-14 by indirect immunofluorescence assay to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

	<u>MoAb</u>	<u>Median Ch. Fluor.</u>
A. RC-2A Untreated Control.	Sal-2	3.6
	FMC-14	482.2
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	FMC-14	286.3
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	FMC-14	332.1

Figure 3.7 A

FMC-14 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

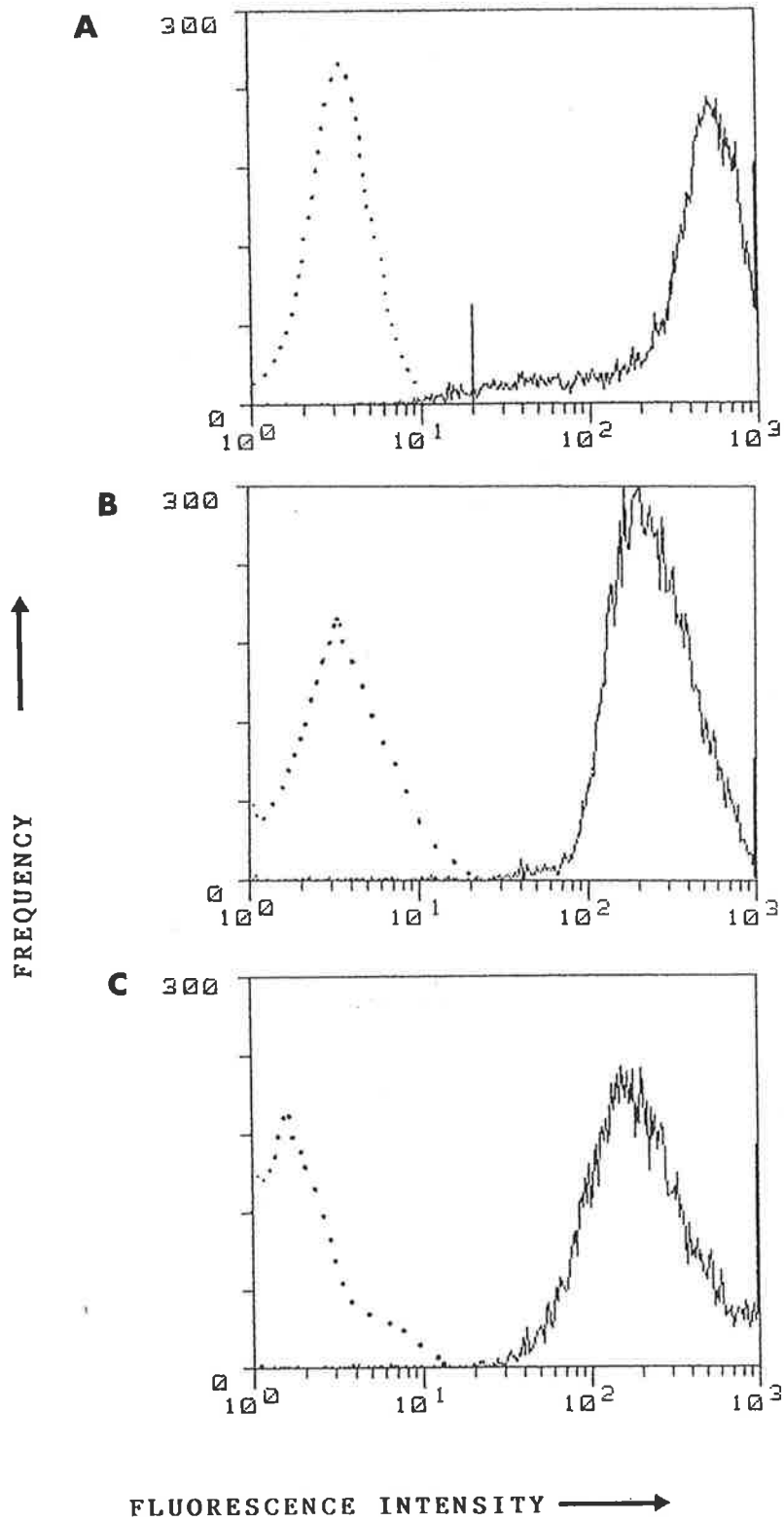


Figure 3.7 B

Histograms showing binding of MoAb anti-Mo1 by indirect immunofluorescence assay to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-3) is shown as a dotted line.

	<u>MoAb</u>	<u>Median Ch. Fluor.</u>
A. RC-2A Untreated Control.	Sal-3	2.5
	anti-Mo1	33.5
B. RC-2A Day 7 TNF- α	Sal-3	2.3
	anti-Mo1	56.3
C. RC-2A Day 7 γ -IFN	Sal-3	4.7
	anti-Mo1	165.2

Figure 3.7 B

♀ Mo1 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

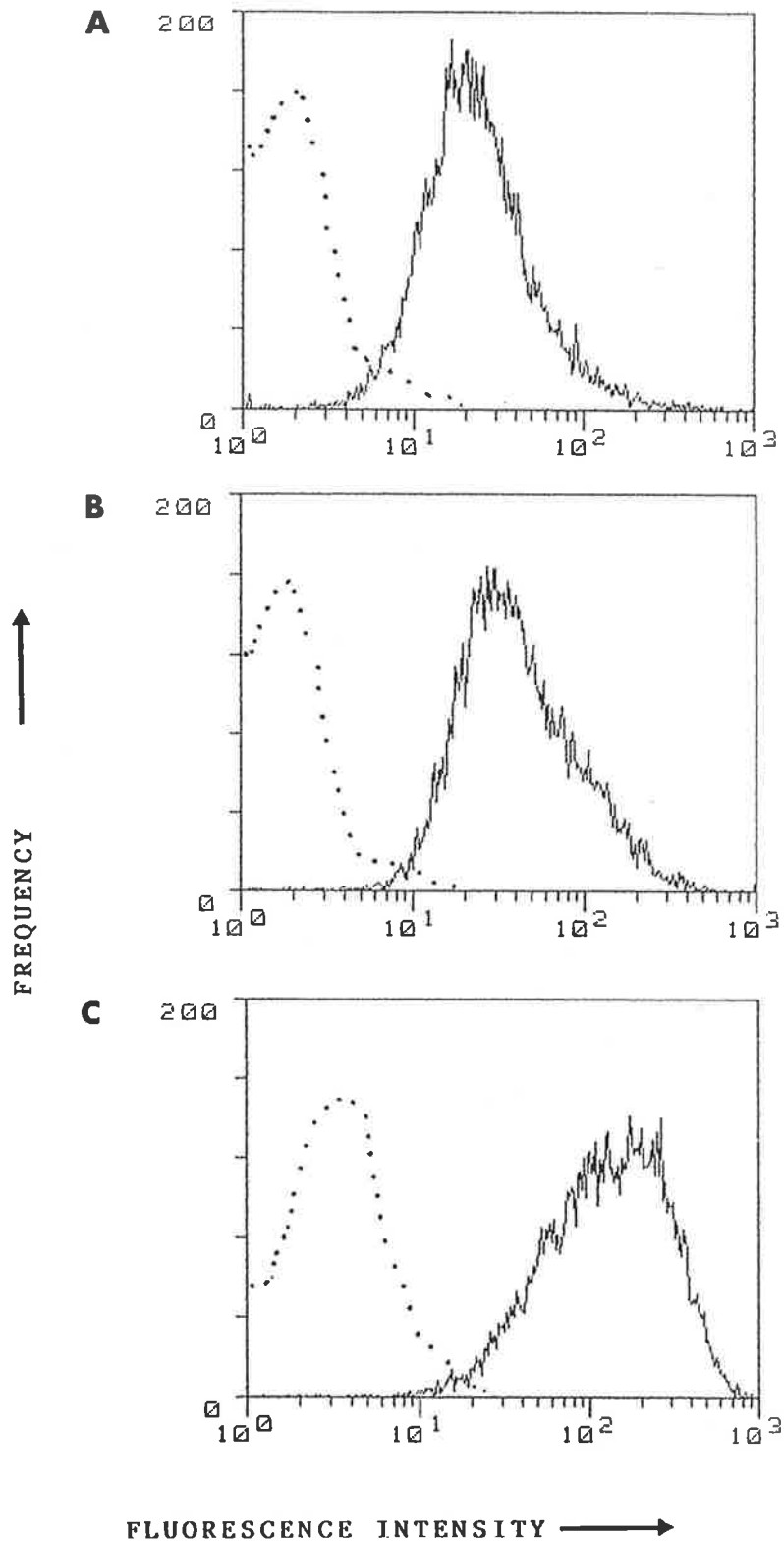


Figure 3.7 C

Histograms showing binding of MoAb anti-Mo2 by indirect immunofluorescence assay to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-3) is shown as a dotted line.

	<u>MoAb</u>	<u>Median Ch.Fluor.</u>
A. RC-2A Untreated Control.	Sal-3	2.5
	anti-Mo2	2.5
B. RC-2A Day 7 TNF- α	Sal-3	2.3
	anti-Mo2	2.3
C. RC-2A Day 7 γ -IFN	Sal-3	4.7
	anti-Mo2	4.7

Figure 3.7 C

Mo2 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

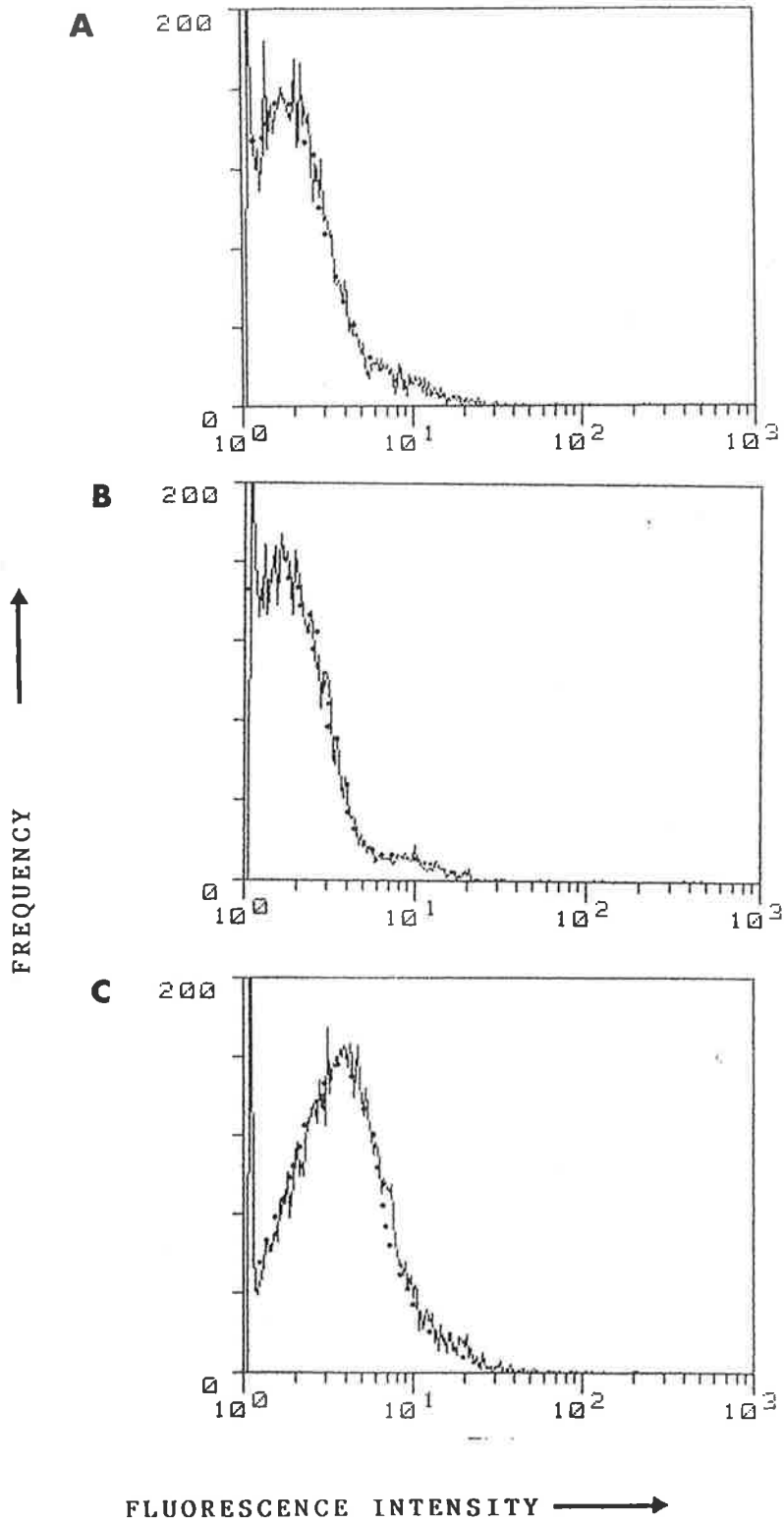


Table 3.2

Assessment of clonogenic potential of RC-2A cells treated for varying times with PHA-LCM.

		COLONIES IN SEMI-SOLID AGAR	
		CELLS SEEDED/PLATE	
		5 x 10 ³	2 x 10 ⁴
DAYS OF CULTURE IN PHA-LCM:			
	0	68±14	248±31
	5	14.4±2.8	71±9
	9	2.4±0.55	8.2±1.4

Clonogenic potential of RC-2A cells after 0, 5 and 9 days in culture with 20% PHA-LCM, determined by ability to form colonies in semi-solid agar. Results are averages ± Standard Error of the Mean of 5 cultures.

Table 3.3

Effect of purified/cloned human growth factors on the clonogenic potential of RC-2A cells evaluated by their ability to form colonies (>40 cells) in semi-solid agar.

Growth Factor	<u>Colonies per 10⁴ cells plated.</u>			
	Expt. 1		Expt. 2	
GM-CSF	104±14.3	(90%)	110±9.2	(81%)
G-CSF	40±5.5	(35%)	93±8.6	(67%)
TNF- α	103±12.6	(90%)	ND	
γ -IFN	12±1.9	(11%)	4±2.1	(<1%)
PHA-LCM	43±4.2	(38%)	4±2.8	(<1%)
none	115±14.4	(100%)	135±13.4	(100%)

RC-2A cells were cultured for 7 days with growth factors, PHA-LCM or alone and re-fed on day 4. The cells were harvested and plated at an appropriate concentration in quad- or quintuplicate in semi-solid agar medium, and supplemented with 100 μ l of RC-2A-CM/plate. Averages of colony counts are presented rounded to the nearest whole colony \pm Standard Error of the Mean. Percentage of control untreated RC-2A colony formation is presented in parentheses.

that used in other experiments. The clonogenicity of cells treated with γ -IFN was significantly less than the untreated controls in two experiments (both experiments $p < 0.001$). Cultures treated with PHA-LCM as a positive control also gave significantly less colonies than the untreated cultures (both experiments $p < 0.001$). The clonogenicity of RC-2A cells treated with G-CSF was also suppressed with respect to untreated controls, but to a lesser extent compared with the effect of γ -IFN (experiment 1, $p < 0.001$, experiment 2, $p < 0.01$). There appeared not to be any suppression of clonogenicity of RC-2A cells treated with GM-CSF (experiment 1, $p > 0.2$, experiment 2, $0.2 < p < 0.1$) or TNF- α ($p > 0.2$).

3.3.6 Ability of cloned/purified growth factors and PHA-LCM to replace the requirement for RC-2A-Conditioned Medium for culture of RC-2A cells in semi-solid agar.

It was found that RC-2A conditioned medium was required for the successful cloning of RC-2A cells in semi-solid agar (see section 3.2.6., table 3.1). As a preliminary experiment, G-CSF, GM-CSF, TNF- α , γ -IFN and PHA-LCM were added to cultures of RC-2A cells in semi-solid agar to investigate the ability of these factors to replace the requirement for RC-2A-CM. The results are summarized in table 3.4., and show that PHA-LCM, GM-CSF and G-CSF all have growth promoting activity for RC-2A cells of about three-quarters of that exhibited by RC-2A-CM when used at the levels indicated in the table. The addition of γ -IFN resulted in the growth of a small number of colonies, about one-fifth of the number in the cultures supplemented with RC-2A-CM. TNF- α appeared to have little or no ability to support the growth of colonies.

Table 3.4

Ability of purified/cloned growth factors to substitute for RC-2A conditioned medium in supporting growth of RC-2A colonies in semi-solid agar.

Growth factor	RC-2A colonies.
GM-CSF (100 μ l)	22 \pm 4.9
G-CSF (20ng)	19 \pm 3.7
TNF- α (100 U)	1 \pm 0.7
γ -IFN (100 U)	6 \pm 1.9
PHA-LCM (100 μ l)	19 \pm 0.7
RC-2A-CM (100 μ l)	27 \pm 3.2
medium (100 μ l)	a few clusters of 4-8 cells.

Results are averages of triplicate assays \pm Standard Error of the Means. Amounts of factor added per plate are in parentheses.

3.3.7 Effect of culture of RC-2A cells in the presence of PHA-LCM on their ability to stimulate in one-way mixed leukocyte culture.

The stimulatory ability of RC-2A cells cultured in the presence of PHA-LCM (i.e. their ability to present allo-antigen to responding peripheral blood T cells from normal donors) showed an increase over that of control RC-2A, such that the incorporation of ^3H -Thymidine was approximately double the control at the optimum stimulator to responder ratio (Figure 3.8). Both were inhibitory at stimulator to responder ratios greater than 1: 2, however the addition of Indomethacin partly abrogated this effect in the case of the induced RC-2A, but not in the control, suggesting that the induced RC-2A cells produce inhibitory prostaglandins.

3.3.8 Effect of anti-RC-2A serum on uptake of ^3H -Thymidine by RC-2A cells.

The effect of anti-RC-2A serum on the uptake of ^3H -Thymidine by RC-2A cells was quite marked, with the uptake increasing to almost 300% of the control value at a dilution of 1/80. Normal mouse serum had no effect on the uptake (see figure 3.9), thus it appears that the antiserum specifically increased the proliferation of RC-2A cells.

3.3.12 Effect of anti-RC-2A serum on bone marrow myeloid progenitor cell growth.

The anti-RC-2A serum, when used at a dilution which gave a maximum increase in the uptake of ^3H -Thymidine by RC-2A cells (i.e. 1/80), was able to stimulate the formation of a small number of predominantly macrophage colonies by bone marrow myeloid progenitors, and to increase the number of clusters of less than 40 cells (see table 3.5). The colonies were smaller in size than those stimulated by PHA-LCM. Normal mouse serum was unable to stimulate the formation of clusters or colonies over the level obtained in negative control cultures containing no added stimulus.

Figure 3.8

^3H -Thymidine incorporation by normal T lymphocytes in response to control (■, □) and day 8 induced (●, ○) RC-2A cells in one way mixed leukocyte culture. Closed figures indicate addition of indomethacin to cultures. Shaded area represents stimulation by allogeneic control mononuclear cells. Responder and stimulator cells alone took up < 750 C.P.M. of ^3H -Thymidine.

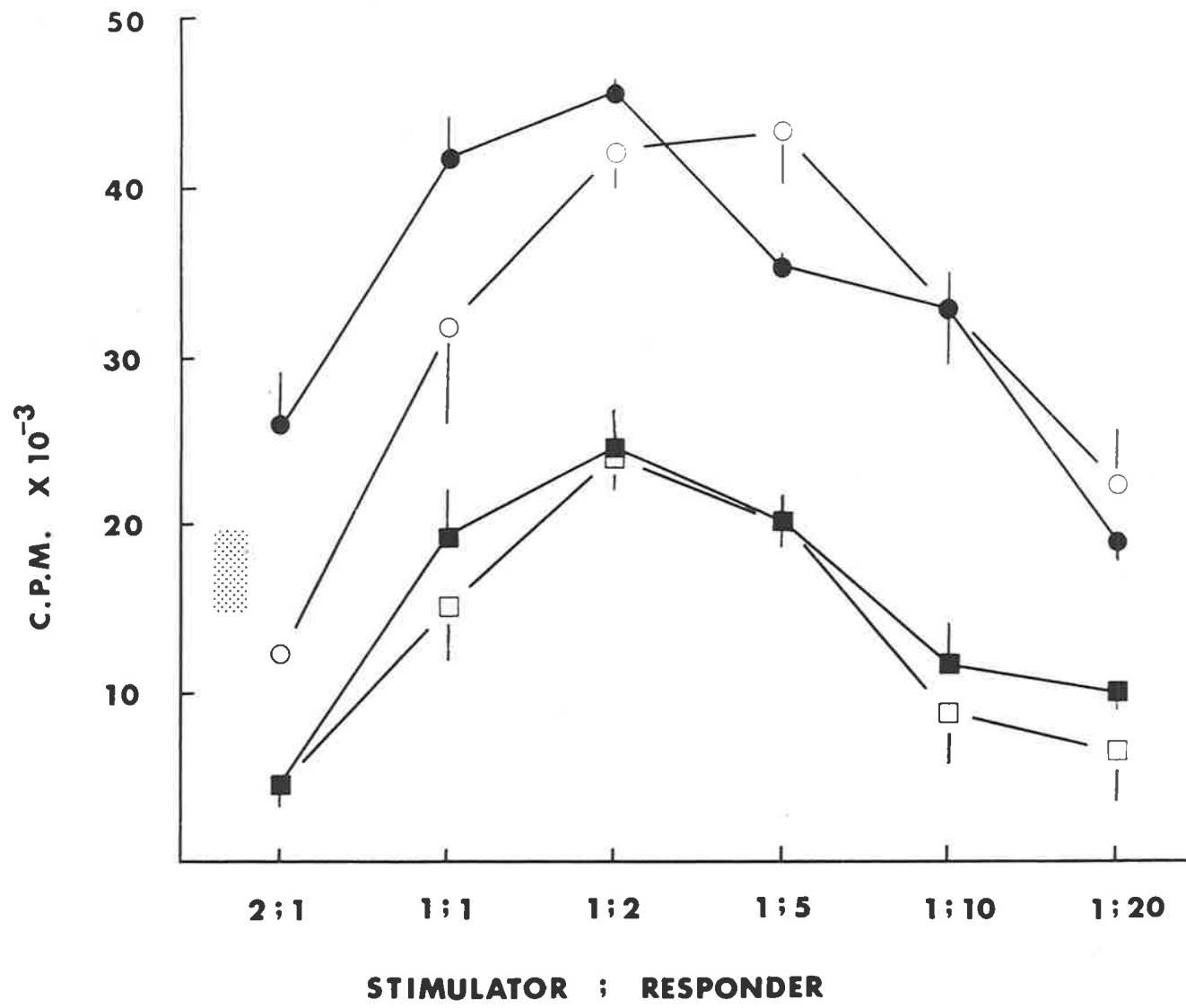


Figure 3.9

Effect of an anti-RC-2A antiserum on the uptake of ^3H -Thymidine by RC-2A cells. After 3 days culture in the presence of dilutions of Normal Mouse Serum (●), anti-RC-2A (■), or RPMI 1640 + 10% FCS (◆), cultures were pulsed with tritiated thymidine for 18 hrs and the incorporated radioactivity counted. Results are averages of triplicate cultures \pm standard deviations.

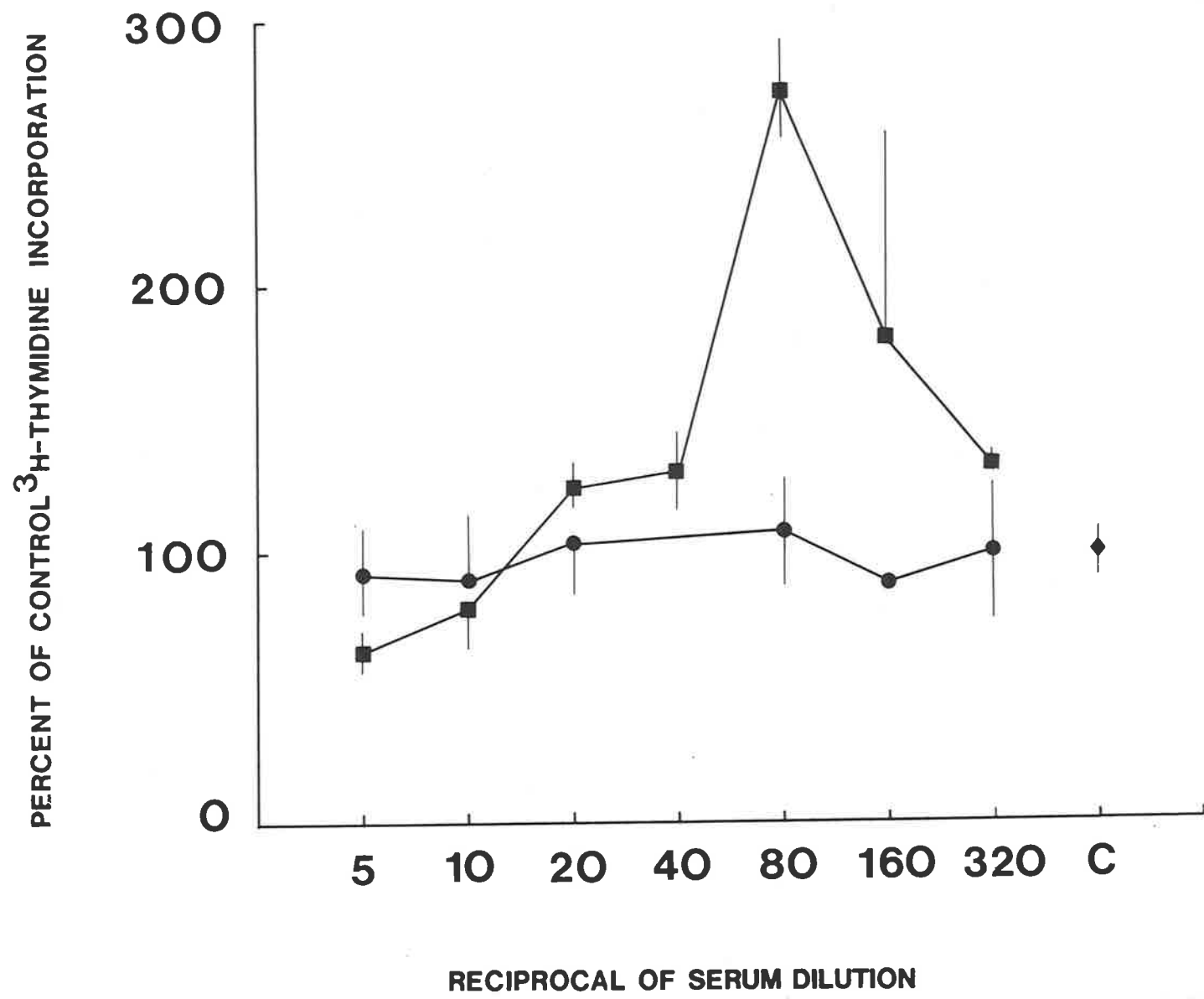


Table 3.5

Effect of anti-RC2A anti-serum on the formation of colonies in semi-solid medium by bone marrow myeloid progenitor cells.

Stimulus	Clusters.	Colonies.	Colony type.
none	17±3.5	-	
PHA-LCM (100µl)	Many	217±18	≈50% Mø, ≈50% Nø, Eø
NMS (1/80)	15±5	-	
α-RC2A (1/80)	86±18	10±4	>90% Mø

Results are averages ± Standard Error of the Means of quadruplicate assays. Colonies were defined as aggregates of > 40 cells, and clusters as aggregates of < 40 cells. 5×10^4 bone marrow mononuclear cells were cultured per plate.

3.4 Discussion.

Leukaemia is characterised by the accumulation of malignant immature haemopoietic cells arising as a result of the transformation of a haemopoietic stem cell and exhibiting a block or arrest in the normal differentiation process. Nevertheless, it has been shown that some leukaemic cell lines and fresh leukaemic cells can be induced to differentiate to a more mature phenotype, with a concomitant decrease in proliferative potential, by treatment in culture with chemical inducers or with biologically derived molecules (see section 1.2). Thus it is possible to at least partially overcome this differentiation block *in vitro*, suggesting that these cells retain some responsiveness to normal biological controls.

Data presented in this chapter has shown that RC-2A, a cell line consisting predominantly of immature myelomonocytic cells, can be induced to differentiate toward macrophages by culturing in the presence of PHA-LCM, which is known to contain a number of biologically active molecules. This process was monitored by several parameters, including histochemistry, cell surface antigen expression and assay of cellular proliferative potential. It was found that RC-2A cells, during the course of culture with PHA-LCM, acquired characteristic features of macrophages, such as strong expression of α -Naphthol Butyrate Esterase and Acid Phosphatase, and increased adherence to plastic after treatment with PHA-LCM. A proportion of RC-2A cells became multinucleated, which has been reported to be a property of cultured human macrophages [Schlesinger *et al*, 1984]. Cultured macrophages exhibit vacuolation [Jacoby, 1965], which is also evident in induced RC-2A cells.

RC-2A cells were found to undergo alterations in the expression of some surface markers detected by monoclonal antibodies. The stage of cell cycle has been shown to influence antigen expression, including that of Ia

[Gesche and Ashman, 1985], however, when taken in the context of cytochemical alterations, these changes in antigen expression provide evidence of true maturational events. Expression of Mo1, an antigen of the lymphocyte functional antigen (LFA) gene family [Todd *et al*, 1981] (C3b1 receptor, CD11b) was found to increase four-fold after 8 days culture of RC-2A cells in the presence of PHA-LCM. Mo1 is found on the majority of myeloid cells in bone marrow and on peripheral blood monocytes, neutrophils and null cells, but not on bone marrow colony forming cells [Foon and Todd, 1986]. Mo1 has been reported to appear on HL-60 cells after treatment with phorbol ester or lymphokines [Todd *et al*, 1981], and to increase on activation of neutrophils [Lanier *et al*, 1985].

The expression of Ia antigen detected by FMC-14 was found to undergo a marked decrease over the period of induction. Fluorescence histograms exhibited a bimodal pattern of antigen expression, delineating two populations of cells, with the proportion of cells in the lower antigen density group increasing from 30 to 90% of the total after 8 days. This result runs counter to the dogma that macrophages express increasing amounts of Ia antigen as they mature, but investigations by Beller and Unanue, 1981, show that in the case of murine peritoneal macrophages, expression of Ia antigen is a transient event which seems to delineate a specific stage in the monocytic lineage, rather than defining a stable Ia⁺ subpopulation.

The expression of the Mo2 antigen was found to be essentially negative for control untreated RC-2A cells, and in cells cultured in the presence of PHA-LCM for up to 4 days. However, after 8 days of induction, RC-2A cells showed binding of anti-Mo2 representing a modest peak shift of 30 channels over negative control binding on a linear scale, with a small subpopulation of cells with a peak channel fluorescence of about 180 appearing, representing 5-10% of the total cells. Anti-Mo2 is a CD.14

monoclonal antibody which binds to peripheral blood monocytes, cultured and peritoneal macrophages. It does not bind to the myeloid cell lines HL-60, U937, KG1 or K562, nor does it bind to bone marrow CFU's, however, treatment of HL-60 cells with lymphokines induces Mo2 expression [Foon and Todd, 1986]. Studies by Morimoto *et al*, 1981 and Todd *et al*, 1983 have indicated that antigen presenting cells are Mo2⁺, as demonstrated by the decreased ability of mononuclear cells depleted of Mo2⁺ cells to present soluble antigen. Increased mixed leukocyte culture stimulation by induced RC-2A cells may correlate with the expression of Mo2. However, at stimulator to responder ratios greater than 1: 2, both control and induced cells showed increasing inhibitory effects. This inhibition was partly overcome by the addition of indomethacin to the culture medium, suggesting that induced RC-2A cells elaborate prostaglandins into the cultures, a property shared by macrophages [Humes *et al*, 1977].

RC-2A cells treated with PHA-LCM showed a progressive decline in growth rate. Clonal assay of the proliferative potential of RC-2A cells demonstrated that during culture in the presence of PHA-LCM, RC-2A cells progressively lost the ability to proliferate, such that after 8 days these cells had approximately 3% of the clonogenic potential of control cultures. This suggests that the decrease in growth rate of cells cultured with PHA-LCM was not merely due to a lengthening of the mitotic cell cycle duration, but due to a commitment step resulting in a loss of proliferative potential. Studies by Metcalf, 1980 and 1982, using the murine myelomonocytic cell line WEHI-3B showed that commitment to differentiation of clonogenic cells, resulting in a decrease in self renewal capacity, was an early and irreversible event, taking place in some cells after only 24 hours exposure to murine post-endotoxin serum as a source of G-CSF. In these studies, it was demonstrated that clonal extinction of the cells responsible for renewal of the cell line population could be achieved. A

study of the ability of murine G-CSF to suppress clonogenicity of HL-60 cells showed a weaker effect than that obtained with WEHI-3B treated with murine G-CSF [Metcalf, 1983].



Investigations of the molecular identity of biologically derived inducers of differentiation of human leukaemic cell lines have shown that at least some of these factors are distinct from the known colony stimulating factors [Olsson *et al*, 1984]. The experiments using cloned/purified human growth factors detailed in section 3.2 emphasise the general observation that single factors rarely are able to induce the full complement of differentiation-associated features in myeloid cell lines (see section 1.2). Gamma-IFN and to a lesser extent G-CSF, are able to suppress clonogenicity in RC-2A cells. The clonogenic suppression by murine G-CSF is well documented [Metcalf, 1980; Metcalf, 1982], and although the clonogenic suppressive effects of γ -IFN are not well documented, it has been reported that this factor suppresses the growth of the myeloid lines U937 and RC-2A in bulk liquid culture [Ralph *et al*, 1983]. Unlike the findings of Begley *et al*, 1987, who reported that human recombinant GM-CSF suppressed clonogenicity in HL-60 cells, this factor did not appear to suppress clonogenic potential of RC-2A cells. It may be noted, however, that clonogenic suppression of HL-60 by GM-CSF was most apparent after 2 to 3 weeks in culture with the factor.

Of the four growth factors investigated, γ -IFN was the only one which increased expression of α -Naphthol-Butyrate esterase activity by RC-2A cells. It was also able to increase the adherence of the cells to plastic, and with prolonged culture without re-feeding, was able to prematurely acidify the culture medium, suggesting upregulation of metabolic activity.

Gamma-IFN and TNF- α , like PHA-LCM, were able to increase the expression of Mo1 and decrease the expression of Ia antigen, but neither induced the expression of Mo2. Koeffler *et al*, 1984 reported that HL-60

cells treated with 100 - 500 U of γ -IFN initially showed a marked increase in Ia expression over 5 days of culture with the factor, but cells decreased their expression of Ia antigen on days 6 and 7 in a bimodal fashion very similar to that exhibited by RC-2A cells treated with PHA-LCM (see figure 3.5). This suggests that Ia expression induced by γ -IFN may be a transient differentiation associated event. When cell lines which are phenotypically more mature phenotype than HL-60, and already express this marker (such as RC-2A) are used, treatment with γ -IFN causes a decrease in Ia expression as cells differentiate to a stage beyond maximum expression of this Ia antigen.

Chapter 6 details further antigen expression studies on RC-2A cells treated with γ -IFN and TNF- α using a panel of five new anti-myeloid monoclonal antibodies. These results show that neither γ -IFN nor TNF- α were able to induce antigenic changes which paralleled those caused by PHA-LCM.

The four growth factors were investigated for their ability to replace RC-2A conditioned medium in supporting the clonal growth of RC-2A cells in semi-solid medium. G-CSF, GM-CSF and to a lesser extent γ -IFN were able to support colony growth. The source of G-CSF was a COS cell supernatant, therefore the possibility that this preparation was able to support colony growth due to a conditioning effect of COS cells cannot be discounted. This preliminary data raises the possibility that autocrine production of growth factors by RC-2A cells may occur. Begley *et al*, 1987 reported that GM and G-CSF initially caused a proliferative burst by HL-60 cells, which may be analogous to the results presented here. The spent culture supernatant from RC-2A cells was assayed for colony stimulating activity in a bone marrow myeloid progenitor assay, but no activity was found (data not shown).

From the preceding results, it appears that PHA-LCM may contain a number of components capable of acting on RC-2A cells, their combined effects giving rise to the differentiated phenotype comprising morphological, cytochemical, clonogenic and surface marker changes. It appears that γ -IFN is a likely to be responsible for the majority of the changes observed, however it seems likely that the differentiation of RC-2A cells by PHA-LCM is a multifactorial process. The work published by Trinchieri *et al*, 1986 demonstrated that γ -IFN and TNF- α act synergistically to induce differentiation of HL-60 cells, and similarly γ -IFN may be acting in a similar way with other factors contained in PHA-LCM to induce differentiation of RC-2A cells.

The ability of a polyclonal antiserum specific for myeloid cell surface components to influence the proliferation of the cell type to which it was raised, and to stimulate the growth of macrophage colonies from bone marrow progenitors, suggests that it may be possible to raise monoclonal antibodies able to exert functional effects on human myeloid cells and aid in the characterization of receptors for growth factors and other biologically active molecules. Such an endeavor has precedent in the work of Lopez and Vadas, 1984; Lopez *et al*, 1985, who reported two monoclonal antibodies (WEM-G1, WEM-G11) which exerted functional effects on neutrophils. Similarly, Lögberg *et al*, 1985 reported the production of monoclonal antibodies exerting functional effects on T lymphocytes. This avenue of investigation is discussed in Chapters 5 and 6.

RC-2A cells showed a marked heterogeneity with respect to both surface marker expression and cytochemical staining, both before and after culture with PHA-LCM. Surface marker analysis indicates that heterogeneity does exist in leukaemic blast populations [Pessano *et al*, 1984]. In this respect RC-2A cells resemble blasts from leukemic patients. This heterogeneity may reflect a lack of stringency of differentiation arrest

[e.g. Greaves, 1982]. The study of normal and leukemic differentiation using cell line models may provide useful information on the mechanisms underlying this process, and in addition such approaches have potential clinical application [Koeffler, 1983].

CHAPTER 4

Development of an improved screening method for monoclonal antibodies binding to cell surface antigens.

4.1 Introduction.

The development of monoclonal antibody technology [Köhler and Milstein, 1975] has enabled immunologists and cell biologists to greatly expand their knowledge of cell surface antigens. However, the screening of hybridoma supernatants for their specificity represents a very large and time consuming proportion of the process. It is desirable to determine the specificity of hybridoma supernatants as early as possible in the process to eliminate the need for maintaining cultures that are not of interest, in view of the time and expense involved. Screening methods that have been employed include whole cell radioimmunoassay [Williams *et al*, 1977], manual indirect immunofluorescence [Murayama *et al*, 1983] and microrosette assays [Parish and McKenzie, 1978] all of which are quite labour intensive. Enzyme linked immunosorbent assays (ELISA), while being less labour intensive, require cell fixation and endogenous enzyme blocking, which may result in the loss of antigenic determinants [Hancock *et al*, 1982; our unpublished results].

Recently, a colorimetric assay has been described, which allows rapid and sensitive assay of antibodies specific for cell surface antigens [O'Neil and Parish, 1984]. This technique uses 96 well microtitre trays coated with a purified antibody to mouse immunoglobulin. These are incubated with test antibody, which binds to the tray. Target cells added to the wells bind if specific antibody is present. Quantitation of bound cells by uptake of Rose Bengal dye and measurement of absorbance with an automated ELISA reader enables rapid determination of antibody specificity and titre.

This chapter concerns the optimization and evaluation of this assay for its suitability as a primary screening assay for hybridomas secreting antibody specific for human cell surface antigens.

4.2 Materials and Methods.

4.2.1 Monoclonal Antibodies and Sera.

All MoAb's used were prepared and cloned in this laboratory (except for FMC 14 [Zola *et al*, 1983], a kind gift of Dr. H. Zola), essentially using the method of Oi and Herzenberg, 1980. MoAb's used as negative controls [Sal-2 (IgG1), Sal-5 (IgG2A)] were raised against cytoplasmic antigens of *Salmonella enteritidis* [O'Connor and Ashman, 1982]. Positive control antiserum was raised against normal human tonsillar lymphocytes in mice. Goat antibody to mouse Ig(G+M) was raised using pooled, purified monoclonal antibodies of various classes. Antibodies were precipitated from the serum with 40% ammonium sulphate and purified by affinity chromatography on columns of purified polyclonal mouse IgG and IgM coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden). The purified antibodies to murine IgG and IgM were pooled and antibody cross reactive with human Ig was removed using a human Ig-Sepharose 4B column (see section 2.2).

Purified goat anti-mouse Ig(G+M) was labelled with fluorescein isothiocyanate (FITC) by the method of Rinderknecht, 1962, and with I^{125} by the method of Salacinski *et al*, 1981. (see sections 2.2.4 and 2.2.5).

4.2.2 Cell Lines.

DAUDI, a human Burkitt Lymphoma derived cell line was obtained from Dr. H. Zola, Flinders Medical Centre, South Australia. The line

was maintained as described in section 2.3.4. Human AML cells used in immunization and screening were prepared and cryopreserved as previously described [O'Keefe and Ashman, 1982] (see sections 2.4.1, 2.3.6 and 2.3.7). Other human cell lines (RC-2A, U937, HL-60, KG-1, K562, BALM-1 and MOLT-4) were obtained and maintained as previously described [O'Keefe and Ashman, 1982b] (see sections 2.3.4 and 2.3.5).

4.2.3 The Rose Bengal Assay.

Flexible 96 round-bottomed well microtitre trays (Linbro, Flow, VA, cat. No. 76-364-05) were coated with goat anti-mouse Ig(G+M) antibody by incubation with 100 μ l per well of affinity purified antibody at 5 μ g/ml in PBS pH 7.4 at 4°C overnight. Trays were washed 3x with isotonic PBS pH 7.4 by flooding and flicking. Undiluted or serial 1/2 dilutions of hybridoma supernatants in PBS pH 7.4 + 0.1% Bovine Serum Albumin (BSA) prepared in separate trays were added in 100 μ l aliquots to the coated trays. The trays were incubated at 4°C for 60 minutes, then washed 3x as previously. Target cells, 2-5x10⁵ in 100 μ l of RPMI 1640 + 10% FCS + 0.1% Sodium Azide (to prevent active adherence or capping), were added to the wells and the trays were incubated for 120 minutes. In later experiments, trays were first incubated for 30 minutes at 37°C, followed by 90 minutes at 4°C. After incubation, excess cells were flicked out and 100 μ l of 0.25% w/v Rose Bengal dye (Faulding, Australia) in PBS was added to each well. Different batches of Rose Bengal were found to vary greatly in their suitability for use in the assay. The trays were washed 3x as above, then 100 μ l of sodium dodecyl sulphate solution (1% w/v in water) was added to wells to lyse bound cells and release the dye. The OD₅₄₀, corresponding to the absorbance maximum of Rose Bengal, was read on a

Titertek Multiskan ELISA reader. Wells having an OD₅₄₀ more than twice that of the negative control wells were considered to be positive.

4.2.4 Indirect Immunofluorescence.

Binding assays to investigate specificity of MoAb's using indirect immunofluorescence were performed as previously described [Gadd and Ashman, 1983] (see section 2.5.1). Manual scoring was performed using an Olympus microscope (Model BH2/BH-RFL-W) with epi-illumination. A minimum of 200 cells per sample were scored. Flow cytometry was carried out at Flinders Medical Centre, South Australia, using a FACS IV (Becton Dickinson). Before analysis, labelled cells were fixed in 1% paraformaldehyde in PBS [Lanier and Warner, 1981] and stored for up to 2 days at 4°C in the dark. Fifty thousand cells were analysed per sample.

4.2.5 Whole Cell Radioimmunoassay.

Whole cell RIA was performed in the same way as indirect immunofluorescence, except that ¹²⁵I-labelled goat anti-mouse Ig(G+M) was added in the second stage instead of FITC-labelled goat anti-mouse Ig(G+M) (see section 2.2.4), and P8 precipitin tubes (Johns Division, Malinckrodt, Australia) were used. All supernatants were assayed in duplicate. After washing away excess radiolabel, cell-associated radioactivity was determined using a Packard auto-gamma spectrometer. Hybridoma supernatants which brought about binding of more than twice as much label as the negative control antibodies were considered positive. Marginal cases were designated (+/-).

4.3 Results.

4.3.1 Optimisation.

It was noted that some human Acute Myeloid Acute Leukaemia (AML) samples gave very low binding in the Rose Bengal Assay (RBA), even though adequate cell numbers were used. To overcome this, an incubation at 37°C for 30 minutes at the cell binding stage was included in the protocol prior to a 90 minute incubation at 4°C. This modification, when used for such AML cell samples, as well as for human cell lines, markedly improved the sensitivity of the assay. This sensitivity increase was most pronounced when low cell numbers were used. With 10⁵ DAUDI cells per well, the sensitivity was eight fold greater than with the original protocol using the same MoAb. Figure 4.1 shows the titration of the MoAb SB1 (IgG1) on DAUDI cells. The concentration of undiluted SB1 was 10µg/ml, thus the end point of 1/64, obtained with the 37°C incubation, was equivalent to 16 ng of MoAb per well. When the entire cell binding incubation was carried out at 4°C, the end point occurred at a dilution of 1/8, corresponding to 125 ng of MoAb per well. The increase in cell binding gained by the inclusion of an incubation at 37°C may be due to increased fluidity of the cell membrane allowing more antigen to come into contact with specific antibody.

4.3.2 Comparison with Whole Cell RIA

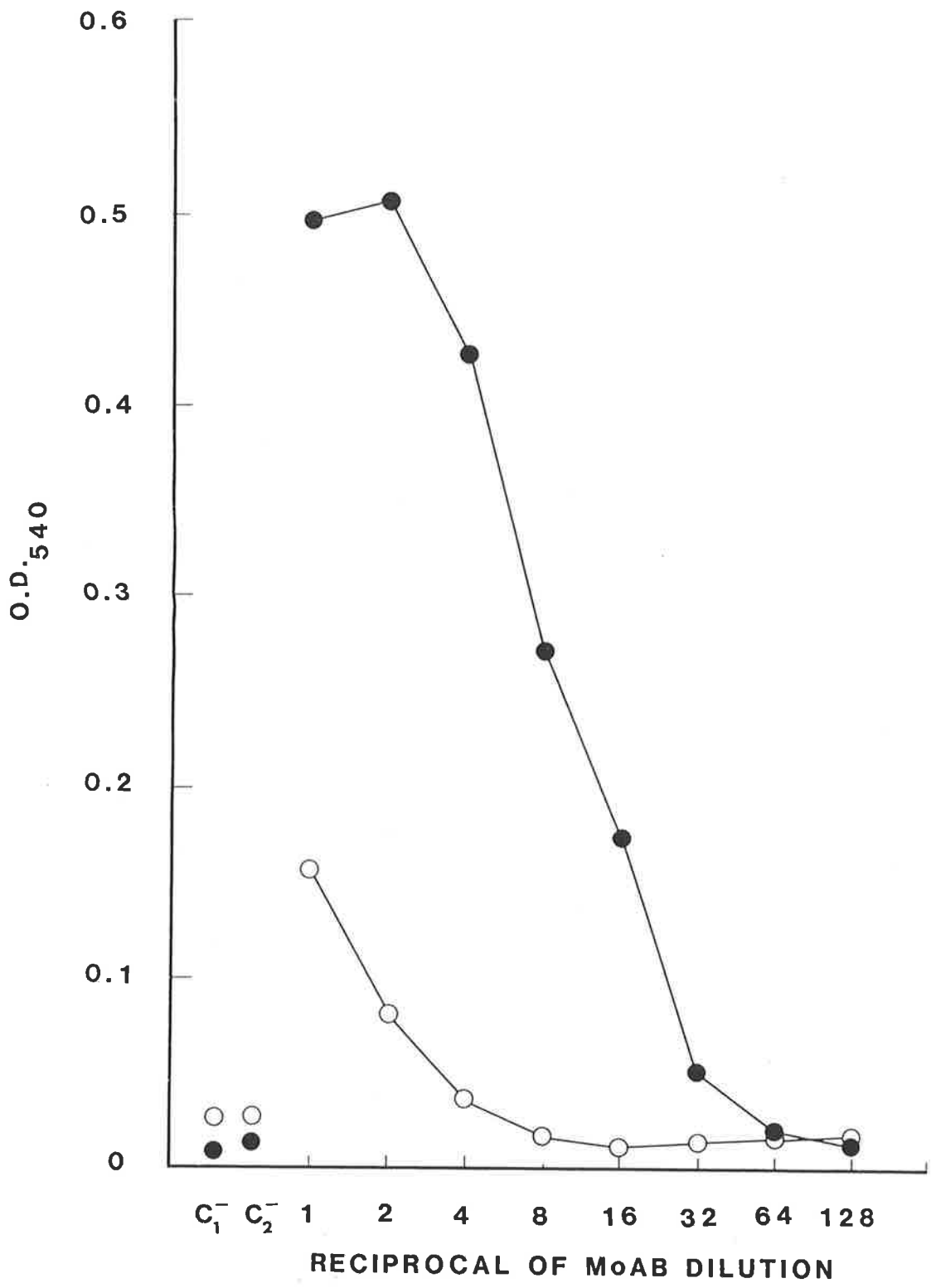
For this comparison, 20 MoAb's of various subclasses were assayed for binding to DAUDI cell surface antigens. Of these 20 MoAb's, 6 were found to be negative and 12 positive in both assays. There were 2 MoAb's which gave discordant results. One MoAb, PA5, was positive in the RBA, and unable to be confidently assigned by RIA, i.e. (+/-).

Figure 4.1

Titration of the monoclonal antibody SB1 (IgG1) on Daudi cells, 10^5 cells per well by the Rose Bengal Assay.

○ : Cell binding step at 4°C for 120 minutes.

● : Cell binding step initially at 37°C for 30 minutes, followed by 4°C for 90 minutes. C⁻¹ and C⁻² are the negative control antibodies Sal-2 (IgG1) and Sal-5 (IgG2a) respectively. Points are the averages of duplicate wells.



Another MoAb, QA4, was negative by RBA and positive by RIA. Subsequent investigation revealed that QA4 had an affinity for the plastic tubes used in the RIA, causing a false positive result. Resuspension of the QA4 labelled cells and transfer to a fresh tube before counting reduced the radioactivity by 60% and resulted in this antibody being classified as negative. In contrast, transfer of cells from other positive and negative control MoAb tests to fresh tubes before counting only resulted in decreases in counts of 10-15%. Comparative data for the RBA and RIA are shown in figure 4.2. Statistical analysis of the data by the Spearman Rank Correlation test [Siegel, 1956] gave a correlation coefficient, r_s , of 0.694, which has a $p < 0.001$.

4.3.3 Comparison with with Manual Indirect Immunofluorescence.

The same 20 MoAb's used in the RIA comparison were tested for binding to DAUDI cells by manual indirect immunofluorescence. The results of the assay in terms of positivity and negativity were in total agreement with the RBA (data not shown). Antibody QA4 was negative, and PA5 was weakly reactive, with about 30% of DAUDI cells scored as positive.

4.3.4 Comparison with Indirect Immunofluorescence Scored by Flow Cytometry.

This comparison, which used 19 of the 20 MoAb's used previously, showed FACS analysis, like manual indirect immunofluorescence, to be in agreement with the RBA in assigning positivity or negativity. MoAb QA4 was again shown to be negative, whereas PA5 showed weak binding to DAUDI cells with a peak channel fluorescence intensity of 38 on a logarithmic scale compared with a negative control peak of 30. Figure 4.3 shows a comparison of data from the RBA and FACS analysis.

Figure 4.2

Comparison between the RBA with RIA of binding of MoAb's to Daudi cells. Cell numbers used were 3×10^5 per well in the RBA and 5×10^5 per tube in the RIA. Points are the average of duplicate assays. Arrowed points PA5 and QA4 are discussed in the text. (Spearman rank correlation coefficient $r_s=0.694$, $p<0.001$).

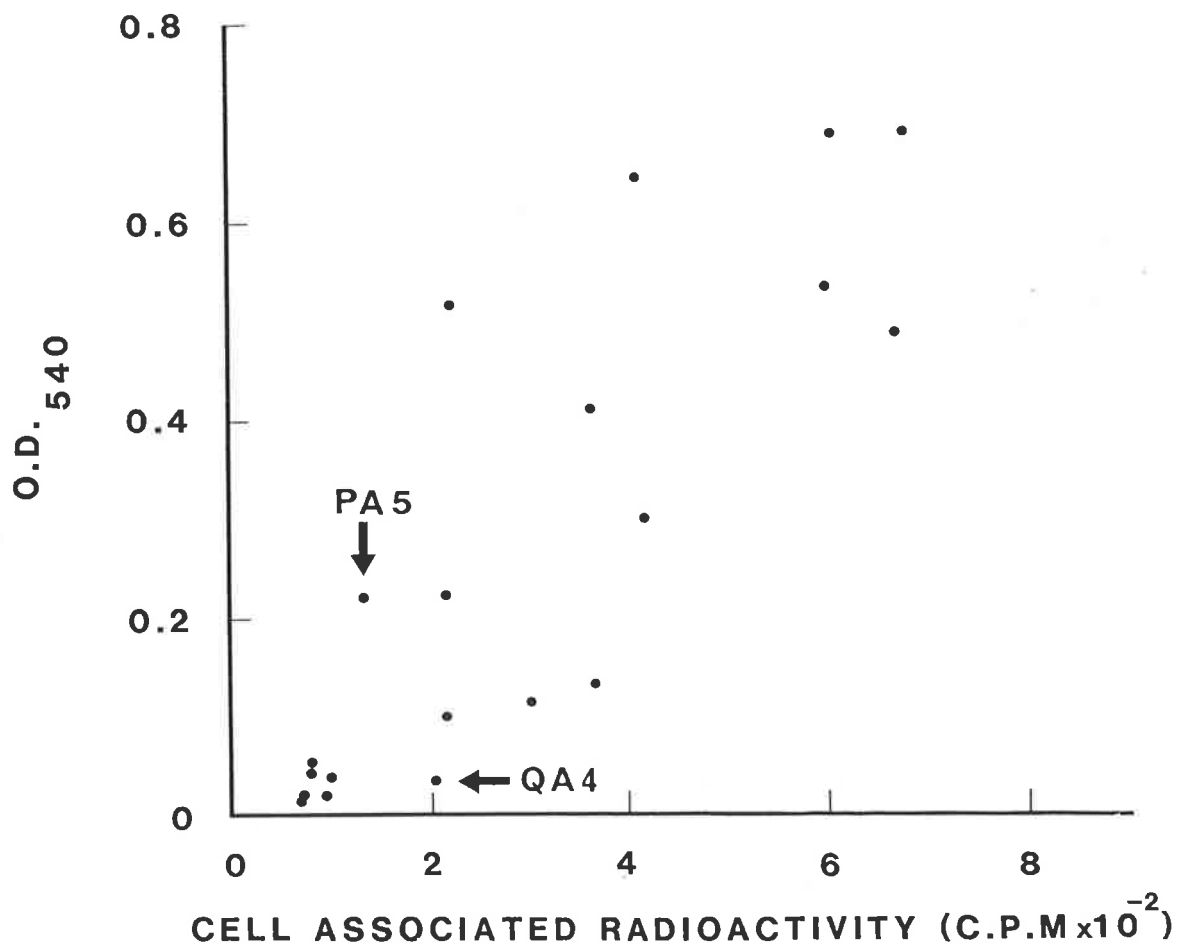
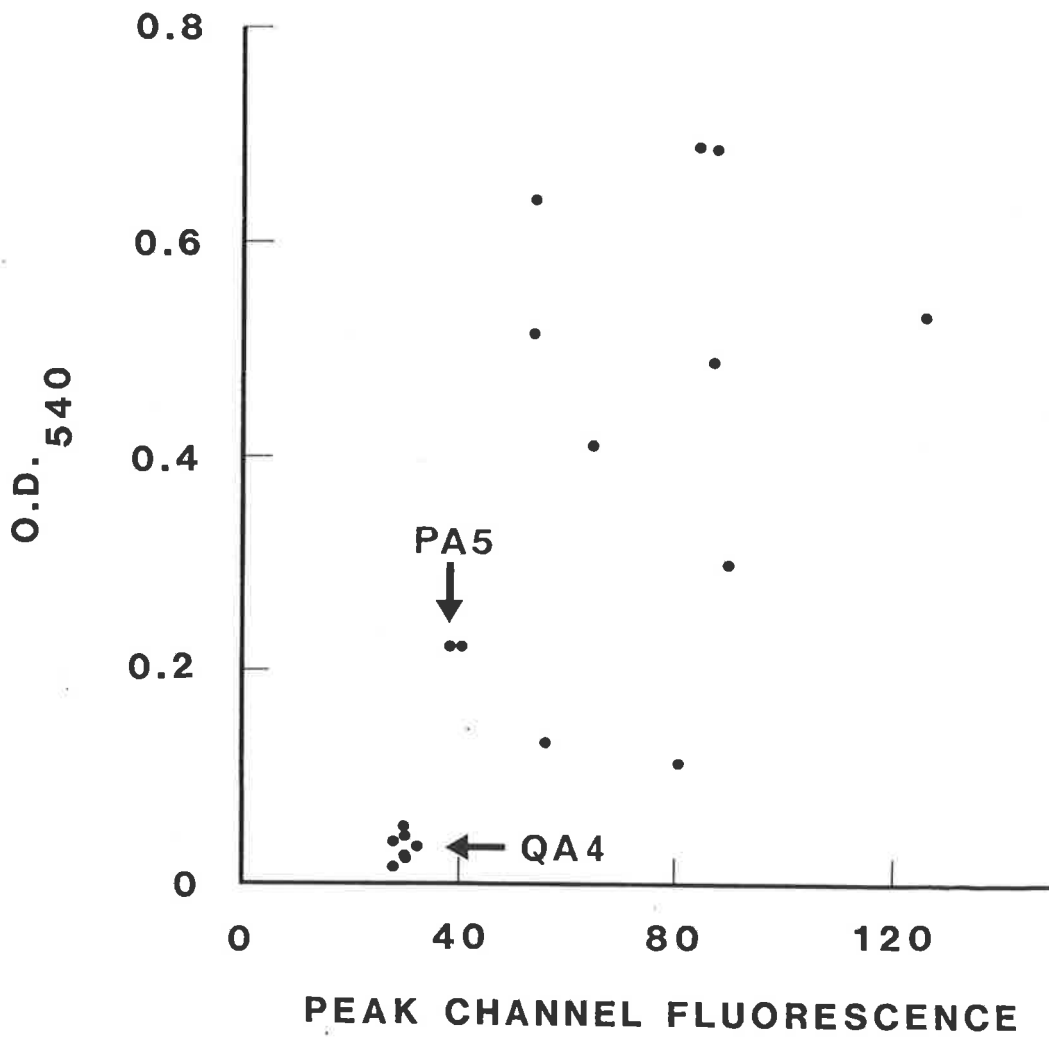


Figure 4.3

Comparison between the RBA with indirect immunofluorescence (with scoring by flow cytometry, logarithmic gain) of binding of MoAb's to Daudi cells. Cell numbers used were 3×10^5 per well in the RBA and 5×10^5 in the immunofluorescence assay. Points are the average of duplicate assays. Arrowed points PA5 and QA4 are discussed in the text. (Spearman rank correlation coefficient $r_s=0.578$, $p<0.01$).



Spearman Rank Correlation coefficient, r_{S} , for this set of data was 0.578, corresponding to $p < 0.01$. The r_{S} for the comparison of the FACS and RIA data was 0.796, corresponding to $p < 0.001$.

4.3.5 Screening of an Anti-AML Fusion: Comparison with RIA

Primary screening of supernatants of hybridomas, which had been prepared from spleen cells of mice immunized with peripheral blood mononuclear cells (containing 86% blasts) from a patient with AML, was carried out simultaneously by RIA and RBA. In addition, the supernatants were screened against an autologous EBV-transformed B cell line, and the DAUDI Burkitt cell line. Figure 4.4 shows a colour photograph of the RBA plates of this assay. Of the 84 supernatants assayed, 21 could be confidently assigned positive according to the RBA results. Of these 21 supernatants, only 11 could be confidently assigned as positive by the RIA, the rest were negative. A further 3 supernatants were (+/-) by RIA, but were negative by RBA. Another supernatant (arrowed in figure 4.5) gave a strong positive result by RIA but was negative by RBA. It was suspected that the reason for this result could be non-specific binding of the MoAb to the tube, as in the case of QA4 (see above), but further investigation of this antibody was not carried out. Figure 4.5 shows a regression plot of the results of this comparison. The correlation coefficient, r_{S} , was 0.912, corresponding to $p < 0.001$. The MoAb's of interest from this fusion were further characterised by manual indirect immunofluorescence. Some clones were apparently unstable and stopped producing antibody, but all those which remained positive by RBA were also positive by indirect immunofluorescence.

Figure 4.4

Photograph of the RBA plates used to screen a fusion for antibodies binding to AML cells (Plate 1), Daudi cells (Plate 2) and to the autologous B cell line (plate 3). See Figure 4.5 for a comparison of RBA data with RIA.

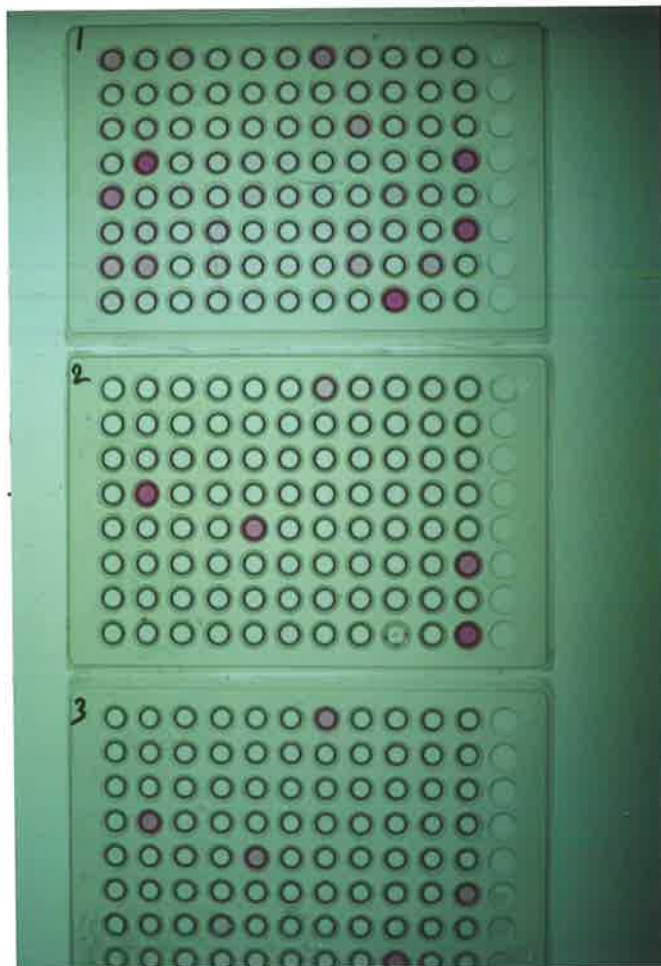
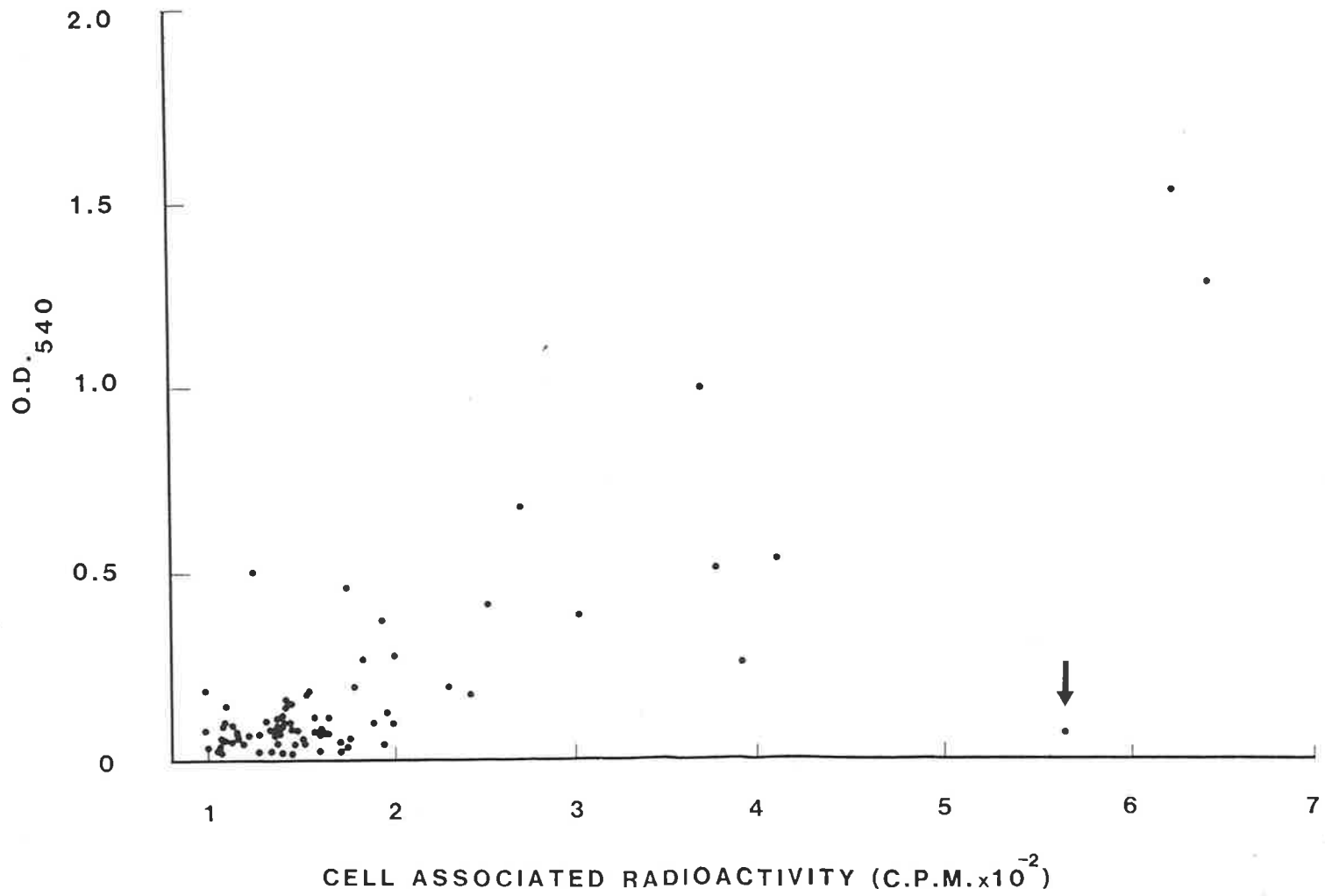


Figure 4.5

Primary screening of hybridoma supernatants against immunizing AML cells by RBA and RIA. The figure shows a regression plot of results obtained by the two methods. Points are the average of duplicate assays. Arrowed point is discussed in the text. (Spearman rank correlation coefficient $r_s=0.912$, $p<0.001$).



4.3.6 Results with Other Human Cell Lines and Cell Types.

A similar comparison has been performed using the human myelomonocytic cell line RC-2A [Bradley *et al*, 1982] (data not shown) with similar success. Other human cell lines which have been used successfully in the RBA include U937, HL-60, KG-1, K562, MOLT-4 and BALM-1. In addition, human cell types such as mononuclear and granulocyte fractions of normal peripheral blood, normal tonsillar lymphocytes, concanavalin A stimulated T cell blasts, normal and leukaemic bone marrow cells and numerous myeloid leukaemia samples have been used successfully in the assay. Some leukaemic samples gave extremely low binding in the assay, which was overcome in most cases by the inclusion of the 37°C binding step, without a subsequent increase in the negative control binding. Other leukaemic cell samples and monocytic cell lines gave high background binding which obscured weak specific MoAb's. It was found that the addition of 5% heat-inactivated normal rabbit serum resulted in a marked decrease in the high backgrounds encountered when such cell types were used as targets. In some instances, the RBA was able to detect MoAb's binding to cells in amounts too low to be easily detected by immunofluorescence with scoring done manually or by flow cytometry. For example, the antibody YB5.B8 [Gadd and Ashman, 1985] gave an OD₅₄₀ of 0.048±0.01, with the negative control antibody Sal-2 giving an OD₅₄₀ of 0.016±0.001 when tested against KG-1 cells (result ± SD of quadruplicate assays), whereas immunofluorescence scored by flow cytometry resulted in superimposable fluorescence histograms, with a barely discernible "tail" of positive cells (figure 4.6). KG-1 cells do express the antigen bound by YB5.B8, as this antibody is able to precipitate a molecule of the appropriate molecular weight from solubilised KG-1 surface membrane by the method described in section

Figure 4.6

Binding of the MoAb YB5.B8 to KG-1 cells assayed by indirect immunofluorescence with flow cytometric scoring.

Median fluorescence intensities (logarithmic gain):

A. YB5.B8 2.14

B. Sal-2 1.92

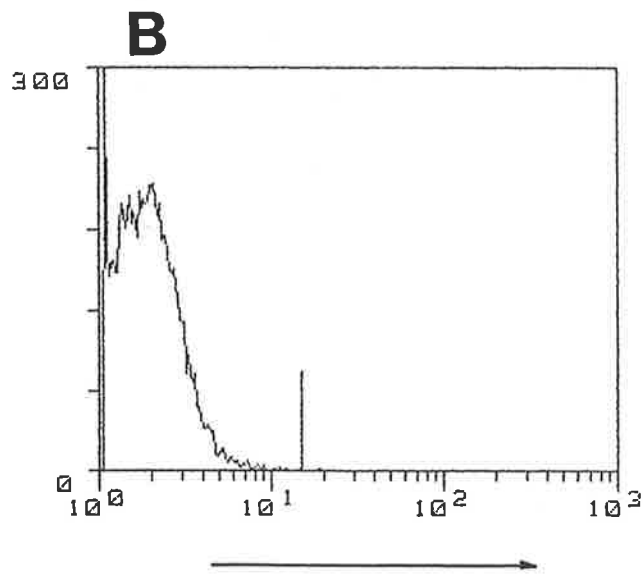
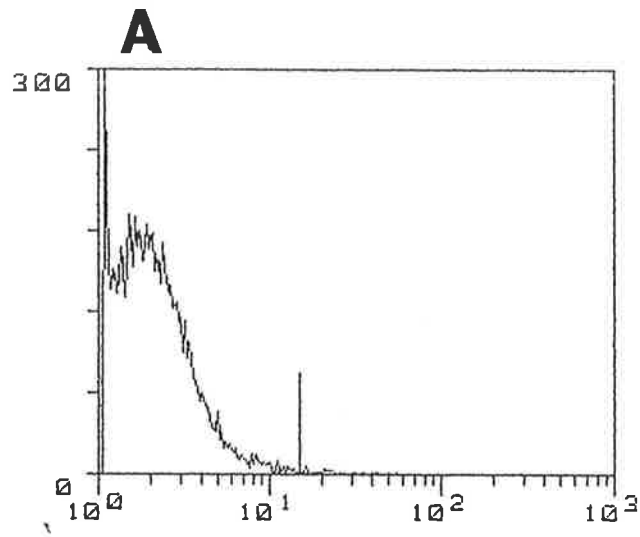
OD₅₄₀ in the RBA (see text):

YB5.B8 0.048 ± 0.01

Sal-2 0.016 ± 0.001

(Results average ± SEM's
of quadruplicate assays)

FREQUENCY



FLUORESCENCE INTENSITY

2.7 (S.R. Cole, unpublished data). Two other monoclonal antibodies detailed in section 5.3.3 and table 5.3 also demonstrate the sensitivity of the RBA.

4.3.7 Detection of MoAb's Specific for Minor Cell Populations.

Mixtures of the human myelomonocytic cell line RC-2A, and the human promyelocytic cell line HL-60 were used to examine the ability of the RBA to detect a MoAb binding to a population of cells making up only a proportion of the cell sample tested. The anti-Ia MoAb FMC-14 binds to RC-2A cells but not to HL-60 cells. A total of 2.5×10^5 cells per well were added to goat anti-mouse Ig(G+M)-coated microtitre trays to which FMC-14 had been bound. The proportion of RC-2A cells in the mixture ranged from 0% to 100%. Table 4.1 shows that the RBA is able to detect MoAb's specific for a minor population of cells comprising 5-10% of the total population.

4.4 Discussion.

The preceding results show that the RBA is able to accurately identify MoAb's specific for cell surface antigens. In a number of cases this assay was able to detect antibodies that may have been overlooked if conventional screening methods had been employed. One of the purposes of developing an improved screening procedure was to maximize the chances of detecting low copy number antigens, and the examples given in section 4.3.6 show that the assay fulfills this requirement. Statistical analysis of the data from experiments in which the RBA was compared with RIA and indirect immunofluorescence revealed a high correlation between the results of the different assays. However, the regression plots appeared to be non-linear, which

Table 4.1

RBA using Mixtures of "Positive" and "Negative" Cells.

% RC-2A	% HL-60	OD ₅₄₀
100	0	1.14
75	25	0.90
50	50	0.73
25	75	0.30
10	90	0.12
5	95	0.05
0	100	0.02

The RBA was carried out on mixtures of RC-2A cells (FMC-14 positive) and HL-60 cells (FMC-14 negative) using the MoAb FMC-14. Cell numbers used were 2.5×10^5 per well in total. Results are averages of duplicate wells. The negative control MoAb Sal-2 (IgG1) gave an OD₅₄₀ of between 0.02 and 0.025 on both cell lines.

may indicate that the results obtained by RBA were not simply a function of antigen density.

Apart from its accuracy, the RBA offers a significant advantage over RIA and immunofluorescence due to its rapidity and non-labour intensive nature. In addition, the RBA overcomes three major disadvantages encountered with the RIA: the non-specific binding of antibodies to the RIA tubes, the poor resolution of weakly binding from negative MoAb's, and as with immunofluorescence, damage to cells due to repeated centrifugation and resuspension.

The RBA has a very low control negative background OD_{540} which allows easier identification of 'weakly positive' MoAb's which cannot always be confidently assigned as positive or negative by RIA. Typically, such antibodies gave results 30 to 60% above background radiolabel binding, whereas in the RBA they gave an OD_{540} of 0.1 - 0.2 compared with a control negative OD_{540} of 0.01 - 0.05. This ability is enhanced greatly by the modified protocol, in which the cell binding step is carried out at 37°C, as the negative control binding is not increased. The necessity for repeated centrifugation and resuspension of the target cells can raise the 'background' in RIA and immunofluorescence due to cell damage. Cell lines, due to their fragility, are especially vulnerable. The RBA avoids this problem as the cells are added at a late stage of the assay.

Whole cell ELISA assays, while rapid to perform, have the disadvantage of requiring fixation of the target cells, which can result in loss of antigenic determinants [Hancock *et al*, 1982] [our unpublished results]. The RBA uses cells in physiologic, buffered medium during the binding process, ensuring retention of antigenic determinants.

The RBA has the ability to detect of the order of 20ng per well of specific antibody to a cell surface antigen. While this is not as sensitive as an optimised ELISA assay, which can detect of the order of 1-10 ng of specific antibody per test well, for the purposes of screening hybridoma supernatants, this level of sensitivity is entirely adequate, as supernatants of antibody producing hybrids usually contain more than 1 μ g of MoAb per ml. The RBA allows detection of MoAb's binding to minor populations in cell samples, which is advantageous when screening hybridoma supernatants against heterogeneous cell samples.

CHAPTER 5

Production of monoclonal antibodies binding to human myeloid
cells.

5.1 Introduction

The state of knowledge of normal myeloid haemopoietic differentiation and its leukaemic counterpart has been enhanced by the production of murine monoclonal antibodies binding to structures on the surface of haemopoietic cells [e.g. Griffin, 1985; Robak and Goldman, 1985; Reinherz *et al*, 1986].

Monoclonal antibodies which bind to structures whose expression is limited to cells of a given lineage or which bind to very immature haemopoietic cell types are of particular interest, as they facilitate the investigation of the relationship between cells of the haemopoietic system. In addition, they have potential for diagnosis and treatment of the myeloid leukaemias [e.g. Ritz and Schlossman, 1982; Drexler *et al*, 1985; Foon and Todd, 1986]. Monoclonal antibodies which exert functional effects on myeloid cells have been only rarely reported (see section 1.3.3.2).

It was proposed to produce murine monoclonal antibodies which were myeloid specific, using the human myelomonocytic cell line RC-2A [Bradley *et al*, 1982] as the immunogen. Preliminary results using a polyclonal murine antiserum raised against RC-2A cells indicated it may be possible to produce MoAb's which alter the proliferative ability of myeloid cells (see sections 3.3.8, 3.3.9), perhaps by binding to the receptor for a growth or differentiation-inducing factor.

This goal was to be approached in the following way;

1. Using a screening protocol which allowed rapid identification of myeloid specific antibodies by simultaneous screening on an autologous B lymphoid cell line as well as the immunizing cell line.

Antibodies which bound to the myeloid cell line but not the B cell line would be considered provisionally myeloid specific; antibodies which bound to both cell lines were to be discarded.

2. Developing a screening assay which was sensitive, accurate and rapid, to maximise the chance of detecting antibodies to antigens of low copy number. It has been demonstrated that antigens with a functional role, for example growth factor receptors, are sometimes present in very small amounts [e.g. Metcalf, 1986]. This was achieved by developing a modification of the RBA described in chapter 4.

3. By simultaneously screening supernatants for their ability to alter ³H-Thymidine incorporation by the immunizing cells. i.e. ability to modulate proliferation.

Cells of the human myelomonocytic cell line RC-2A were used to immunize mice for the production of monoclonal antibodies binding to myeloid specific antigens. Hybrid supernatants were screened for binding to the immunising cell line and the autologous EBV B cell line Cess B [Bradley *et al*, 1982]. This strategy was employed to identify antibodies with specificity for myeloid cells, by allowing antibodies to ubiquitous "species" specific antigens to be screened out early in the process. Hybridoma supernatants were also screened for their ability to modify the proliferation of RC-2A cells.

5.2 Materials and Methods.

5.2.1 Immunisation of mice.

Two mice were immunized by the peritoneal route with 10^7 washed RC-2A cells in sterile PBS. Forty days later, each mouse was injected with a further 10^7 RC-2A cells by the same route. After a period of 21

days, mice were injected with 10^7 RC-2A cells via the dorsal tail vein. Three days later, mice were sacrificed and their spleens removed aseptically for preparation of spleen cells required for fusion.

5.2.2 Preparation of single-cell suspensions from mouse spleen and thymus.

Spleens or thymuses were removed aseptically and washed in serum free medium. The tissue was chopped into small pieces using sterile surgical scissors and placed in 5 ml of serum free RPMI 1640 medium. The tissue was gently homogenized using a loose-fitting ground-glass homogenizer and the debris was allowed to settle for 2-3 minutes. The debris-free single cell suspension was decanted and the cells were washed three times by repeated centrifugation (200g for 5 min) and resuspension in medium with or without FCS, depending on the final use to which the cells would be put.

Lysis of erythrocytes from spleen cell suspensions was achieved by suspending cells in a hypotonic solution. Stock solutions A and B (see section 2.1) were mixed at a ratio of 9:1 just before use and put on ice. Pelleted, washed spleen cells were resuspended in this solution at a concentration of 2×10^7 cells/ml and incubated for 10 min on ice. The intact cells were recovered by centrifugation and the cells were washed three times in medium as previously described, and cell number estimated using a haemocytometer and viability assessed by trypan blue exclusion.

5.2.3 Production of monoclonal antibody secreting murine hybridomas.

The method of Oi and Herzenberg, 1980 was essentially used as described. Spleen cell suspensions of immunized BALB/c mice were made

and the erythrocytes were removed by hypotonic lysis (see section 5.2.2). Routinely, $1-3 \times 10^8$ cells per spleen were obtained.

The fusion partner was the murine myeloma cell line x63-Ag-8.653 which produces no immunoglobulin of its own (Kearney *et al*, 1979) Myeloma cells were harvested from logarithmic phase cultures and had a viability of over 90%, as judged by trypan blue exclusion. Cells from each spleen were mixed separately with myeloma cells so that the ratio of spleen to myeloma cells was 5:1. The mixtures of cells were centrifuged in 50 ml conical Falcon tubes (Becton Dickinson) at 200g for 5 min and all but 1 ml of the supernatant aspirated. The pellet of cells was resuspended in the 1 ml and re-centrifuged to ensure even distribution of the fusion partners, and the supernatant aspirated fully.

The agent used for fusion (PEG; polyethylene glycol) was prepared just before use by autoclaving 2g of PEG (BDH), which was allowed to cool to approximately 45°C, before the adding 2 ml of serum free RPMI 1640. After thorough mixing, this solution was held at 37°C until use. One ml of the PEG solution was added to each pellet of fusion partner cells, stirring in dropwise over one min, followed by a 2 min incubation at 37°C. The PEG and cell mixture was diluted gently over 2 min with 2 ml of serum-free RPMI 1640, then with a further 7 ml over 2-3 min. The cells were then pelleted by centrifugation at 200g for 5 min, and resuspended in medium containing 2% v/v 50x HAT (Hypoxanthine/Aminopterin/Thymidine; Flow Laboratories) and 10% v/v FCS (HAT medium) to a density of 2.5×10^6 cells per well.

One ml of the suspension was plated per well in an appropriate number of 24 well tissue culture trays (Costar #3524) i.e. 2 trays per 10^8 spleen cells used in the fusion. The trays were incubated in a fully humidified atmosphere of 5% CO₂ in air at 37°C. After 4 days, 1

ml of HAT medium was added to each well. Cultures were left undisturbed other than to check for contamination until macroscopic hybrid colonies were observed, usually from day 8 onwards. When most wells had visible hybrid growth, but before acidification of the medium occurred, aliquots of supernatant were removed using sterile technique for assay against the immunizing cells. Hybrids which were producing antibodies of interest were cloned by limit dilution and then grown up in medium containing 2% v/v 50x HT (Hypoxanthine/Thymidine; Flow Laboratories) and 10% v/v FCS (HT medium) to obtain frozen stocks. (see sections 5.2.6 and 5.2.7)

5.2.4 Screening by RBA.

Aliquots were removed from hybrid containing wells on day 15 for assay by the RBA (see section 4.2.3). Target cells were used at 2×10^5 cells per well. Stocks of RC-2A and Cess B, which had been grown up and cryopreserved as assay stocks, were thawed and used in the screening assay (see sections 2.3.4, 2.3.5, 2.3.6 and 2.3.7).

5.2.5 Screening by ^3H -Thymidine uptake assay.

The assay described in section 3.2.12 was used to screen sterile supernatants for their ability to affect the proliferation of RC-2A cells. Briefly, 50 μl of each supernatant was plated in triplicate with 4×10^4 RC-2A cells in a final volume of 200 μl of RPMI 1640 + 10% FCS in 96 well tissue culture plates (Linbro, round bottomed well, USA). Supernatants from some wells containing no visible hybrids were also assayed to control for the possible effects of HAT medium on ^3H -Thymidine uptake by RC-2A cells. After 3 days in culture, wells were pulsed with 1 μCi of ^3H -Thymidine for 18 hours, harvested and counted as previously described in section 3.2.10.

5.2.6 Limit dilution cloning of antibody-producing murine hybridomas.

The method used for the cloning of hybrids was essentially as described by Oi and Herzenberg, 1980. Young BALB/c mice (4-10 weeks old) were used to make single cell suspensions of thymocytes to act as feeder cells during cloning (see section 5.2.2). Approximately 230×10^7 viable hybridoma cells were placed in 4.6 ml of HT medium containing 10^7 thymocytes/ml. Aliquots (0.1 ml) of this suspension were placed into the first 36 wells of a 96 x 0.2 ml flat bottomed tissue culture plate (Linbro, USA) (i.e. average of 5 cells/well). To the remaining 1 ml suspension, 4 ml of the thymocyte suspension was added. This was placed in 0.1 ml aliquots in the next 36 wells (i.e. average of 1 cell/well). To the remaining 1.4 ml a further 1.4 ml of thymocyte suspension was added, and 0.1 ml aliquots of the resulting mixture were placed in the final 24 wells (i.e. average 1 cells/2 wells). The cloning plates were incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air undisturbed for 7 days other than to check for contamination. When hybrid colonies became visible, a further 0.1 ml of HT medium was added to wells. When colonies covered about 1/3 of the well, 0.1 ml of supernatants from wells exhibiting clonal growth and some samples from non-clonal areas (see section 5.3.3) were aspirated using sterile pasteur pipettes and assayed for antibodies binding to the immunising cells. Clones of interest were expanded into 24 well Costar plates, with 10^6 thymocytes per ml to act as feeder cells. From this point onwards, clones were expanded and cryopreserved as in sections 5.2.7 and 2.3.6, taking particular care not to cross-contaminate cultures.

5.2.7 Expansion of hybrids.

Hybridoma cultures producing antibodies of interest were expanded when confluent growth of hybrid cells was observed over at least 2/3 of the bottom of a well in the 24 well plates. The cells were resuspended and the contents of the well (2 ml) placed in the well of a 6 well Costar dish. To each well, 3 ml of HT containing 10^6 BALB/c thymocytes per ml (see section 5.2.2) was added. Between 1 and 4 days later, depending on the growth rate of the hybrid cells, a further 5 ml of HT medium was added. When these cultures reached an appropriate density (i.e. when the medium turned orange), 8 ml of the 10 ml was harvested and transferred to a 30 ml Costar dish. Eight ml of HT medium was added back to the 10 ml wells and up to 24 ml of HT medium was placed in the 30 ml dish. This procedure continued until there were enough cells to freeze down stocks of at least 2 ampoules of each hybrid expanded, with each ampoule containing $5-10 \times 10^6$ cells (see section 2.3.6). The supernatants from these uncloned hybrids was retained to ensure these stocks were still producing antibody and also allowed comparison with the properties of the antibodies produced by cloned hybrids. The rate at which hybrids grew varied considerably, so the process of expansion was matched with the growth of each hybrid to ensure that neither overgrowth or "stalling" of cultures due to overdilution occurred.

5.2.8 Determination of immunoglobulin sub-class by radial immunodiffusion.

Supernatant samples were first concentrated approximately five to ten-fold by centrifugation in Centriflo CF-25 membrane cones (Amicon Corp., USA) at 400g in a MSE minor S bench centrifuge for 25 min.

Glass microscope slides (50 x 75mm, Esco or Corning) were washed with alcohol, dried and then pre-coated with 0.1% agarose in PBS/Az. A solution of 1% w/v agarose in PBS/Az was melted at 100°C and 10ml was carefully pipetted onto each coated slide. After setting the agarose by incubation at 4°C for 1 hr, rosettes were cut into the gels using a perspex template and a gel punch (Biorad). The well dimensions were chosen to allow a volume of 10µl to be placed in each well. Into each central well was pipetted 10µl of subclass-specific goat anti-mouse immunoglobulin (Meloy, Springfield, USA), which was specific for mouse IgM, IgG1, IgG2a, IgG2b, or IgA. Concentrated supernatants and standard immunoglobulin solutions were pipetted into the wells surrounding the goat anti-mouse subclass reagent-containing well. The slides were incubated at 4°C for 48hr in humidified atmosphere and were then inspected for lines of immunoprecipitated immunoglobulin between the wells. To detect very weak immunoprecipitation, slides were first washed to remove non-precipitated protein by immersion in saline for 30 min, then dried by overlaying with Whatman No. 1 filter paper, paper towel and a heavy glass plate. The paper towel was changed several times until no more fluid was extracted. This procedure was repeated 5 times, with a final overnight soaking in distilled water. The slides were air dried and stained for 30 min in 0.15% w/v Coomassie Blue G-250 stain in methanol:acetic acid:water (5:1:5 v/v/v), and destained with overnight in the same solvent.

5.2.9 Determination of immunoglobulin concentrations.

The quantity of immunoglobulin in supernatants was determined using a competitive enzyme linked immunosorbent assay. Flexible plastic 96 well trays (Linbro cat. no. 76-364-05) were coated overnight with the appropriate immunoglobulin subclass by placing

100 μ l of a 20 μ g/ml solution in PBS/Az into wells and incubating overnight at 4°C in a humidified atmosphere. Some wells were left uncoated as negative controls. Coating solution was aspirated from the wells at the end of the incubation period and the wells washed three times by flooding with PBS and flicking. To block non-specific binding sites, 0.2 ml of a 0.3% w/v solution of gelatin in PBS/Az was added to all wells and incubated for 2hr at room temperature. Plates were washed three times as above. Serial 1/2 dilutions of standard immunoglobulin of the appropriate subclass, ranging from 50 down to 0.1 μ g/ml were made, and 50 μ l aliquots were added to duplicate wells of immunoglobulin coated plates. Fifty μ l aliquots of unknowns (hybridoma supernatants) were also plated in duplicate wells, using serial 1/2 dilutions and starting with a 1/2 dilution. The diluent used was PBS/Az containing 0.05% Tween 20 and 0.1% BSA. Controls used were diluent only in coated wells for maximum binding, and diluent only in uncoated wells for blanks. Fifty μ l of alkaline phosphatase conjugated goat anti-mouse immunoglobulin (diluted 1/500 in Enzyme Diluent, see section 2.1) was added to each well, and plates were incubated overnight at room temperature in a 5% CO₂ atmosphere. The liquid was aspirated from the wells and then they were washed 4 times with PBS/Az containing 0.05% Tween 20, and once in saline by flooding and flicking. Two hundred μ l of substrate solution (1 mg/ml p-nitrophenylphosphate in Assay Buffer (see section 2.1) was added to wells and plates were incubated for 3-4 hr at 37°C in a humid atmosphere. The OD₄₀₅, corresponding to p-nitrophenol, was read on a Titertek Multiskan Micro-elisa reader. The absorbance was plotted against the logarithm of the reciprocal of the dilution of the immunoglobulin standards and unknowns. The immunoglobulin concentrations of the unknowns was calculated by interpolating the the

dilutions of standards and unknowns required to give 50% inhibition of binding of the alkaline phosphatase-conjugated goat anti-mouse immunoglobulin to the coated wells.

5.3 Results.

5.3.1 Screening by RBA.

Hybridoma supernatants from 72 wells were screened at 15 days post-fusion for antibody binding to RC-2A cells and the Cess B autologous EBV B-cell line by the RBA (see section 4.2.3). Hybrids were considered to be producing specific antibody if supernatants gave more than double the OD₅₄₀ of wells that contained negative control anti-Salmonella MoAb's Sal-2 (IgG1), Sal-5 (IgG2a) and Sal-3 (IgM). A positive control MoAb, FMC-14, known to bind strongly to both cell lines was also included in all RBA screens.

Of the 72 hybrid supernatants screened, 39 contained antibody which bound to RC-2A cells and 23 which bound to Cess B cells. Eighteen of those binding to RC-2A cells did not bind to Cess B, and thus appeared to have specificity for myeloid cell-surface structures. Two supernatants contained antibody which bound to Cess B but apparently not to RC-2A.

5.3.2 Screening for ability to alter ³H-Thymidine uptake by RC-2A cells.

It was found that about one-half of these supernatants caused changes of up to 50% in the uptake of ³H-Thymidine by RC-2A cells, however it appeared that supernatants which came from wells with dense growth were more likely to give decreased uptake. Upon further testing of later cultures, it was found that cultures rarely gave the same

result as the initial screen (data not shown). It appeared that this type of assay was not suitable as an initial screen for proliferative effects by hybridoma supernatants. The fluctuating results may have been caused by breakdown products liberated into the cultures by the dying parental cells or densely growing hybrid cells. The presence of HAT medium did not appear to inhibit the uptake of ^3H -Thymidine to an extent that would make the assay too insensitive, which is in agreement with similar assay used by Lögberg *et al*, 1985.

The five hybrids chosen for further investigations (see section 5.3.3) were tested for their ability to alter proliferation. For this purpose, cultures were established from cells in logarithmic growth, and supernatants were harvested before any sign of overgrowth occurred. In addition, supernatants were titrated from 1/4 to 1/32 in 1/2 serial dilutions in an attempt to minimise the possibility of any non-specific effects obscuring specific alterations in ^3H -Thymidine uptake. Results of triplicate wells are shown in table 5.1. These results showed no evidence of alteration in proliferation of RC-2A cells by any of the five MoAb's.

5.3.3 Limit dilution cloning of antibody secreting hybridoma cells.

The eighteen hybridomas identified as having specificity for myeloid cells by the RBA were cloned by limit dilution (see section 5.2.5) as soon as practicable after primary screening to ensure monoclonality, and to prevent the antibody secreting hybrids from being overgrown by non-antibody producing variants.

Supernatants from wells which contained hybridoma colonies that had a probability of 95% or greater of being monoclonal, based on a Poisson distribution of hybrid cells in the plating procedure (i.e. less than 1/3 of the wells of a given dilution having colonies

Table 5.1

Effect of supernatants of cloned hybridomas on the uptake of ^3H -Thymidine by RC-2A cells. Results expressed as counts per minute of triplicate cultures \pm standard deviations.

Dilution	1/4	1/8	1/16	1/32
<u>MoAb</u>				
4B4. E11	73787 \pm 9561	66658 \pm 21921	65168 \pm 19885	69160 \pm 15363
4B5. F5	72199 \pm 17032	66768 \pm 17587	72604 \pm 23912	82987 \pm 4719
4C5. D12	86521 \pm 4283	60887 \pm 17167	66868 \pm 27544	61442 \pm 17569
5A2. G5	744471 \pm 3944	69709 \pm 4964	65233 \pm 7039	75550 \pm 855
5A4. C5	77334 \pm 7030	67907 \pm 12904	64491 \pm 15033	72972 \pm 8146
<hr/>				
Dilution	1/16	1/32	1/64	1/128
<u>Serum</u>				
NMS	83580 \pm 3665	73813 \pm 16348	70805 \pm 9792	68507 \pm 8240
α -RC-2A	63485 \pm 4367	71120 \pm 7621	115325 \pm 12078	143078 \pm 17652
Control; RPMI 1640+10%FCS		73824 \pm 11747		

growing), as well as some supernatants from non-clonal regions were assayed for binding to RC-2A and Cess B cells by RBA.

Of the 18 hybridomas cloned, 2 failed to give any colonies, 3 failed to yield any positive clones, and 1 retested positive on the B cell line Cess B. Five of the remaining hybrids were chosen to be retained for further investigations. The decision to retain the five hybridomas (designated 4B4, 4B5, 4C5, 5A2 and 5A4, based on the position of the hybrid in the original 24 well plate.), was made on binding data shown in chapter 6, which demonstrated that the antibodies bound to five different structures. Table 5.2 shows the results of screening of the supernatants from hybrid containing wells of the plates used to clone the hybridomas 4B4, 4B5, 4C5, 5A2 and 5A4. The wells from which hybrids were expanded were chosen to ensure their monoclonality. Clones of four of the hybrids were expanded from areas of the cloning plate which exhibited less than 6% occupancy of wells within that dilution. The hybrid 5A4 failed to give antibody producing clones in the "clonal" region of the plate, therefore cryopreserved cells expanded from a positive well (C5) of the primary cloning plate were recloned. The clone which was expanded from the secondary cloning plate, 5A4.C5.E11, will hereafter be designated 5A4.C5 to denote its original source. The supernatant from 4B4.E11 hybrids in later cultures gave a slightly different binding pattern to the original supernatant, so this hybrid was recloned from an early stock of 4B4.E11 to ensure monoclonality. The clone 4B4.E11.D12 was chosen for expansion as it was monoclonal and exhibited the original binding pattern. This clone will hereafter be referred to as 4B4.E11 to denote its original source.

Table 5.2

Cloning of hybridomas.

MoAb	Dilution	Occupancy	Positive on RC-2A	Clone Selected
4B4	0.5/well	1/24	1/1	4B4.E11 (recloned)
4B4.E11	1/well	19/36	9/9	4B4.E11.D12
	0.5/well	4/24	4/4	
4B5	1/well	1/36	1/1	4B5.F5
	0.5/well	1/24	1/1	
4C5	5/well	13/36	4/4	4C5.D12
	1/well	2/36	2/2	
5A2	0.5/well	1/24	1/1	5A2.G5
5A4	5/well	36/36	5/6	5A4.C5 (recloned)
	1/well	4/36	0/4	
	0.5/well	1/24	0/1	
5A4.C5	1/well	6/36	5/6	5A4.C5.E11

5.3.4 Immunoglobulin sub-classes of monoclonal antibodies.

All of the five MoAb's retained for further study (see section 5.3.3) only gave precipitin lines in immunodiffusion assay against an IgG1-specific reagent. No lines were observed in immunodiffusion assay against reagents specific for other subclasses, therefore it is concluded that the only detectable immunoglobulin subclass produced by the hybridomas is IgG1.

5.3.5 Establishment of frozen stocks of hybridoma cells, and supernatants.

Cultures of clones designated 4B4.E11, 4B5.F5, 4C5.D12, 5A2.G5 and 5A4.C5 were expanded until each was contained in approximately 200 ml and was growing exponentially. At this stage, hybridoma cells were harvested by centrifugation and, for each hybridoma, 3 to 6 ampoules containing $5-10 \times 10^6$ cells were cryopreserved as described in section 2.3.6. The spent medium from these cultures was collected, 0.1% NaN_3 added as a preservative, and these stocks were stored at 4° C. Table 5.3 shows a comparison of binding data of the five antibody supernatant stocks to RC-2A and Cess B cells by RBA, and also compares peak channel fluorescence intensities due to antibody binding to RC-2A cells in an indirect immunofluorescence assay scored by flow cytometry (representative example of 3 separate assays). Note the small peak channel fluorescence shifts given by the antibodies 4B4.E11 and 4C5.D12, in comparison to the increase in OD_{540} over the negative control MoAb Sal-2 in the RBA.

Table 5.3

Comparison between the RBA and Indirect Immunofluorescence assay of binding of MoAb's to RC-2A cells.

MoAb	Rose Bengal Assay		Indirect Immunofluor.	
	OD ₅₄₀		OD ₅₄₀ Test	Median Ch. Fluor.
	Cess B	RC-2A	OD ₅₄₀ Sal-2 RC-2A	(Logarithmic gain) RC-2A
4B4. E11	0.017	0.194	4.9	4
4B5. F5	0.020	0.321	8.3	51
4C5. D12	0.018	0.217	5.4	12
5A2. G5	0.023	0.674	16.9	150
5A4. C5	0.020	1.246	31.2	58
Sal-2	0.018	0.040	1	3

5.3.6 Concentrations of immunoglobulin in MoAb stocks.

The concentration of immunoglobulin in supernatant stocks was assayed by competitive ELISA (see section 5.2.9). The results obtained

were;	4B4.E11	25 µg/ml
	4B5.F5	96 µg/ml
	4C5.D12	11 µg/ml
	5A2.G5	15 µg/ml
	5A4.C5	16 µg/ml

5.4 Discussion.

This chapter describes the production, screening and cloning of monoclonal antibodies binding to human myeloid associated antigens.

The screening of the hybridoma supernatants was carried out using a new colorimetric assay. Apart from rapidity and ease of performance, the Rose Bengal Assay compared favorably in terms of sensitivity and specificity with other screening assays commonly used. The RBA in some cases was able to detect binding of MoAb's to myeloid cells that were either barely detectable or undetectable by indirect immunofluorescence scored by flow cytometry. Examples of such antibodies include YB5.B8 binding to KG-1 cells (see section 4.3.6), and 4B4.E11/4C5.D12 binding to RC-2A cells (see section 5.3.5).

The ³H-Thymidine uptake assay for detection of antibodies exerting a proliferative effect on the immunizing cell line was found to be inappropriate as a primary screening procedure. The assay appeared to be sensitive to the state of growth of cultures tested, perhaps due to cellular breakdown products in dense cultures. Subsequent titration of supernatants from exponentially growing

hybridoma cultures demonstrated that the five tested had no activity in this system.

Five hybridomas produced from a single fusion of spleen cells from mice immunized with human myelomonocytic RC-2A cells were cloned by limit dilution, and their subclasses determined. Clones were cultured to produce stocks of antibody-containing supernatants and cryopreserved cells. Antibody stocks were prepared, and the concentration of immunoglobulin in them was determined in preparation for further investigations of cell binding patterns and molecular identity of the antigens detected by the five antibodies. These studies are covered in Chapter 6.

CHAPTER 6

Characterization of monoclonal antibodies binding to human
myeloid cells.

6.1 Introduction.

Monoclonal antibodies which bind to human myeloid cell surface antigens have been produced by a number of laboratories and reported in the literature. International workshops have been held for the purposes of defining groups of antibodies binding the same structures, similar structures or unique structures in an attempt to clarify a sometimes confusing literature (see section 1.3).

The five MoAb's described initially in Chapter 5 have been characterized according to the cell types they bound, and by the molecular weights of the antigens they recognised on RC-2A cells. This approach was taken to assess the usefulness of these antibodies in facillitating the investigation of the relationships between the cells of the human haemopoietic system. Binding of these antibodies to a limited panel of peripheral blood AML samples was also undertaken in this laboratory as a preliminary step in determining their value as immunodiagnostic reagents. Furthermore, the antibodies were screened for their ability to block or enhance differentiation of myeloid progenitor cells and RC-2A cells.

Many of the MoAbs which have been reported in the literature to bind to human myeloid cells have been shown to recognize common structures which are highly immunogenic in mice [Reinherz *et al*, 1986; McMichael *et al*, 1987], and have been grouped according to binding and other data into clusters recognizing the same or similar structures. The five antibodies were studied in sufficient detail so that they could be provisionally grouped according to workshop clusters. If the antibodies did not appear to be classifiable within cluster groups,

the binding data would then allow comparisons with antibodies which are so far unclustered to be made.

6.2 Materials and Methods.

6.2.1 Estimation of molecular weights of antigens detected by anti-myeloid MoAb's.

MoAbs were used to immunoadsorb antigen from solubilized biotinylated cell surface membranes of RC-2A and Cess B (autologous B cell line) cells as described in section 2.7. Immunoadsorbed protein surface antigen was separated according to molecular weight by SDS Polyacrylamide Gel Electrophoresis using the procedure described in sections 2.7.2 and 2.7.3. The separated proteins were transferred to nitrocellulose sheet electrophoretically as detailed in section 2.7.4, and biotin-labelled protein was detected enzymically using a horseradish peroxidase/streptavidin conjugate, as described in section 2.7.5.

6.2.2 Binding to Human haemopoietic cell lines.

The cell lines used in binding studies were obtained and maintained as described in sections 2.3.4 and 2.3.5. The Rose Bengal assay was used to investigate the binding of the antibodies described in chapter 5 and were carried out as detailed in section 4.2.3.

6.2.3 Binding to normal peripheral blood leukocytes and erythrocytes and bone marrow nucleated cells.

Binding of antibodies to peripheral blood elements was investigated. Suspensions of peripheral blood mononuclear cells and granulocytic cells were prepared from normal donors as in sections

2.4.1 and 2.4.2. Indirect immunofluorescence with scoring carried out manually or by flow cytometry was performed as in section 2.5. The Rose Bengal Assay was carried out as described in section 4.2.3. Haemagglutination assays on human erythrocytes were performed as described in section 2.5.4.

Cloned normal cytotoxic human T lymphocytes which had been activated by exposure to alloantigens on RC-2A cells and maintained using the method of Van de Griend *et al*, 1984, were provided by D. Rofe, and MoAb binding to these cells was assayed by RBA.

Peripheral blood leukocytes and normal bone marrow nucleated cells were isolated by density gradient centrifugation, as described in section 2.4.2 and 2.4.5 respectively. Cytocentrifuge smears prepared as in section 2.4.7 were assayed for binding of the 5 antimyeloid MoAb's by an alkaline phosphatase-anti alkaline phosphatase (APAAP) immunoassay, with Sal-2 (IgG1) used as a negative control, using the method described in section 2.8.2.

6.2.4 Complement mediated lysis of cord blood haemopoietic progenitor cells.

For each test or control antibody, 200 μ l of hybridoma supernatant (dialysed/filter sterilized) was added to 800 μ l of medium containing 10⁶ cord blood mononuclear cells prepared by bouyant density centrifugation (see section 2.4.6) in sterile plastic 10 ml V-bottomed tubes (Disposable Products, Adelaide), and incubated on ice for 1 hr.

Control antibodies used were; a negative control of the same subclass (Sal-2, IgG1), a positive control anti-HLA-DR antibody (FMC-14, IgG1), or with no antibody or complement. In one experiment, a 1/500 dilution of a polyclonal murine antiserum to the myeloid cell

line RC-2A (see section 3.2.11) was used as a further positive control.

After the incubation, the tubes were centrifuged at 200g for 5 min, and the supernatant aspirated. The pellets were resuspended in 1 ml of medium and centrifuged once more for 5 min at 200g. After aspiration of the supernatant, 1 ml of a 1/500 dilution of a 1mg/ml rabbit anti-mouse immunoglobulin preparation (described in section 2.2) was added and the tubes incubated for a further 1 hr on ice. The tubes were centrifuged for 5 min at 200g, the supernatant aspirated, and the pellets resuspended in 1 ml of a 1/10 dilution of rabbit complement (low cytotoxicity to human, Pel-Freez) in medium. The dilutions of the rabbit anti-mouse immunoglobulin and the rabbit complement were chosen as they exhibited minimal toxicity to human cells at these levels, whilst test antibodies were still able to lyse RC-2A cells at these levels (data not shown). The tubes were incubated in a 37°C water bath for 45 min, centrifuged for 5 min at 200g, then plated out in semi-solid medium as described in section 2.6.2. Five plates per antibody or control were prepared, and 75µl of PHA-LCM (prepared as in section 2.6.1) was added to each plate as a source of colony stimulating factor.

After incubation, agar plugs were dried onto glass slides and stained as described in section 2.6.3. Aggregates of more than 40 cells were regarded as colonies. The staining of cells within colonies allowed them to be grouped into four types; macrophage, neutrophil, eosinophil and macrophage/neutrophil.

6.2.5 Immunohistochemistry.

Cryostat sections of normal human tonsil and small intestine were a kind gift of Dr. G. Mayrhofer and L. Spargo in this department.

Smears of RC-2A cells were prepared by cytocentrifugation onto glass slides (see section 2.4.7). Binding of the 5 anti-myeloid MoAb's was assessed by an immunoperoxidase assay, the methodology of which is detailed in section 2.8.1. In addition, Sal-2 was used as a negative control and 1B4 (anti-HLA, L.K. Ashman, not published) as a positive control (both IgG1).

6.2.6 The effects of purified growth factors and PHA-LCM on the surface marker expression of RC-2A cells.

RC-2A cells were treated with TNF- α and γ -IFN for 7 days in culture as described in section 3.2.4, and with PHA-LCM as described in section 3.2.3. Immunofluorescence studies of the binding of FMC-14 (Ia)(IgG1), anti-Mo1 (CD11b), anti-Mo2 (CD14) (both IgM) and the 5 anti-myeloid antibodies described in chapter 5 (all IgG1), in addition to the negative control antibodies Sal-2 (IgG1) and Sal-3 (IgM) were performed on untreated cells and PHA-LCM, TNF- α or γ -IFN treated cells as described in section 2.5. Insufficient quantities of the growth factors GM-CSF and G-CSF (used in section 3.2.4) were available to assess their effects on surface marker expression by RC-2A cells.

6.2.7 Effects of antibodies on differentiation of RC-2A cells and cord blood myeloid progenitor cells.

One ml cultures of RC-2A cells at a density of 2×10^5 /ml were set up in 24 well tissue culture trays (Costar). Duplicate cultures were set up with or without 10% v/v PHA-LCM. One hundred μ l of antibody containing supernatants (dialysed/filter sterilized) were added to duplicate cultures. Sal-2 was included as a negative control. Trays were incubated in a fully humidified atmosphere of 5% CO₂ in air for 7 days. The wells were inspected for adherent cells, then cells were

harvested, cytospin smears made (see section 2.4.7) and stained for α -Naphthol-Butyrate esterase activity (see section 3.2.5).

Twenty five μ l of antibody containing supernatants were added to quadruplicate 1 ml cultures of cord blood progenitor cells which were incubated and stained as in section 2.6. A sub-optimal level of 50 μ l of PHA-LCM was used as a source of colony stimulating factors (see section 2.6.1), and a duplicate set of cultures were set up with no PHA-LCM to assess the growth promoting activities of the MoAbs alone. The antibody YB5.B8 was included as a positive control, as it was able to inhibit colony formation by approximately 50% under these conditions (A.B.Lyons, L.K. Ashman, not published), and Sal-2 was included as a negative control (all IgG1).

6.2.8 Binding of antibodies to peripheral blood leukocytes of AML patients.

The antibodies were screened on a limited panel of 18 AML, 16 ALL and 3 CLL samples by Ms S Cooper in this laboratory. Mononuclear fractions were prepared by bouyant density centrifugation (see section 2.4.1) and antibody binding assessed by indirect immunofluorescence scored by flow cytometry (see sections 2.5.1, 2.5.3). MoAbs were considered to be positive if more than 6% of the cells exhibited fluorescence above the 99% cut-off of the negative control.

6.3 Results.

6.3.1 Molecular weights of antigens detected by anti-myeloid MoAb's.

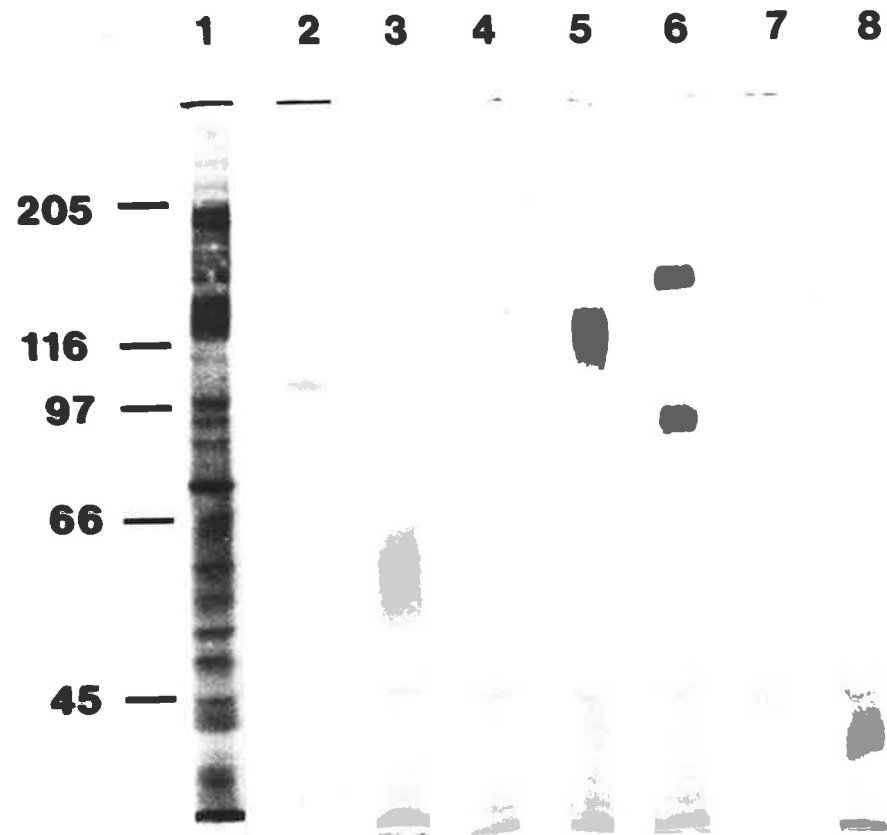
Figure 6.1 shows a composite photograph of biotinylated cell surface proteins from RC-2A cells immunoabsorbed by the 5 MoAbs described in Chapter 5. Four of the 5 antibodies reproducibly gave bands, demonstrating these antibodies bound to proteins of different molecular weights. The antibody 4C5.D12 was unable to immunoabsorb detectable protein components. This may indicate that this antibody detects a non-protein determinant, that the epitope it binds is destroyed by detergent solubilization process, or that the structure recognized is not biotinylated. The positive control anti-HLA antibody 1B4 (IgG1, not published) immunoabsorbed two proteins, one of which had the expected molecular weight of approximately 45 kd. The smaller β_2 microglobulin component of the HLA-A,B,C molecule of 12 kd was not resolved by the 7.5% gel and was detected at the dye front. The negative control IgG1 antibody Sal-2 gave no bands.

The 5 anti-myeloid antibodies, 1B4 and Sal-2 were used to immunoabsorb biotinylated cell surface proteins from the autologous EBV-transformed B cell line Cess B. None of the 5 anti-myeloid antibodies produced any bands, whereas the anti-HLA antibody 1B4 produced two bands in the same positions as described for RC-2A (data not shown).

The logarithm of the molecular weights of the proteins used as standards (see section 2.7.4) were plotted against the R_f (relative mobility) of their respective bands (R_f = distance travelled from origin / distance travelled by dye front) to obtain standard curves for gels (not shown). The estimated molecular weights of the

Figure 6.1

Composite photograph of protein bands immunoabsorbed by antimyeloid monoclonal antibodies. Track 1: Total biotinylated surface membrane proteins from RC-2A cells, Track 2: 4B4.E11 (100 kd), Track 3: 4B5.F5 (50-60 kd), Track 4: 4C5.D12 (no band obtained), Track 5: 5A2.G5 (125 kd), Track 6: 5A4.C5 (95,160 kd), Track 7: Sal-2 (negative control), Track 8: 1B4 (α -HLA-A,B,C positive control).



immunoabsorbed proteins from RC-2A cells were obtained from the standard curve after calculating their R_f values.

Estimates of molecular weights of antigens obtained by immunoabsorbtion with 5 anti-myeloid MoAb's were;

4B4.E11	100 kd
4B5.F5	50-60 kd
4C5.D12	no bands.
5A2.G5	125 kd
5A4.C5	95,160 kd heterodimer.

6.3.2 Binding to human haemopoietic cell lines.

Table 6.1 shows the binding characteristics of the 5 anti-myeloid MoAb's to five myeloid (RC-2A, K562, KG-1, HL-60 and U937), two B cell lymphoid (Cess B, Balm-1), two T cell lymphoid (Molt-4, HSB2) and one null cell (Nalm-6), human haemopoietic cell lines. The RBA was used in all cases.

6.3.3 Binding to normal human peripheral blood leukocytes and erythrocytes, and bone marrow nucleated cells.

Fluorescence histograms of a typical example of an experiment in which binding of these antibodies to peripheral blood leukocytes assayed by indirect immunofluorescence scored by flow cytometry is shown in figures 6.2 A-6.2 F (Shown at the end of this chapter). Table 6.2 A shows summarized binding data for the 5 anti-myeloid antibodies to normal peripheral blood elements. The data for binding to lymphocytes, monocytes and granulocytes are median fluorescence channels taken from the legends to figures 6.2 B-6.2 F. Resolution of the three populations was based on the flow cytometric gating windows

Table 6.1

Binding of anti-myeloid MoAb's to human haemopoietic cell lines.

Cell lines: MoAb	MYELOID					LYMPHOID				NULL
	RC-2A	K562	KG-1	HL-60	U937	Cess B	BALM-1	Molt-4	CCRF-HSB2	NALM-6
4B4.E11	+	-	-	-	-	-	-	-	-	-
4B5.F5	+	-	-	-	-	-	-	-	-	-
4C5.D12	+	-	-	-	-	-	-	-	-	-
5A2.G5	+	+ w	+	-	+	+w /- #	-	+	+	-
5A4.C5	+	-	+	+ w	+ w	-	-	-	-	-
Sal-2	-	-	-	-	-	-	-	-	-	-

Note; w- denotes weak binding of approximately twice negative control.

#- 5A2.G5 intermittently gave positive binding Cess B by RBA, however this antibody was unable to immunoadsorb a protein band from this line (see section 6.3.1)

Table 6.2 A

Binding of anti-myeloid MoAb's to normal blood elements.

MoAb	Median Channel Fluorescence (Log scale)				HA titre
	Ungated	Monocytes	Granulocytes	Lymphocytes	Erythrocytes
4B4.E11	6.9	15.1	6.4	2.5	-
4B5.F5	14.9 (3, 50)	47.2	18.1	3.3	-
4C5.D12	6.3	5.2	2.9	2.6	-
5A2.G5	105.3 (4, 220)	262.2	66.9	4.5 (87%) 38.8 (13%)	-
5A4.C5	137.9 (2, 450)	436.4	495.1	2.7 (88%) 53.6 (12%)	-
Sal-2	7.5	6.5	5.5	2.9	-
DA1.F9	ND	ND	ND	ND	>1/64

Figures in parentheses give either positions of peaks (for ungated cells). The cells of the lymphocyte gate were negative for the first 3 antibodies, however 5A2.G5 and 5A4.C5 gave an unresolved peak or shoulder of positive cells. To clarify this, gated cells are shown as two populations, the first having fluorescence less than the 99% cutoff of the negative control, the second greater than the 99% cutoff. This data is expressed as a percentage of the total (in parentheses) and the median fluorescence of this population. This table is in part derived from the histograms in Figures 6.2 B-F.

shown in figure 6.2 A. Assay of the ungated, monocyte and lymphocyte populations was performed on mononuclear fractions of human peripheral blood obtained using the method described in section 2.4.1. The granulocyte assay was performed on a purified granulocyte population prepared from the same blood samples as described in section 2.4.2. Binding to erythrocytes was assayed by haemagglutination using the method described in section 2.5.4.

Table 6.2 B shows a summary of the data obtained by APAAP assay of peripheral blood leukocytes and bone marrow nucleated cells. Photographs of stained cell smears are shown in figures 6.3 A-F and 6.4 A-F, which are of peripheral blood leukocytes and bone marrow nucleated cells respectively (Shown at the end of this chapter).

Table 6.2 C Shows results of a typical RBA of antibody binding to peripheral blood mononuclear and granulocyte fractions, and of cultured T cells.

6.3.4 Complement mediated lysis of myeloid progenitor cells from cord blood.

Figures 6.5 A-B show photographs of myeloid colonies grown in semi-solid agar, which have been stained according to the method in section 2.6.3. The colonies are identified by the presence of α -naphthol acetate esterase (brown, macrophage), chloroacetate esterase (blue, neutrophil), luxol fast blue (green, eosinophil) or mixed (brown/blue, macrophage/neutrophil) (Shown at the end of this chapter).

Of the five MoAb tested, only 4B4.E11 and 4C5.D12 gave killing of colony forming cells by complement mediated lysis. Expression of the results as a breakdown of colony types cultured (see tables 6.3 A and B), showed that 4B4.E11 gave particularly effective killing of

Table 6.2 B

Binding of anti-myeloid MoAb's to normal blood and bone marrow elements by APAAP.

MoAb	Peripheral Blood	Bone Marrow
4B4.E11	Nø +++ Mø +	Myeloblast + w Promyelocyte + w Myelocyte/metamyelocyte ++ Late metamyelocyte/'Band' +++
4B5.F5	Nø + w Mø + w	very weak, mainly late Metamyelocytes?
4C5.D12	no apparent binding.	no apparent binding
5A2.G5	Nø + w Mø + Pl ++	Monocytic +? Late metamyelocytes + w Platelets ++
5A4.C5	Nø +++ Mø +++	Myelocyte + Late Metamyelocytes +++
Sal-2	no binding.	no binding.

Number of plusses gives subjective appraisal of staining.
Nø - neutrophil, Mø - monocyte, Pl - platelet

Table 6.2 C

Binding of anti-myeloid MoAb's to normal blood leukocyte fractions and activated T cells assayed by RBA.

MoAb	PB MNC fraction.	PB PMN fraction	Activated T cells.
4B4.E11	+	+	-
4B5.F5	+	+/-	-
4C5.D12	-	-	-
5A2.G5	+	+	+
5A4.C5	+	+	+
Sal-2	-	-	-

Binding considered to be positive when OD_{540} is greater than twice the negative control value.
 +/- denotes a marginal case.

Table 6.3 A

MoAb/Complement mediated lysis of cord blood myeloid progenitor cells- Expt. 1.

MoAb	Colony Type					Total
	M	G	GM	Eo		
4B4.E11	8.8±1.1 (41)	0.5±0.3 (13)	2.5±0.5 (63)	0.5±0.3 (20)	12.3±1.3	(39)
4B5.F5	22.0±1.8	3.8±0.5	4.3±0.3	2.3±0.3	32.0±1.3	(102)
4C5.D12	10.6±0.7 (49)	3.2±0.4	2.4±0.25 (60)	1.6±0.3 (64)	17.8±0.7	(56)
5A2.G5	20.6±1.0	3.0±0.5	3.6±0.7	1.6±0.5	28.6±1.6	(90)
5A4.C5	21.0±1.5	3.5±0.8	4.0±0.6	2.5±0.3	31.0±2.7	(98)
FMC 14	2.4±0.3 (11)	0.0±0 (0)	0.0±0 (0)	0.2±0.2 (8)	2.6±0.4	(8)
Sal-2	21.5±1.7	4.0±0.7	4.0±0.4	2.5±0.7	31.8±3.3	(100)
Untreated	22.25±2.2	4.5±0.6	4.3±0.5	2.8±0.5	33.8±3.4	(106)
α-RC-2A	0.0±0	0.0±0	0.0±0	0.0±0	0.0±0	(0)

Results are average colony counts of quadruplicate cultures ± Standard Error of the Mean.
 Figures in parentheses are percentages of control colony counts.
 M - Macrophage colony, G - Neutrophil colony, GM - mixed Neutrophil/Macrophage colony,
 Eo - Eosinophil colony.

Table 6.3 B

MoAb/Complement mediated lysis of cord blood myeloid progenitor cells- Expt. 2

MoAb	Colony Type					Total
	M	N	GM	Eo		
4B4.E11	4.0±1 (42)	0.2±0.2 (8)	4.6±1	0.2±0.2 (8)	9.0±1.7	(47)
4B5.F5	8.4±1.3	4.4±0.6	6.6±0.5	1.2±1.3	21.0±1.3	(110)
4C5.D12	3.3±1.1 (34)	3.5±1.2	3.5±0.9	1.0±0.5 (42)	11.3±3.4	(59)
5A2.G5	8.2±1.6	1.8±1.3	5.0±0.6	1.8±0.4	16.8±2.7	(88)
5A4.C5	7.8±1.3	2.5±0.5	5.5±0.7	1.3±1.5	17.0±3.7	(89)
FMC 14	2.2±0.7 (23)	0.6±0.3 (23)	0.4±0.5 (9)	0.8±0.4 (33)	4.0±1.5	(21)
Sal-2	9.6±2	2.6±0.8	4.6±0.8	2.4±0.7	19.2±3.7	(100)

Results are average colony counts of quadruplicate cultures ± Standard Error of the Mean.
 Figures in parentheses are percentages of control colony counts.
 M - Macrophage colony, G - Neutrophil colony, GM - mixed Neutrophil/Macrophage colony,
 Eo - Eosinophil colony.

granulocytic progenitors, moderate killing of macrophage progenitors and variable killing of macrophage/neutrophil progenitors. 4C5.D12 appeared to have a different pattern of binding, with moderate killing of macrophage progenitors, variable killing of macrophage/neutrophil and eosinophil progenitors, and no killing of neutrophil progenitors. It must be stressed that as the total numbers of colonies are quite small, such a breakdown of figures does not necessarily give statistically significant comparisons. Nonetheless, it appears that the two antibodies bind to different subgroups of myeloid progenitor cells. The positive control anti-Ia antibody FMC-4 gave moderate to strong killing of progenitors. This agrees with the observation of Koizumi *et al*, 1982, that cord blood progenitors appear to express less Ia antigen than bone marrow progenitors. The positive control anti-RC-2A serum gave complete killing of progenitors (expt. 1) and the negative control gave similar colony counts to experiments using no antibody or complement (expt. 1).

6.3.5 Immunohistochemistry.

Figures 6.6, 6.7 and 6.8 show photographs of immunoperoxidase stained RC-2A cell smears, tonsil and small intestine (Peyer's patch) sections respectively (Shown at the end of this chapter). Initial experiments with blocking of endogenous peroxidase with acid ethanol or methanol/hydrogen peroxide of RC-2A cell smears (see section 2.8.1) demonstrated that epitopes recognised by the antibodies were destroyed or at least markedly diminished by blocking. The decrease in endogenous peroxidase obtained by blocking was therefore negated by a lowered 'signal to noise' ratio. The blocking was therefore omitted, but as the endogenous peroxidase was mostly restricted to eosinophils in both types of sections, specific staining was relatively easy to

Table 6.4

Immunoperoxidase staining of tissue sections- Summary of binding patterns.

MoAb	Tonsil	Small Intestine
4B4.E11	Scattered cells in epithelium, -consistent with Langerhans cells. Germinal centres of B follicles, -consistent with tingivle body Mø, (not dendritic cells) Loose lymphoid T dependant area, -consistent with Mø related cells	Tingivle body Mø Mø-like cells in T dep. areas Mø in <i>lamina propria</i> of villi.
4B5.F5	Similar to above, however preference for scattered cells in loose lymphoid area. Very weak staining.	Very little staining.
4C5.D12	No staining.	No staining.
5A2.G5	Endothelium- vascular, lymphatic, high-wall endothelial cells in T-dep. area.	Endothelium.
5A4.C5	Germinal centres of B follicles, -consistent with follicular dendritic cells Loose lymphoid T dependant area, -consistant with Mø related cells.	Follicular dendritic cells. (Doesn't appear to stain Mø or Langerhans cells)

identify. The epitope bound by 4C5.D12 appeared not to survive fixation, and it has therefore been omitted from the photographs of figures 6.7 and 6.8. Table 6.4 summarizes the binding pattern of antibodies to the tissues.

6.3.6 Binding to RC-2A cells treated with inducers of differentiation.

Figures 6.9 A-F show fluorescence histograms of the binding of antibodies to RC-2A cells cultured with PHA-LCM for 4 and 8 days, and figures 6.10 A-H show binding of antibodies to RC-2A cells treated with γ -IFN and TNF- α for 7 days (Shown at the end of this chapter). This data is summarized in table 6.5. One striking change is the massive increase in binding of 4C5.D12 by γ -IFN treated cells, and to a lesser extent by TNF- α treated cells. In an attempt to determine the molecular weight of the antigen detected by 4C5.D12, γ -IFN treated cells were surface biotinylated and immunoadsorbed as described in sections 6.2.1 and 2.7., but again this antibody failed to give a protein band (data not shown). Other changes include decreased Ia expression after treatment by both factors, and increase in 5A4.C5 binding by both factors. The overall changes in binding caused by PHA-LCM treatment were markedly different to the changes caused by treatment with γ -IFN and TNF- α , such that neither factor alone could account for the effects of PHA-LCM (see chapter 3).

6.3.7 Functional effects of antibodies on RC-2A cells and myeloid progenitor cells.

None of the antibodies affected the expression of α -Naphthol Butyrate esterase by PHA-LCM treated RC-2A cells, nor were any able to induce the expression of the enzyme. The antibody 5A4.C5 altered the appearance of PHA-LCM treated cells in culture, such that the cells no

Table 6.5

Binding of MoAb's to RC-2A cells treated with inducers of differentiation.

Monoclonal antibody binding: Median channel fluorescence (Log. scale).

MoAb:	4B4.E11	4B5.F5	4C5.D12	5A2.G5	5A4.C5	FMC-14	Sal-2	α -Mo1	α -Mo2	Sal-3
RC-2A (control)	4.1	50.7	12.3	149.4	58.4	678.5	2.9			
RC-2A (Day 4)	5.0	58.4	10.1	142.8	121.9	540.4	3.0	See Chapter 3 Figures 3.5, 3.6		
RC-2A (Day 8)	6.9	76.3	16.3	72.3	320.9	329.8	5.4			
Change	-	↑	-	↓	↑	↓	-	↑	↑	-
RC-2A (control)	2.0	18.7	6.1	68.2	23.8	482.2	3.6	33.5	2.5	2.5
RC-2A (+TNF- α)	16.6	6.8	71.7	42.4	597.5	286.3	4.3	56.3	2.3	2.3
Change	↑	↓	↑	↓	↑	↓	-	↑	-	-
RC-2A (+ γ -IFN)	7.4	104.2	171.9	254.0	54.8	332.1	2.5	165.2	4.7	4.7
Change	↑	↑	↑	↑	↑	↓	-	↑	-	-

longer displayed characteristic adherence properties (see figure 3.4), but rather appeared as small agglutinated clumps of cells. The large increase in expression of the antigen bound by 5A4.C5 when RC-2A cells are treated with PHA-LCM (see table 6.5) may allow the agglutination of the cells by 5A4.C5, giving rise to the different appearance of the cells in culture.

None of the antibodies were able to alter the number of colonies grown from cord blood mononuclear cells. Cultures containing the five antibodies, the negative control Sal-2 or no antibody grew 27 ± 3 colonies per plate, whereas plates with YB5.B8 added grew 17 ± 3 colonies per plate (results average \pm Standard error of the means). Cultures with antibody but not PHA-LCM added grew no colonies, demonstrating that the MoAbs had no growth promoting activity in this system.

6.3.8 Binding of antibodies to myeloid leukaemic cells.

Table 6.6 shows the binding of antibodies to peripheral blood mononuclear cells of leukaemia patients. The antibodies 4B4.E11 and 4C5.D12 bound to no specimens. Generally, the AML specimens bound all or none of the remaining three antibodies (4B5.F5, 5A2.G5, 5A4.C5). The leukaemias with obvious monocytic involvement bound all three. Of the lymphoid leukaemias, 4B5.F5 bound to one ALL specimen, and 5A2.G5 bound to one CLL specimen. As blast cell counts were not taken into account, residual normal cells could account for the binding to the lymphoid specimens.

6.4 Discussion.

The characteristics of the five anti-myeloid monoclonal antibodies described in this chapter are summarized here.

Table 6.6

Binding of antibodies to leukaemic specimens—Summary of immunofluorescence assays.

MoAb:	4B4.E11	4B5.F5	4C5.D12	5A2.G5	5A4.C5
AML (M1/M2)	0/12	4/12	0/12	7/11	7/12
AMML/AMoL (M4/M5)	0/6	6/6	0/6	6/6	6/6
ALL	0/16	1/16	0/16	0/16	0/12
CLL	0/3	0/3	0/3	1/3	0/3

4B4.E11:

The 100 kd protein bound by 4B4.E11 is present on monocytes and neutrophils, and APAAP results show that it is expressed with increasing density from myeloblasts through to mature neutrophils. The appearance of the antigen at such an early stage is confirmed by the MoAb/complement mediated cell lysis results, which show effective killing of granulocytic progenitor cells and to a lesser extent macrophage and neutrophil/macrophage progenitors. Assay of binding of this MoAb to granulocytes by immunofluorescence assay shows this structure is barely expressed by granulocytes, however the very bright staining by APAAP of neutrophils suggests that this antigen may be predominantly intracellular. This antibody did not bind to any human haemopoietic cell lines tested, apart from RC-2A. The expression of the antigen detected by this antibody was increased by treatment of RC-2A cells with both γ -IFN and TNF- α . This antibody does not appear to be identifiable with any workshop antibody cluster. It bound to none of the 18 acute myeloid leukaemias tested. A recently reported antibody (12B1, Farace *et al*, 1986) which binds to a structure of similar molecular weight demonstrated a quite different binding pattern. 12B1 bound to HL-60 and K562 cells, and to dendritic cells in tonsil cells, whereas 4B4.E11 bound to neither line, and bound to tingivle body macrophages, Langerhans-like cells and macrophage-like cells in the loose lymphoid tissue of tonsil. It appears that this antibody differs to anti-myeloid MoAb's reported to date.

4B5.F5:

The 55 kd structure detected by 4B5.F5 is present on monocytes and to a lesser extent on neutrophils. It was not able to inhibit myeloid progenitor cell growth by MoAb/complement mediated cell lysis.

The antibody did not bind to any human haemopoietic cell lines tested except RC-2A, and expression of the structure on RC-2A cells increased markedly after treatment with γ -IFN, slightly after PHA-LCM treatment, but decreased when treated with TNF- α . The antibody stained macrophage-like cells in the loose lymphoid area of tonsil. 4B5.F5 bound to a proportion of M1/M2 AML's, and to all M4/M5 subtypes tested. The molecular weight of the antigen detected by this antibody and its binding pattern suggest that it may be a CD14 type antibody of the second group which includes FMC-17 (Brooks *et al*, 1983) but not Mo2 (Todd *et al*, 1981b), based on binding to transfectants (McMichael *et al*, 1987).

4C5.D12:

Apart from binding to RC-2A and to myeloid progenitor cells (mainly macrophage and eosinophil), binding of this antibody to other cell and tissue types was not detectable by any of the assays employed. This antibody detects a labile determinant which does not survive conventional immunohistochemical fixation techniques. A molecular weight for this structure was not able to be determined, suggesting it is not a protein structure, or at least cannot be biotinylated using the method described in section 2.7. One interesting observation was the massive increase in binding of the antibody to RC-2A cells treated with γ -IFN or TNF- α . This antibody appears to be distinct from any anti-myeloid antibodies reported to date.

5A2.G5:

The 125 kd protein detected by 5A2.G5 is found on monocytes, neutrophils, platelets, a proportion of lymphocytes perhaps

corresponding to LGL's and to activated T lymphocytes. The antibody binds to most of the myeloid cell lines tested, as well as two T cell lines. RC-2A cells treated with PHA-LCM or TNF- α have decreased binding of the antibody, whereas γ -IFN treatment results in an increase in binding of this antibody. In tissue sections this antibody stains vascular and lymphatic endothelium. This antibody binds to a proportion of M1/M2 AML's and to all the M4/M5 subtypes tested. The characteristic binding data obtained suggests that 5A2.G5 is similar to SM \emptyset (Hogg *et al*, 1984) and the CD31 antibodies such as TM3 (Ohto *et al*, 1985) or SG134 (Goyert, in McMichael *et al*, 1987).

5A4.C5:

This antibody immunoadsorbs two proteins of 95 and 160 kd found on monocytes, neutrophils and a subpopulation of lymphocytes, possibly LGL's, as well as on activated T lymphocytes. It binds to a number of myeloid cell lines, but not to any lymphoid cell lines tested. APAAP assay of binding to peripheral blood leukocytes and bone marrow nucleated cells shows specificity for more mature myeloid cells, which is confirmed by the fact that this antibody was unable to inhibit myeloid progenitor cell growth after MoAb/complement mediated cell lysis. In tonsil and small intestine tissue sections, this antibody stained cells of the follicular dendritic type in germinal centres, and macrophage-like cells in the loose lymphoid area of tonsil. The antibody demonstrated a similar pattern to 4B5.F5 and 5A2.G5, showing variable binding to M1/M2 subtypes and binding to all M4/M5 subtypes tested. The molecular weights of species detected by this antibody, in addition to the pattern of binding obtained, suggest this antibody is of the CD11b type, such as exemplified by Mo1 (Todd *et al*, 1981b).

The five anti-myeloid monoclonal antibodies characterized here recognize five distinct structures, based on binding patterns and molecular weight estimations of the antigens they detect. Two of the antibodies appear to be unique, with no antibodies reported to date having the same binding characteristics. Interestingly, these two antibodies would have perhaps gone undetected if indirect immunofluorescence had been the hybridoma screening method employed, rather than the rose bengal assay. In addition, these two antibodies were the only ones out of the five which were able to inhibit myeloid colony formation after MoAb/complement mediated cell lysis.

The other three MoAb's appeared to be recognizing structures commonly detected by a number of antibodies reported in the literature. This reinforces the concept that the immunity of the mouse is directed mainly against a limited number of strongly immunogenic structures. To enable MoAb's which bind to rare or weakly immunogenic determinants to be identified early in the process of monoclonal antibody production, such a screening protocol can be successfully employed.

One aim of this project was to attempt to produce antibodies which exerted a functional effect on myeloid cells, perhaps by interaction with growth factor receptors. It became apparent that the cell proliferation assay employed was unsuitable for the task at hand, consequently no monoclonal antibodies with such properties were isolated. The results obtained with a polyclonal antiserum suggests that the feasibility of such an attempt warrants further investigation using a different screening procedure.

Further characterization of these antibodies will enable their usefulness in immunophenotypic analysis of normal and leukaemic haemopoietic differentiation to be assessed.

Figure 6.2 A

Gating patterns used to delineate populations of monocytes, granulocytes and lymphocytes using flow cytometry, enabling binding of anti-myeloid MoAb's to be assessed. These gates were used for the fluorescence histograms of figures 6.2 B-F.

B: Monocytes- Volume: 125-160
S.S.C: 95-160

C: Granulocytes- Volume: 115-165
S.S.C: 155-215

A: Lymphocytes- Volume: 85-120
S.S.C: 20-135

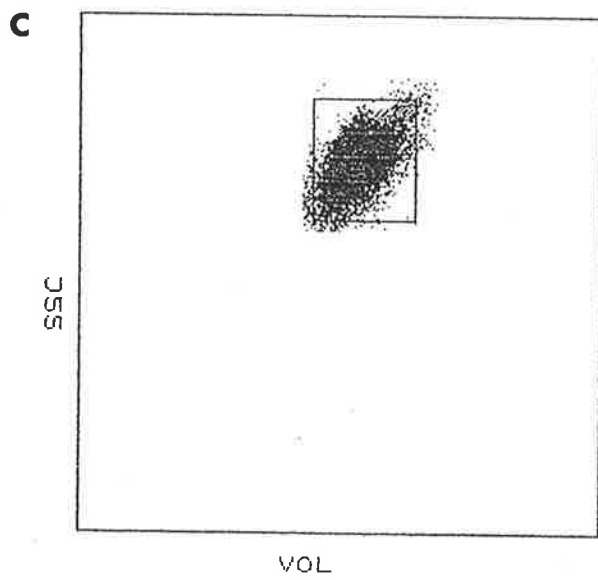
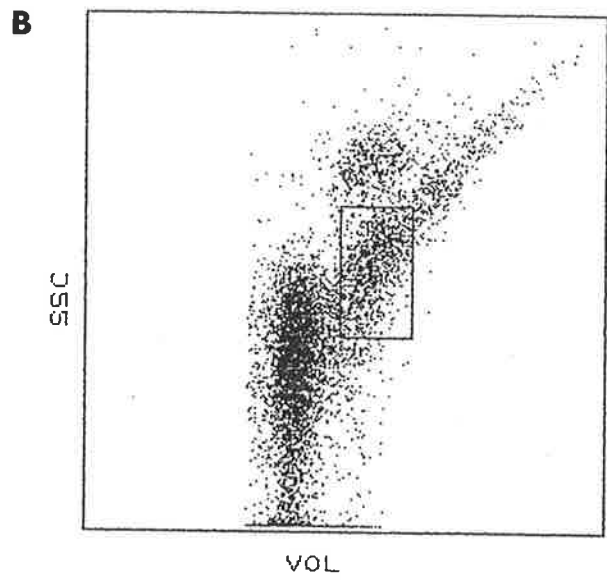
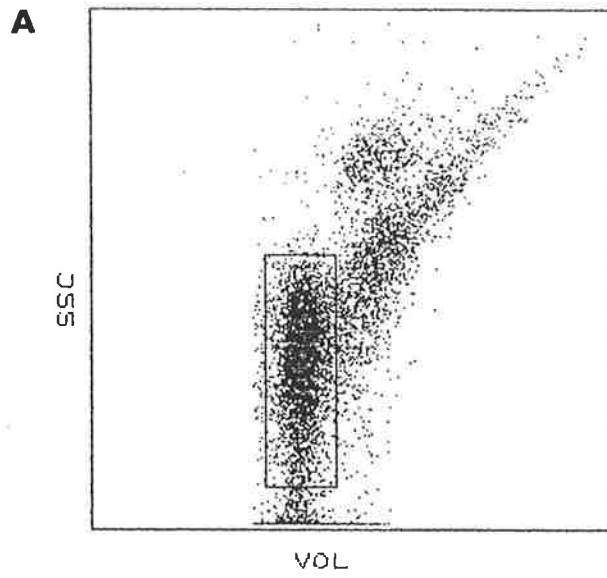
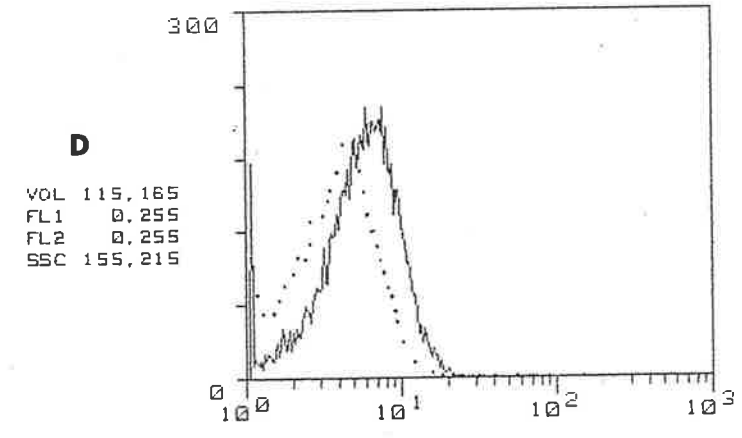
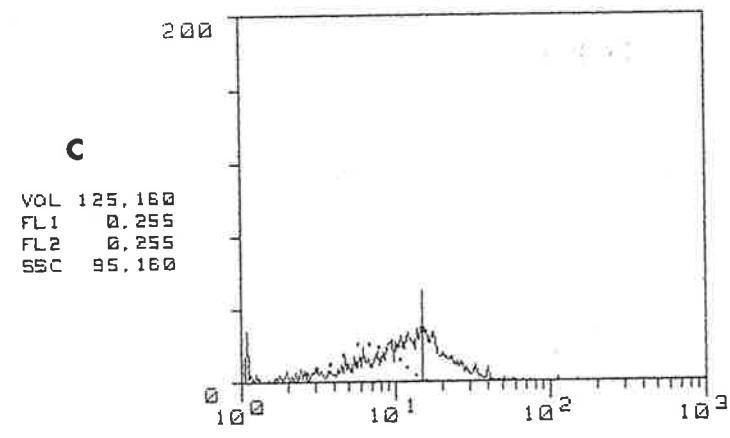
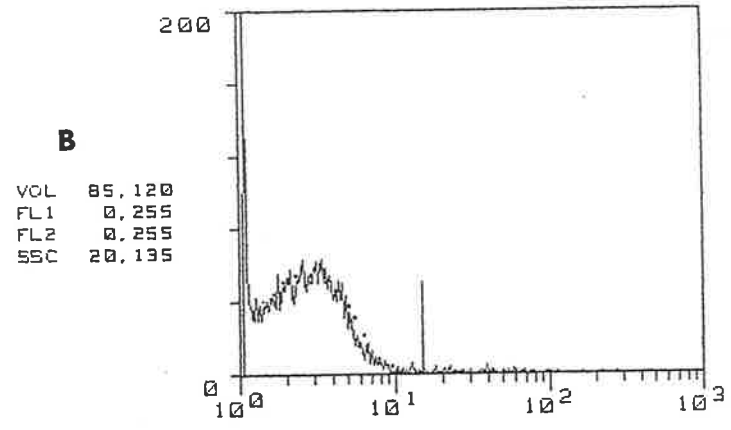
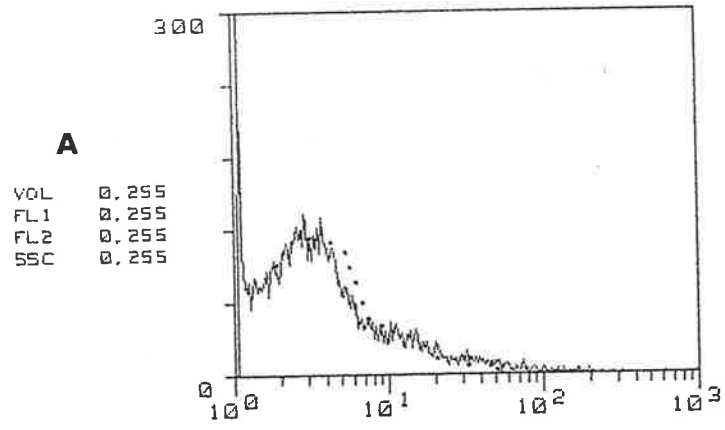


Figure 6.2 B

Histograms showing binding of MoAb 4B4.E11 to peripheral blood leukocytes assayed by indirect immunofluorescence with scoring by flow cytometry, using the gating criteria shown in Figure 6.2 A. Negative control antibody (Sal-2) binding is shown as a dotted line.

	<u>MoAb</u>	<u>Median Fluorescence Channel.</u>
A. Ungated.	Sal-2	7.5
	4B4.E11	6.9
C. Monocytes	Sal-2	6.5
	4B4.E11	15.1
D. Granulocytes	Sal-2	5.5
	4B4.E11	6.4
B. Lymphocytes	Sal-2	2.9
	4B4.E11	2.5

4B4.E11 -BINDING TO PERIPHERAL
BLOOD LEUKOCYTES



FREQUENCY ↑

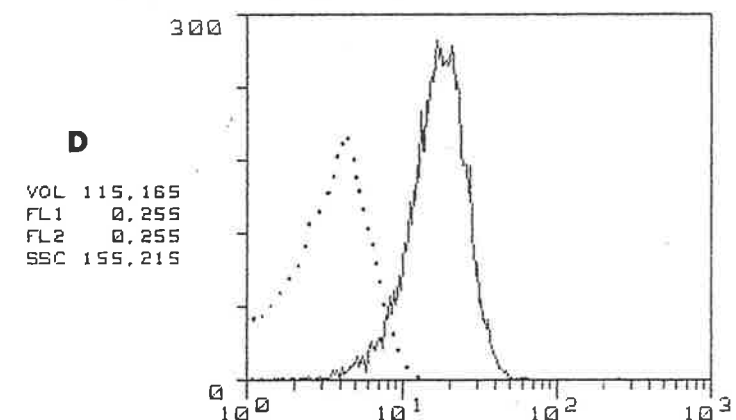
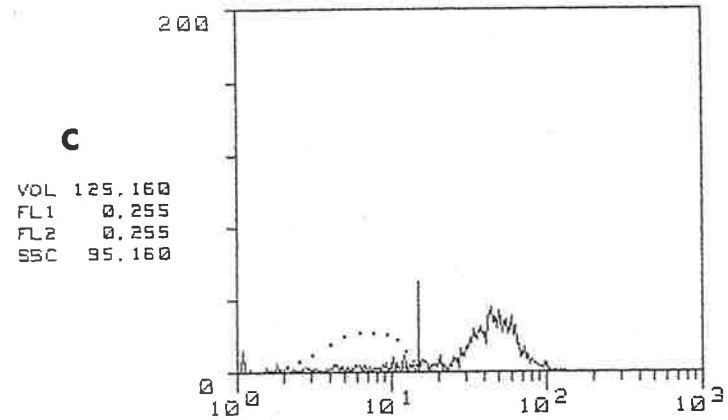
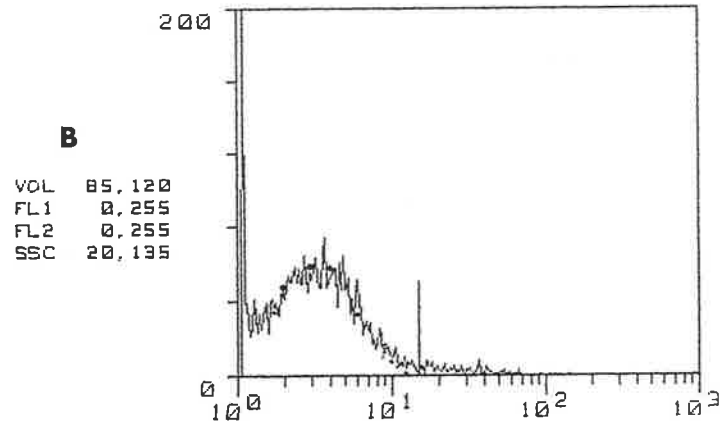
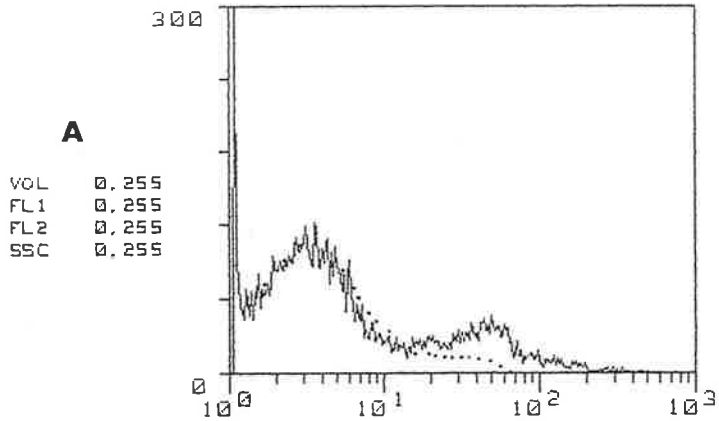
FLUORESCENCE INTENSITY →

Figure 6.2 C

Histograms showing binding of MoAb 4B5.F5 to peripheral blood leukocytes assayed by indirect immunofluorescence with scoring by flow cytometry, using the gating criteria shown in Figure 6.2 A. Negative control antibody (Sal-2) binding is shown as a dotted line.

	<u>MoAb</u>	<u>Median Fluorescence Channel.</u>
A. Ungated	Sal-2	7.5
	4B5.F5	14.9 (peak channels 3, 50)
C. Monocytes	Sal-2	6.5
	4B5.F5	47.2
D. Granulocytes	Sal-2	5.5
	4B5.F5	18.1
B. Lymphocytes	Sal-2	2.9
	4B5.F5	3.3

4B5.F5 -BINDING TO PERIPHERAL
BLOOD LEUKOCYTES



FREQUENCY ↑

FLUORESCENCE INTENSITY →

Figure 6.2 D

Histograms showing binding of MoAb 4C5.D12 to peripheral blood leukocytes assayed by indirect immunofluorescence with scoring by flow cytometry, using the gating criteria shown in Figure 6.2 A. Negative control antibody (Sal-2) binding is shown as a dotted line.

	<u>MoAb</u>	<u>Median Fluorescence Channel.</u>
A. Ungated	Sal-2	7.5
	4C5.D12	6.3
C. Monocytes	Sal-2	6.5
	4C5.D12	5.2
D. Granulocytes	Sal-2	5.5
	4C5.D12	2.9
B. Lymphocytes	Sal-2	2.9
	4C5.D12	2.6

4C5.D12 -BINDING TO PERIPHERAL
BLOOD LEUKOCYTES

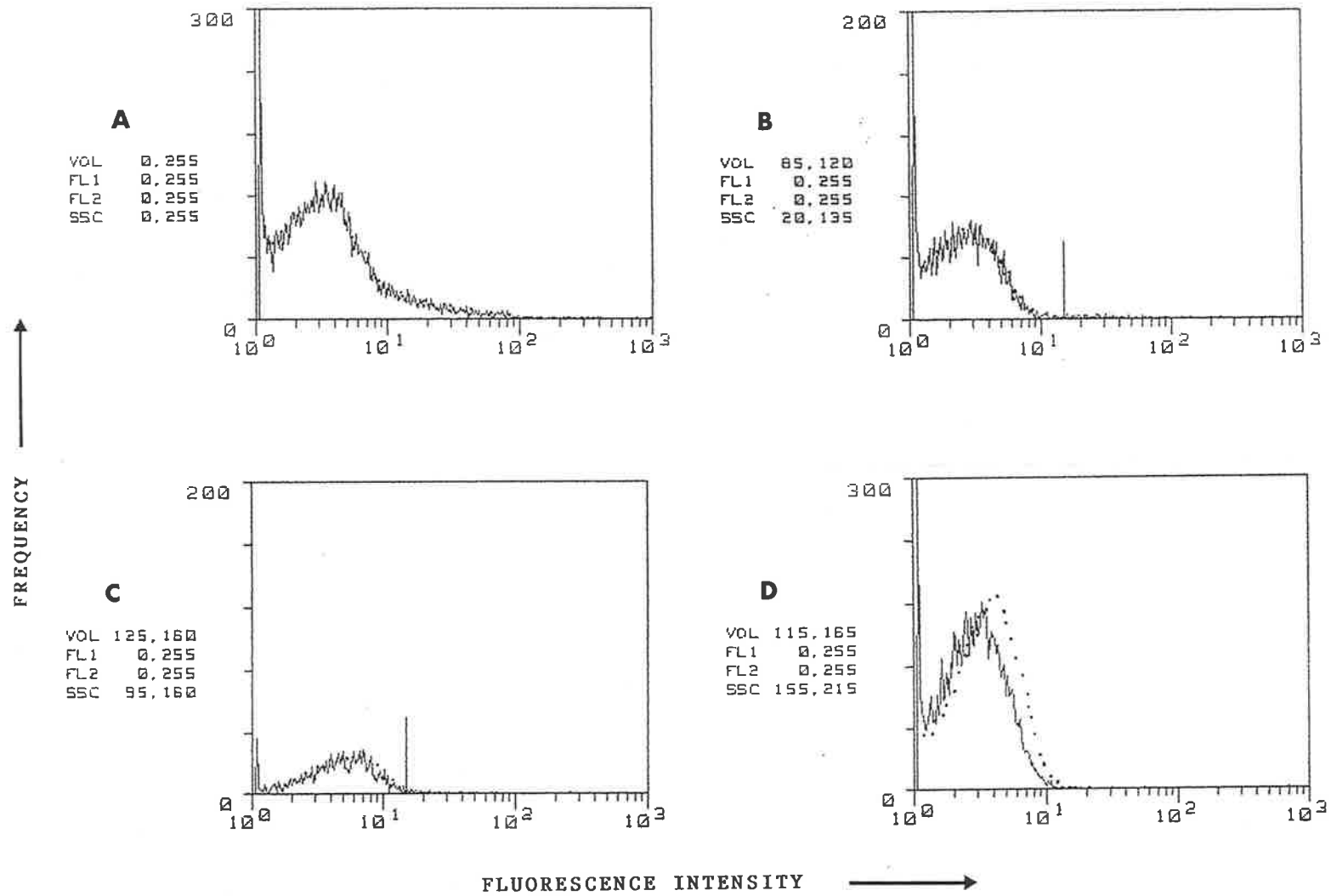


Figure 6.2 E

Histograms showing binding of MoAb 5A2.G5 to peripheral blood leukocytes assayed by indirect immunofluorescence with scoring by flow cytometry, using the gating criteria shown in Figure 6.2 A. Negative control antibody (Sal-2) binding is shown as a dotted line.

	<u>MoAb</u>	<u>Median Channel Fluorescence.</u>
A. Ungated	Sal-2	7.5
	5A2.G5	105.3 (peak channels at 4, 220)
C. Monocytes	Sal-2	6.5
	5A2.G5	262.2
D. Granulocytes	Sal-2	5.5
	5A2.G5	66.9
E. Lymphocytes	Sal-2	2.9
	5A2.G5	4.5 (87% of events)
		38.8 (13% of events)

5A2.G5

-BINDING TO PERIPHERAL
BLOOD LEUKOCYTES

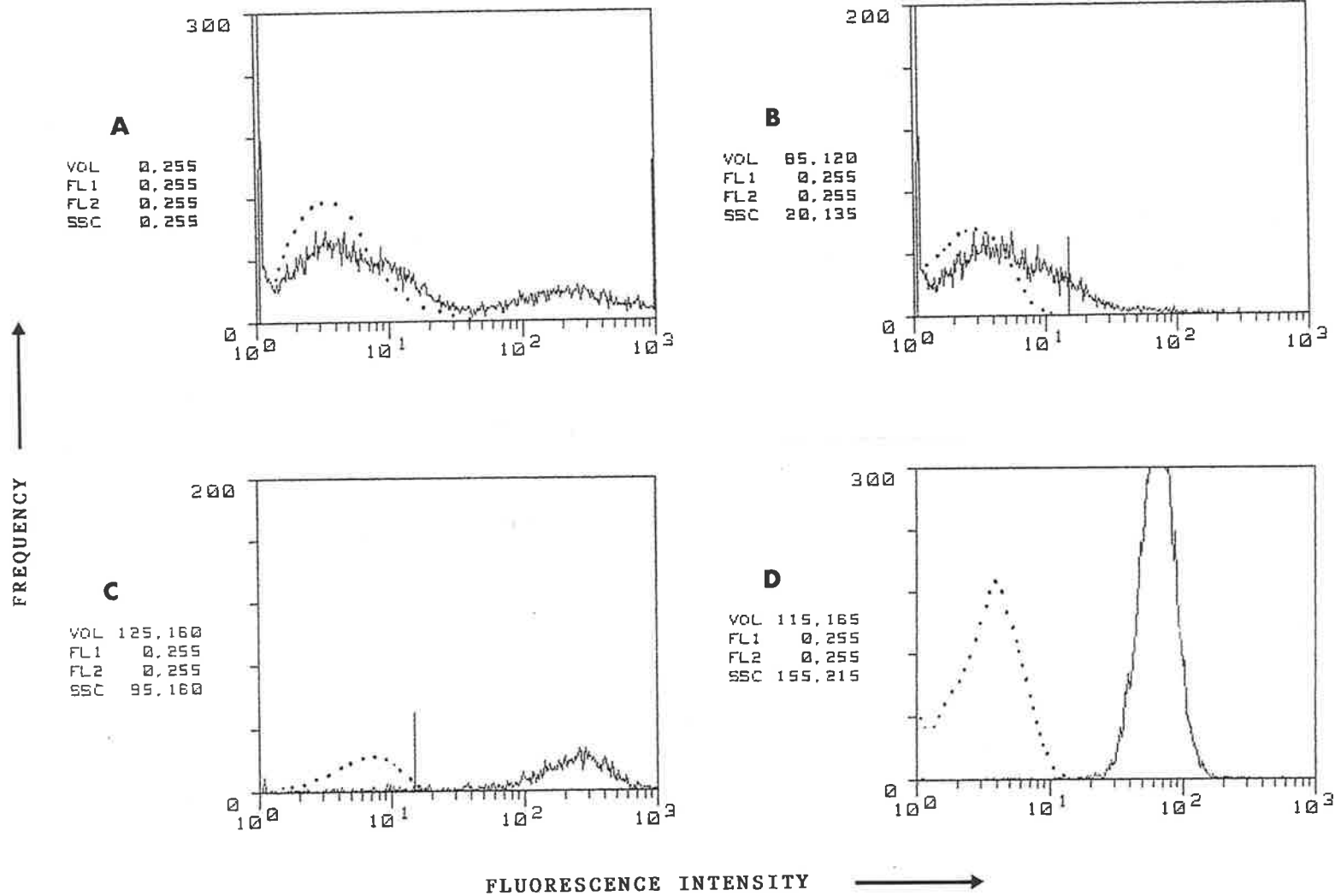


Figure 6.2 F

Histograms showing binding of MoAb 5A4.C5 to peripheral blood leukocytes assayed by indirect immunofluorescence with scoring by flow cytometry, using the gating criteria shown in Figure 6.2 A. Negative control antibody (Sal-2) binding is shown as a dotted line.

	<u>MoAb</u>	<u>Median Channel Fluorescence.</u>
A. Ungated	Sal-2	7.5
	5A4.C5	137.9 (peak channels at 2, 450)
C. Monocytes	Sal-2	6.5
	5A4.C5	436.4
D. Granulocytes	Sal-2	5.5
	5A4.C5	495.1
B. Lymphocytes	Sal-2	2.9
	5A4.C5	2.7 (88% of events)
		53.6 (12% of events)

5A4.G5

-BINDING TO PERIPHERAL
BLOOD LEUKOCYTES

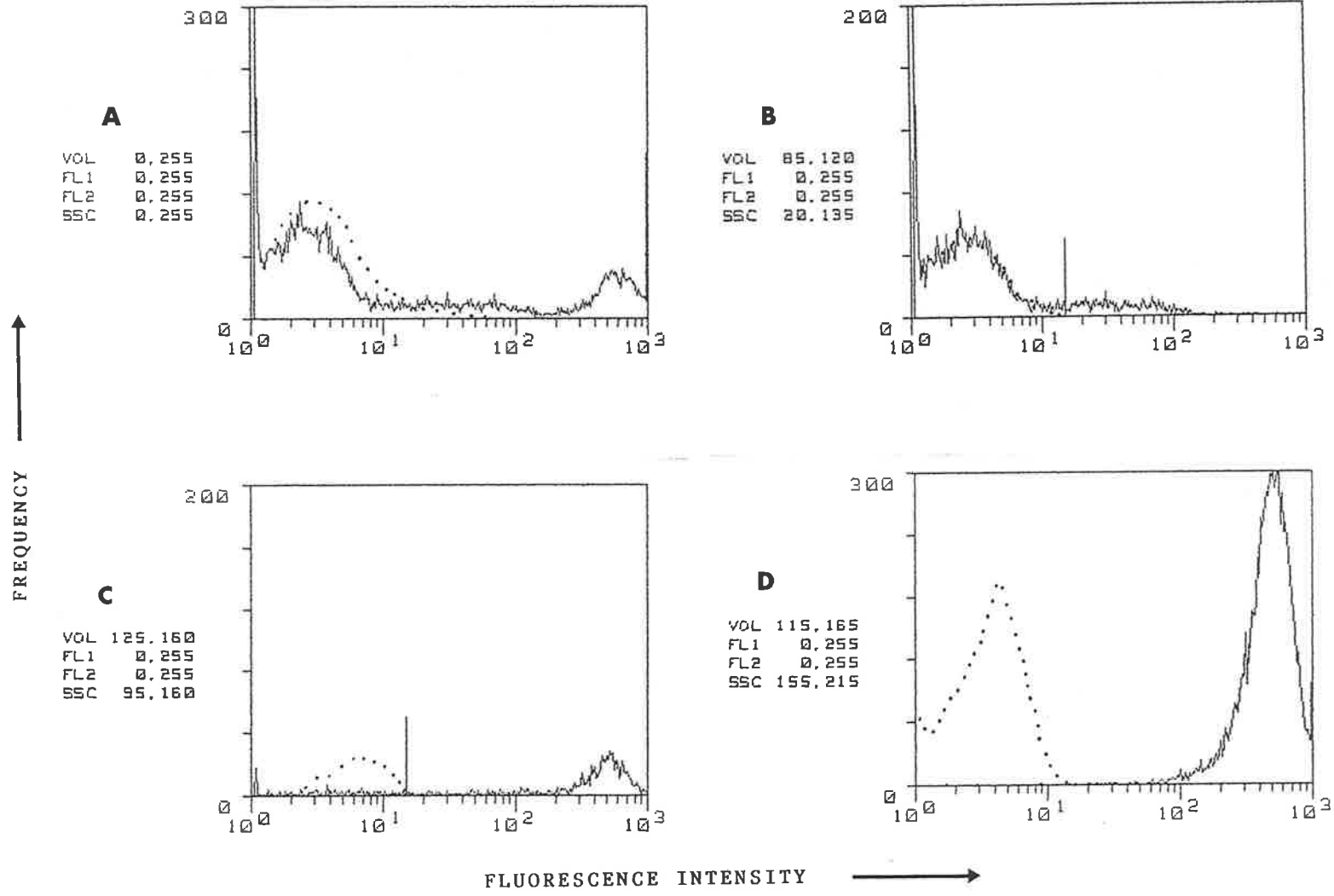


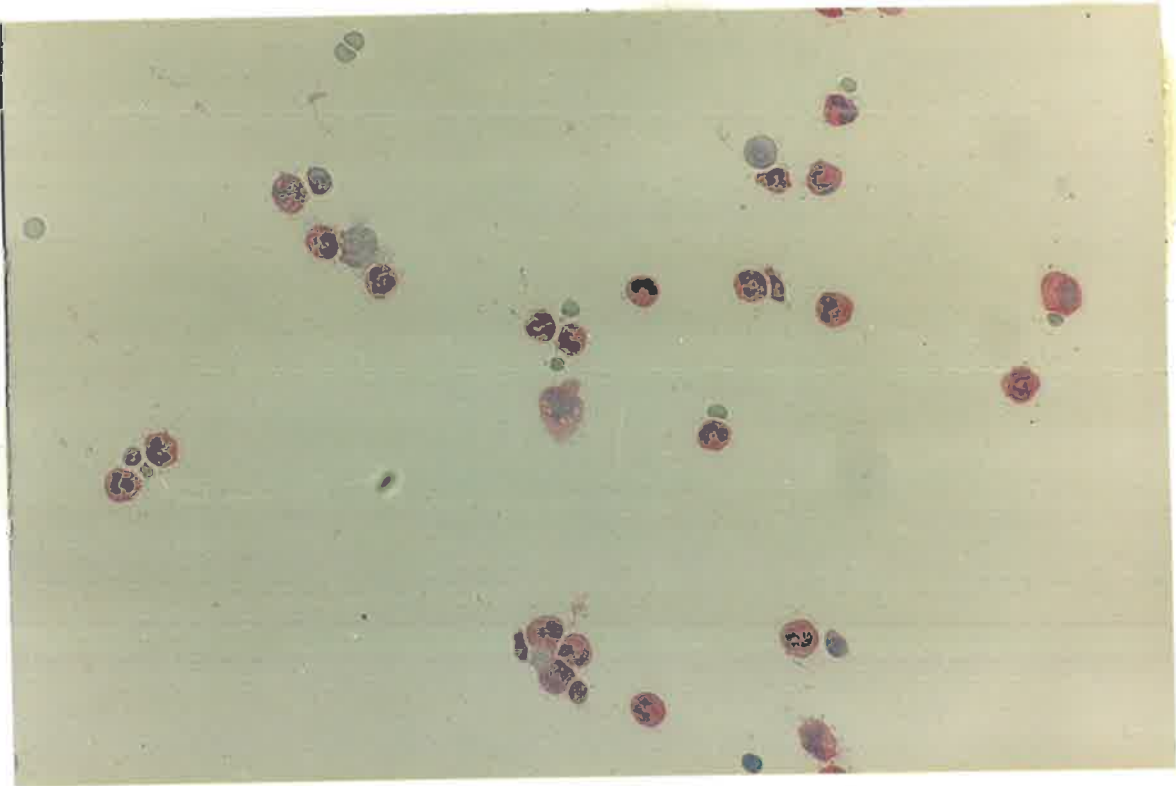
Figure 6.3

Binding of monoclonal antibodies to peripheral blood leukocytes
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

A. 4B4.E11

B. 4B5.F5

A



B

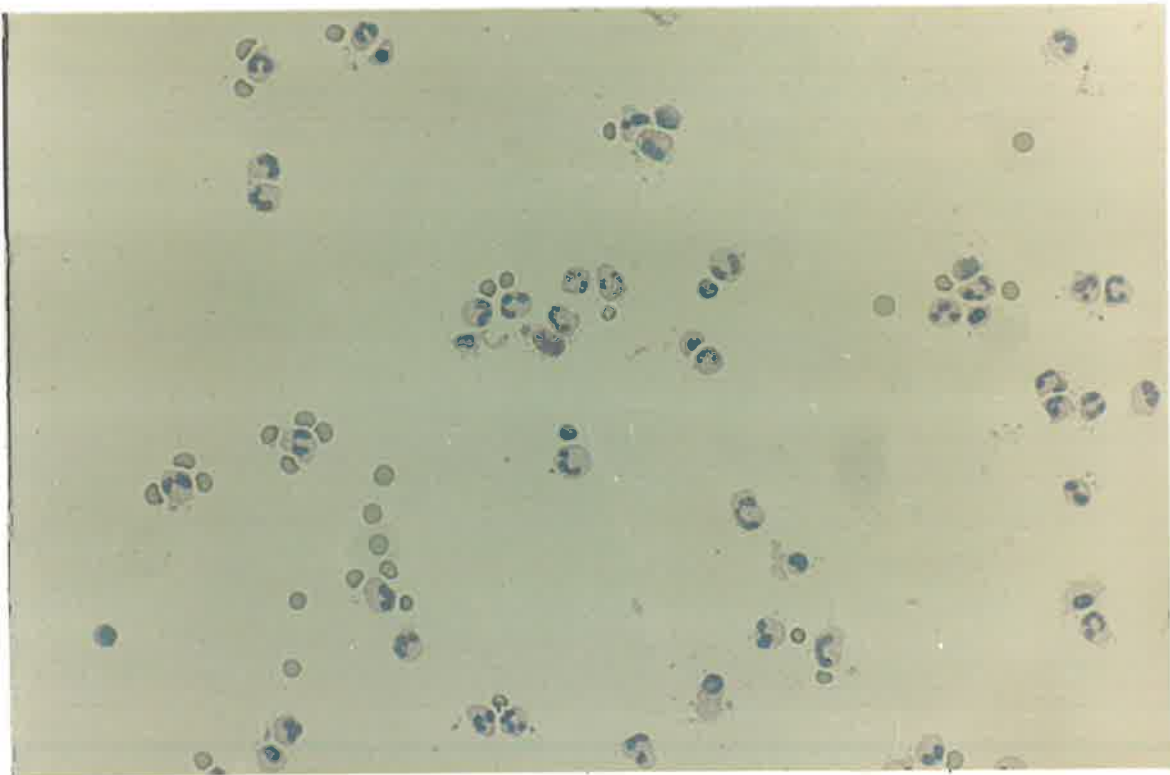


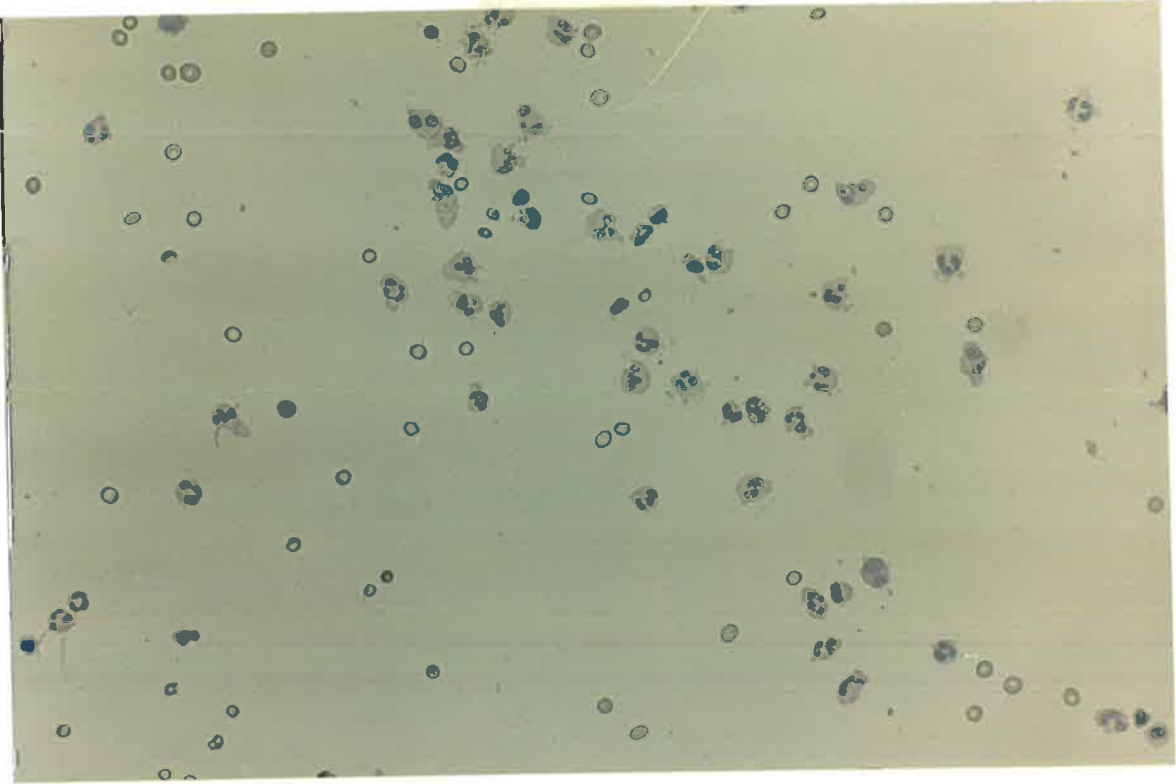
Figure 6.3

Binding of monoclonal antibodies to peripheral blood leukocytes
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

C. 4C5.D12

D. 5A2.G5

C



D

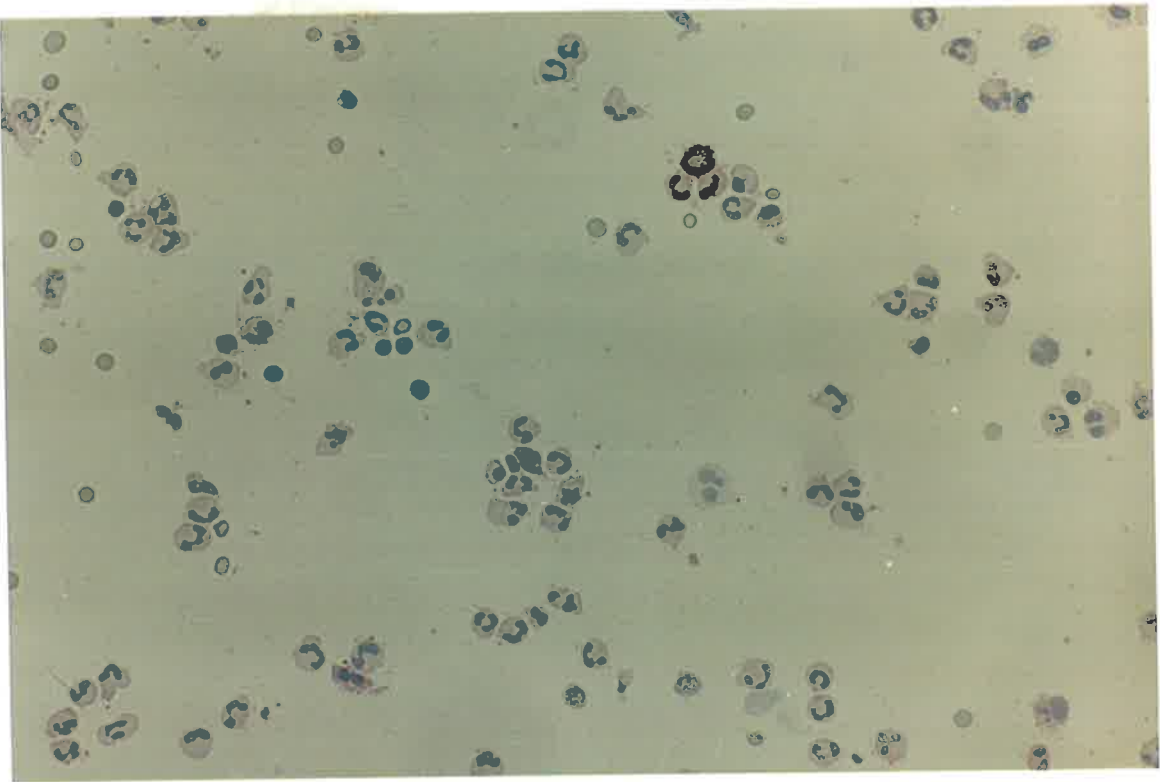


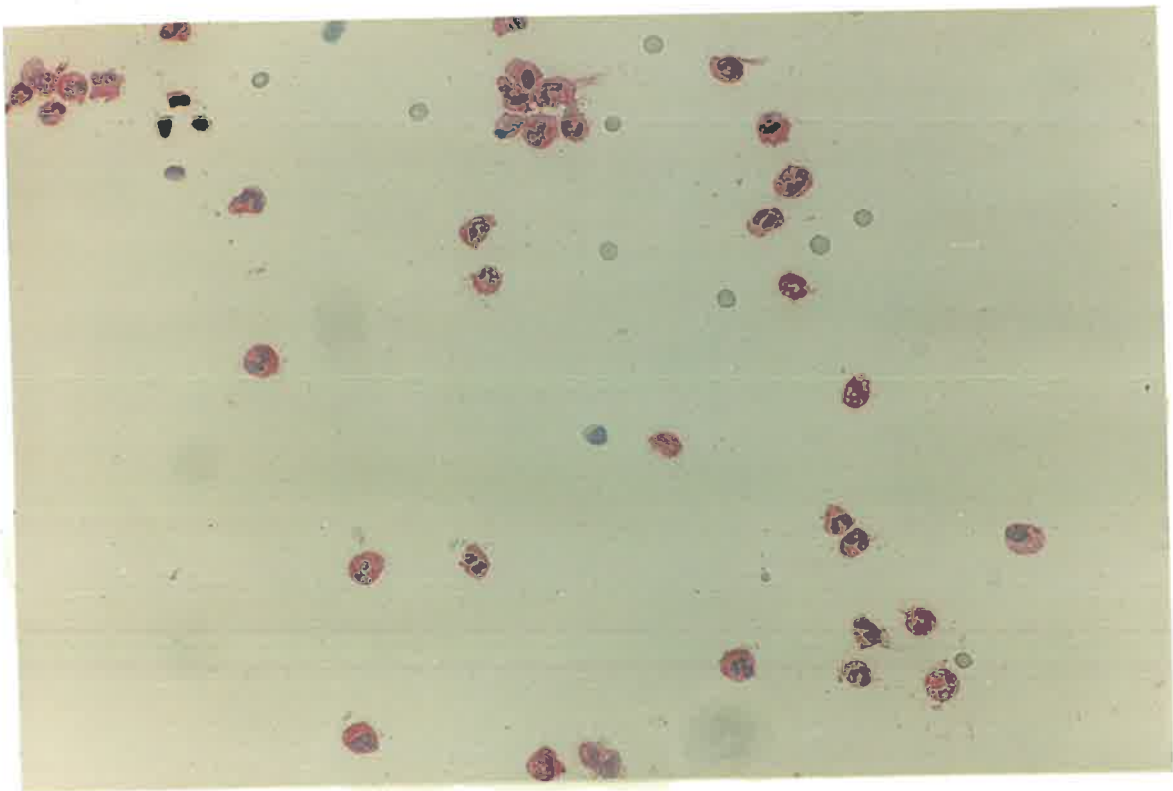
Figure 6.3

Binding of monoclonal antibodies to peripheral blood leukocytes
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

E. 5A4.C5

F. Sal-2

E



F

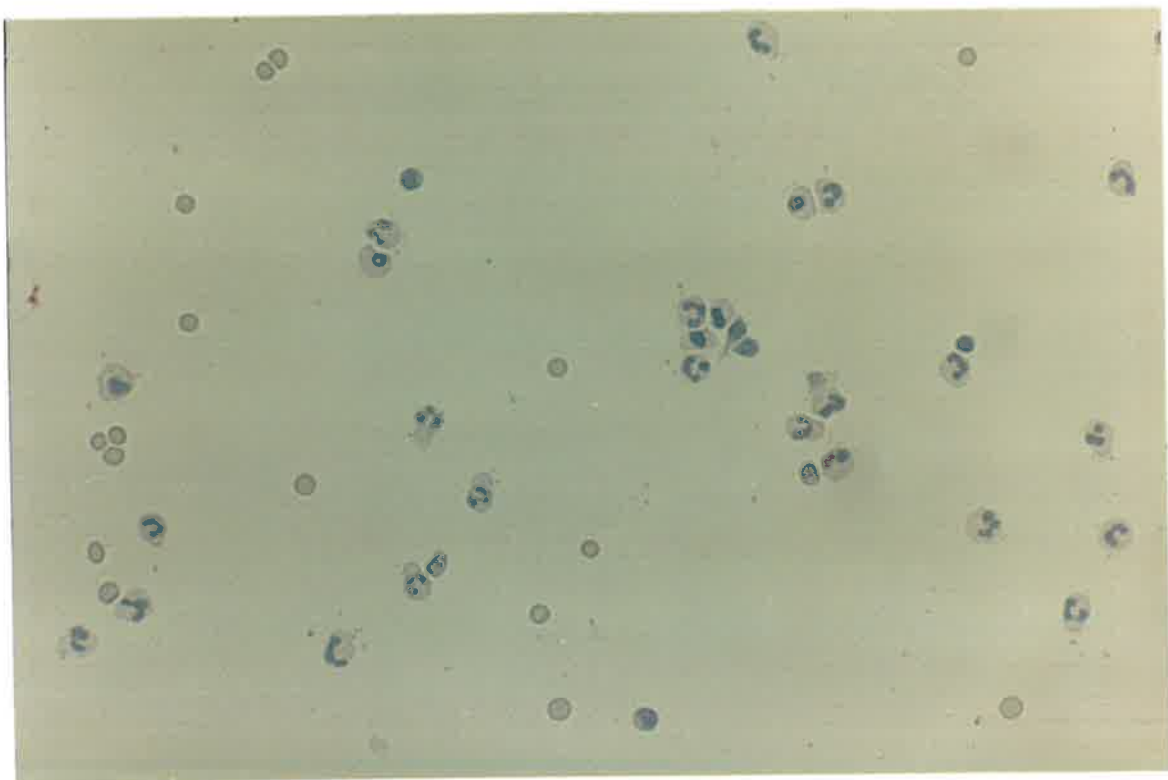


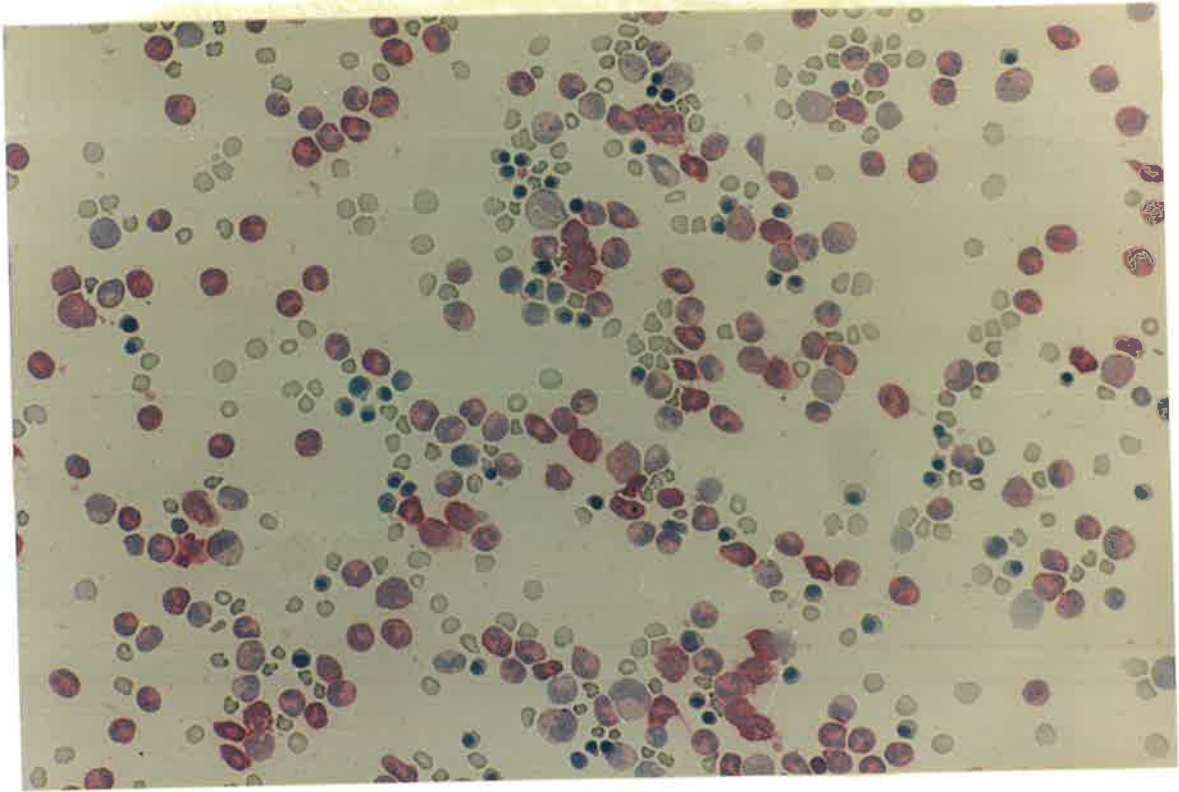
Figure 6.4

Binding of monoclonal antibodies to bone marrow nucleated cells
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

A. 4B4.E11

B. 4B5.F5

A



B

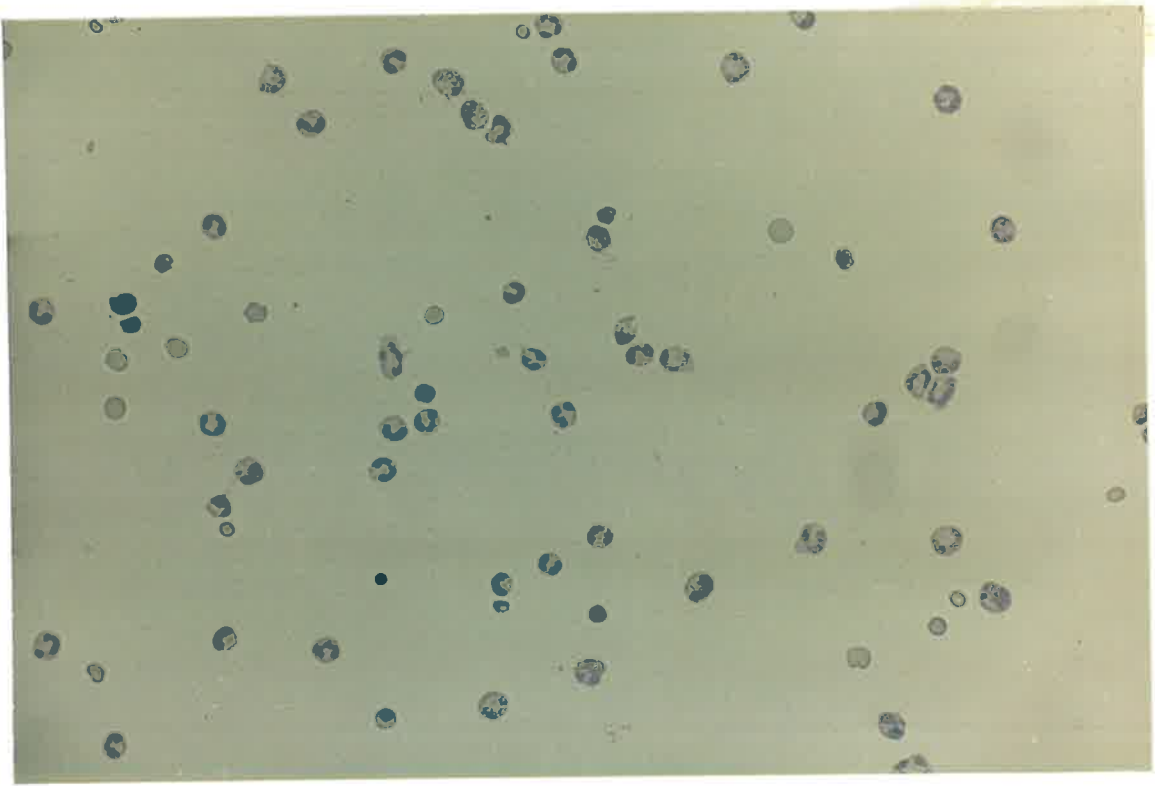


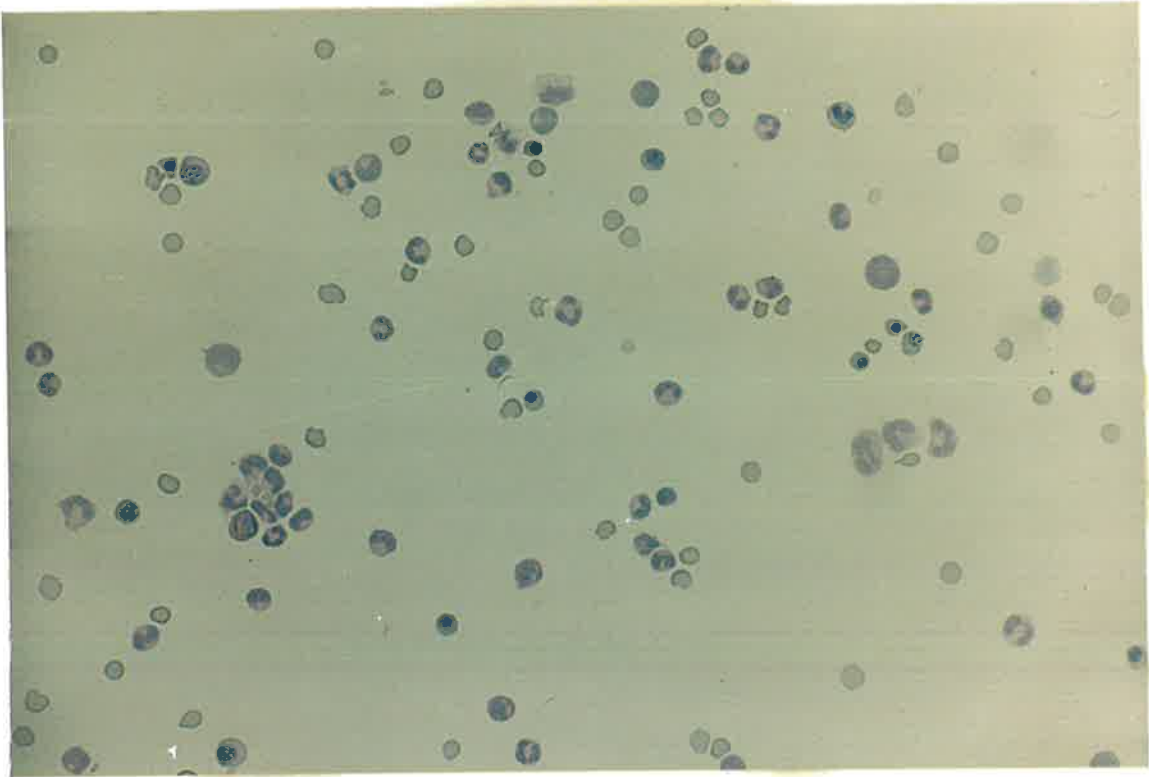
Figure 6.4

Binding of monoclonal antibodies to bone marrow nucleated cells
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

C. 4C5.D12

D. 5A2.G5

C



D

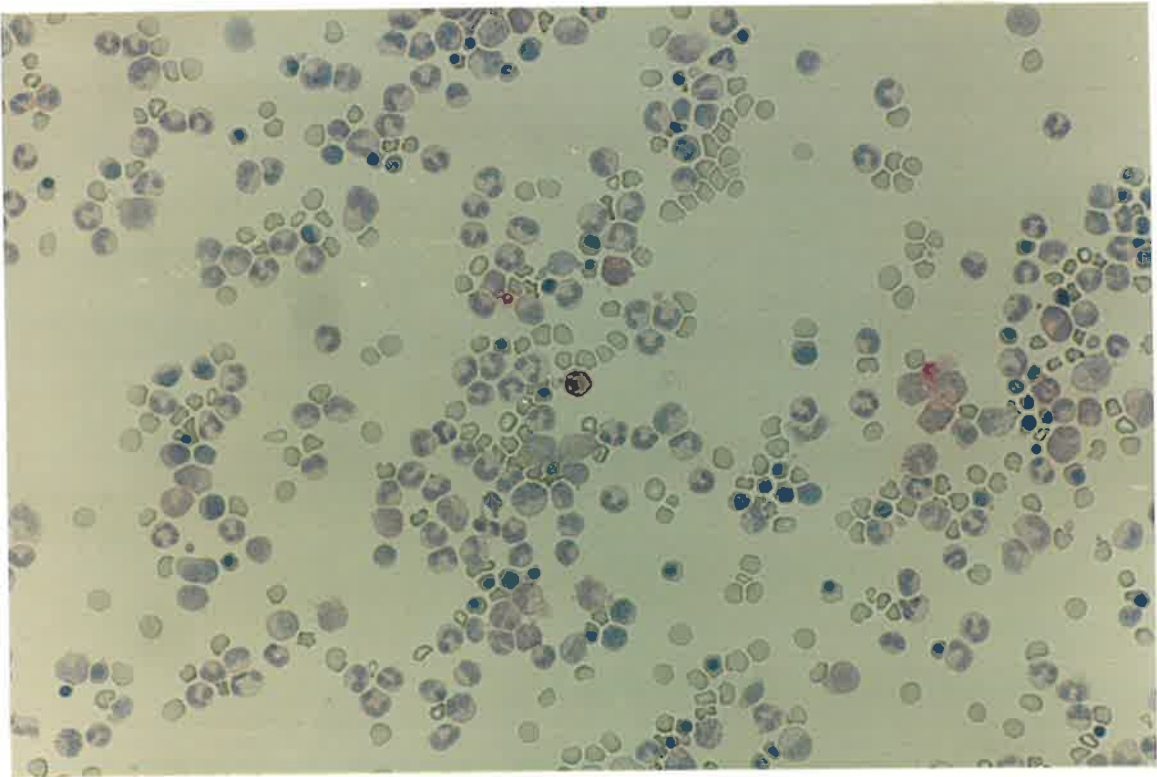


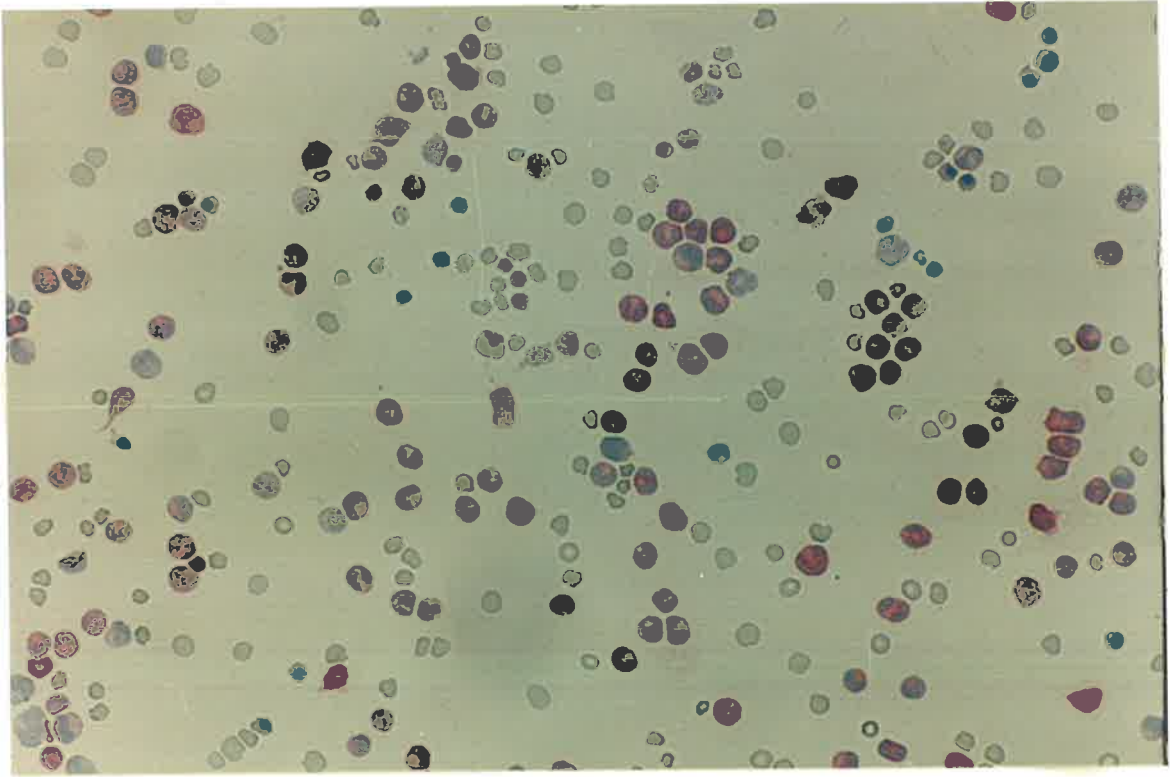
Figure 6.4

Binding of monoclonal antibodies to bone marrow nucleated cells
assayed by Alkaline phosphatase/anti-alkaline phosphatase
immunocytochemistry (APAAP)

E. 5A4.C5

F. Sal-2

E



F

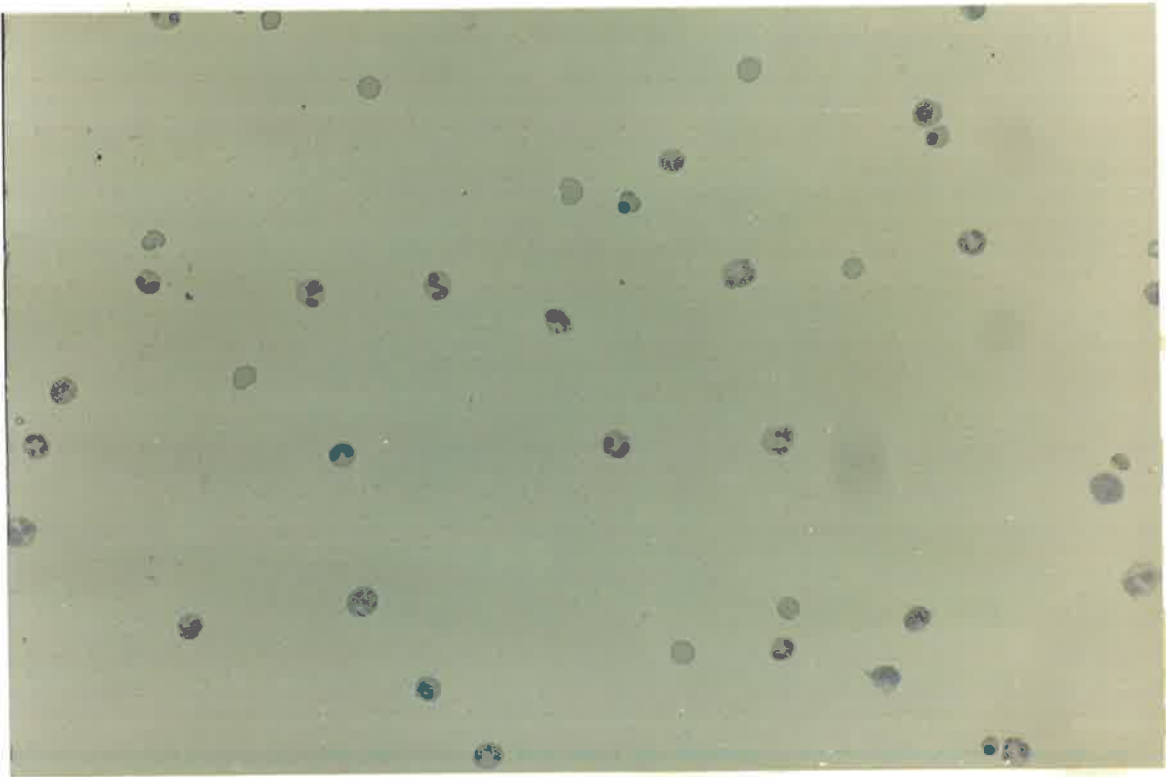


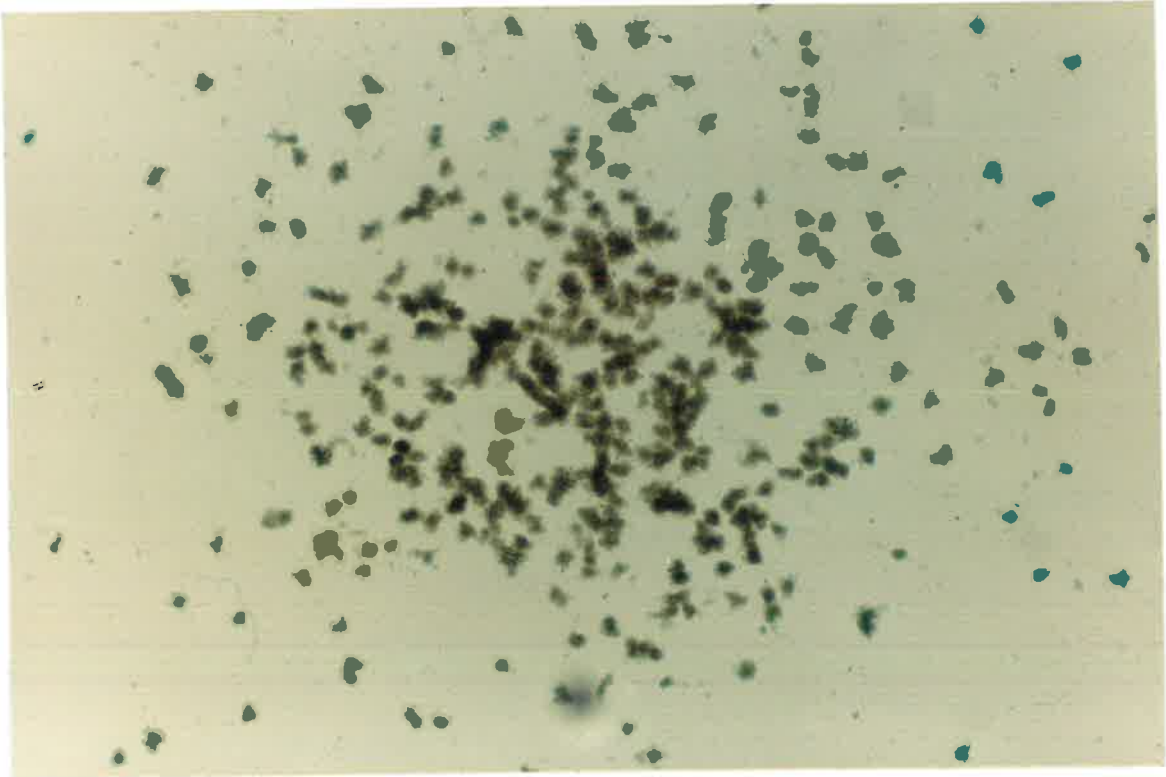
Figure 6.5

Photographs of stained myeloid colonies grown from cord blood progenitors.

A. Macrophage

B. Neutrophil

A



B

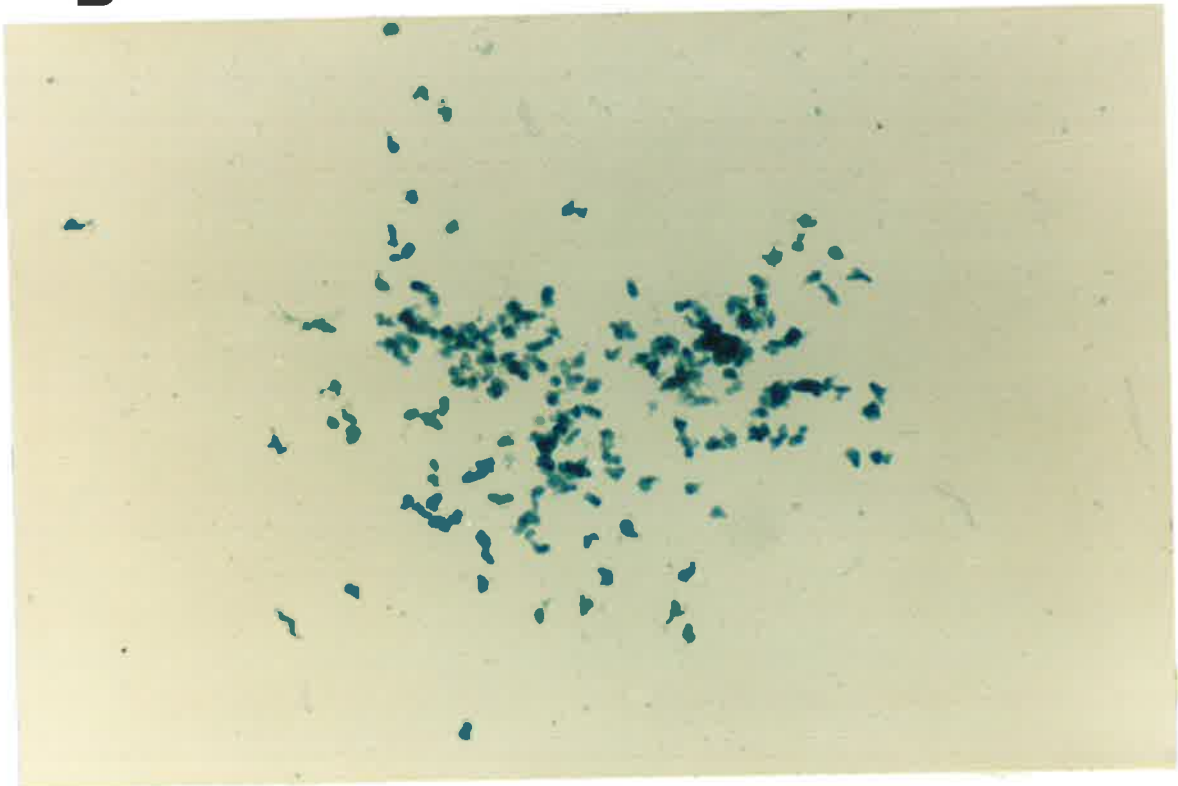
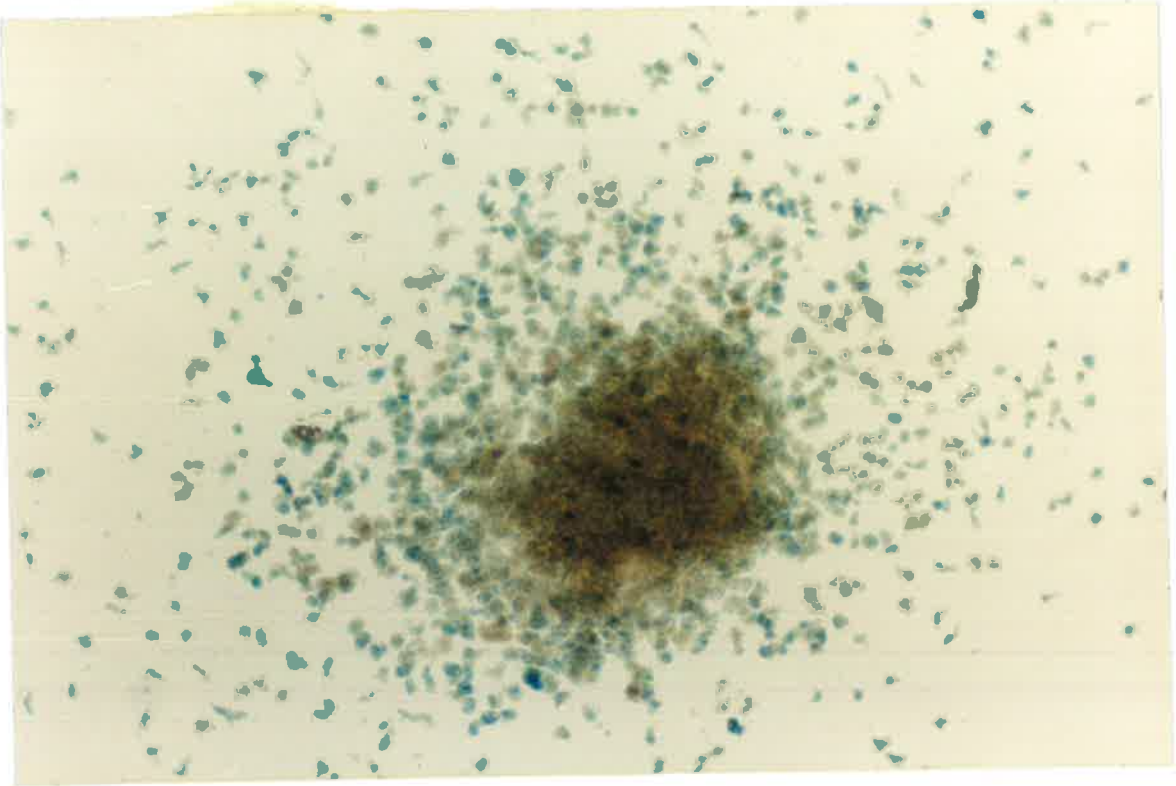


Figure 6.5

Photographs of stained myeloid colonies grown from cord blood progenitors.

- D. Eosinophil
- C. Mixed macrophage/Neutrophil

C



D

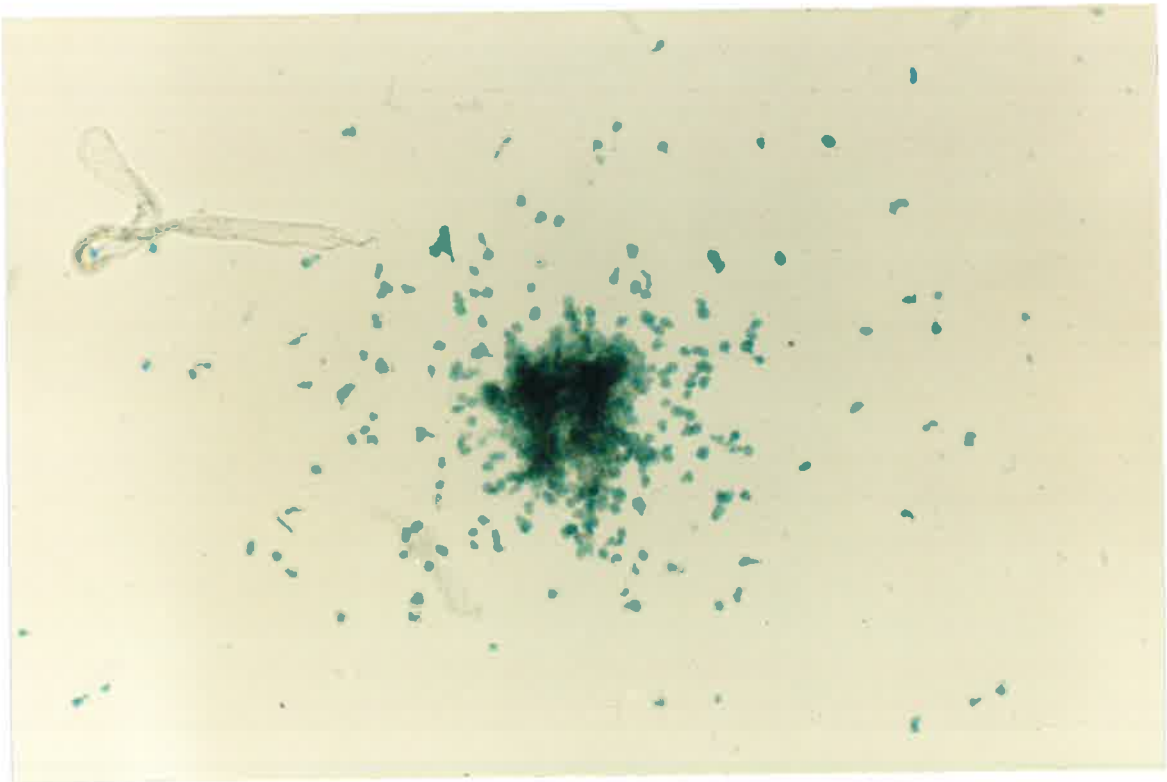
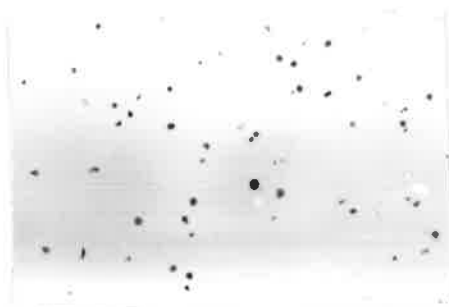


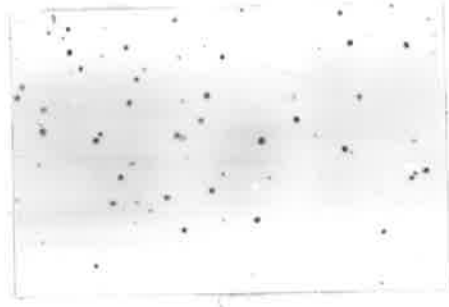
Figure 6.6

Immunoperoxidase assay of binding of monoclonal antibodies to RC-2A cells. Photographs are approximately x 100 and were taken through an Olympus BH-2 microscope using an Olympus (C35AD/PM-10AD/PM-CBAD) camera system and Kodak technical pan film. A blue (LBD-2) filter was used to emphasise the brown stain.

FIGURE 6.6
Binding to RC-2A Cells- Immunoperoxidase



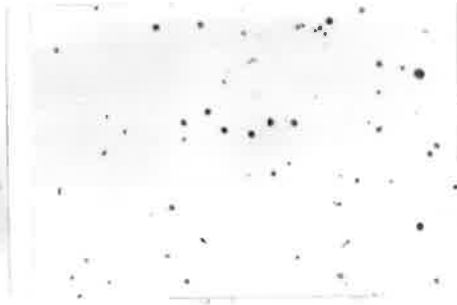
4B4.E11



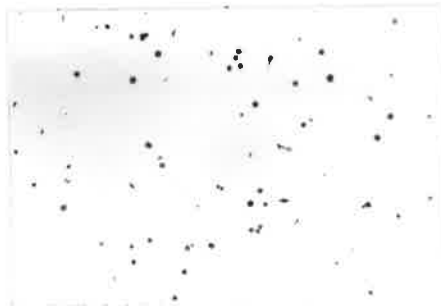
4B5.F5



4C5.D12



5A2.G5



5A4.C5



Sal-2

Figure 6.7

Immunoperoxidase assay of binding of monoclonal antibodies to human tonsil sections. Photographs were taken through an Olympus BH-2 microscope using an Olympus (C35AD/PM-10AD/PM-CBAD) camera system and Kodak technical pan film. A blue (LBD-2) filter was used to emphasise the brown stain. The x10 and x40 refer to the power of the objective used.

x40 - Follicle

x40B - Interfollicular area

FIGURE 6.7 - Tonsil

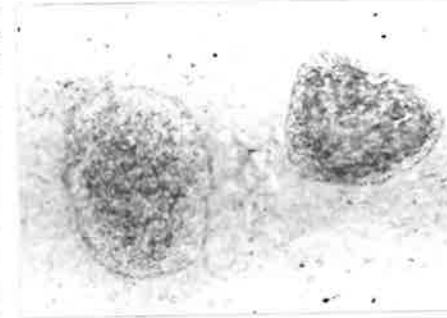
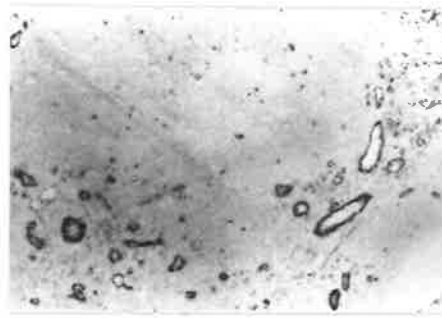
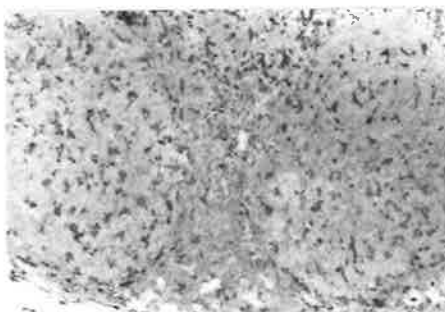
4B4.E11

4B5.F5

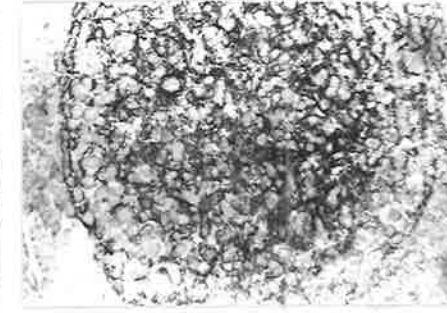
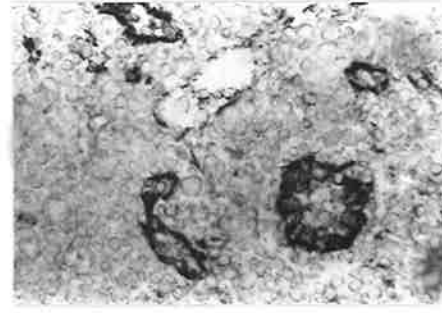
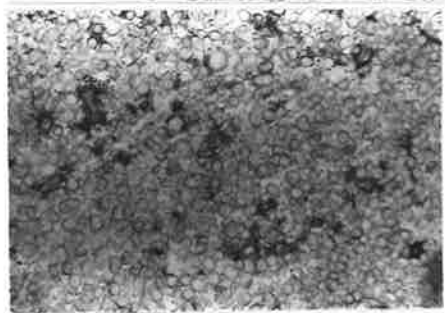
5A2.G5

5A4.C5

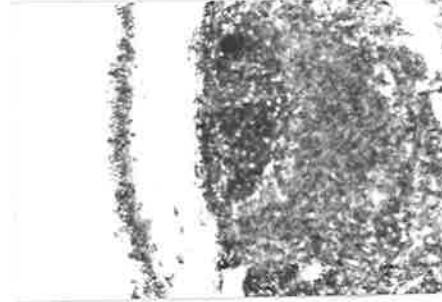
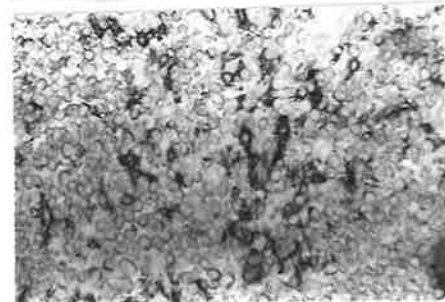
x10



x40



x40
B



↑
C- x10
Sal-2

↑
1B4 C+ x10
KHLA-ABK

Figure 6.8

Immunoperoxidase assay of binding of monoclonal antibodies to human small intestine sections. Photographs were taken through an Olympus BH-2 microscope using an Olympus (C35AD/PM-10AD/PM-CBAD) camera system and Kodak technical pan film. A blue (LBD-2) filter was used to emphasise the brown stain. The x10 and x40 refer to the power of the objective used.

x40 - Follicle

x40B - Interfollicular area

FIGURE 6.8 -Small Intestine

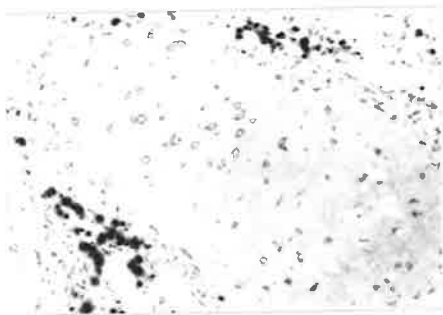
4B4.E11

4B5.F5

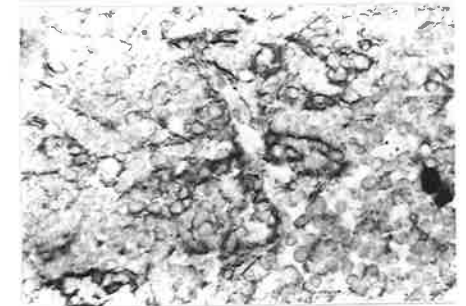
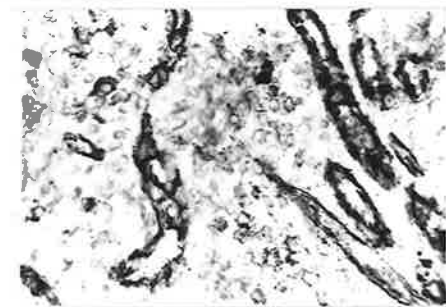
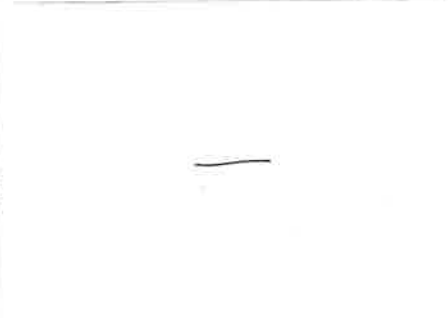
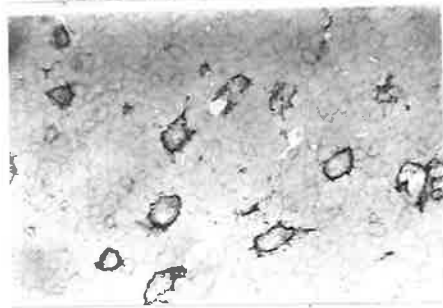
5A2.G5

5A4.C5

x10

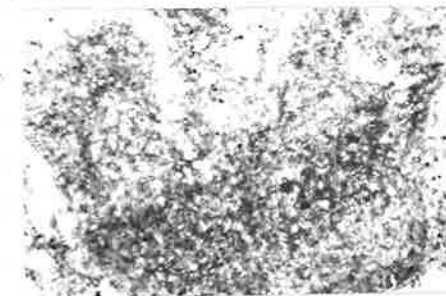
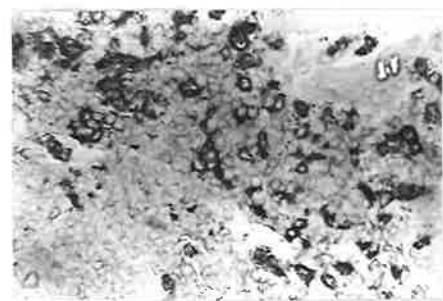


x40



x40

B



↑
C- x10

Sal-2

↑
1B4 C+ x10

α HLA -ABC

Figure 6.9 A

Histograms showing binding of MoAb 4B4.E11, by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	4B4.E11	4.1
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	4B4.E11	3.0
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	4B4.E11	6.9

Figure 6.9

4B4.E11 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

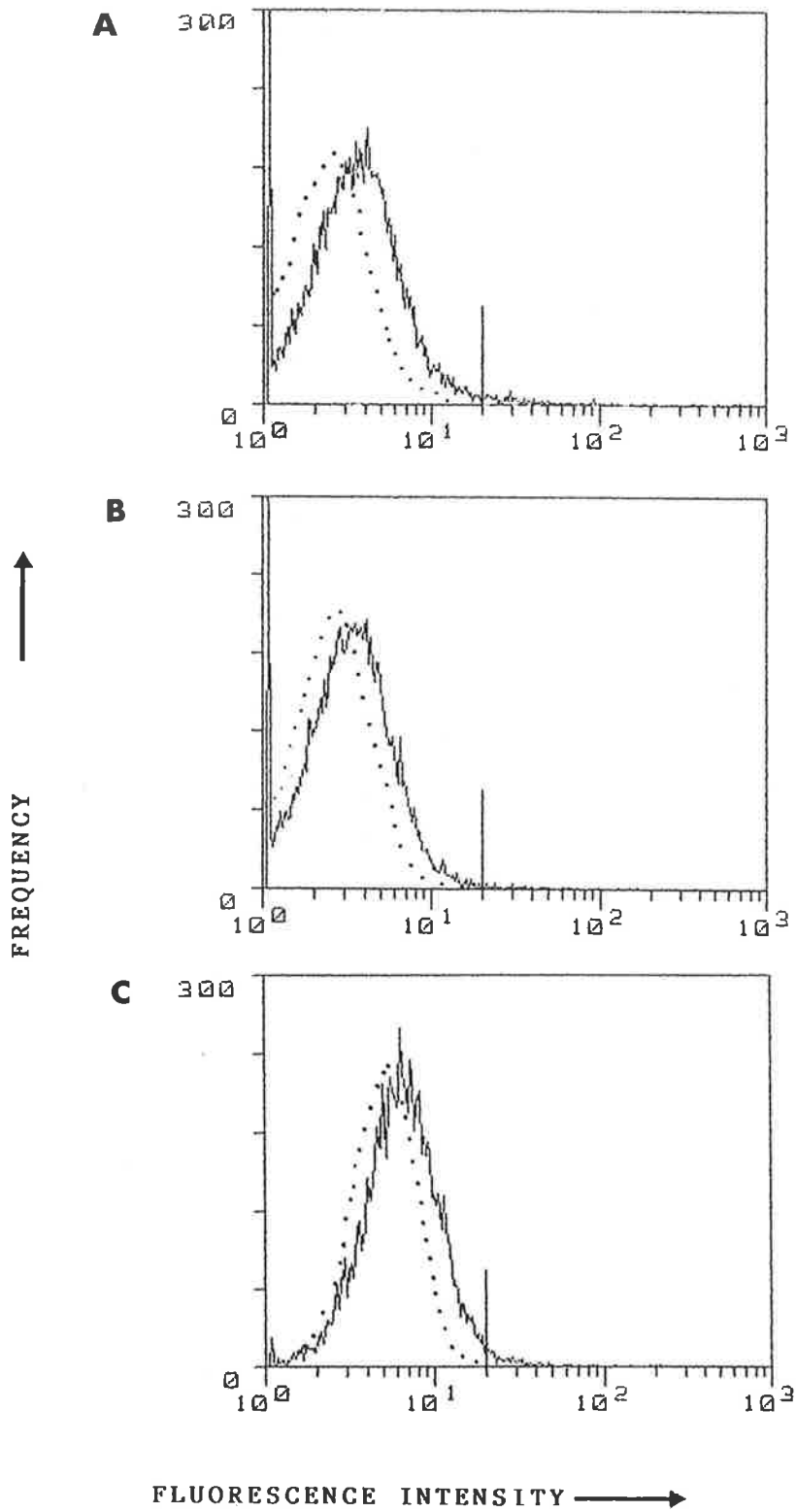


Figure 6.9 B

Histograms showing binding of MoAb 4B5.F5, by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	4B5.F5	50.7
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	4B5.F5	58.4
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	4B5.F5	76.3

Figure 6.9

4B5.F5 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

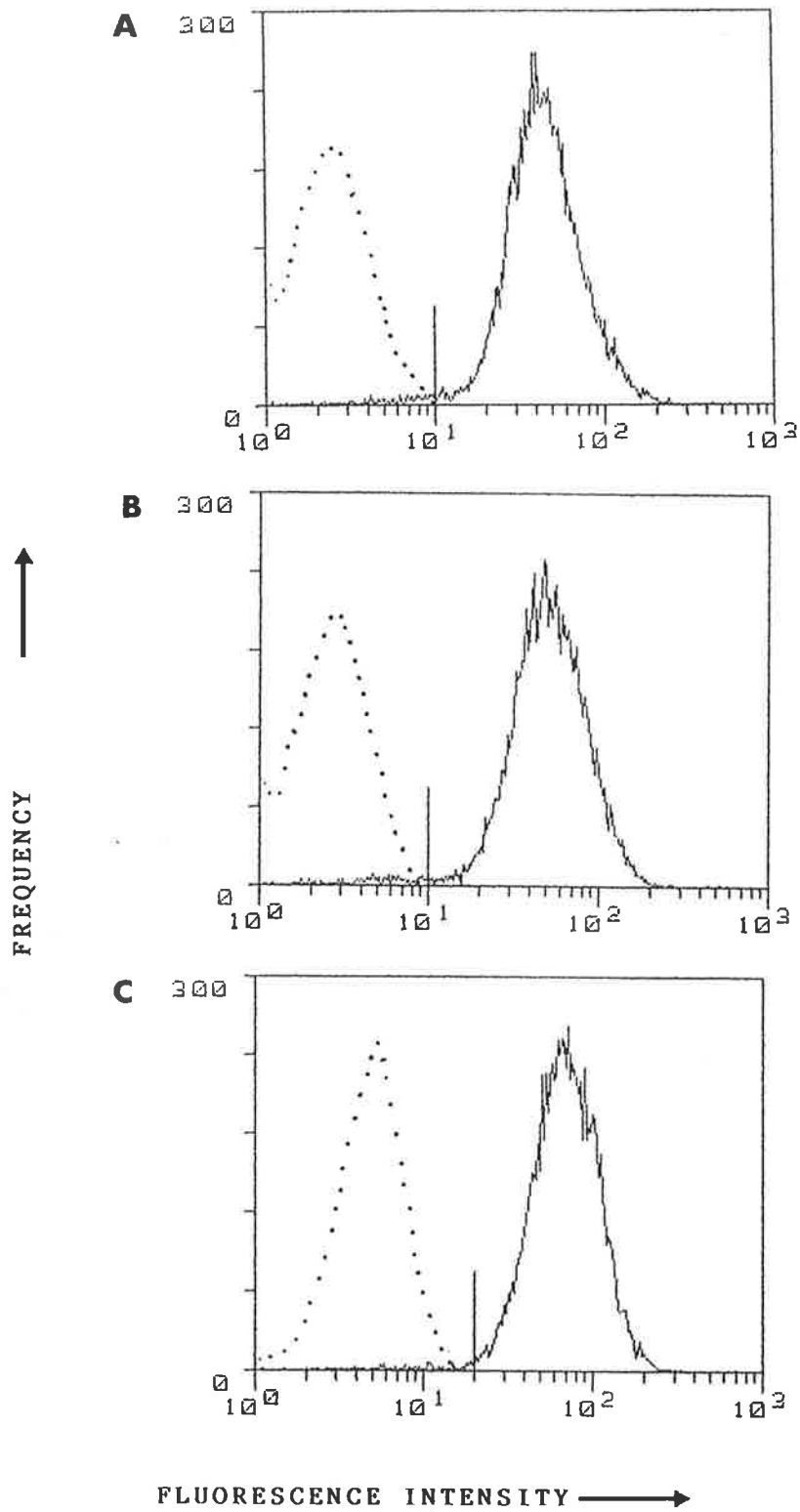


Figure 6.9 C

Histograms showing binding of MoAb 4C5.D12, by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	4C5.D12	12.3
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	4C5.D12	10.1
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	4C5.D12	16.3

Figure 6.9

4C5.D12 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

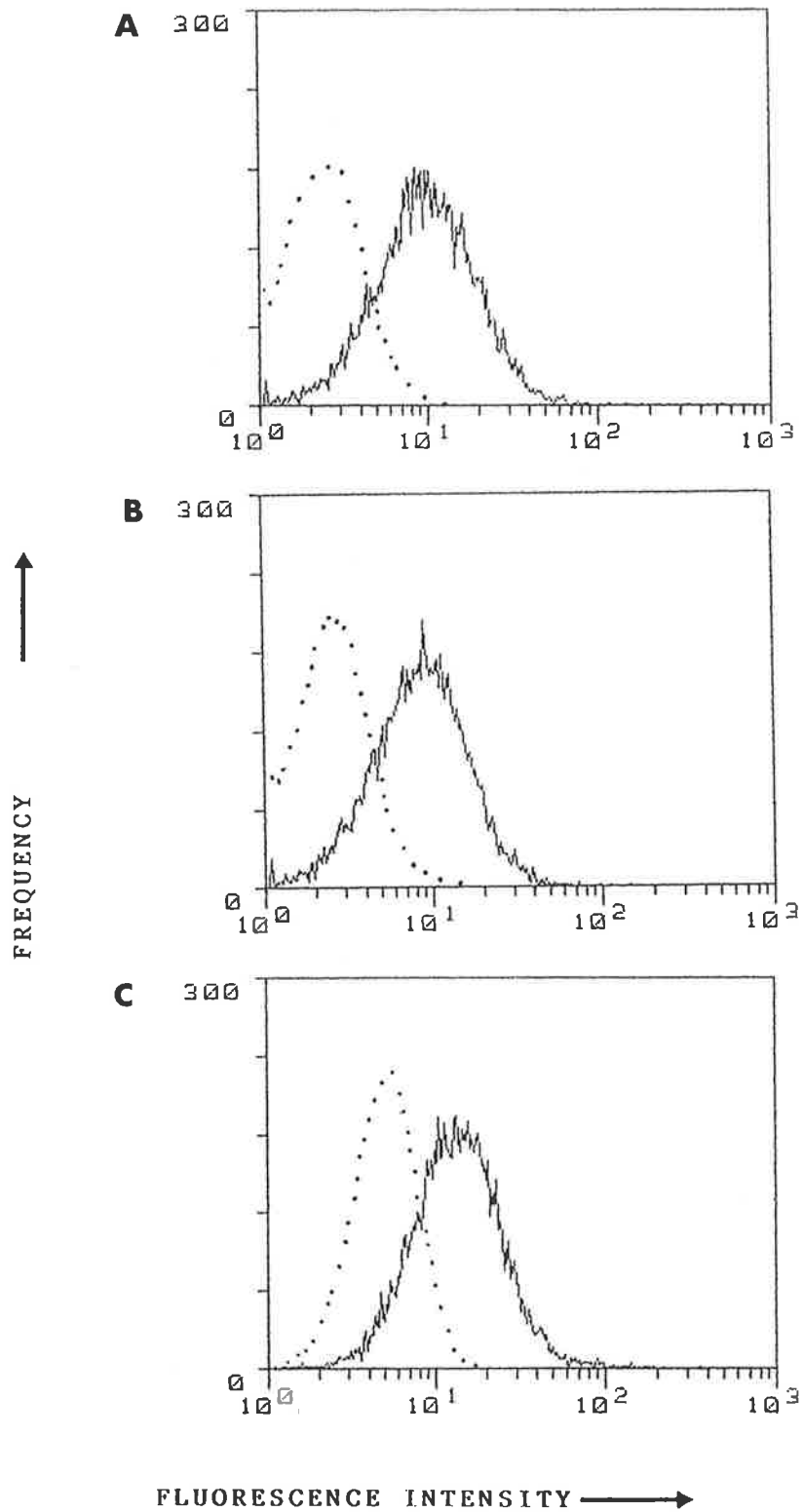


Figure 6.9 D

Histograms showing binding of MoAb 5A2.G5, by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	5A2.G5	149.4
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	5A2.G5	142.8
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	5A2.G5	72.3

Figure 6.9

5A2.G5 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

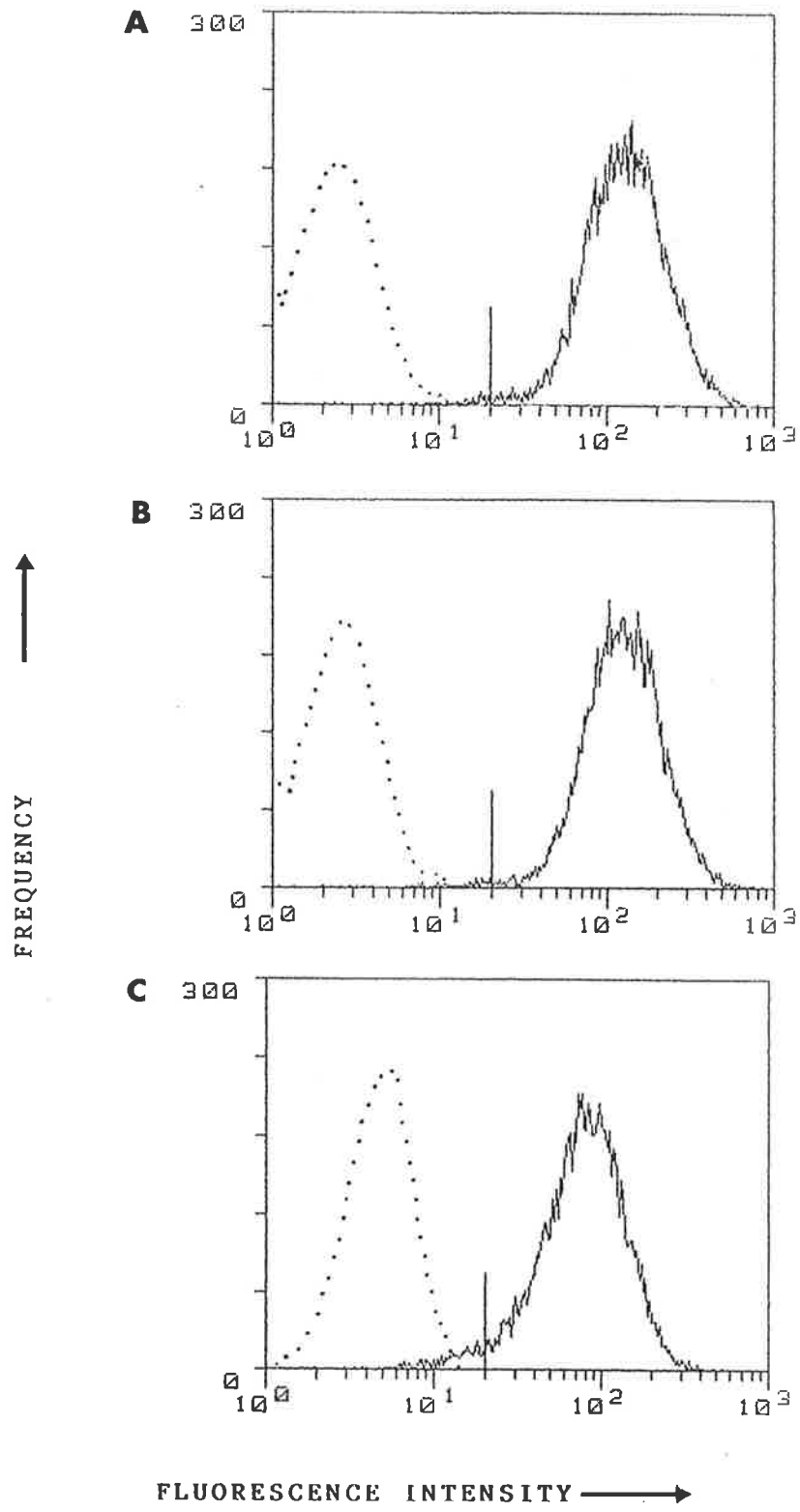


Figure 6.9 E

Histograms showing binding of MoAb 5A4.C5, by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	5A4.C5	58.4
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	5A4.C5	121.9
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	5A4.C5	320.9

Figure 6.9

5A4.C5 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

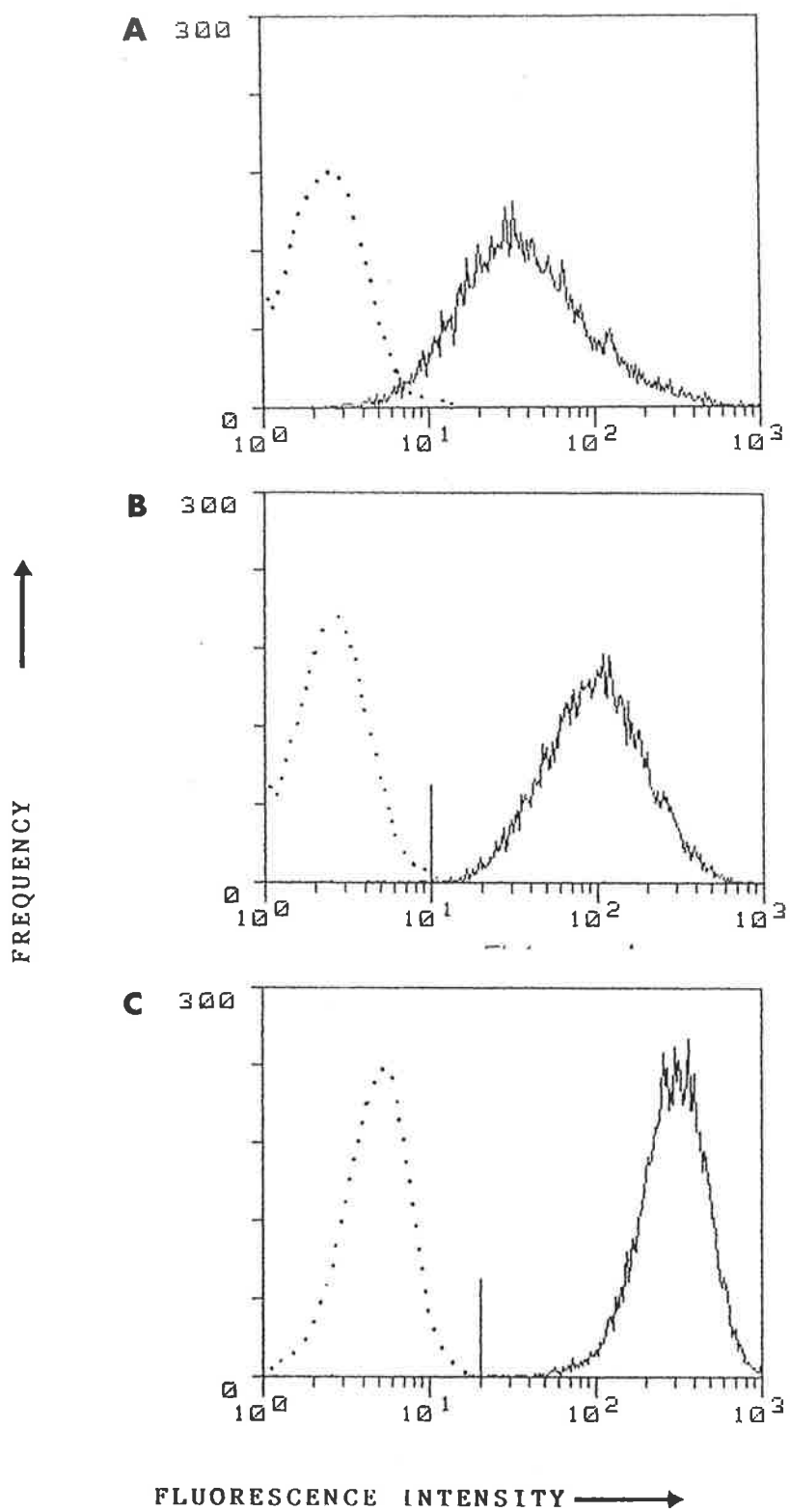


Figure 6.9 F

Histograms showing binding of MoAb FMC-14 (anti-Ia), by indirect immunofluorescence assay with scoring by flow cytometry, to RC-2A cells which have been induced to differentiate in culture with 20% v/v PHA-LCM for zero, four and eight days. Negative control antibody (Sal-2) binding is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	2.9
	FMC-14	678.5
B. RC-2A Day 4 PHA-LCM	Sal-2	5.0
	FMC-14	540.4
C. RC-2A Day 8 PHA-LCM	Sal-2	5.4
	FMC-14	329.8

Figure 6.9

FMC-14 - BINDING TO PHA-LCM
TREATED RC-2A CELLS.

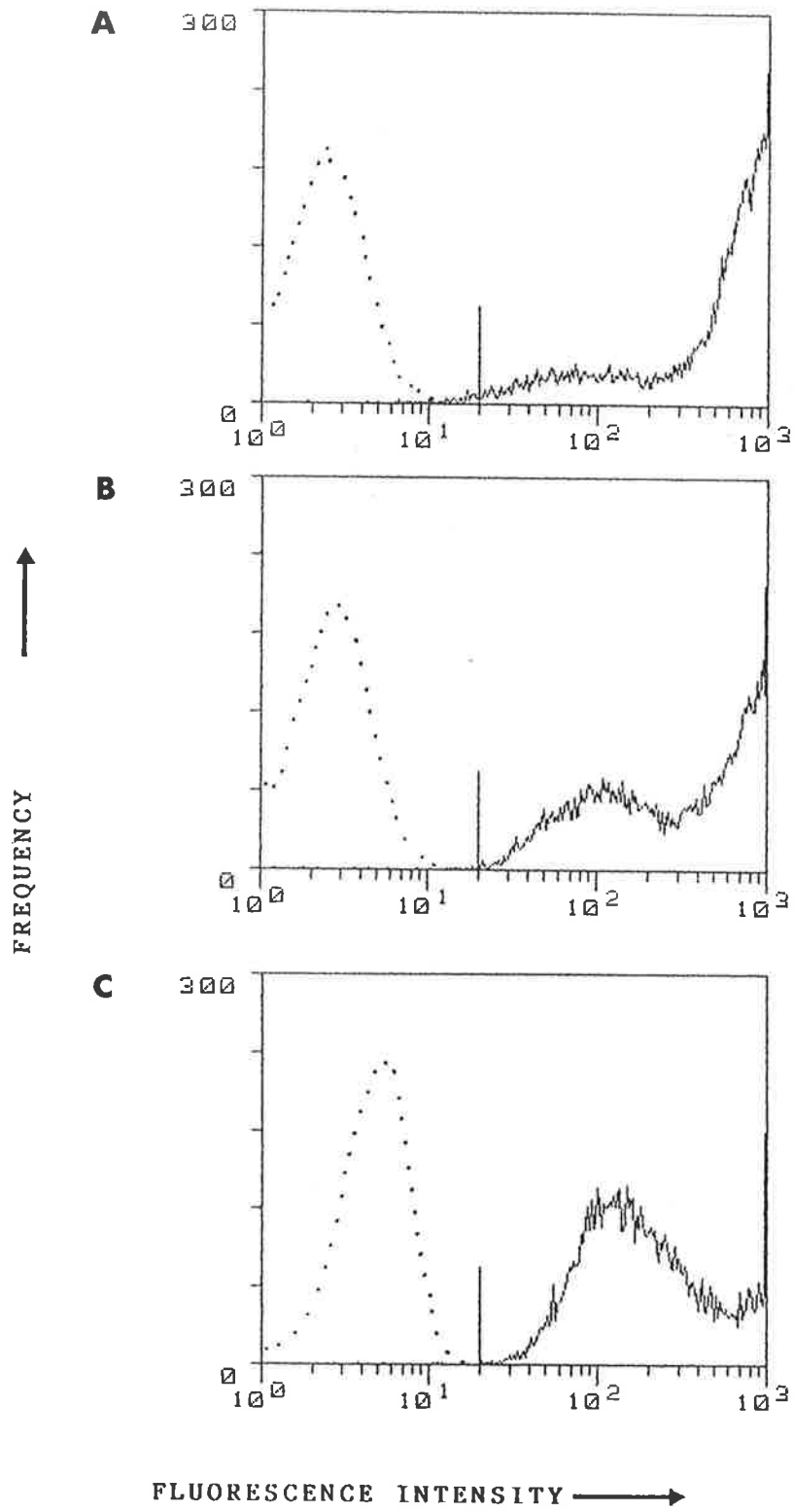


Figure 6.10 A

Histograms showing binding of MoAb 4B4.E11, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	4B4.E11	2.0
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	4B4.E11	16.6
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	4B4.E11	7.4

Figure 6.10 A

4B4.E11 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

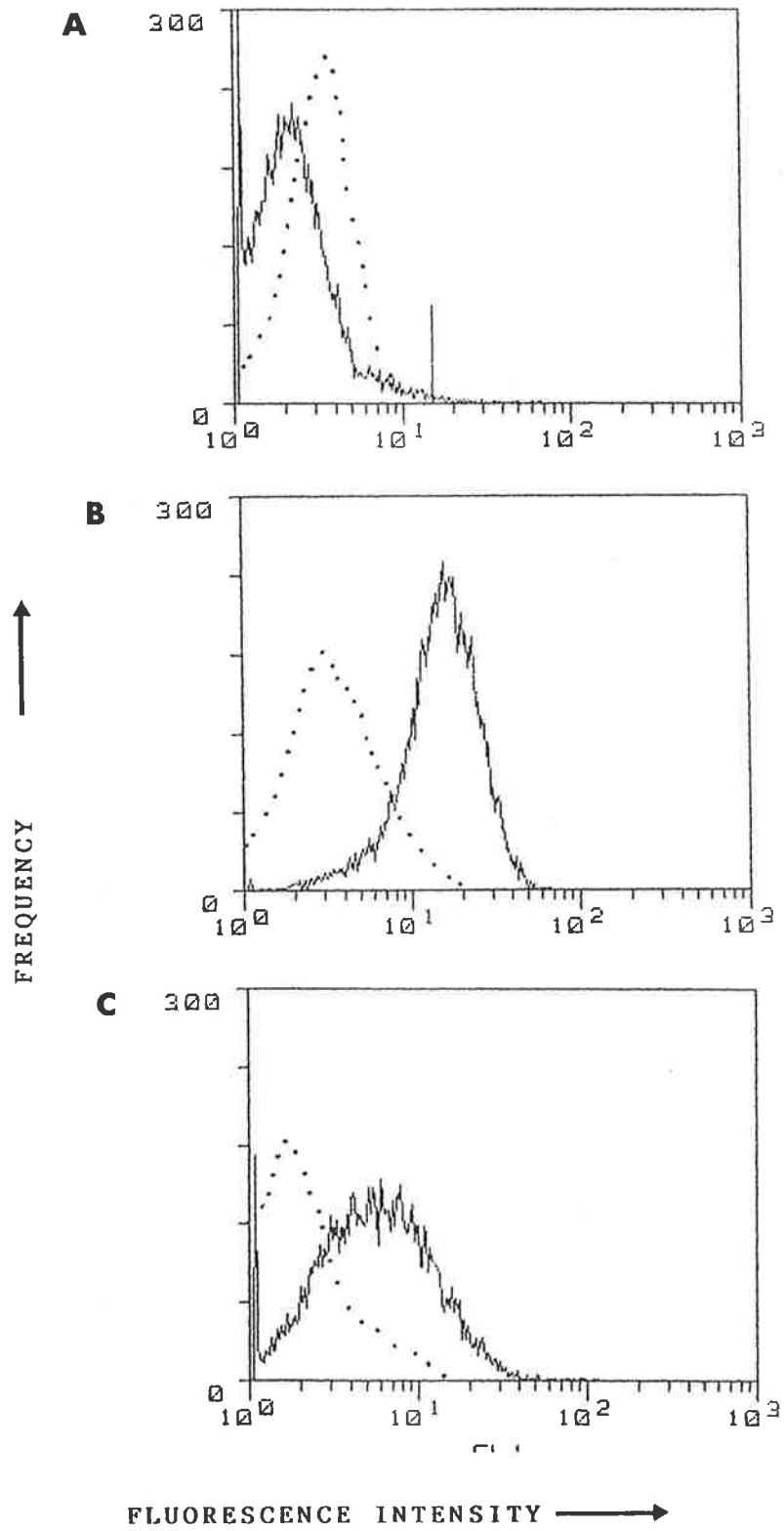


Figure 6.10 B

Histograms showing binding of MoAb 4B5.F5, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	4B5.F5	18.7
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	4B5.F5	6.8
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	4B5.F5	104.2

Figure 6.10 B

4B5.F5 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

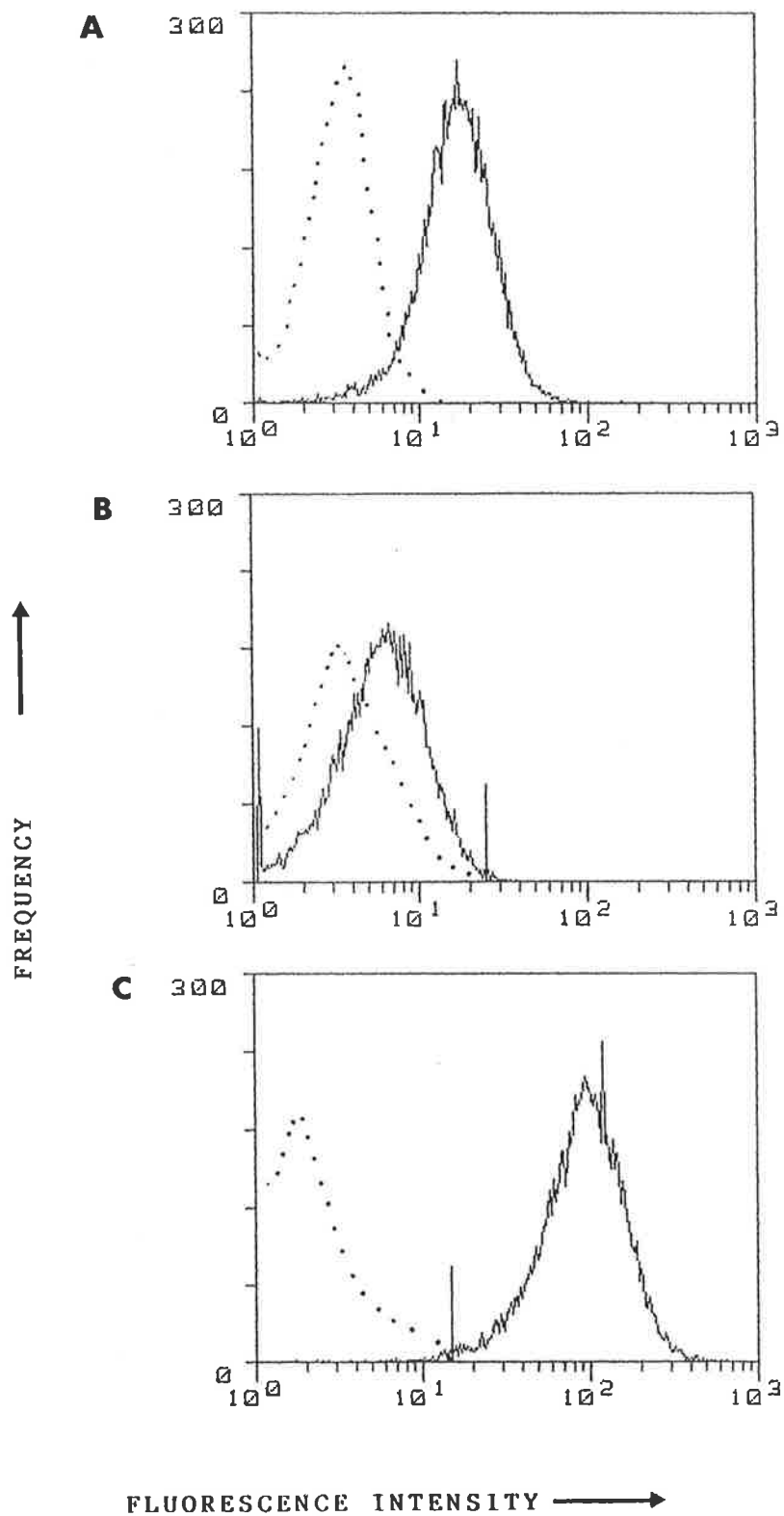


Figure 6.10 C

Histograms showing binding of MoAb 4C5.D12, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	4C5.D12	6.1
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	4C5.D12	71.7
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	4C5.D12	171.9

Figure 6.10 C

4C5.D12 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

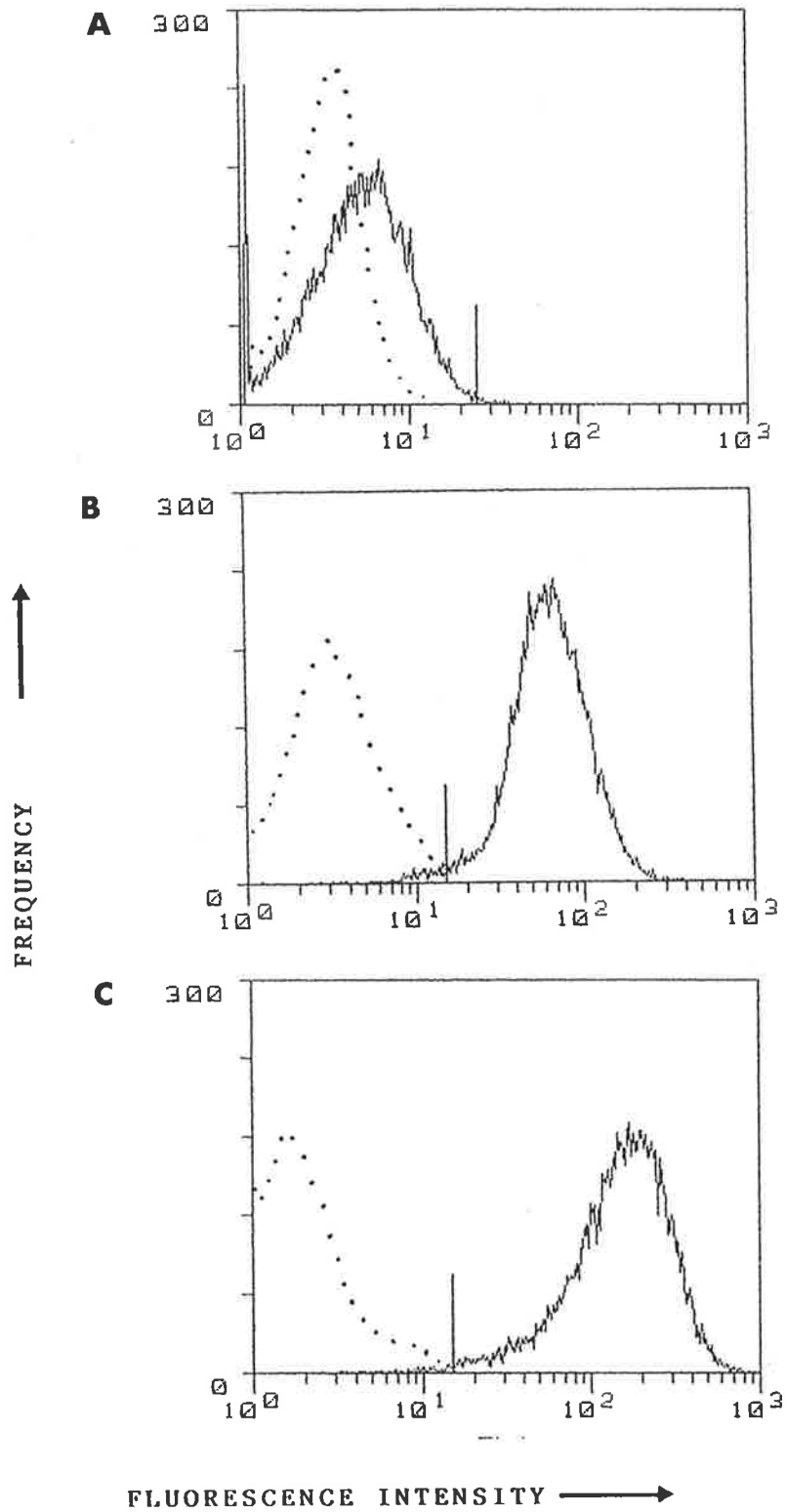


Figure 6.10 D

Histograms showing binding of MoAb 5A2.G5, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	5A2.G5	68.2
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	5A2.G5	42.4
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	5A2.G5	254.0

Figure 6.10 D

5A2.G5 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

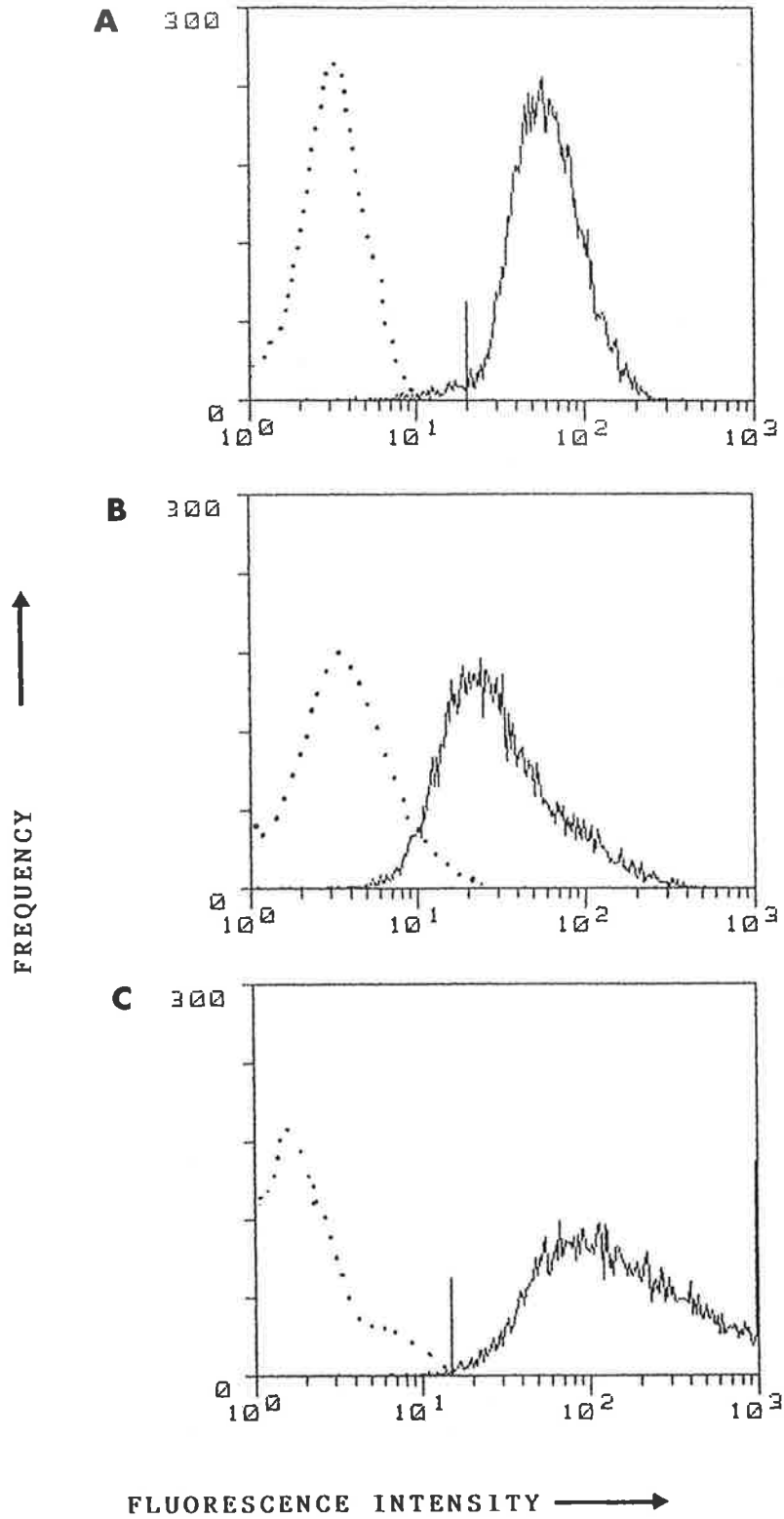


Figure 6.10 E

Histograms showing binding of MoAb 5A4.C5, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	5A4.C5	23.8
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	5A4.C5	597.5
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	5A4.C5	54.8

Figure 6.10 E

5A4.C5 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

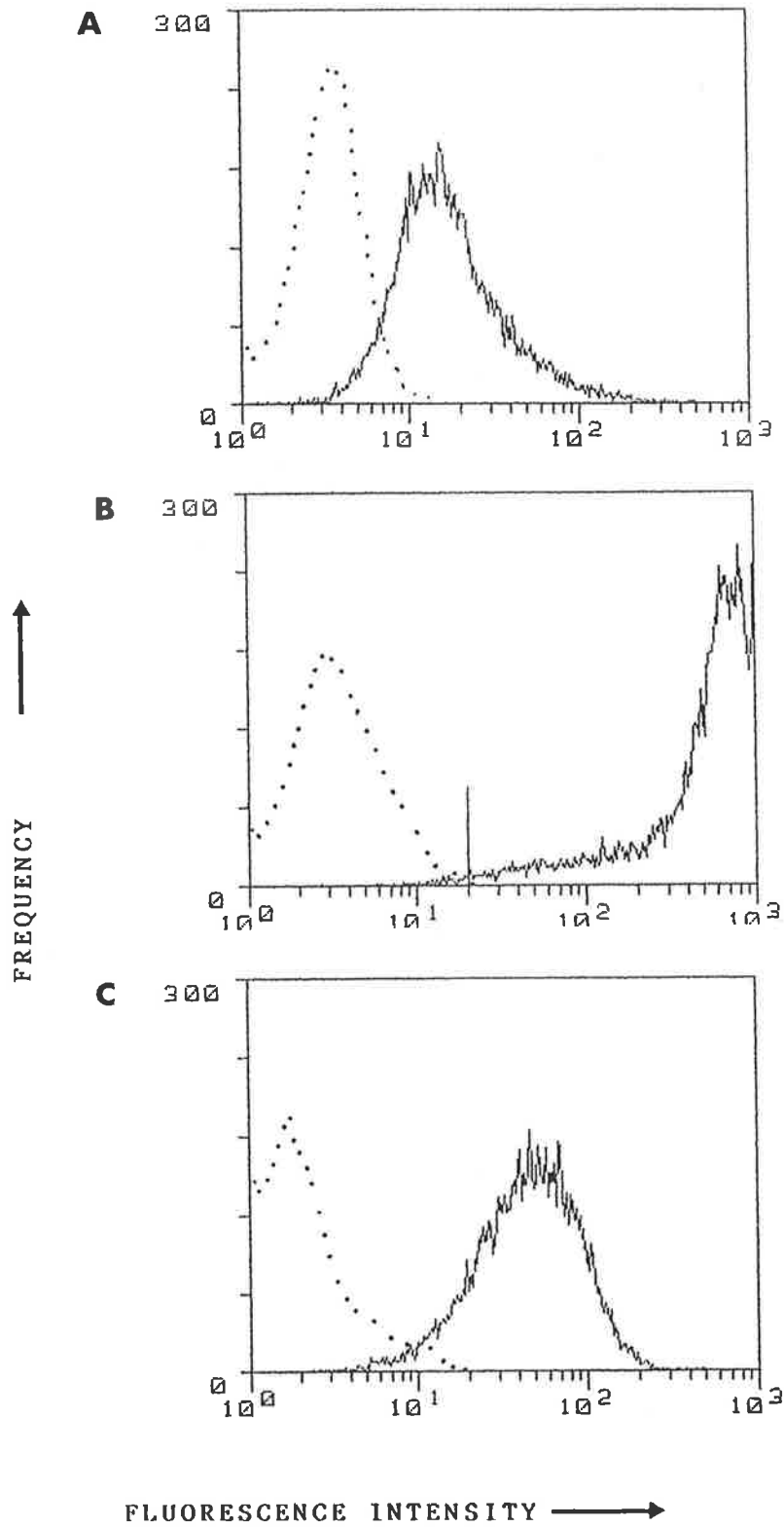


Figure 6.10 F

Histograms showing binding of MoAb FMC-14, by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-2) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-2	3.6
	FMC-14	482.2
B. RC-2A Day 7 TNF- α	Sal-2	4.3
	FMC-14	286.3
C. RC-2A Day 7 γ -IFN	Sal-2	2.5
	FMC-14	332.1

Figure 6.10 F

FMC-14 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

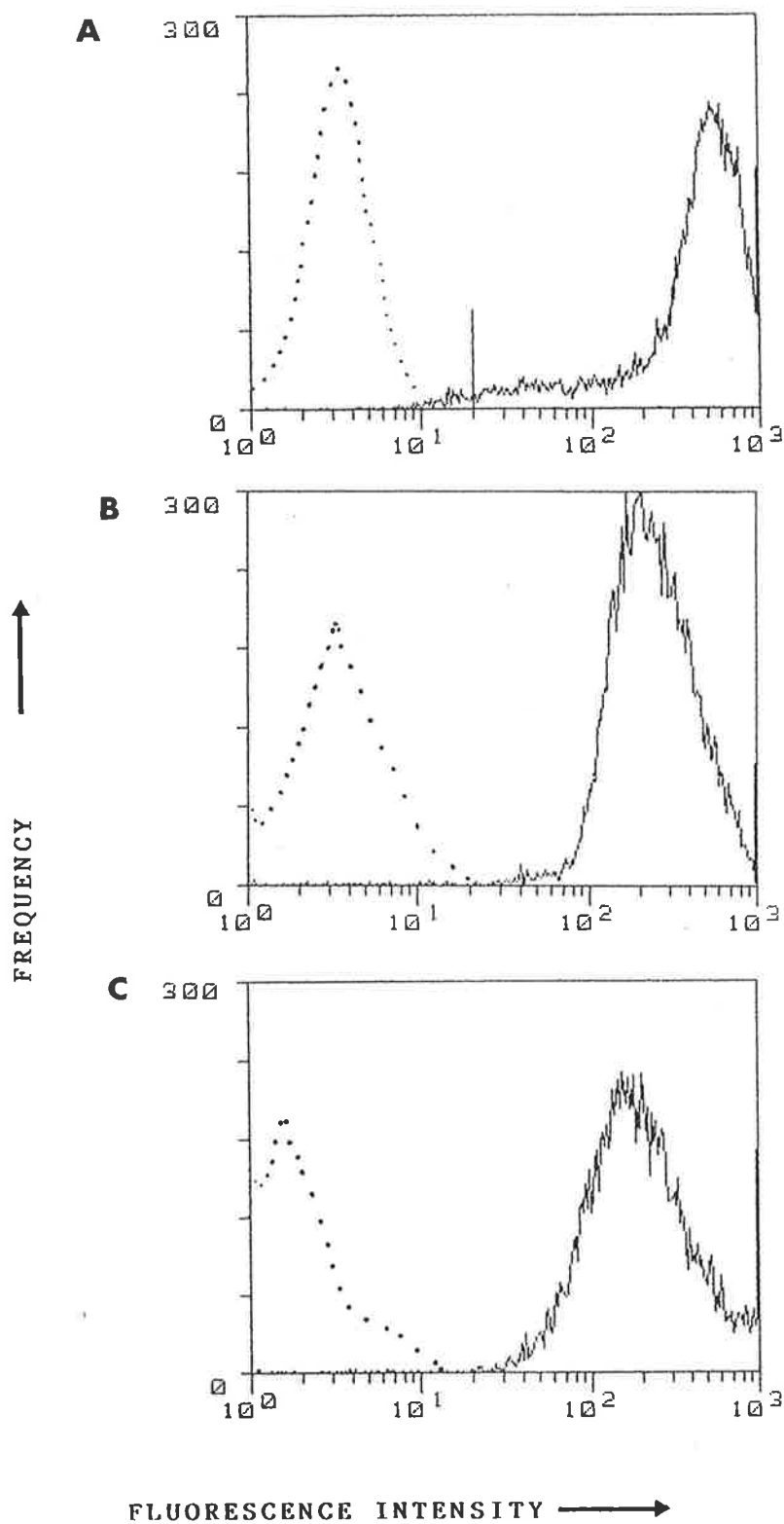


Figure 6.10 G

Histograms showing binding of MoAb anti-Mo1 (DC11b), by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-3) is shown as a dotted line.

A. RC-2A Untreated Control,	Sal-3	2.5
	anti-Mo1	33.5
B. RC-2A Day 7 TNF- α	Sal-3	2.3
	anti-Mo1	56.3
C. RC-2A Day 7 γ -IFN	Sal-3	4.7
	anti-Mo1	165.2

Figure 6.10 G

Mo1 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS

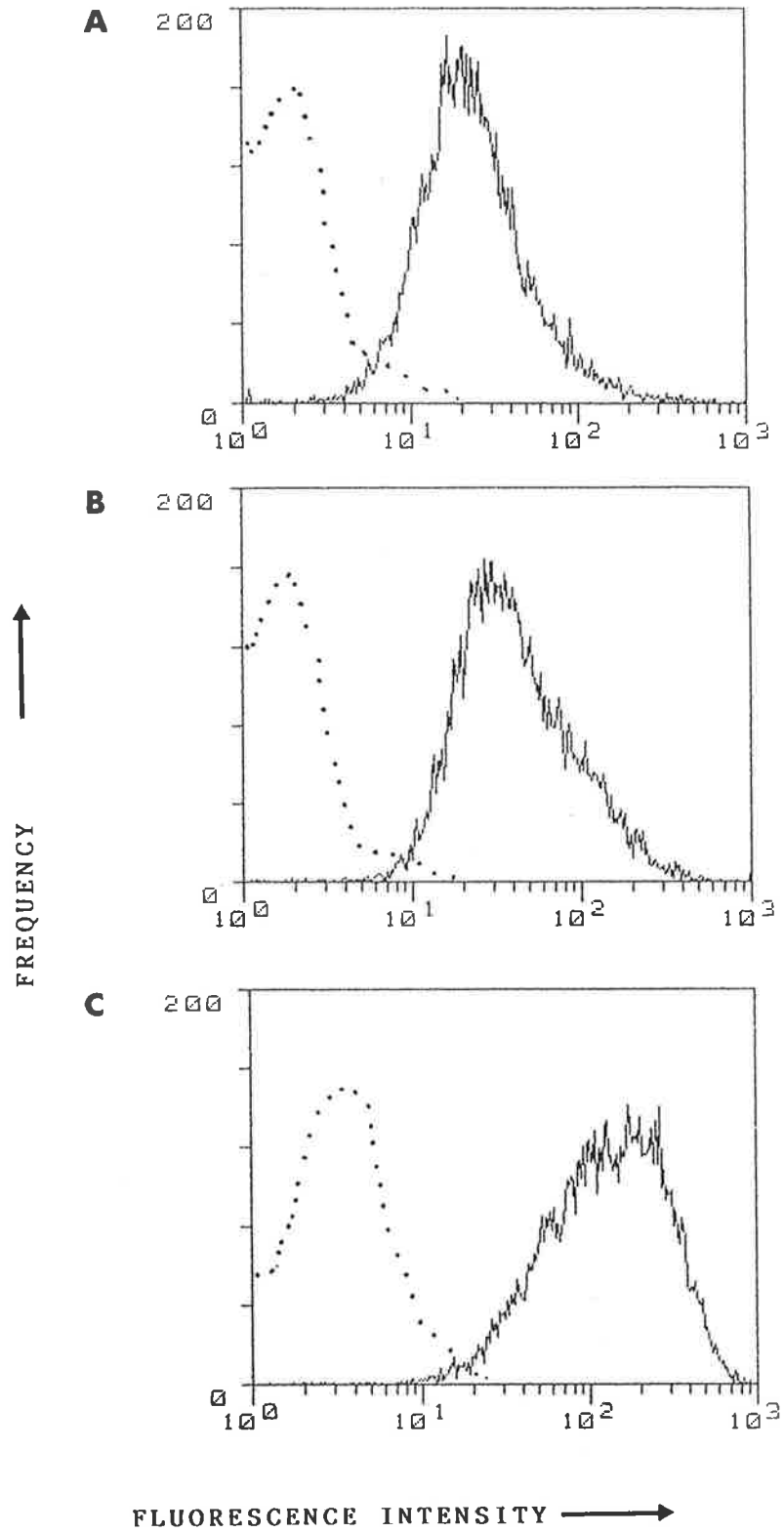


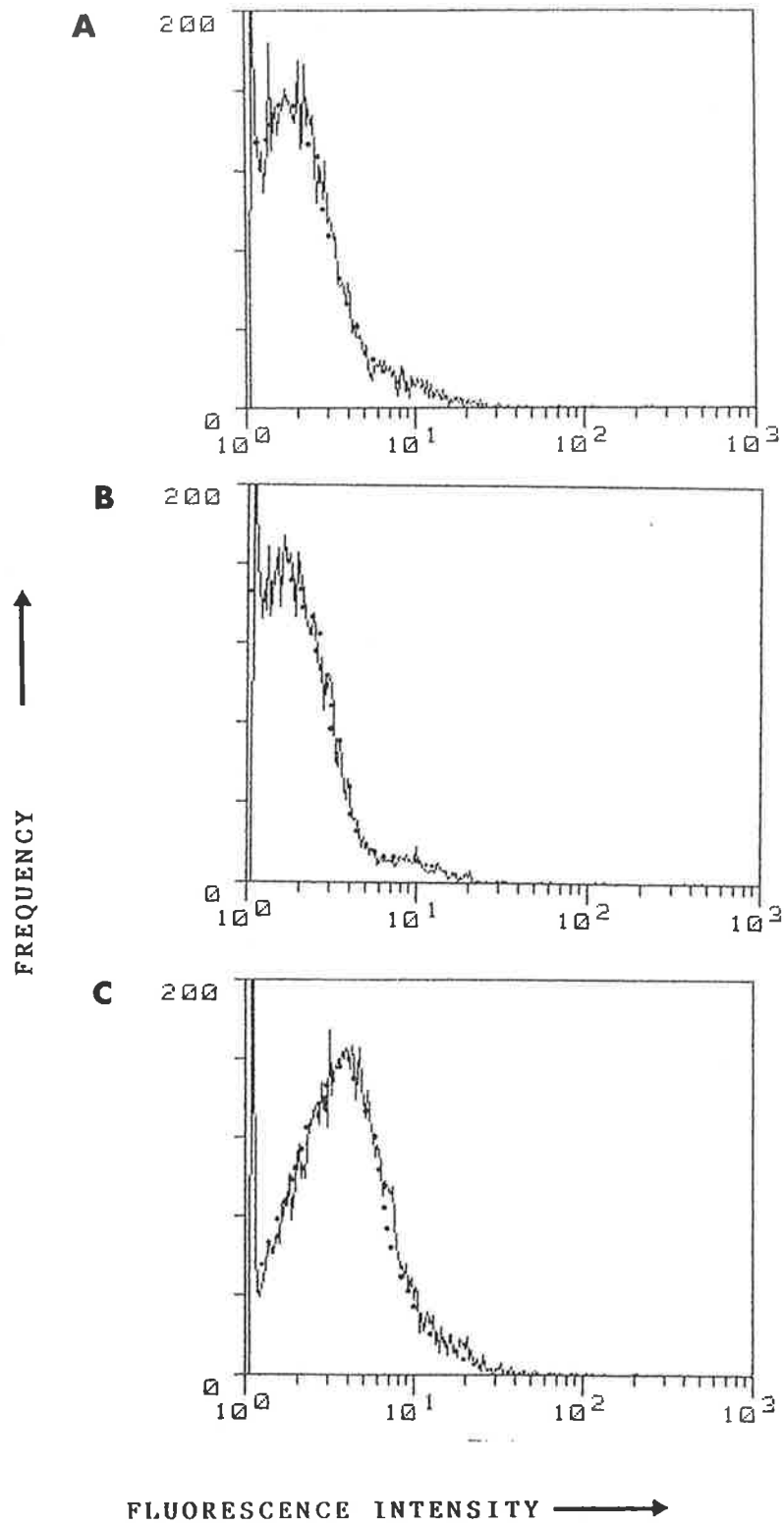
Figure 6.10 H

Histograms showing binding of MoAb anti-Mo2 (CD14), by indirect immunofluorescence with scoring by flow cytometry, to control untreated RC-2A cells and RC-2A cells treated with the inducers of differentiation TNF- α and γ -IFN for 7 days in culture. Negative control antibody (Sal-3) is shown as a dotted line.

A. RC-2A Untreated Control.	Sal-3	2.5
	anti-Mo2	2.5
B. RC-2A Day 7 TNF- α	Sal-3	2.3
	anti-Mo2	2.3
C. RC-2A Day 7 γ -IFN	Sal-3	4.7
	anti-Mo2	4.7

Figure 6.10 H

Mo2 - BINDING TO TNF AND
GAMMA IFN TREATED RC-2A CELLS



CHAPTER 7

General discussion.

General Discussion.

The aim of this project was to investigate the surface antigenic makeup of human myeloid cells, and to investigate the expression of surface molecules in relation to the differentiation stage of myeloid cells. This was to be achieved in the following ways:

1. By investigation of a model system of differentiation using a human myeloid cell line able to be induced to differentiate in response to biologically derived factors.

2. By development of a more sensitive hybridoma screening assay for the detection of monoclonal antibodies binding to cell surface structures, which would maximise the probability of isolating antibodies to low copy-number antigens. In addition, this would increase the feasibility of detecting antibodies with functional effects on myeloid cells, such as those recognizing growth or differentiation factor receptors.

3. By raising murine anti-myeloid monoclonal antibodies to surface structures on the cells of the inducible cell line, and investigation of the expression of the antigens detected by such antibodies during differentiation of this cell line. In addition, antibodies would be characterized as fully as possible, in terms of binding to haemopoietic cells and tissues, and the molecular characterization of the antigens to which they bound.

The cell line RC-2A was derived from the peripheral blood of a patient with acute myelomonocytic leukaemia [Bradley *et al*, 1982]. The predominant cells in the population are of immature monocytic appearance. Investigations showed that the cells of this line would differentiate towards macrophages in response to biologically derived molecules found in PHA-LCM. Changes induced in these cells were increased α -Naphthol-Butyrate esterase and acid phosphatase expression, adherence to plastic and morphological changes consistent with macrophage differentiation. PHA-LCM treated RC-2A cells showed a progressive decline in growth rate, and a loss of clonogenic potential as assayed by ability to form colonies in semi-solid agar.

Changes in the expression of cell surface antigens detected by the monoclonal antibodies FMC-14 (α -Ia), anti-Mo1 (CD 11b) and anti-Mo2 (CD 14) revealed a pattern consistent with macrophage differentiation, with Mo1 increasing markedly and Mo2 increasing to a lesser extent. The expression of Ia antigen underwent a decrease, perhaps a surprising result, given that macrophage differentiation is usually thought to be associated with increasing Ia expression. However, investigation of the murine system by Beller and Unanue, 1981 suggests that Ia expression is a transient event in monocytic cell development. RC-2A cells induced to differentiate with PHA-LCM show an increased ability to stimulate in a one way mixed leukocyte culture, which may correlate with Mo2 expression, as Morimoto *et al*, 1981, amongst others, have demonstrated antigenic presenting ability to be a property of a Mo2⁺ subset of macrophages.

Investigations into the identity of the factors responsible for the differentiation of RC-2A cells using four purified/cloned human growth factors (G-CSF, GM-CSF, γ -IFN and TNF- α) revealed that none of

these used alone would result in the full acquisition of differentiated characteristics by RC-2A cells. Of the four factors, only γ -IFN was able to induce the expression of α -Naphthol-Butyrate esterase. γ -IFN also markedly reduced the clonogenic potential of RC-2A cells, as did G-CSF to a lesser extent. Ability to alter antigen expression was investigated in the cases of γ -IFN and TNF- α , but not with the other two due to the scarcity of the material. These two factors were able to cause an increase in expression of Mo1, a decrease in Ia, but no induction of Mo2 was observed. Again, the expression of Ia has been reported to be enhanced by γ -IFN (see section 3.4), however treatment of HL-60 cells with this molecule initially induces the expression of Ia, but as the culture time in the presence of γ -IFN is prolonged, density of Ia decreases [Koeffler *et al*, 1984]. Both γ -IFN and TNF- α caused marked alterations in expression of antigens detected by the antibodies described in chapters 5 and 6, but neither pattern of expression matched that which resulted after culture in the presence of PHA-LCM. Many investigators have found that differentiation of myeloid cell lines is a multifactorial process, and single factors do not induce the full differentiated phenotype (see section 1.2).

As a preliminary step to assess the feasibility of raising monoclonal antibodies to growth or differentiation factor receptors on myeloid cells, a polyclonal antiserum directed against RC-2A cells was raised in mice. This antiserum was adsorbed against the autologous B cell line Cess B, in order to remove antibodies which recognized common species specific antigens. In effect, this procedure mimicked the planned protocol for screening hybridomas against myeloid antigens viz. binding to RC-2A, non-binding to Cess B, resulting in an

antiserum with the sum of specificities expected of monoclonal antibodies screened in that way. It was found that this antiserum was able to enhance the proliferation of RC-2A cells as demonstrated by a three fold increase in the uptake of tritiated thymidine by cells incubated with an appropriate dilution of the antiserum. At the same dilution, this antiserum was able to support the growth of a limited number of predominantly macrophage colonies from bone marrow progenitor cells, suggesting that the polyclonal antiserum contains a proportion of antibodies which mimic growth factors.

To further increase chances of detecting monoclonal antibodies detecting weakly expressed antigens, such as receptors, an improved screening assay was developed which was based on the colorimetric method of O'Neil and Parish, 1984. The Rose Bengal Assay (RBA) was modified to greatly increase its sensitivity, and then compared with the commonly used hybridoma screening methods indirect immunofluorescence (IF) and whole cell radioimmunoassay (RIA). In this regard, the assay was at least as sensitive as IF with flow cytometric scoring, however, during the course of subsequent screening it became apparent that MoAbs which would have been overlooked by routine IF or RIA screening were detected by RBA. For example, when screening of a fusion was carried out simultaneously using RBA and RIA, the RBA was able to identify 21 positively binding supernatants, whereas the RIA only identified 11 (see chapter 4). Similarly, the antibodies 4B4.E11 and 4C5.D12 described in chapters 5 and 6 gave strong signals in the RBA, but very weak fluorescence in IF. The RBA uses microtitre trays and is adaptable to semi-automation, therefore taking far less time than IF or RIA. It is also able to detect binding of antibodies to minor cell populations of the order of 5 - 10% of total. Another

advantage of the RBA was that no procedures were required that could potentially damage either cells or antigenic determinants, such as repeated cell centrifugation or cell fixation. These investigations showed that the RBA was a suitable assay for the detection of low copy number antigens.

The cell line RC-2A was used to raise monoclonal antibodies to myeloid antigens. This line was chosen because it could be induced to differentiate in response to biologically derived factors, and therefore was a good candidate for attempting to raise monoclonal antibodies which bound to the receptor(s) involved in this process, as well as to other myeloid antigens. The inducibility of the line allowed investigation of the changes in expression of myeloid antigens detected by such monoclonal antibodies, and the availability of the autologous B cell line Cess B facilitated the exclusion of antibodies recognising common species specific antigens. Hybridoma supernatants were screened simultaneously for their ability to alter the proliferation of RC-2A cells by a tritiated thymidine uptake assay. It was found that this assay was unsuitable for the screening of hybridoma supernatants, as the density of culture of the hybrids appeared to influence the uptake of thymidine.

Screening by RBA resulted in a number of hybrids producing myeloid specific antibodies being identified. Of these, five were chosen for further investigation, which included molecular weight estimation of the antigens they bound, and the expression of these antigens by human haemopoietic cell lines, normal and leukaemic haemopoietic cells and tissues. Three of the five antibodies appeared to be the same as antibodies in the international workshop clusters CD11b (5A4.C5), CD14 (4B5.F5) and CD31 (5A2.G5). The other two

(4B4.E11 and 4C5.D12) appear to have binding characteristics not previously reported. Interestingly, MoAb/complement mediated cell lysis experiments revealed the presence of the structures recognised by these two antibodies on myeloid progenitor cells. To date, 4C5.D12 has only been detected on the immunizing RC-2A cells and myeloid progenitor cells.

During the course of the investigations reported in this thesis, most of the aims of the project were met. The attempts to produce antibodies which exert functional effects on myeloid cells, while not being successful, indicate the feasibility of such an approach. With the development of a more suitable assay, coupled with the screening of a large number of hybrids, I feel that this line of investigation is worth pursuing further. Some approaches to the problem of developing a suitable assay may involve measurement of perturbations of the protein kinase C system, or changes in calcium fluxes across the cell membrane.

The antibodies produced appear to be useful in the localization of macrophage related cells in tissues. Their markedly different patterns of binding in the tissues studied so far suggest they define functionally distinct populations of cells of the mononuclear phagocyte lineage. The antibodies may find use in the investigation of altered cell distribution in pathological conditions. For example, it is intended to study the expression of the markers in sections of grafted kidney undergoing rejection. It is interesting to speculate on the binding of 5A2.G5 (which binds to activated T cells) in such cases. Their usefulness in the immunophenotypic analysis of leukaemia is still being assessed, however, in common with CD14-like antibodies, 4B5.F5 appears to identify AML with monocytic involvement, as does the

CD31-like antibody 5A2.G5. Further screening will determine if this antibody is of use in subtyping certain lymphoid leukaemias, based on its cell line binding data, and its binding to activated T cells and a subpopulation of lymphocytes.

Further screening will need to be carried out to determine if the antibodies 4B4.E11 and 4C5.D12 bind to any AML samples. The investigation of binding of these antibodies to progenitors earlier than GM-CFU's would also be useful.

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