IDENTIFICATION OF MURINE HAEMOPOIETIC STEM CELLS WITH MONOCLONAL ANTIBODIES

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IDENTIFICATION OF MURINE HAEMOPOIETIC STEM CELLS WITH MONOCLONAL ANTIBODIES

Identificatie van hemopoëtische stamcellen van de muis met monoklonale antilichamen

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Aan mijn ouders

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ABBREVIATIONS

Av	avidin
Av/FITC	avidin conjugated(/) to fluorescein isothiocyanate
Av/PE	avidin conjugated to phycoerythrin
Av/TxR	avidin conjugated to Texas Red
BFU-E	burst forming unit-erythrocyte
blagra	blast cells and granulocytes
BP-filter	bandpass-filter
C'	(guinea pig) complement
CFC-C	colony forming cell-culture
CFC-S	colony forming cell-spleen
CFU-Baso	colony forming unit - basophilic granulocyte
CFU-C	colony forming unit-culture; general indication for committed progenitor cells
CFU-C2	PMUE responsive monocyte/macrophage progenitor
CFU-E	colony forming unit-erythrocyte
CFU-EO	colony forming unit-eosinophilic granulocyte
CFU-G	colony forming unit-granulocyte (neutrophils)
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	colony forming unit-granulocyte, monocyte/macrophage
CFU-M	colony forming unit-monocyte/macrophage
CFU-Meg	colony forming unit-megakaryocyte/platelet
CFU-S	colony forming unit-spleen
CSF/CSF-1	colony stimulating factor/colony stimulating factor-1
DAG	differentiation antigen
ELISA	enzyme linked immunosorbent assay
Epo	erythropoietin
FACS	fluorescence activated cell sorter
FLS	forward light scatter
FU	5-fluorouracil
GARA/FITC	goat-anti-rat immunoglobulin antibody conjugated to fluorescein isothiocyanate
GLISA	gold linked immunosorbent assay
GM-CSF	granulocyte macrophage-colony stimulating factor
H-1	hemopoietin-1.
H-2	murine major histocompatibility complex
HAT-medium	hypoxanthine, aminopterine, thymidine containing medium
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HH	Hanks' balanced salt solution bufferend with HEPES
HPP-CFC	high proliferative potential-colony forming cell
HSA	HH supplemented with serum and sodium azide
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.s.	intrasplenic
i.v.	intravenously

LCA	leukocyte common antigen
LP-filter	long pass filter
LPP-CFC	low proliferative potential - colony forming cell
MA	metrizamide
MCA	monoclonal antibody
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MHSC	multipotent hemopoietic stem cell
MRA	marrow repopulating ability
MSCM	mouse spleen cell conditioned medium
NBM	normal bone marrow
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
PEG	polyethyleneglycol
PHSC	pluripotent haemopoietic stem cell
PLS	perpendicular light scatter
PMUE	pregnant mouse uterus extract
PYR	1-pyrenebutyryl
RELACS	Rijswijk Experimental Light Activated Cell Sorter
Rh123	rhodamine 123
RPA	radioprotective ability
SF	synergistic factor
TFR	transferrin receptor
TOF	time of flight
WGA	wheat germ agglutinin

CHAPTER 1

INTRODUCTION

HAEMOPOIESIS

Haemopoiesis is the process by which very large numbers of a wide variety of mature blood cells are generated. Haemopoietic cells can be found in many tissues of the body. In the adult animal, the bone marrow, one of the largest organs in the body, is the primary site of haemopoiesis (Metcalf and Moore, 1971). The bone marrow is a complex tissue composed of cells belonging to multiple haemopoietic differentiation lineages of various stages of differentiation, although most bone marrow cells are mature cells. In the bone marrow cavities all morphologically recognizable blood cells are produced, i.e., erythrocytes, megakaryocytes/platelets, monocytes/macrophages, neutrophilic-, eosinophilic- and basophilic-granulocytes, B-lymphocytes and T-lymphocytes. Once released into the circulation the mature cells exhibit highly specialized functions and fulfil the vital roles of oxygen and carbon dioxide transport (erythrocytes), blood clotting (platelets), antibody production (B-lymphocytes), cell mediated immunity (T-lymphocytes), and response to invading organisms and their by-products (granulocytes and macrophages).

Most of the mature blood cell types in the circulating blood have only a short life span, varying from several hours (monocytes, granulocytes; Springer et al., 1979; Whetton and Dexter, 1986) to several weeks (erythrocytes; Metcalf, 1984). This "programmed" cell death necessitates the constant production of very large numbers of new blood cells throughout life, even in unstressed (steady state) situations. The continuous replacement of terminally differentiated cells is regulated with great precision and the levels of circulating cells are maintained within narrow limits of variation (Metcalf, 1985b). Yet, under conditions of haemopoietic stress, like bleeding or infection, the haemopoietic system is capable to respond rapidly to the altered demands by transiently altering the output of cells of the appropriate type(s) (Metcalf, 1985b; Whetton and Dexter, 1986; Clark and Kamen, 1987). Derangements of the complex process of blood cell formation do occur and result in a range of diseases from anaemia to leukaemia (Metcalf, 1985b; Quesenberry and Levitt, 1979).

Clinical and experimental data suggest that all the mature haemopoietic cell types are derived from the same ancestral cell, i.e., the pluripotent haemopoietic stem cell (PHSC) (McCulloch, 1983; Metcalf, 1984). PHSC are believed to mediate long-term haemopoietic reconstitution after bone marrow transplantation (Van Bekkum et al., 1981) and are characterized by extensive proliferative capacity, the ability to generate progeny committed to one or more of any of the myeloid and lymphoid differentiation lineages and the ability for self-renewal. The ability for self-renewal does not decline during the natural life span (Schofield, 1978) and makes the PHSC essentially immortal. However, it is still not known if the self-renewal of PHSC under

normal physiological conditions leads to the production of daughter cells that are exactly identical to the parental cell.

The existence of PHSC is recognized for many years and was first predicted from studies assessing the reconstitutive capacity of transplanted bone marrow cells following total body irradiation of mice (Jacobson et al., 1949; Lorenz et al., 1951; Vos et al., 1956; Ford et al., 1956; Nowell et al., 1956). Convincing evidence for the existence of cells which can give descendants in all haemopoietic differentiation lineages was obtained using radiation induced chromosomal translocations as lineages markers (Wu et al., 1968; Abramson et al., 1977) and, more recently, retroviral integration sites (Dick et al., 1985; Lemischka et al., 1986; Keller et al., 1985; Williams et al., 1984).

Based on the results obtained with both in vivo and in vitro clonal assays which will be discussed in the next sections, many investigators divide the haemopoietic system into three compartments of increasing maturity and size (Watt et al., in press; Till, 1976; Ogawa et al., 1983; Till and McCulloch, 1980). These three compartments comprise: 1) the PHSC, which are not usually undergoing mitotic divisions during normal haemopoiesis but have an extensive capacity to generate; 2) committed progenitor cells, which form a transient population and are predominantly dividing and proliferating, but have lost the capacity of self-renewal. They are "programmed" to differentiate in only one or two lineages. These committed progenitor cells generate and are thought to be primarily responsible for maintaining the level of 3) mature, morphologically recognizable haemopoietic cells. This subdivision represents an abstraction, since development is likely to be a process of continuous change rather than a series of discrete steps, except perhaps at the level of the genome. Thus, each cellular subclass is likely to be heterogeneous in composition (Till, 1976; Botnick et al., 1979). The three compartments are depicted conventionally as "lineage-diagrams", an example of such a diagram is shown in Fig. 1.1. It is assumed that the transition between compartments are unidirectoral, always leading towards increasing specialization and an associated loss of proliferative potential.

Regulation of haemopoiesis appears to depend on interactions among haemopoietic cells, stromal elements and a variety of growth factors (McCulloch, 1983; Dexter, 1982; Metcalf, 1984). Despite of the fact that a number of models has been proposed concerning the regulation of self-renewal and commitment of PHSC (reviewed by Metcalf, 1984) it is still not known whether the above mentioned interactions direct commitment to particular lineages or whether the commitment process occurs at random or is programmed and microenvironmental factors merely influence the probability of self-renewal or differentiation commitment. However, it is obvious that a balance must be maintained between the three cellular compartments. If too many cells were to differentiate the haemopoietic system would soon undergo exhaustion; if too few were to differentiate there would not be sufficient input into the progenitor cell compartment to meet the demands (Chervenick and Boggs, 1971).

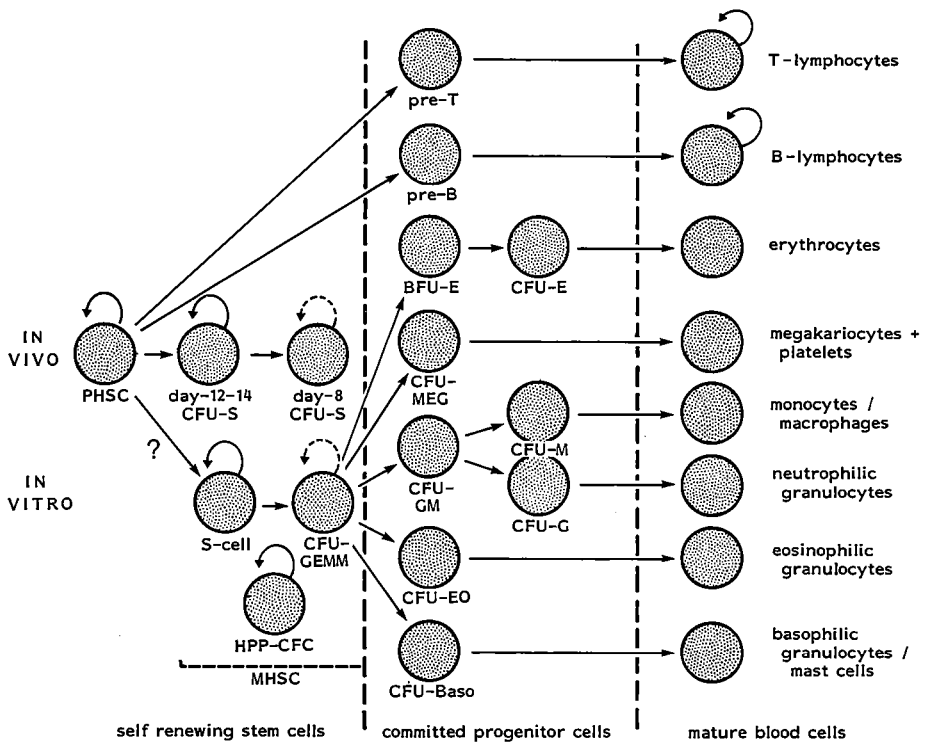


Figure 1.1:
A schematic representation of haemopoietic stem cell differentiation. The capacity of pluripotent and multipotent stem cells for self-renewal is indicated by dashed lines (low capacity) and solid lines (high capacity). The morphologically recognizable but immature cells in each differentiation lineage are not shown. For details, see text.

IN VIVO ASSAYABLE HAEMOPOIETIC STEM CELLS

Much of the knowledge and understanding of the complex process of haemopoiesis has been, and still is, derived from the development and application of a series of both in vivo and in vitro clonal assays for haemopoietic cells (reviewed by Metcalf, 1984). In 1961, Till and McCulloch described the first clonal assay, i.e., the spleen colony forming assay. These authors discovered that when haemopoietic cells are transplanted into lethally irra-

diated mice, nine days post transplantation discrete macroscopic colonies could be detected on the spleens of the recipients. The number of spleen colonies appeared to be linearly related to the number of transplanted cells (Till and McCulloch, 1961). Later it was shown by injection of bone marrow cells with unique radiation induced chromosome markers, that cells present in an individual spleen colony carried the same unique marker (Becker et al., 1963, Wu et al., 1967). This suggested that spleen colonies were derived from a single cell, thus were of clonal origin.

Analysis of the cellular content of spleen colonies has shown that erythroid-, granulocytic-, megakaryocytic-, and monocytic cells were consistently present, either as pure populations or in varying mixtures (Fowler et al., 1967; Lewis and Trobaugh, 1964; Curry and Trentin, 1967). This multiplicity of cell lineages present in spleen colonies indicated that spleen colonies were derived from a pluripotent cell operationally called colony forming cell-spleen (CFC-S). In contrast with cells of the different myeloid lineages, cells of the T-lymphocytic lineage have never been demonstrated in spleen colonies (Metcalf, 1984), whereas the presence of B-lymphoid cells is still a controversial issue (Paige et al., 1979; Lala and Johnson, 1978).

It was shown in serial retransplantation experiments of individual spleen colonies that a proportion of these spleen colonies contained cells that could form new spleen colonies (Siminovitch et al., 1963; Lewis and Trobaugh, 1964). These observations led to the conclusion that some of the original CFC-S had the capacity to renew themselves. In addition, it has been demonstrated that cells from a single spleen colony could reconstitute the complete haemopoietic system (both myeloid and lymphoid) of lethally irradiated animals (Trentin and Fahlberg, 1963; Till and McCulloch, 1980; Wu and Liu, 1984). In many respects, therefore, the CFC-S possess the characteristics of pluripotent haemopoietic stem cells (PHSC).

It was recognized that following bone marrow transplantation only a fraction (3-25%) of the total number of CFC-S reaches the spleen and forms a colony, the remainder entering other tissues such as the bone marrow, liver and lungs (Quesenberry and Levitt, 1979; Lahiri and Van Putten, 1969; 1972; Lahiri et al., 1970; Till and McCulloch, 1972; Schooley, 1966; Siminovitch et al., 1963). The fraction that settles in the spleen and forms a colony is called the spleen seeding efficiency (f-factor) and can be determined by secondary transplantation of the spleen shortly (2-48 hours) after marrow injection. Since only a fraction of the CFC-S reaches the spleen and gives rise to a colony, the number of spleen colonies is called the number of colony forming unit-spleen (CFU-S) and the colony forming assay is called the CFU-S assay. The development of the CFU-S assay made it for the first time possible to quantitate the frequency of PHSC in various haemopoietic populations and has been proven invaluable for characterizing the PHSC in terms of their biological and physical properties and cell surface characteristics (see later section). However, the pluripotency of CFU-S and hence the usefulness of the CFU-S assay as the endpoint for PHSC has been, and still is, a matter of debate. For instance, it has been observed after injection of bone marrow cells with an unique chromosomal marker (Wu et al., 1968; Lemischka et al., 1986) that cells repopulating the thymus, lymph nodes and spleen of lethally irradiated recipients carried the same unique marker as cells present in spleen colonies. Together with the absence of at least one

class of lymphoid cells in spleen colonies, this led some investigators to conclude that CFU-S are not the ultimate PHSC (Metcalf and Moore, 1971; Schofield, 1978; Ogawa et al., 1983; Curry and Trentin, 1967; Trentin et al., 1967; Curry et al., 1967). In this view, CFU-S and lymphoid cells are both derived from a more primitive stem cell, the true PHSC (Fig. 1.1). The CFU-S are regarded as multipotent haemopoietic stem cells (MHSC) with the capacity to generate cells of all myeloid lineages and the capacity for self-renewal. It should be realized, however, that the absence of lymphoid cells in spleen cell colonies could also be the result of the lack of the appropriate microenvironment for lymphoid development (Wolf and Trentin, 1968; Metcalf, 1984).

More data suggesting that CFU-S were not the ultimate PHSC have been obtained. It has been reported that a loss of the repopulating ability of a graft could occur with (initially) little change in the number of CFU-S (Ogden and Micklem, 1976; Ross et al., 1982). In addition, it was shown by Boggs et al. (1982) that to cure genetically anaemic W/W^V mice, less bone marrow cells were needed than was expected on the basis of the number of CFU-S in the inoculum. Analysis of bone marrow regeneration following treatment of mice with 5-fluorouracil suggested the existence of a more primitive stem cell, i.e., the pre-CFU-S and that the CFU-S assay does not adequately predict haemopoietic reconstitution (Hodgson and Bradley, 1979; 1984; Hodgson et al., 1982; Van Zant, 1984).

A part of the observations described above suggesting that CFU-S are not the ultimate PHSC can be explained by the finding that, in contrast with what was initially assumed, the CFU-S form a heterogeneous population in which a hierarchy consists of cells with differing self-renewal capacities and with differing abilities to allow prolonged survival of irradiated mice (Rosendaal et al., 1976; 1979; Hodgson and Bradley, 1979; Hellman et al., 1978). The quality of the CFU-S appears to depend on the day the CFU-S are examined (Magli et al., 1982; Wolf and Priestley, 1986). It is now generally accepted that spleen colonies visible 7 to 8 days post transplantation (day-7-8 CFU-S) are derived from different cells than the spleen colonies visible after 12 to 14 days (day-12-14 CFU-S) (Magli et al., 1982). This is consistent with the early observations of Siminovitch et al. (1963) who showed that day-8-10 CFU-S did not contain new CFU-S upon retransplantation, whereas most day-14 CFU-S did. In other words, day-8 CFC-S have no self-renewal ability, while day-12-14 CFC-S do have the ability to renew themselves and therefore must represent more primitive stem cells. Besides the difference in self-renewal capacity both types of CFC-S appear to differ in a number of physical properties (Worton et al., 1969b; Haskill et al., 1970; Metcalf et al., 1971), differentiating potentials (Mulder et al., 1985; Mulder, 1986), sensitivity to a number of cytostatic drugs (Schofield and Lajtha, 1973; Botnick et al., 1979; Hodgson and Bradley, 1979; 1984; Johnson and Nicola, 1984; Ross et al., 1982), cell cycle status (Baines and Visser, 1983) and cell surface properties (Harris et al., 1984a; Berman and Basch, 1985; Mulder et al., 1985; Mulder, 1986). Based on these differences it was suggested that day-8 CFC-S are the progeny of day-12 CFC-S.

At present, the relation between CFU-S and PHSC is still not clear although it seems that at least a proportion of the CFU-S (day-12-14 CFU-S) is closely related to, and might even be identical with the PHSC.

IN VITRO ASSAYABLE HAEMOPOIETIC STEM CELLS

Following the description of the CFU-S assay, a series of semi-solid culture techniques in which the progeny of the individual stem cells can be recognized as clones or colonies and thus can be enumerated and analyzed has been developed (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; reviewed by Metcalf, 1984). These in vitro clonal assays revealed the existence of different types of haemopoietic stem cells each with unique properties.

The first haemopoietic stem cells that became assayable in vitro were the committed progenitor or the precursor cells. Assays for this type of stem cells depend on the identification of colonies consisting of mature cells formed from a single progenitor cell and the time in culture at which these appear. The progenitor cells that give rise to a colony in methylcellulose or soft-agar are referred to as colony forming units - culture (CFU-C). CFU-C are restricted in their differential capacity to only one or two lineages (in case of granulocytes and macrophages; see Fig. 1.1) and produce colonies consisting only of one (or two) mature cell type(s). From this it is generally concluded that CFU-C have no or very limited self-renewal capacity (Sutherland et al., 1970; Metcalf and Moore, 1971; Metcalf et al., 1979; Suda et al., 1983; Whetton and Dexter, 1986) and act merely as an amplification compartment for the production of the mature cells. Even under steady state conditions most CFU-C, in contrast with CFU-S, are actively dividing and proliferating (Lajtha et al., 1969; Rickard et al., 1970). Thus, committed progenitor cells are capable of extensive clonal expansion to supply the need of the haemopoietic system, but they need to be replenished from the self-renewing stem cell pool.

CFU-C most likely represent the precursors for the immature blood cell types and form the earliest committed progenitor cells in each lineage (Metcalf, 1984). Lineage restricted progenitors are identified by the type of progeny they produce in the presence of specific growth stimulating preparations. The eight groups of mature haemopoietic cells (Fig. 1.1) are thought to originate from lineage restricted progenitors and CFU-C of all myeloid lineages have now been identified. Progenitors for T- and B-lymphocytes cannot be identified in these clonal assays (Watt et al., in press). The generation of T- and B-lymphocyte colonies has been reported (Metcalf et al., 1975; Rosenszajn et al., 1975), but the cells that produced these colonies showed functional and morphological properties of relatively mature cells.

The survival, proliferation and differentiation of the committed progenitor cells appears to be absolutely dependent on the presence of a group of well defined lineage specific growth factors (Dexter and Spooncer, 1987; Whetton and Dexter, 1986; Metcalf, 1985b; Nicola, 1987a). Haemopoietic growth factors or colony stimulating factors (CSFs) are soluble glycoproteins which exert their stimulating activity at very low concentrations (10^{-11}-10^{-13} M) (Metcalf, 1985c; 1986b). Some of the CFSs will be discussed in more detail in Chapter 6.

CFU-C, like CFU-S, are a heterogeneous population which is reflected by the amount of progeny they produce (50-10,000 cells/colony) and the concentration and the type of growth factor(s) needed to stimulate colony

formation (Bol et al., 1979; Bol and Williams, 1980; Gregory, 1976; Wagemaker, 1980).

In the late 1970s an in vitro assay was described that allowed the detection of colonies in which commitment to one (or two) differentiation lineage(s) had not yet occurred (Johnson and Metcalf, 1977; Hara and Ogawa, 1978). These colonies appeared to contain cells of the granulocytic (neurophils, eosinophils and basophils/mast cells), erythroid, megakaryocytic and macrophage lineages (Metcalf et al., 1979; Johnson et al., 1980; Schrader et al., 1981). Hence, the stem cell that generated these colonies was called CFU-GEMM (Fauser and Messner, 1979). Some controversial data indicate that the human CFU-GEMM may also have the potential to develop into T-lymphocytes (Messner et al., 1981). CFU-GEMM derived colonies have been obtained from a variety of mammalian species including mouse, rat, cat, dog, sheep, cow and man (Dexter and Spooncer, 1987). Colony formation by CFU-GEMM is stimulated by (a source of) interleukin-3 (IL-3).

Further analysis of the cells present in CFU-GEMM derived colonies showed that besides a variety of mature cells and committed progenitor cells these colonies may also contain CFU-GEMM, indicating that CFU-GEMM have self-renewal ability (Humphries et al., 1981; Metcalf et al., 1979; Johnson, 1980). Together with the variety of cell types present in CFU-GEMM derived colonies this indicated that the CFU-GEMM are multipotent haemopoietic stem cells (MHSC). The self renewing capacity of CFU-GEMM appeared to be relatively low and not all CFU-GEMM derived colonies gave rise to new colonies upon replating (Humphries et al., 1981; Metcalf et al., 1979). It has also been shown, by transplanting CFU-GEMM derived colonies into lethally irradiated mice, that some CFU-GEMM colonies contain a low frequency of CFU-S (Humphries et al., 1979; Johnson, 1980; Metcalf et al., 1979; Johnson and Nicola, 1984).

The exact relationship between CFU-S and CFU-GEMM is not (yet) clear but these cells clearly represent overlapping populations (Ogawa et al., 1983; Nakahata and Ogawa, 1982a; Dexter et al., 1984a). It has even been suggested, on the basis of similarities in frequency, sedimentation velocity and proliferative state, that CFU-GEMM and day-8 CFU-S might be the same (Hara and Ogawa, 1978; Johnson, 1980; Till and McCulloch, 1980; Metcalf, 1984).

In 1982 another in vitro detectable MHSC type was discovered (Nakahata and Ogawa, 1982b; Keller and Phillips, 1982). This MHSC gives rise to colonies in methylcellulose cultures stimulated with (a source of) IL-3. Colonies appear after 6-18 days (Suda et al., 1983). The formed colonies are small and the cells within such colonies show no sign of terminal differentiation but all have the morphology of blast cells. Hence, the colonies are called blast cell colonies. The cell that gives rise to these blast cell colonies is called S-cell or CFU-"stem" (Nakahata and Ogawab, 1982; Ogawa et al., 1983). S-cells are not actively cycling and are thus relatively resistant to treatment with 5-fluorouracil (Suda et al., 1983; Nakahata and Ogawa, 1982b).

Replating of these blast cell colonies resulted in a high frequency of secondary blast cell colonies, indicating a high self-renewal capacity of S-cells (Nakahata et al., 1982). In addition, analysis of individual blast cell colonies showed that these colonies also contain high numbers of CFU-GEMM

and day-8-9 CFU-S (Nakahata and Ogawa, 1982b; Keller and Phillips, 1982) and a variety of more restricted progenitor cell types (Suda et al., 1983; 1985). No evidence is yet available regarding the potential of lymphoid differentiation by S-cells (Williams et al., 1987b). Together with the observation that replating of CFU-GEMM derived colonies resulted in only a few secondary CFU-GEMM derived colonies but not in the formation of blast cell colonies (Ogawa et al., 1983) this indicated that S-cells must be more primitive multipotent stem cells than CFU-GEMM. It has been suggested that S-cells might be the precursors of the more numerous CFU-GEMM (Nakahata et al., 1982; Nakahata and Ogawa, 1982b; Ogawa et al., 1983; Keller and Phillips, 1982; Metcalf, 1984). Since CFU-GEMM and day-8 CFU-S appeared to be very similar cell types (see above) this indicates that S-cells must also be more primitive than day-8 CFU-S (Nakahata and Ogawa, 1982b; Ogawa et al., 1983). It has also been shown by Keller et al. (1984) that the spleen colony forming cells present in blast cell colonies could not give rise to day-12 CFU-S. This makes the relation between S-cells and day-12 CFU-S unclear, although it is evident that both cells must be closely related in the hierarchy of haemopoietic stem cells.

Another type of primitive haemopoietic stem cells has been described in studies on the regenerative capacity of bone marrow cells from mice treated with 5-fluorouracil and has been identified by its requirements for two or more growth stimuli in order to generate colonies in agar cultures (Bradley and Hodgson, 1979; Bradley et al., 1980; Bertoncello et al., 1985; 1986; 1987; 1988; McNiece et al., 1986; 1987). One of these stimuli must be a source of macrophage-colony stimulating factor (M-CSF), whereas the other must be an enhancing- or synergistic-factor. Growth factors and growth enhancing factors will be discussed in more detail in Chapter 6. These stem cells produce very large colonies consisting of thousands of macrophages which demonstrates the extensive proliferative capacity of these stem cells. The stem cells giving rise to these huge colonies are therefore called high proliferative potential-colony forming cells (HPP-CFC). Initially, it was believed that HPP-CFC were primitive macrophage progenitors but a recent report by McNiece et al. (1987) suggests that HPP-CFC derived colonies can be replated and in addition to macrophages also may contain cells of the granulocytic- and megakaryocytic-lineage but no erythroid cells. Thus, HPP-CFC may represent another MHSC type. This was also suggested by earlier data. It appeared that HPP-CFC are not actively cycling (Baines and Visser, 1983; Hodgson and Bradley, 1979; 1984). In addition, it has been proposed that HPP-CFC are more primitive cells than day-8-10 CFU-S on basis of their relatively resistance to 5-fluorouracil (Bradley et al., 1980) and are closely related to day-13 CFU-S because in experiments to purify these cells both were enriched to the same degree (Baines et al., 1984). Furthermore, a strong correlation between the HPP-CFC content and the capacity of a bone marrow graft to repopulate the marrow of lethally irradiated mice has been found (Hodgson et al., 1982; Bertoncello et al., 1985). Cells with marrow repopulating ability (MRA) are regarded to be more primitive than day-12-13 CFU-S (Hodgson and Bradley, 1979; 1984; Hodgson et al., 1982; Bertoncello et al., 1985; Van Zant, 1984). Thus, the exact relationship between HPP-CFC and the other multipotent haemopoietic stem cells and the pluripotent haemopoietic stem cell is as yet unclear.

From the above described studies it is clear that there exist several different haemopoietic stem cells capable of self-renewal albeit to a different extent. The observation that of the different MHSC types not all cells have the same self-renewal capacity, reflects the heterogeneity and also the complexity of the haemopoietic stem cell compartment. The process of differentiation commitment is still poorly understood, but an attractive theory on basis of data derived from both in vivo and in vitro clonal assays holds that haemopoietic stem cell commitment is a random process (Till et al., 1964; Nakahata et al., 1982; Ogawa et al., 1983) and that stem cells are arranged such that more primitive cells have a higher self-renewal capacity than do more differentiated cells (Hellman et al., 1978; Rosendaal et al., 1979; Ogawa et al., 1983).

ENRICHMENT AND PURIFICATION OF HAEMOPOIETIC STEM CELLS

Traditionally, haemopoietic stem cells have been identified by morphological criteria (Van den Engh, 1976). The different mature haemopoietic cell types all have their characteristic morphology (Bessis, 1977). This approach has been of limited value when applied to haemopoietic stem cells. PHSC and the various types of MHSC and committed progenitor cells cannot be distinguished from each other on basis of morphological criteria (Van den Engh et al., 1981; Spivak et al., 1985; Williams et al., 1987b; Till and McCulloch, 1980). It has been postulated that both CFU-S and committed progenitors may resemble traditional lymphocytes (Yoffey, 1973), while others have suggested that they may be blast cells in appearance (Van Bekkum et al., 1971; 1979). The lack of a definitive description of the morphology of the various haemopoietic stem cells is mainly due to the very low frequency of these cells in the bone marrow (Fitchen et al., 1981; Metcalf and Moore, 1971; Boggs et al., 1982; Lord and Spooncer, 1986; Van Bekkum, 1977; Van Bekkum et al., 1979; Dicke et al., 1985), and the very heterogeneous cellular composition of this tissue (see Fig. 1.1). The frequency of haemopoietic stem cells is too low to make any direct measurements of their properties (Visser and Bol, 1981; Lord and Spooncer, 1986). In fact, the quantities and qualities of the haemopoietic stem cells can still be determined only retrospectively from their abilities to reconstitute the haemopoietic system of lethally irradiated recipients or from their abilities to form colonies in vivo and in vitro.

Colony forming assays have the disadvantage that they take at least seven days and that not the stem cells themselves but their (mature) progeny is analyzed. In addition, there are often a number of cellular stages between the measured progeny and the ancestral stem cells (McCulloch, 1983). Thus, the critical events during the initial stages of differentiation cannot be studied or manipulated using colony forming assays. Highly purified stem cell suspensions would allow direct analysis of the early molecular, biological and biochemical events taking place in haemopoiesis and further definition of the properties of the various stem cells. These suspensions will also be helpful in investigations and possible manipulations of the conditions which lead to self-renewal, commitment or differentiation of the various stem cell types and in exploring the relationship between PHSC, MHSC and CFU-C (Harris et al., 1984a; Bertoncello et al., 1985). In addition, studies on the

mode of action of various haemopoietic growth factors and the role of accessory cell populations can be studied with purified stem cells (Williams et al., 1987b; Strife et al., 1987; Civin et al., 1987; Hoang et al., 1983).

A major drawback in the development of procedures that would allow the resolution and purification of the various haemopoietic stem cells has been, and still is, the paucity of these cells in haemopoietic tissues and the lack of specific distinguishing probes (Hoang et al., 1983; Mouchiroud et al., 1985; Van den Engh et al., 1981). Past attempts to obtain various haemopoietic stem cell types in pure populations have relied on differences in physical characteristics between cells of different maturation stages (Shortman, 1972; Moore et al., 1972). Differences in sedimentation velocity at unit gravity (Worton et al., 1969a; Miller and Phillips, 1969; Miller, 1973), buoyant density (Turner et al., 1967; Shortman, 1968; Bol et al., 1977), adherence to glass beads (Metcalf et al., 1971) and cell electrophoresis (Bol, 1980; Bol et al., 1981) have been used, but none of these rather nonspecific techniques resulted in such a resolution and concentration sufficient for useful practical application (Van den Engh, 1981; Fitchen et al., 1981; Lord and Spooncer, 1986). Equilibrium density gradient centrifugation, however, is a frequently employed separation technique in combination with others. Another currently used technique for the separation of cells is counterflow centrifugal elutriation (Pretlow and Pretlow, 1979; Inoue et al., 1981). Using this technique the cells are separated on basis of cell size. To provide a more homogeneous starting population and to improve the separation, counterflow centrifugal elutriation has been mostly used to separate and purify stem cells from mice treated with cytostatic drugs (Nijhof and Wierenga, 1984; Williams et al., 1987) or endotoxin (Ploemacher et al., 1987; Ploemacher and Brons, 1988a; 1988b).

More specific and better separation and purification can be obtained with immunological procedures such as immune adherence (panning) (Hoang et al., 1983; Mouchiroud et al., 1985), immune- rosetting (Harris et al., 1983; 1984a; Koizumi et al., 1985; Miller et al., 1985b), complement mediated cytotoxicity (Boswell et al., 1984), affinity chromatography (Mattson et al., 1987) and, more recently, immunomagnetic depletion (Kanourakis and Bol, 1987). All these techniques allow for the rapid and bulk separation of cells and are based on the binding of a (monoclonal) antibody to the wanted (positive selection) or unwanted cells (negative selection). The purity of the resulting cell suspensions using these procedures is still relatively low.

A very powerful tool for the identification, isolation, and purification of haemopoietic stem cell subpopulations is flow cytometry and electronic cell sorting using a fluorescence activated cell sorter (FACS). Unlike the other cell separation procedures described above flow cytometry allows to make rapid quantitative multiparameter measurements of individual cells in large numbers (Shapiro, 1985; Van den Engh et al., 1980; Van den Engh and Visser, 1984; Parks et al., 1986). For instance, flow cytometry makes it possible to analyze the size, shape and structure (light scatter intensities) and viability of individual cells and to combine these measurements with those quantifying the cell surface-, cytoplasmic-, or nuclear-fluorescence intensities. This makes it possible to evaluate discrete populations of rare cells, like haemopoietic stem cells in such a heterogeneous tissue like bone marrow (Van den Engh et al., 1979; 1980; Visser et al., 1978; Van den

Engh and Visser, 1984; Herzenberg et al., 1976; Loken et al., 1987a, 1987b; Parks et al., 1986; Herzenberg and Herzenberg, 1978).

An important feature of cell sorting by flow cytometry is that the cells are inspected individually before they are sorted, which means that high purities of the wanted cells can be obtained. In addition, the sorting criteria can be based on any combination of measured parameters. Furthermore, it is possible to isolate defined populations of viable cells for functional analysis like colony formation and repopulation of lethally irradiated animals. Flow cytometry and cell sorting either alone or in combination with any of the bulk separation procedures described above have been successfully used to highly enrich and separate murine and rat haemopoietic stem cell subpopulations on the basis of their light scatter properties, lectin-binding, nucleic acid content, metabolic state, and most widely by their affinity for various (combinations of) (monoclonal) antibodies directed against cell surface structures (Harris et al., 1984a; 1985; Muller-Sieburg et al., 1986; Visser et al., 1984; Bauman et al., 1986; Bauman and Chen, 1987; Watt et al., 1983; in press; Palavicini et al., 1985a; 1985b; 1987; Lord and Spooncer, 1986; McCarthy et al., 1987; Castagnola et al., 1981; Goldschneider et al., 1980; Bertoncello et al., 1985; 1986; 1987; Visser and de Vries, in press; and many others). In Chapters 5 and 6 of this thesis the use of flow cytometry and cell sorting for purification and enrichment of murine haemopoietic stem cells will be discussed extensively.

CELL MEMBRANE STRUCTURES ON HAEMOPOIETIC STEM CELLS

The regulation of self-renewal, commitment and differentiation of haemopoietic stem cells is still poorly understood but appears to depend on interactions among haemopoietic stem cells, stromal elements and a variety of growth factors (Metcalf, 1984; Dexter, 1982; McCulloch, 1983). It is obvious that determinants expressed on the cell membrane must play an important rôle in the control of these complex processes (Watt et al., in press; Berridge, 1982; Fitchen et al., 1981). Thus, knowledge of the composition of the cell surface membrane of haemopoietic stem cells might result in a better understanding of the events taking place during the early steps of haemopoiesis. It can be assumed that the presence of certain cell surface determinants is more related to the functional capacities of a cell than features like cell size and density (Van den Engh et al., 1980). Evidence for this was obtained in studies on the cell surface characteristics of lymphocytes. Now more than 50 antigenic determinants have been described on murine lymphocytes (Morse III et al., 1987). Although many of these antigenic determinants are not specific for lymphocytes and are also expressed on other cell types, the identification of some of these structures have led to the description of discrete functional subsets of cells (Hibbs et al., 1984; Habu and Okumura, 1984; Ceredig et al., 1985; Cantor and Boyse, 1975; Cantor et al., 1976).

The success in the segregation of functional lymphocyte subsets on the basis of recognition of distinct cell surface determinants suggested that a similar approach could be used to segregate and purify various morphologically indistinguishable stem cell subsets. In earlier studies polyclonal

antisera and complement mediated cell lysis were used to determine the expression of certain antigenic determinants on CFC-S and different types of committed progenitor cells. It was found that CFU-S express antigenic determinants associated with mouse brain (Golub, 1972), sperm and testis (Berridge and Okech, 1980), lymph nodes, thymocytes, peritoneal macrophages and platelets (Berridge and Okech, 1979), embryo (Price et al., 1979), endogenous xenotropic C- type virus (Staber et al., 1978), Abelson murine leukaemia virus (Risser, 1979) and class II and various class I H-2 antigens (Russel and Van den Engh, 1979; Kincade et al., 1980; Fitchen and Ferrone, 1981). Some of these antisera have been used in attempts to enrich for mouse CFU-S, but none of these attempts were very successful (Monette, 1979; Price et al., 1979; Van Bekkum et al., 1979) and it is not clear to what extent any of these detectable surface antigens may be uniquely expressed on CFU-S and not also on other haemopoietic (stem) cell types (Till and McCulloch, 1980).

A diffference was observed between CFU-S and CFU-C with regard to the expression of brain associated determinants (Van den Engh and Golub, 1974). CFU-S were killed by treatment with anti-brain serum and complement, whereas CFU-C were not. Therefore, it was believed that CFU-S and CFU-C could be distinguished on basis of reactivity with anti-brain serum. Later, using flow cytometry, however, it was found that anti-mouse brain serum did not react with CFU-S (Van den Engh et al., 1980).

On basis of various patterns of reactivities two hypotheses with regard to cell surface marker expression on haemopoietic stem cells have been proposed. The first hypothesis proposed by Davis (1975) is that none of the cell surface markers of mature cells is expressed on the PHSC. The second model proposed by Till (1976) predicts that PHSC, because of their pluripotentiality, co-express antigenic determinants of all haemopoietic lineages at low and perhaps nonfunctional levels. In Fig. 1.2 a schematic representation of the two hypotheses is shown. According to the hypothesis of Till differentiation of stem cells could involve a progressive restriction in the expression of cell surface markers, with a specialization in the expression of the particular markers characteristic of a single pathway of differentiation and a concomitant disappearance of irrelevant markers. The validity of this hypothesis is supported by the description of multiple lineage marked leukaemic cells, but has never been verified in normal haemopoiesis (Ferrero et al., 1985). A major obstacle to testing both hypotheses is that low level expression of antigenic determinants or other structures on rare cells like haemopoietic stem cells is difficult to establish (Watt et al., in press).

Progress in the understanding of the surface characteristics of haemopoietic cells has been advanced by the introduction of the technology for monoclonal antibody (MCA) production (Köhler and Milstein, 1975) and flow cytometry. MCAs have several advantages over conventional polyclonal antisera (Galfre and Milstein, 1979). Their homogeneity, high specificity and unlimited availability are the most pronounced advantages. The MCA technology especially in conjunction with flow cytometry has led to the description of numerous antigenic determinants expressed on haemopoietic cells of different maturation stages and differentiation lineages (Ledbetter and Herzenberg, 1979; Springer et al., 1978a; b; Trowbridge, 1978; Austyn and Gordon, 1981; Watt et al., 1983; 1984; and many others).

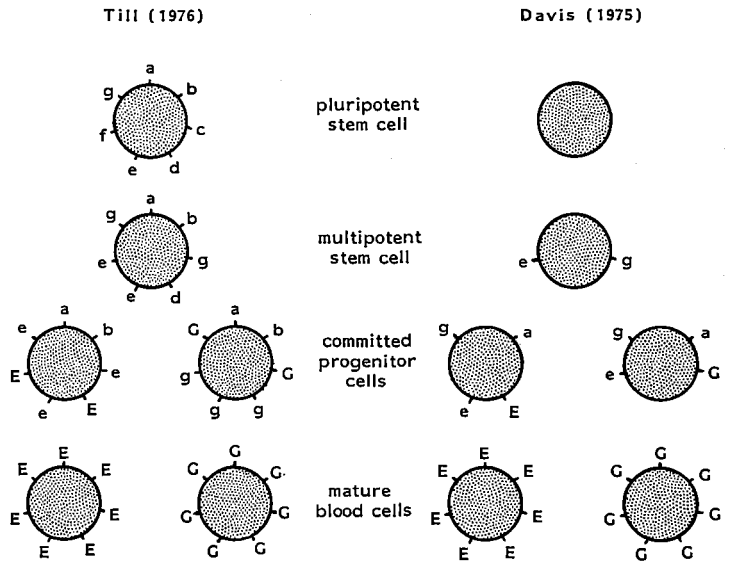


Figure 1.2:
A diagrammatic illustration of the two hypotheses proposed regarding the expression of cell surface markers on haemopoietic stem cells. Lower case letters represent markers present at low levels or of limited functional capacity, upper case letters represent the converse. For simplicity two of the possible pathways of differentiation (G: granulocytes, E: erythrocytes) from the pluripotent haemopoietic stem cell are shown. (Partly adapted from Till, 1976).

To date a great number of MCAs directed against the various mature haemopoietic cell types has been described. Very few MCAs appear to be specifically directed against cells of a certain lineage or a particular stage within that lineage (Harris et al., 1985). Despite many efforts and in contrast with what was proposed by Till (1976) (see Fig. 1.2), MCAs specifically directed against a subpopulation of murine haemopoietic stem cells have not yet been described (Bertoncello et al., 1987; Watt et al., in press).

From these studies it appeared that some antigenic determinants expressed at early stages of differentiation are lost during the course of differentiation, whereas other antigenic determinants become increasingly expressed (Bertoncello et al., 1987; Williams et al., 1985). In addition, some antigenic determinants are only transiently expressed during differentiation within a certain lineage, whereas others are expressed in the same densities

throughout differentiation (Van den Engh et al., 1983). Antigenic determinants that appeared to be specific for a particular cell lineage and that remain conserved on all cell types of that lineage throughout differentiation (cell lineage antigens) have also been described (Berridge, 1979).

The knowledge of the cell surface structure of haemopoietic cells has proven to be a very powerful tool for the identification, segregation and purification of subsets of haemopoietic stem cells. Subsets of stem cells could be identified, isolated and significantly purified on basis of quantitative differences in the expression of antigenic structures and carbohydrate moieties (identified by lectins) using a cell sorter and combinations of fluorescent probes (Nicola et al., 1980; 1981; Visser and Bol, 1981; Visser et al., 1984; Bauman et al., 1986; Bauman and Chen, 1987; Hoang et al., 1983; Watt et al., 1983; 1984; in press; Bertoncello et al., 1986; 1987; Muller-Sieburg et al., 1986; and many others).

OBJECTIVES AND OUTLINE OF THIS THESIS

The work described in this thesis deals with the production and application of monoclonal antibodies to characterize murine haemopoietic stem cells. Monoclonal antibodies directed against determinants uniquely expressed on various classes of haemopoietic stem cells should provide valuable tools for analyzing the early differentiation steps in the stem cell compartment at the molecular level, for elucidating the relationhip between the various stem cell classes and for purifying the various stem cell types. In the following chapter the experimental approaches used are described. Chapter 3 deals with attempts to produce stem cell specific monoclonal antibodies. The characterization of the monoclonal antibodies and their possible usefulness in haemopoietic studies are described in chapter 4. Using one of the produced monoclonal antibodies a widely applicable multiparameter flow cytometric procedure for the identification and purification of murine haemopoietic stem cells is described in chapter 5. In chapter 6 various purification procedures for haemopoietic stem cells are compared. It appeared that the highly purified day-12 CFU-S suspensions are heterogeneous, therefore the usefulness of the CFU-S assay as endpoint-assay for pluripotent haemopoietic stem cells is discussed. In the final chapter, the results will be considered in a broader perspective and possible approaches for further study will be outlined.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

The mice and rats that were used in this study were bred at the Radiobiological Institute TNO. They were maintained under specific pathogen free conditions until the initiation of the experiments. Thereafter, the animals were housed in conventional animal quarters, except for mice used in the 30-day survival studies (Table 6.2). In these experiments the mice were housed in a sterile hood.

In Table 2.1 the mouse strains used in this thesis are listed. In most experiments, BC3 mice were used, unless stated otherwise. Mice, 7-9 weeks old, were used as bone marrow or other organ and tissue donors throughout this study. Mice, 11-16 weeks of age, were used as recipients in the CFU-S assay and 30-day survival experiments. Both male and female mice were used but within single experiments, there was a restriction to one sex.

Table 2.1

MOUSE STRAINS USED IN THIS STUDY

mouse strain	abbreviation	H-2 haplotype
(C57Bl/Rij x C3H/Law)F1	BC3	b/k
(C57Bl/Rij x CBA/Rij)F1	BCBA	b/q
C57Bl/Rij	C57BL	b
C3H/Law	C3H	k

Rats were used for immunization purposes (Chapter 3). Only female rats, varying in age between 8 and 16 weeks at the time of the first immunization, were used in the present study. In Table 3.1, the used rat strains are listed. In most cases, BN rats were used.

PREPARATION OF CELL SUSPENSIONS

Single cell suspensions were prepared in Hanks' Balanced Salt solution (HBSS) (Laboratoires Eurobio, Paris, France) buffered at pH 6.9 with 10 mM HEPES buffer (Merck). This solution is abbreviated as HH (HBSS + HEPES) and had an osmolarity of 300-305 mOsm/kg. Organs and tissues were removed

after asphyxiation of the animals with CO_2. Bone marrow cells were obtained by flushing the femurs or tibias with 1 ml of ice-cold HH using a 25 gauge needle adapted to a syringe. A single cell suspension was obtained by pipetting and subsequent filtration through a six-layer nylon sieve.

Fetal liver cells were obtained from 17 days old C57Bl fetuses by gently pressing the liver through a nylon sieve with a spatula after premoistering the sieve. Single cell suspensions of thymus and spleen were prepared in the same way as fetal liver cell suspensions. Peripheral blood leukocytes (PBL) were prepared from whole blood obtained by orbital puncture of anesthesized (aether) animals. Clotting of the blot was prevented by adding tromboliquine (2% v/v, Organon Technika, Oss, The Netherlands) to the collection tube. After dilution of the blood with an equal volume of HH erythrocytes were lysed using a 150 mM NH_4CL solution buffered at pH 7.0. Peritoneal exudate cells (PEC) were isolated by injection of 5 ml ice-cold sucrose solution (0.34 M) into the peritoneal cavity. After gentle massage of the abdomen for 3-4 min, the cells were isolated by withdrawing the fluid into the syringe.

All cell suspensions were washed with HH containing either 5% fetal calf serum (FCS, Seralab) or newborn calf serum (NCS; Seralab) and 0.02% (v/v) sodium azide (Merck) at 300 g for 7 min at 4°C. This medium is abbreviated HSA (HH + serum + azide). Cells were resuspended in HSA prior to counting. Nucleated cell counts were performed in a Bürker haemocytometer using Türk's staining solution. All cell suspensions were kept on melting ice until use. For all experiments at least two mice were used to minimize variation due to individual differences.

IRRADIATION OF MICE

For CFU-S assays and 30-day survival experiments mice were subjected to lethal total body irradiation. Irradiations were performed with a ^{137}Cs gamma-cell-20-small-animal irradiator (Atomic Energy of Canada). A maximum of 25 mice was irradiated at a time in an animal container flushed with air. The gamma-dose rate at the position of the mice was 0.83 Gy/min. BC3 and C3H mice received a total dose of 9.25 Gy, BCBA mice received a total dose of 9.0 Gy. Bone marrow cell transplantations were given 2 to 8 hours after irradiation. Without bone marrow transplantation the lethally irradiated recipients died around the 15th day post-irradiation.

THE SPLEEN COLONY ASSAY

The number of spleen colony forming cells in various bone marrow suspensions was determined according to the assay system described by Till and McCulloch (1961). Appropriate numbers of cells, estimated so as to give approximately 10 colonies per spleen, were suspended in HH and injected in 0.5 ml volumes into the lateral tail vein of lethally irradiated syngeneic recipients. Irradiated mice which had not been transplanted with haemopoietic cells served as controls for endogenous colony formation. Seven to ten mice were used per experimental group. After eight (or seven, Table 6.6) to twelve days the mice were killed, their spleens removed and fixed in

Tellyesniczky's solution. Macroscopically visible nodules were scored as spleen colony forming units (CFU-S). Spleen colonies counted at different days are referred to as day-8 CFU-S, day-10 CFU-S and day-12 CFU-S.

30-DAY SURVIVAL ASSAY

Unseparated or sorted bone marrow cells were transplanted at several dilutions in groups of ten lethally irradiated syngeneic recipient mice. A dilution of the same suspensions was used for determination of the day-12 CFU-S content of the graft (Table 6.2). The proportion of mice surviving 30 days after irradiation and transplantation was determined for each dilution. The number of bone marrow cells and the number of day-12 CFU-S needed for survival of 50% of the animals was calculated from these data by probit analysis.

CLONAL ASSAYS IN VITRO

A. Agar cultures

A detailed description of the in vitro assay for colony forming cells is given by Bol and Williams (1980). Bone marrow cells were suspended in supplemented Dulbecco's modified eagle medium (Gibco M07-2501) (Bol et al., 1979) containing 0.3% agar (Bacto agar, Difco Laboratories) and 20% of a mixture of 2 volumes of horse serum (HS, non-commercial batch or obtained from Seralab) and 1 volume of FCS (Seralab). Immediately before use 1.5 mM L-glutamine was added to the medium. Aliquots of 1 ml containing 5×10^4 unseparated bone marrow cells, or the indicated number of sorted cells were plated into 35 mm Petri dishes (Falcon, 1008). Prior to plating, solutions of one or more types of growth stimulating factor preparations were added to the dishes. The total volume of these preparations never exceeded 50 µl. Each experimental group was tested in triplicate.

To stimulate colony formation by primitive macrophage progenitors, i.e., CFU-C2 (Bol and Williams, 1980; Bol et al., 1979), 25 µl of a crude pregnant mouse uterus extract preparation (PMUE) was added to the cultures (Bradley et al., 1971; Stanley et al., 1972). The cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. In the experiments depicted in Table 6.4 cultures were stimulated with 10 units (U) recombinant murine IL-3 (7.5 µl per culture dish). One unit of IL-3 is defined as the quantity needed for 50% proliferation of the IL-3 dependent cell line DA-1 (see Table 4.4). IL-3 was a gift from Dr. L. Dorssers. Recombinant human IL-1α (Genzyme Corporation, Boston, MA, U.S.A.) was used at a concentration of 10 U/ml (10 µl/culture dish). In these experiments (Table 6.4) colonies were scored after 14 days.

Colonies (more than 50 cells) were counted using an inverted microscope at a 30-fold magnification.

B. Methylcellulose cultures

The method employed for serum-free methylcellulose cultures of haemopoietic progenitors was described in detail before (Merchav and Wagemaker,

1984; Merchav, 1986). The serum-free medium consisted of Dulbecco's medium (Dulbecco's MEM, Gibco 430-1600) supplemented with amino acids, vitamin B12, biotin, Na-pyruvate, glucose, $NaHCO_3$ and antibiotics (α-medium, 300 mOsm/kg). This α-medium was supplemented with 1% (w/v) delipidated, deionized (Worton et al., 1969a) bovine serum albumin (BSA; Fraction V, Sigma), 4×10^{-6} M iron-saturated human transferrin (Boehring-Mannheim), 10^{-7} M $Na_2SeO_3.5H_2O$ (Merck), 10^{-4} M β-mercaptoethanol (Merck), 1.5×10^{-5} M linoleic acid (Merck) and cholesterol (Sigma), nucleosides (10 µg/ml each), and 0.8% (w/v) methylcellulose (Methocel A4M Premium grade, Dow Chemical).

One ml aliquots of this medium supplemented with the appropriate amount of bone marrow cells and the appropriate (combinations of) growth factors were plated in 35 mm Petri dishes (Falcon 1008). Each experimental group was tested in duplicate. Cultures were incubated at 37°C in a fully humidified atmosphere of 7.5% CO_2 in air. Colonies were scored after 7-14 days, except BFU-E which were scored after 10-14 days. Colony formation by granulocyte-macrophage progenitor cells (CFU-GM) was stimulated with recombinant murine GM-CSF at a predetermined optimal concentration. In addition, the culture medium was supplemented with 10^{-6} M hydrocortisone sodium succinate (Sigma). Colony formation by macrophage progenitor cells (CFU-M) was stimulated with a partly purified preparation of CSF-1. CSF-1 was purified from PMUE as described by Merchav and Wagemaker (1984) using affinity chromatography (Concanavalin-A (ConA) - Sepharose) and ion exchange chromatography (DEAE-Sepharose, pH 8.0). The formation of colonies by BFU-E (burst forming unit-erythrocyte) was stimulated with a combination of 20% of a crude preparation of ConA stimulated mouse spleen cell conditioned medium (MSCM) (Wagemaker and Visser, 1980) and a partly purified preparation of erythropoietin (Epo). Epo was purified as described by Merchav (1986). BFU-E were assayed in medium supplemented with 0.2 M hemin (Photoporphirin IX, Sigma).

In the experiment shown in Table 6.5 cultures were stimulated with 10 U IL-3, 10 U IL-1 and the same concentration of CSF-1 and Epo + hemin as used to assay CFU-M and BFU-E, respectively.

SUSPENSION CULTURES

Suspension cultures of sorted bone marrow cell fractions (see Table 6.6 and 6.7) were performed in serum-free medium as described by Merchav (1986). The indicated number of cells were cultured in plastic round bottom tubes (Falcon, 2057) containing 1 ml of the above described modified Dulbecco's medium, supplemented with 10^{-6} M hydrocortisone sodium succinate (Sigma) and 10^{-6} M isoproterenol (Sigma). The cultures were stimulated with a saturating dose of IL-3 (10 U/ml), IL-1 (10 U/ml) or CSF-1 (the same batch and concentration as used for methylcellulose cultures). Loosely capped tubes were incubated at 37°C, 7.5% CO_2 in a fully humidified atmosphere for a period of four days. Then, the cultures were terminated by adding 5 ml ice-cold HH to the tubes. The diluted suspensions were either injected intravenously (i.v.) in 0.5 ml volumes into lethally irradiated syngeneic recipients for enumeration of the CFU-S content (Table 6.6) or

were washed and suspended in modified Dulbecco's medium and cultured in methylcellulose medium to determine the CFU-M, CFU-GM and BFU-E content of the cultures (Tables 6.7).

The standard deviations of the in this study presented CFU-S and in vitro clonogenic progenitor cell counts were calculated on basis of the assumption that colony counts are Poisson distributed (see Van den Engh, 1976; Bol, 1980).

EQUILIBRIUM DENSITY CENTRIFUGATION

As the first step in stem cell purification procedures and in the preparation of immunization suspensions, bone marrow cells were separated using a discontinuous metrizamide (Nyegaard, Oslo, Norway) density gradient as described earlier (Visser et al., 1984). In brief, discontinuous gradients were prepared by first pipetting 1 ml of a high density metrizamide (MA) solution (1.100 g/cm^3, dissolved in HH + 1% BSA, pH 6.7, 300 mOsm/kg) containing 5-6 x 10^7 bone marrow cells in a round bottom tube (Falcon, 2057). On top of this solution, 3 ml of a MA solution with intermediate density (1.078 g/cm^3) was layered. Finally, 1 ml of the low density solution (1.055 g/cm^3) was put on top of the intermediate solution. The cells were centrifuged for 10 min, 4°C, at 1000 g. The cells in the low density fraction and from the interface between the top and intermediate layer (low density cells) were collected, washed, centrifuged and counted. The yield of low density cells in the present study was 10 ± 5% of all nucleated cells.

IN VIVO TREATMENT OF MICE WITH 5-FLUOROURACIL

Seven to eight weeks old mice were injected i.v. with 150 mg 5-fluorouracil (FU, Sigma) per kg body weight. FU was dissolved in HH at a concentration of 7.5 mg/ml. Mice were injected with a volume determined by their weight (in grams) x 0.02 ml. After 7 days the mice were sacrificed and bone marrow was isolated. Low density FU treated bone marrow cell fractions were used for immunizations of rats (Tables 3.4 and 3.5).

INTRASPLENIC IMMUNIZATION OF RATS

For intrasplenic (i.s.) immunization of rats, the animals were anaesthetized by intraperitoneal (i.p.) injection of avertine (Janssen Pharmaceutics, Beerse, Belgium). Immediately before use, the stock solution of avertine (1 g/ml) was 40-fold diluted in prewarmed (37°C) HH. Rats received 0.1 ml of this avertine solution per 100 g body weight. The fur from the left side was shaved and the abdomen swabbed with 70% ethanol. First, a 10-15 mm long skin incision was made followed by incision of the muscle layers and peritoneum. The spleen was exposed by gently lifting its lower pole with the aid of forceps. A thin needle (0.35 mm diameter) was inserted deeply into the spleen and the cells were injected (100-150 µl) as the needle was pulled

out, in order to ensure that the antigen was distributed through most of the spleen (Spitz et al., 1984). After injection, the spleen was carefully pushed back into the peritoneal cavity. Subsequently, the peritoneal and muscular walls were sutured with thread and the skin was closed with 2-4 wire stitches. One to three months later the rats were boosted in the same way. Four days after the final immunization, the animals were killed and the spleen removed. The spleen was used in fusion experiments. The intrasplenic (i.s.) immunization was performed in a laminar airflow to prevent infection of the animal.

In the experiments shown in Figures 3.2 and 3.3 some rats were immunized by i.p. or i.v. administration of murine bone marrow cells. For i.p. and i.v. immunization the appropriate number of cells was injected in a volume of 0.5-1 ml HH.

FUSION PROTOCOL

Fusion was performed 4 days after the final immunization according to standard procedures (Goding, 1980). The spleen of the immunized rat was asceptically removed and a single spleen cell suspension was prepared in HEPES buffered RPMI-1640 (RPMI, Gibco) by pressing the spleen (using a spatula) through a steel sieve. The suspension was transferred to a conical 50 ml plastic tube (Falcon, 2070), dispersed by repeated pipetting and allowed to settle for 5 minutes. The supernate was then removed and transferred to another tube and RPMI was added to a final volume of 50 ml. The cells were spun down at 300 g for 10 min at room temperature, resuspended in 50 ml RPMI and spun again. Finally, the cells were resuspended in 20 ml RPMI and counted. Prior to fusion, myeloma cells (see Table 3.2) were grown in RPMI supplemented with 10% FCS (Seralab), 3 mM L-glutamine (Gibco), 1 mM MEM-sodium pyruvate (Gibco), 0.05 mM β-mercaptoethanol (Merck) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco). This medium is designated as RPMI-total. Two weeks before a fusion experiment the myeloma cells were expanded (150 cm^2 flasks, Falcon) in RPMI-total and kept at low density (half confluency) to be sure that on the day of fusion they were in the logarithmic growth fase. Myeloma cells were collected and washed twice with RPMI. The cells were then resuspended in 20 ml RPMI and counted.

In most cases the number of myeloma cells determined the "size" of the fusion. In a typical experiment 1.5×10^8 spleen cells were fused with $5\text{-}7.5 \times 10^7$ myeloma cells in the following way: The appropriate number of spleen cells was added to the myeloma cells and the cells were centrifuged. After removing the supernate, 1 ml of a prewarmed solution of 40% (w/v) PEG 4000 (Merck) was added slowly over one minute with constant agitation and careful stirring. Another ml of 40% PEG 4000 was then added slowly over the next minute. Subsequently, 1 ml of RPMI was added over the next minute followed by another 8 ml of RPMI over the next three minutes. The fused cells were incubated for 5 minutes at 37°C before they were centrifuged at 300 g for 5 min. The fused cells were resuspended at a density of $1\text{-}2.5 \times 10^6$ spleen cells per ml selective medium. The cells were then plated into the wells (100 µl/well) of 96-wells microtiter plates (Mikrotest III,

Falcon) and were cultured at 37°C, 5% CO_2 and 100% humidity. Selective medium consisted of RPMI-total supplemented with 10% HECS (human endothelial cell culture supernatant, Astaldi et al., 1980; Costar), 5% HS (either a noncommercial batch or obtained from Seralab) and HAT; a combination of Hypoxantine (0.1 mM, Merck 4517), Aminopterine (0.4 µM, Sigma A-2255) and (desoxy) Thymidine (0.016 mM). HAT was used as selective medium in the fusion experiments shown in Table 3.3. In the fusion experiments listed in Table 3.5, a combination of azaserine (1 µg/ml; Sigma) and hypoxanthine was used as selective agents. After 3-4 days, 100 µl of fresh selective medium was added to the wells. After another 3-4 days half the medium was removed and an equal volume (100 µl) of fresh selective medium was added, etc. After 2-4 weeks when growing hybridoma clones became visible, either aminopterine or azaserine was ommitted from the selective media.

Antibody producing hybridoma clones were subcloned by limiting dilution at average densities of 0.5 cells/well and 2 cells/well in RPMI total supplemented with 10% HECS, 5% HS and either HT (in case of HAT selection) or H (in case of selection with azaserine). Antibody producing subclones were expanded in RPMI-total, frozen and stored in liquid nitrogen.

LABELLING OF BONE MARROW CELLS WITH MONOCLONAL ANTIBODIES

For flow cytometric analysis (screening), 10^6 bone marrow cells were washed with HSA and centrifuged once at 300 g for 7 min at 4°C. The cells were then resuspended in 50 µl of spent hybridoma culture supernatant and incubated on melting ice for 45 min. Subsequently, the cells were washed and centrifuged twice with ice-cold HSA. The cells were subsequently resuspended in 50 µl of a 50-fold dilution (in HSA) of goat-anti-rat/IgG (H+L)/FITC (GARA/FITC, affinity purified, TAGO Inc., Burlingame, CA, U.S.A.) and incubated for 30 min on melting ice. The labelled cells were washed with HH and centrifuged once. Finally, the cells were resuspended in HH. Labelling of cells was done either in plastic tubes (Falcon 2058) or in wells of 96-wells round-bottom microtiter plates (Greiner). When the labelling was done in tubes, cells were washed with a volume of 1 ml HSA or HH. When this was performed in microtiter plates, the cells were washed with 200 µl of HSA or HH. After the last wash, the cells were resuspended in 100 µl HH and transferred to a plastic tube containing 900 µl of HH.

For CFU-S purification purposes cells were labelled with 50 µl of the appropriate dilution (1 : 20 in HSA) of biotinylated anti-H-$2K^k$ antibody (α-H-$2K^k$-biotin) (Becton Dickinson Monoclonal Center Inc., Mountain View, CA, U.S.A.) and either 50 µl of the appropriate dilution (100x) of fluorescein-isothiocyanate labelled avidin (Av/FITC, Sigma, Deisenhofen, West-Germany) or 50 µl of avidin conjugated with phycoerythrin (1:50 in HSA) (Av/PE, Becton Dickinson, Immunocytometry Systems, Mountain View, CA, USA). Anti-H-$2K^k$ was biotinylated as described by Bauman et al. (1985).

Bone marrow cells were also labelled with 50 µl of a hundred-fold diluted mouse MCA directed against Thy-1 (α-Thy-1.1, New England Nuclear, NEI 014) and goat-anti-mouse/Ig/FITC (GAM/FITC, Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 50-fold in HSA (see Fig. 5.4). Besides this indirect labelling of bone marrow cells, cells were

also stained directly with FITC conjugated MCAs. MCA 15-1.1 (this thesis) and anti-GM1.2 (New England Nuclear, NEI 031) were conjugated to FITC as described below. Labelling of bone marrow cells with directly conjugated antibodies involved a 45 min incubation on melting ice, followed by two wash steps with HH. Anti-GM1.2/FITC was diluted 5 times and 15-1.1/FITC 10 times in HSA.

In chapter 3 (Figures 3.2 and 3.3) bone marrow cells were incubated with 50 µl of diluted heat inactivated rat serum instead of hybridoma culture supernatant. Serum incubated cells were subsequently stained with GARA/FITC as described for cell suspensions incubated with hybridoma supernatant.

CONJUGATION OF MCAs WITH FITC

Equal volumes of saturated ammonium sulphate and supernatant containing the MCA of interest were mixed. After 1-2 hours at room temperature (with occasional shaking), the suspension was centrifuged at 12,000 g for 30 min and the supernate removed. The pellet was dissolved in 40% ammonium sulphate and centrifuged again. The pellet was then resuspended in 1/5 - 1/10 of the original (supernatant) volume of distilled water and dialyzed against distilled water for 48 hours at 4°C. The content of the dialysis bag was centrifuged at 1200 g for 10 minutes and the supernate was collected and lyophilized. A solution was made containing 5 mg lyophilized protein per ml Phosphate Buffered Saline (PBS, pH 7.2). Per ml protein solution 0.1 ml of a 0.5 M $NaHCO_3$ solution, pH 9.5 was added, the pH checked and, if necessary, adjusted to pH 9.0 by adding $NaHCO_3$.

Under continuous stirring, 10 µl of a stock solution of FITC isomer-1 (1 mg/ml DMSO, Nordic Immunological Laboratories, Tilburg, The Netherlands) was slowly added per mg protein. The mixture was incubated (continuous stirring) for 3 hours in the dark at room temperature. During this period the pH was repeatedly checked and, if necesary, adjusted to pH 9.0. The mixture was then loaded on a PD-10 Sephadex column (Pharmacia 17-08500) and eluted with PBS.

FLOW CYTOMETRIC ANALYSIS OF CELLS

Cell suspensions were analyzed using a modified fluorescence activated cell sorter (FACS-II, Becton Dickinson, Sunnyvale, CA, U.S.A.). The machine was adapted to measure two light signals perpendicular (70-110 degrees) to the laser beam by introducing an achromatic beam splitter and a second photomultiplier. Forward light scatter (FLS) was collected over the angles 0.5-13 degrees with respect to the axis of the laser beam. Perpendicular light scatter (PLS) intensity was measured by an S-11 type photomultiplier. FITC (green) fluorescence intensity was measured through a combination of a broad band multicavity interference filter (520-550 nm transmission; Pomfrett Research Optics Inc., Stamford, CT, U.S.A.) and a 520 nm cut-off filter (Ditric Research Optics Inc., Hudson, MA, U.S.A.) by a S-20 type photomultiplier. The laser was tuned at 488 nm with an output

of 0.5 W. FLS and PLS signals were linearly amplified. The amplification of the FLS was adjusted such that the peak of the lymphocytes in the pulse height-analyzer histogram was at channel 100. PLS amplification was adjusted so that the peak of the lymphocyte cluster (Figure 4.1) was at channel 25. FITC-fluorescence was logarithmically amplified (T. Nozaki, Stanford, CA, U.S.A.). The sheat fluid consisted of 0.9% (w/v) saline.

For screening of hybridoma culture supernatants electronic windows were set to select each of the four major cell clusters distinguishable on basis of FLS and PLS intensities (Fig. 4.1). The fluorescence distribution of 5×10^3 events falling within each cluster were stored in a histogram and compared with the fluorescence distribution of the negative control (incubated with GARA/FITC only) by means of the ANNA software package developed in the Radiobiological Institute TNO.

To determine the reactivity of MCAs with CFU-S or in vitro clonogenic progenitor cells of various differentiation lineages first electronic windows were set to select the blast cells or the blast cells + granulocytes (Fig. 4.1, Van den Engh et al., 1979). Then, depending on the fluorescence distribution, two or more fractions differing in fluorescence intensities were selected by setting electronic windows (see Figs. 4.5 and 4.6) and the subpopulations sorted. For sorting experiments, the sheat fluid consisted of HH (without phenol red). Cells were sorted into 15 ml glass tubes, containing 0.5 ml HH + 10% FCS. The walls of the tubes were rinsed prior to and after the sort with HH + 10% FCS. During the sorts the collection tubes and the tube containing the unsorted cells were cooled (4°C). The sorted cells were assayed for their CFU-S or in vitro clonogenic progenitor cell content as described above.

PURIFICATION OF CFU-S USING A FACS-II

In this study CFU-S were purified using three different procedures (Fig. 6.1). The first step in all three procedures consisted of the separation of low density cells using a discontinuous density metrizamide gradient as described above with one modification. During the centrifugation the cells were labelled with the FITC-labelled lectin wheat germ agglutinin (WGA/FITC; Polysciences Inc., Warrington, PA, U.S.A.). For this purpose, 3 µl of a WGA/FITC stock solution (1 mg/ml) was added to 14 ml of the intermediate density solution. (In CFU-S purification experiments normally 4 gradients were processed simultaneously.) During the centrifigation only low density cells travel through the WGA/FITC containing layer to the top layer, as a consequence of which they are labelled. The low density cells were subsequently analyzed on the FACS and the WGA/FITC positive cells with medium to high FLS and low PLS intensities (blast cell cluster) were sorted as described earlier (Visser and Bol, 1981). The in this way selected cells comprised between 4-8% of all low density cells.

CFU-S were further purified according to the procedure described by Visser et al. (1984). First, WGA/FITC was removed from the cells by incubation (15 min, 37°C) in 10 ml of an isotonic solution of 0.2 M N-acetyl-D-glucosamine (Polysciences Inc., Warrington, PA, U.S.A.). Subsequently, the cells were centrifuged at 300 g for 7 min, resuspended in 1 ml HH trans-

ferred to a plastic tube and centrifuged again. The cells were then labelled with α-H-2K^k-biotin and Av/FITC and sorted again. In this second sort 20-30% of the most brightly fluorescent cells with the same FLS and PLS intensities as during the first sort were selected. In chapter 5 (Table 5.2) Av/PE was used to detect bound α-H-2K^k-biotin instead of Av/FITC. This procedure yielded typically about 1 x 10^5 cells.

Another way to purify CFU-S employed here, was by means of two subsequent WGA/FITC sorts as described by Lord and Spooncer (1986). After the first WGA/FITC sort the cells were transferred to a plastic tube (Falcon 2058) and immediately sorted again using the same FLS, PLS and FITC-fluorescence window settings as in the first sort. In this second sort, 60 - 80% of the cells were sorted. Selection of CFU-S in this way yielded typically 2 x 10^5 cells.

The third procedure to select CFU-S was developed in the present study (chapters 5 and 6) and involved selection on basis of MCA 15-1.1 fluorescence intensity in a second sort. First WGA/FITC conjugates were removed from the cells which were then labelled with 15-1.1/FITC. In the second sort the 15-1.1 negative cells with the same FLS and PLS intensities as in the first sort were selected. In this second sort between 40 and 50% of the cells were isolated resulting in the ultimate selection of 1-2 x 10^5 cells.

STAINING OF CELL SUSPENSIONS WITH RHODAMINE 123 (Rh123)

Cells were labelled with Rh123 as described by Bertoncello et al. (1985). Rh123 (Eastman Kodak, Rochester, New York, U.S.A.) was dissolved in distilled water (1 mg/ml). Bone marrow cell suspensions sorted on basis of α-H-2K^k or 15-1.1 fluorescence were incubated in 10 ml HH + 5% FCS and 0.1 µg/ml Rh123 for 15-20 min at 37°C in the dark. The cells were washed once with HH + 5% FCS and resuspended in HH at a concentration of 1-3 x 10^6 cells per ml. Cells isolated on basis of two sequential WGA/FITC sorts were stained with Rh123 while at the same time WGA/FITC was removed. To the sorted cells 10 ml of a solution containing 0.2 M N-acetyl-D-glucosamine + 5% FCS and 0.1 µg/ml Rh123 was added. After 15-20 min at 37°C in the dark, the cells were washed as described above. Rh123 stained samples were sorted on the FACS-II using the same FLS and PLS windows as in the previous sorts and the same optical filters as used for FITC fluorescence measurements. Rh123 stained samples were processed in subdued light.

PURIFICATION OF CFU-S USING A ONE SORT PROCEDURE

Purification of CFU-S in a one sort procedure, employing two different fluorochromes was done using the Rijswijk Experimental Light Activated Cell Sorter (RELACS, Stokdijk et al., 1985). The RELACS is a two laser flow cytometer and allows for 8 parameter measurements (for details about the RELACS, see Herweijer, 1988).

Low density bone marrow cells were separated and at the same time labelled with either WGA/FITC or with WGA conjugated to the red fluorescent dye Texas Red (Titus et al., 1982; WGA/TxR, Molecular Probes, Eugene,

OR, U.S.A.) as described before. WGA/TxR was used at a 2000-fold dilution in HSA (final concentration 0.5 µg/ml). Subsequently, the WGA stained cells were labelled with α-H-2K^k-biotin and Av/PE. When cells were labelled with WGA/FITC only one laser of the RELACS was used (Coherent CR6, Coherent, Palo Alto, CA, U.S.A.). The laser was operated at 488 nm and 0.4 W. FLS and PLS intensities were measured through 488 nm band pass (BP) filters (Melles Griot, Irvine, CA, U.S.A.) and were linearly amplified. Green FITC fluorescence (WGA) and orange PE fluorescence (α-H-2K^k) signals were separated with a 570 nm dichroic mirror (Zeiss, West Germany) and measured through a 530 nm and 577 nm BP filter, respectively (Corion, Hollistone, MA, U.S.A.). Fluorescence signals were measured using logarithmic amplifiers. Spill over of green fluorescence into the detector used for the orange fluorescence was corrected as described in chapter 5.

When cells were labelled with WGA/TxR both lasers were used. The second laser of the RELACS (Spectra Physics 2020-05, Mountain View, CA, U.S.A.) was used to pump a tunable dye laser (Spectra Physics 375) circulating rhodamine 6G. The dye laser emitted 590 nm light (0.3 W) and was used to excite TxR. TxR fluorescence was measured using two RG630 nm long pass (LP) filters (Schott Optical Glass Inc., Duryea, PA, U.S.A.) and was logarithmically amplified.

Selection of the cell population that is enriched for CFU-S is described in detail in chapter 5. Briefly, after exclusion of aggregates consisting of two or more cells, the blast cells were selected by means of electronic window settings including cells with low to intermediate FLS and low PLS intensities. Then, a window was set to select the WGA positive blast cells. This was done in the same way for samples labelled with WGA/FITC or WGA/TxR. Finally, a window was set to select the 20-30% most strongly α-H-2K^k positive cells. In this way about 0.1-0.4% of all nucleated bone marrow cells were selected. The selected cells were sorted and assayed for their CFU-S content (Table 5.1).

THREE COLOUR IMMUNOFLUORESCENCE FLOW CYTOMETRIC SCREENING OF MCAs FOR REACTIVITY WITH CFU-S

The above described procedure was modified to allow <u>in vitro</u> screening of MCAs for reactivity with CFU-S. Unseparated or low density bone marrow cells were stained in the following way: First, 10^6 bone marrow cells were resuspended in 50 µl of spent hybridoma culture supernatant to which the appropriate dilution of α-H-2K^k-biotin was added (see above). After incubation on ice the cells were washed twice with HSA. The pelleted cells were then resuspended in 50 µl of the appropriate dilution of GARA/FITC (to detect MCA) and Av/PE. After incubation and washing of the cells they were resuspended in 1 ml HH supplemented with WGA/TxR (1:2000) or WGA conjugated to the blue fluorescent dye 1-pyrenebutyryl (WGA/PYR, Molecular Probes, Junction City, OR, U.S.A.). WGA/PYR was diluted 2500-fold (final concentration 0.4 µg/ml HSA). After 10-15 min incubation at room temperature the cells were analyzed on the RELACS. The first laser was tuned as described above. In case, WGA/PYR was used, the second laser of the RELACS was tuned to emit UV light with lines at 351 nm and 364 nm. In

case WGA/TxR was used the machine was tuned as described above. A total of 10^5 nucleated cells was analyzed (erythrocytes were excluded from the analysis by setting a threshold after the FLS amplifier). Of each cell six parameters were measured and stored simultaneously in a list mode file in the memory of an HP9000-220 microcomputer. The recorded parameters were time of flight (TOF), FLS, PLS, FITC fluorescence (MCA) and PE fluorescence (α-H-$2K^k$) generated by the first laser and TxR fluorescence (WGA) or PYR fluorescence (WGA) generated by the second laser (see chapter 5). The data were analyzed using the ELDAS software package written in the Radiobiological Institute TNO. Analysis was done as described in detail in chapter 5. In brief, first CFC-S were selected as described above, then the FITC fluorescence (MCA) of the selected cell population was analyzed and compared with a sample treated in the same way except that MCA was ommitted (see Figs. 5.4 and 5.5).

DETERMINATION OF THE IMMUNOGLOBULIN (SUB)CLASS OF MCAs

The immunoglobulin (Ig) (sub)class of the produced MCAs was determined by an Ouchterlony immunodiffusion reaction in 1% (w/v) agarose, dissolved in veronal buffer, pH 8.4 (12 g/liter 5.5 diethylbarbituric acid - sodium salt and 4.4 g/liter 5.5 diethylbarbituric acid). The central well was filled with ten-fold concentrated culture supernatant (10 µl/well), the surrounding wells were topped with undiluted rabbit- or goat-anti-rat Ig (sub)class specific antisera (Nordic, Tilburg, The Netherlands): RARA/IgG1, IgG2a, IgG2b, IgA (Fc-part), IgE (Fc-part) and GARA/IgG2c and IgM (Fc-part). After diffusion for 24 hours at 4°C, the slides were placed in distilled water during 2x one hour. Subsequently, the slides were stored in PBS for 24 hours after which they were air-dried and stained with Coommassie Brilliant Blue Staining solution.

GOLD LINKED IMMUNOSORBENT ASSAY (GLISA)

Terasaki flat-bottom tissue culture plates (Greiner 726180, Nuertingen, FRG or Nunc, Roskilde, Denmark) were pretreated with poly-L-lysine (Sigma, P2636) suspended in PBS (0.01 mg/ml) as described by Lansdorp et al. (1980). Then, unseparated bone marrow cells were added to the wells or WGA positive low density blast cells were sorted into the wells (1000/well) using a modified automated microscope stage positioner as described by Visser et al. (1984). The cells were spun to the bottom of the wells at 100 g for 1 minute and fixed (10 minutes, room temperature) with 0.025% (v/v) glutaraldehyde (in PBS, Merck, 4239). Plates were then washed 3 times with PBS and could be stored for several weeks at 4°C by adding PBS supplemented with 200 µg/ml gelatin (Merck 4072) and 0.1% NaN_3 (see Lansdorp et al., 1980).

Screening was done by adding 10 µl of spent hybridoma culture supernatant to the wells after the plates had been washed 3x with PBS and 1x with PBS supplemented with 0.2% (v/v) Tween-20 (PBS-T). The plates were incubated for one hour at room temperature and were then washed 4 times

with PBS and once with PBS-T. Subsequently, 10 µl of a 1:20 dilution (in HSA) of a goat-anti-rat Ig serum conjugated with 5 nm colloidal cold particles (GARA/gold, Janssen Pharmaceutics) was added to the wells and the plates were incubated for another 30 min at room temperature. The plates were washed 3 times with PBS, once with PBS-T and finally with PBS and fixed again with 2% glutaraldehyde for 15 min at room temperature (10 µl/well). After the plates had been washed twice with 0.2 M citrate buffer, pH 3.85, ten µl/well of a silver enhancing solution (77 mM hydroquinone, 5.5 mM silver lactate in 0.2 M citrate buffer, Janssen Pharmaceutics) was added. After 4-6 minutes at room temperature the reaction was stopped by adding 10 µl/well fixing solution (Janssen Pharmaceutics). The fluid was removed and the wells examined for silver precipitates on the surface of the cells using an inverted microscope.

ANTIBODY DEPENDENT AND COMPLEMENT MEDIATED CYTOTOXICITY ASSAY

In the antibody dependent cytotoxicity assay, 10^6 bone marrow cells were incubated with hybridoma culture supernatant as described before. Subsequently, equivalents of 5×10^4 cells were injected into lethally irradiated recipients for determination of the CFU-S content. Injected unlabeled bone marrow cells served as control (100% recovery).

In the complement mediated cytotoxicity assay, 10^6 unseparated bone marrow cells were incubated with hybridoma culture supernatant followed by incubation (30 min, 37°C) with an appropriate dilution of a noncommercial batch of guinea pig complement (1:10 dilution in HH, 0.5 ml/10^6 cells).

The cells were washed once with HH. The pelleted cells were then resuspended at a concentration equivalent to 1×10^5 cells/ml HH. Subsequently, lethally irradiated recipients were injected with 5×10^4 cells for determination of the CFU-S content. Bone marrow cells incubated with HSA, instead of MCA, and complement were injected and served as reference (100% recovery) for the determination of the expression of the antigenic determinant recognized by the MCAs on CFU-S. Mice were killed after 8, 10 and 12 days for spleen colony counts (Table 4.3).

MORPHOLOGICAL DIFFERENTIATION

Cell suspensions from bone marrow (subpopulations) and other organs and tissues were deposited (750 rpm, 5 min, Shandon Cytospin-2) onto glass slides (10^4 cells/slide in 50 µl HSA), previously coated by centrifugation of 50 µl HSA. The microscope slides were air-dried and subsequently stained with May/Grünwald Giemsa staining solution according to standard procedures. Stained slides were examined using an inverted microscope. Of each slide 100 cells were counted.

CHAPTER 3

SETTING UP THE FUSION SYSTEM

INTRODUCTION

Köhler and Milstein (1975) first reported the derivation of immortalized cell lines (hybridomas) capable of unlimited production of monoclonal antibodies (MCAs) of desired, predefined specificity. Since that time many people have adapted the hybridoma technology and the technique has moved into almost every area of biomedical research (Goding, 1980; Galfre and Milstein, 1982).

The great advantage of the hybridoma technology is that unlimited quantities of homogeneous, exquisitely specific antibodies can be produced, even if the immunizing antigen is not pure, or is present in only low amounts, for instance, on minor cell populations (Goding, 1980; Köhler, 1986; Galfre et al., 1977; Springer et al., 1978a; Springer et al., 1979).

The technique is based on the fusion between cells from an in vitro adapted, enzyme deficient myeloma cell line, and spleen cells from an immunized animal. Fusion is reached by incubation of the myeloma-spleen cell mixture for a short time in the presence of a fusing agent like polyethylene glycol (PEG, Pontecorvo, 1976). Hybrids between myeloma and spleen cells can be selected from the two parental components as the only cells that actively multiply in selective medium (for details, see later section). Once the hybrids are growing well, the cultures are tested for the production of the desired antibody. Individual clones, that secrete the desired antibody, can be selected and isolated from the positive cultures by replating and can be expanded either in vivo or in vitro. All antibodies produced by an isolated clone have the same unique specificity and are thus of monoclonal origin. A general scheme for the production of MCAs is shown in Figure 3.1.

The advent of hybridoma technology has proven to be very important and successful in defining cell surface antigens on cells belonging to the immunohaemopoietic system of mice and man (Goding, 1980; Galfre and Milstein, 1982; Ledbetter and Herzenberg, 1979; Griffin et al., 1984; Hogarth et al., 1980; Lachmann et al., 1980). In the mouse, more than fifty different antigenic complexes on haemopoietic cells have been described by means of MCAs raised by allogeneic (mouse) or xenogeneic (rat) immunization (Shen, 1981; Hogarth and McKenzie, 1981; Morse III et al., 1987). Many of the antigens recognized by these MCAs are differentiation antigens (DAG). DAG are cell surface antigens of which the expression can be turned on and off during cellular differentiation with regard to the specific physiological functions at that differentiation stage. DAG are either specific for individual cell types, at a particular differentiation stage, or common to sets or subsets of cells belonging to a certain cell lineage (Wang and Krüger, 1985; Swirsky et al., 1983; Williams et al., 1979). MCAs against DAG have contributed to the identification and phenotyping of leukaemias, and are used as probes in the study of normal haemopoiesis (Chatenoud, 1986; Cote et al., 1983;

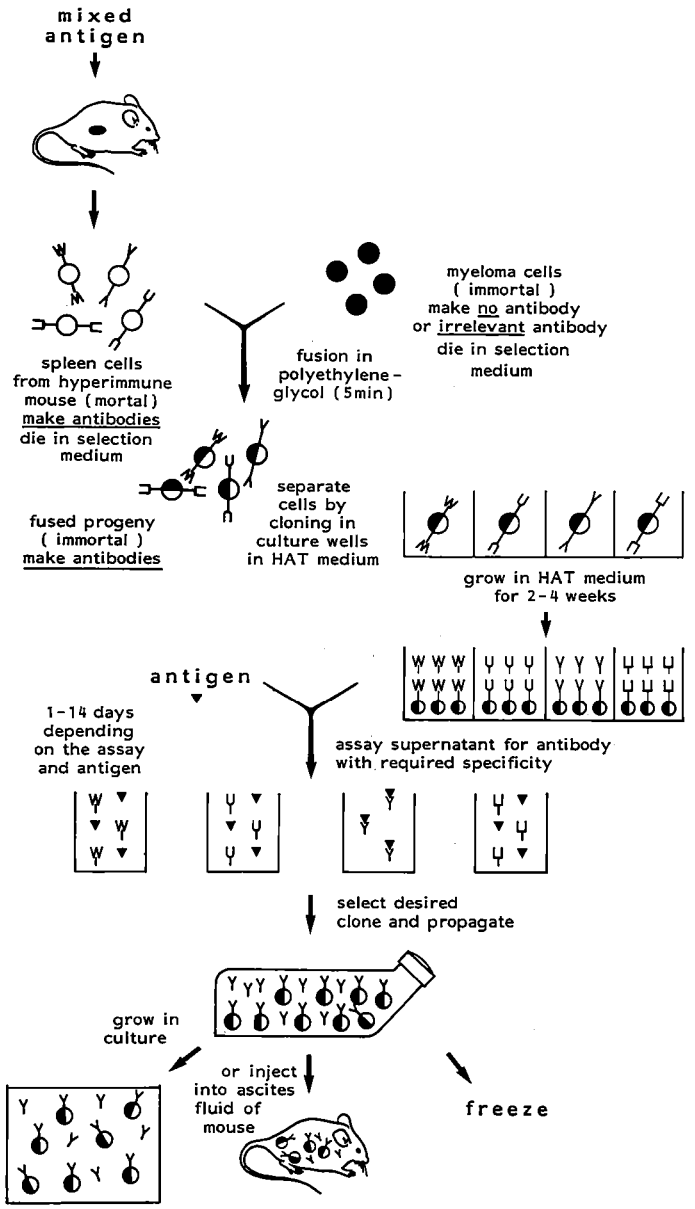


Figure 3.1:
Schematic representation of the general procedures for monoclonal antibody production. Adapted from Campbell (1984).

Hokland et al., 1986; Janossy et al., 1978; Takei, 1984; Griffin et al., 1984). From these studies it was clear that most antigenic surface markers are not specific for one single type of leukaemia, or one individual subset of normal haemopoietic cells. In the mouse and human haemopoietic system, MCAs have been described that react with antigenic determinants present on pluripotent haemopoietic stem cells (PHSC) and early committed progenitor cells, but none of these MCAs react exclusively with these cell types. All MCAs reactive with stem cells or progenitor cells are also reactive with other, more mature cells. Many of these recognized antigens are DAG (Griffin et al., 1984; Springer et al., 1978a; 1978b; 1979; Hogarth et al., 1980; Shen, 1981; Davignon, 1981; Kürzinger et al., 1981; Trowbridge, 1978; Gaulton et al., 1985; Chan and Takei, 1986; and many others).

In this chapter, aspects concerning the production of MCAs which would be exclusively specific for antigenic determinants on murine PHSC or early committed progenitor cells will be discussed. First, factors determining the choice of the animals to be immunized and the myeloma fusion partners will be discussed. Furthermore, attention will be payed to the importance of the purity (content of stem cells) of the immunization suspensions and the route and dose of immunization with regard to the frequency of stable antibody producing hybridomas. Finally, some of the results will be discussed.

ANIMALS USED FOR IMMUNIZATION

The first developed and still most commonly used system for the production of MCAs employs spleen cells of (hyper) immunized mice that are fused with mouse myeloma cells (Köhler and Milstein, 1975; Ware et al., 1984). The choice of mice was mainly based on the availability of a number of good mouse myeloma cell lines. This will be discussed in a later section.

Many cell surface antigens of the mouse have been discovered by immunization of mice with cells from mice belonging to other strains (allogeneic immunization). A disadvantage of allogeneic immunization is that strong immune responses can be elicited only against polymorphic antigenic systems like for instance antigens of the major histocompatibility complex (MHC) and that only a minority of cell surface antigenic determinants are subject to polymorphic variation (Springer, 1980; Springer et al., 1978b). Against monomorphic antigenic molecules, only weak immune responses or no responses at all are elicited by means of allogeneic immunization (Köhler and Shulman, 1978; Springer et al., 1978b).

Xenogeneic immunization has the advantage over allogeneic immunization that responses can be elicited against both polymorphic and monomorphic determinants (Omary et al., 1980). In xenogeneic immunizations most molecules are potentially antigenic, because many commonly occurring homologous proteins have diverged significantly during the course of evolution. The difference between homologous proteins from different species will be greater than between the polymorphic variants in one species. Therefore, the xenogeneic antibody response will be stronger (expressed as the antibody concentration in serum) and directed against a much wider range of

antigenic determinants than the allogeneic immune response (Springer et al., 1978a; 1978b; Springer, 1980). For this reason, in this study rats were used for xenogeneic immunization. In addition, it has been reported that in many cases immunized rats showed stronger immune responses than mice (Westerwoudt, 1985).

Significant differences in the strength of the immune response between animals of different inbred strains have been reported upon challenging with the same immunogen (Spitz et al., 1984). Springer et al. (1978b) found a six-fold difference between the strength of the immune response of rats from the DA and AO strain after immunization with the same antigen. In order to establish which rat strain gave the strongest immune response, rats of three different strains (Table 3.1) were intravenously (i.v.) immunized (lateral tail vein) with 10^7 nucleated bone marrow cells of BC3 mice. At the peak of antibody production, i.e., ten days after immunization, the rats were bled (orbital puncture), and, subsequently, serum was isolated and inactivated (56°C, 30 min). Appropriate dilutions of the sera were incubated with BC3 bone marrow cells, stained and analyzed on a FACS-II (Chapter 2). The percentage of fluorescent cells was determined and regarded as a measure of the strength of the immune response. Results are shown in Figure 3.2. A clear difference in the percentage of antibody binding cells is observed between the sera of BN rats and those of SD or WAG rats. BN rats showed a stronger immune response than SD and WAG rats.

Table 3.1

RAT STRAINS USED IN THIS STUDY

rat strain	abbreviation	histocompatibility antigen haplotype		
		Rt-1 (Ag-B)	Rt-2 (Ag-C)	Rt-3 (Ag-D)
BN/BiRij	BN	n	a	b
SD/Rij	SD	u	b	b
WAG/Rij	WAG	u	b	b

Only female rats, 8-16 weeks of age were used.

Because the strength of the antibody response appears to be the critical factor in the production of specific antibody producing hybridomas (Springer et al., 1978b; Galfre and Milstein, 1982; Kilmartin et al., 1982; Lane, 1985), it was decided to use BN rats for immunization.

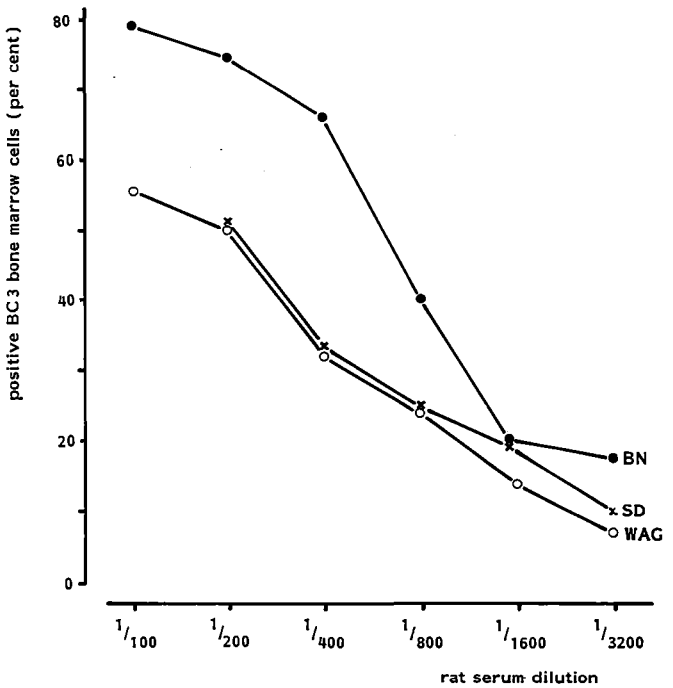


Figure 3.2:
Comparison of the immune response of three different rat strains. Female BN, SD and WAG rats were injected (i.v.) with 10^7 nucleated murine (BC3) bone marrow cells. After 10 days sera were collected (orbital puncture) and tested for reactivity with BC3 bone marrow cells on a FACS as described in Chapter 2.

MYELOMA CELL LINES

The success of a fusion, reflected in the number of growing hybridomas producing the specific MCA, is not only dependent on the immune state of the animal from which the spleen is taken but also on the myeloma cell line used for fusion (Reading, 1982; Kearny et al., 1979). Myeloma cells can be cultured indefinitely because a myeloma is a transformed immortalized tumour of the immune system (Milstein, 1986).

There are three major considerations in choosing a specific myeloma cell line as fusion partner. First, the expression of a drug resistance gene for selection of unfused myeloma cells. Second, the presence or lack of production of heavy and light chains of immunoglobulin (Ig) molecules by the

myeloma cells. Third, the number and stability of the hybridomas formed by fusion with the myeloma cells.

The myeloma lines used in this study (Table 3.2) were all selected for resistance to the purine analogue 8-azaguanine. The resistant cells lack functional activity of the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), required in the salvage pathways of nucleotide biosynthesis (Littlefield, 1964). The HGPRT deficient cells can still synthesize the necessary bases for RNA and DNA synthesis via de novo pathway. In 1962, Szybalski et al. described the selective HAT-medium containing hypoxanthine, aminopterin and thymidine. Aminopterin (an analogue of folic acid) blocks de novo synthesis of purines and pyrimidines. To survive in the presence of aminopterin, cells must be able to synthesize these nucleotides by utilizing an exogeneous source of hypoxanthine and thymidine (provided in HAT-medium) via the salvage pathways. Myeloma cells die in HAT-medium, since they lack HGPRT activity and cannot use the salvage pathways. Normal spleen cells have functional HGPRT activity and therefore can survive in HAT-medium. However, the normal parental spleen cell is terminally differentiated and has a finite short life span. In HAT-medium unfused myeloma and spleen cells and hybrids between two or more myeloma cells and between two or more spleen cells will die within a few days. Only hybrids between myeloma cells (providing tumour cell growth properties) and spleen cells (providing HGPRT activity and the genetic information for the required antibody) are able to grow in HAT-medium (Littlefield, 1964).

Table 3.2

MYELOMA CELL LINES USED IN THIS STUDY

myeloma line	abbreviation	origin species/strain	Ig-chain expression
SP2/0 Ag14	SP2	mouse/BALB/c	none
YB Ag1-2.3	Y3	rat/Lou/M/Wsi	kappa
YB 2/0	Y0	rat/Lou/M/Wsi	none

Nowadays, HAT-medium is the most commonly used selective medium in hybridoma technology. Another selective medium consisting of hypoxanthine and azaserine has been described. The selective mechanism exploits deficiency of the enzyme adenine-phosphoribosyltransferase (APRT) in myeloma cells. Azaserine blocks the pathway of de novo synthesis of purine nucleotides of 8-azaguanine resistant cells, but not of pyrimidine nucleotides and consequently thymidine need not be included in the medium (Foung et al., 1982). Selection with azaserine is reported to result in higher frequencies of stable antibody producing hybridomas than selection with

aminopterin which is photosensitive and highly toxic (Campbell et al., 1986; Horenstein et al., 1987; Westerwoudt, 1985). Both selection media were used in the present studies.

Hybridomas codominantly express the Ig-molecule chains of both parental cells. If the myeloma line used expresses both heavy and light chains of an Ig-molecule, the hybrids will express four chains, two of each parent. In this case, sixteen combinations of the four different Ig-chains are possible, resulting in ten different Ig-molecules. Just one out of the sixteen combinations will be made up of the desired heavy and light chains of the parental spleen cell (Shulman et al., 1978). The affinity and frequency of MCAs with the desired specificity obtained in fusions between Ig-molecule producing myeloma cells and spleen cells will be lower than in fusions in which mutant myeloma cells are used which only express Ig-light chains. The amount of irrelevant antibodies in the latter kind of fusions will be lower than in the former case. At least twenty five percent of the produced antibodies will be made up of the desired heavy and light chains of the parental spleen cells (Galfre and Milstein, 1982). Mutant myeloma cell lines that do not express any Ig-chain, will give rise to hybridomas which only express the antibody of the parental spleen cell. The use of such (non-producer) myeloma lines is recommended by many investigators, because they are obviously the most suited lines for the production of stable hybridomas, secreting (only) the desired, specific MCAs (Galfre and Milstein, 1982; Hudson and Hay, 1981; Lemke et al., 1979; Milstein, 1986; Ozata and Sachs, 1981; Reading, 1982; Stähli et al., 1980).

The first established myeloma lines suited for fusion were of mouse origin. Most, if not all, murine myeloma lines known today, are derived from the MOPC BALB/c plasmacytoma (Potter, 1972; Clark et al., 1983; Köhler and Milstein, 1976). In the present study, the widely used non-producer line SP2 was employed (Shulman et al., 1978). It has been shown that this line is well suited for fusions with spleen cells of mice, rats, humans, frogs and birds, but stable hybrids are only rarely obtained in other than rodent-rodent fusions (Galfre and Milstein, 1982; Hudson and Hay, 1981; Lemke et al., 1979; Köhler and Shulman, 1978; Misra et al., 1981; Ozata and Sachs, 1981; Reading, 1982; Stähli et al., 1980).

In contrast with mouse myeloma cell lines, rat myeloma cell lines are comparatively rare. Only three rat myeloma cell lines suited for fusions with both rat and mouse spleen cells have been described (Kilmartin et al., 1982; Galfre et al., 1979; Bazin, 1982). Two of the three rat myeloma lines were used in the present studies. The kappa-light chain producing line Y3 was the first line used for fusions (Galfre et al., 1979) and is still commonly used. From this line a non-producer mutant line called Y0 was derived (Kilmartin, 1982). All rat myeloma cell lines are derived from spontaneously occurring ileocecal immunoplasmacytomas in rats of the Lou strain (Bazin et al., 1973).

There is no consensus of opinion in the literature about the best choice of a myeloma line for the production of stable rat-anti mouse antibody producing hybridomas, except that, if possible, non-producer myeloma lines should be used.

In general, the use of myeloma cells of the same species and, when possible, of the same strain as the immunized animal is recommended,

because in xenogeneic fusions the hybridomas tend to lose chromosomes more rapidly and more frequently than in intraspecies fusions, resulting in lower frequencies of stable antibody producing hybridomas (Bazin, 1982; Clark et al., 1983; Galfre and Milstein, 1982; Galfre et al., 1979; Goding, 1980; Hokland et al., 1986; Köhler and Shulman, 1978; Milstein, 1986; Nowinski et al., 1980; Weiss and Green, 1967). On the other hand, many investigators successfully used interspecies fusions between rat spleen cells and mouse myeloma cells, for the production of stable antibody producing hybridomas (Misra et al., 1981; Ledbetter and Herzenberg, 1979; Lemke et al., 1979; Springer et al., 1978b; Takei, 1984; Shen, 1981; Stern et al., 1978; Koeslter et al., 1984; Trowbridge, 1978; Gaulton et al., 1985; Kearny et al., 1979; Hemmi et al., 1985; and many others).

FUSION EXPERIMENTS WITH DIFFERENT MYELOMA CELL LINES

To find out which of the available myeloma cell lines resulted in the highest percentage of growing and stable antibody producing hybridomas, fusion experiments were performed to compare the quality of the three myeloma cell lines.

A total of eight fusion experiments were performed between spleen cells of the same rat and cells of the three myeloma cell lines. In five of the experiments no growing hybrid clones could be detected with any of the myeloma cell lines. The results of the experiments in which growth was detectable are shown in Table 3.3. The fusion frequency (percentage of growing hybridomas) with both allogeneic (Y3 and Y0) and xenogeneic (SP2) myeloma lines was very low. In fusions in which Y3 was used no growing hybridoma clone was observed in any of the 1020 seeded wells. Only in one fusion experiment in which Y0 was used as fusion partner, growing hybridomas were observed in a low frequency. The highest fusion frequency was obtained in xenogeneic fusions with SP2 cells. In all three fusion experiments with this cell line, growth was observed. The fusion frequency showed a great variability, as was also reported by others (Lemke et al., 1979; Abrams et al., 1983; Kilmartin et al., 1982), and was rather low.

The frequency of initially antibody producing hybridomas in the fusion with Y0 was 100%, while the mean frequency of initially antibody producing hybridomas in the fusions with SP2 was 43%. These percentages are in agreement with those observed by Clark et al. (1983). They reported that the proportion of hybridomas expressing antibodies was greater than 90% in rat x rat fusions, 60% in mouse x mouse fusions (using a variety of myeloma lines) and somewhat lower than 60% in rat spleen x mouse myeloma fusions.

In both intra- and interspecies fusions the initially producing hybridomas were very unstable. In the fusion with Y0 only 5% (one clone) could be rescued by subcloning. Ninety five % of the initially positive clones either stopped growing (they probably lost the chromosome containing the HGPRT enzyme gene), or ceased antibody production, upon further growth and subcloning. In just one of the three fusion experiments with SP2, a low percentage (6%, 2 clones) of the initially antibody producing hybridomas appeared to be stable clones. All the other initially positive hybridomas (in all three experiments) were lost due to either cell death or loss of antibody production.

Table 3.3

FUSIONS IN WHICH DIFFERENT MYELOMA CELL LINES WERE USED

fusion	wells containing hybrids/ total wells seeded* no.	%	antibody initial positive hybrids/ total hybrids no.	%	stable positive hybrids/ total positive hybrids no.	%
BN x SP2	6/960	1	5/6	83	0/5	0
x Y0	20/480	4	20/20	100	1/20	5
x Y3	0/660	0				
BN x SP2	83/360	23	15/83	18	0/15	0
x Y0	0/360	0				
x Y3	0/360	0				
BN x SP2	113/1260	9	32/113	28	2/32	6
x Y0	0/720	0				

*1-2.5 x 10^5 spleen cells per well. Spleen cell : myeloma cell ratio: 2:1.
Immune spleen cells were fused with the myeloma cell lines as described in Chapter 2. Initial positive hybrids represents the number of wells in which the hybridoma(s) produced antibody as determined in the initial screening round using the FACS (Chapter 2).
Stable positive hybrids: hybridomas of which the supernatant contained antibody in 4-5 independent sequential screening assays.

In this study, no difference in the frequency of stable antibody producing hybridomas was found between xenogeneic (SP2) and allogeneic (Y0) fusions. This is in agreement with data in the literature indicating that fusions between rat spleen cells and mouse myeloma cells cab be equally successful as rat x rat fusions (Köhler and Shulman, 1978; Ledbetter and Herzenberg, 1979; Misra et al., 1981; Hemmi et al., 1985; Bazin, 1982; Westerwoudt, 1985).

Because of the slightly higher fusion frequency, the similar frequency of stable antibody producing hybridomas, and the fact that the nonadherent SP2 cells are easier to handle than the adherent Y0 cells, it was decided to use SP2 myeloma cells in further fusion experiments. Results of these fusions are discussed in a later section.

ANTIGENICITY AND PURITY OF THE IMMUNOGEN

Besides the choice of the animal used for immunization and the myeloma cell line used as fusion partner, another major factor that influences the success of a fusion is the immunogen itself and the schedule and dose of immunization (Dresser, 1986; Spitz et al., 1984).

It is reported that, in general, cell surface antigens are highly immunogenic when presented on intact cells (Goding, 1980; Taggart and Samloff, 1982; Westerwoudt, 1985). Cell surfaces are complex mosaics of many different immunogenic glycoproteins and glycolipids. Some of these determinants are immunodominant and give strong immune responses even when present in only trace amounts, while others are minor antigens and hardly elicit an immune response (Galfre and Milstein, 1982). Therefore, it is rather difficult (especially in xenogeneic immunizations) to obtain highly specific MCAs which recognize individual poorly represented cell surface molecules (Springer et al., 1978a; 1978b; Stern et al., 1978).

One of the great advantages of the hybridoma technology is that each hybrid produces a single MCA specific for a single antigenic determinant, regardless the complexity and the impurity of the antigen or the overall immune response of the spleen cell donor. The idea exists that all specificities can be rescued by cell fusion methods if an immune response can be elicited (Goding, 1980; Köhler, 1986; Stern et al., 1978; Lachmann et al., 1980). An example of the irrelevance of the purity of the immunogen and the usefulness of the hybridoma technique for the identification of antigens present in low amounts on minor subpopulations of cells is reported by Springer et al. (1979). They obtained a MCA (M1/70) specific for an antigenic determinant (MAC-1) only present on macrophages, whereas macrophages comprised only 3% of the cells present in the immunization suspension.

Although purity of the immunogen per se is not necessary, it is obvious that the purer the immunogen, the higher the chance of obtaining immunogen specific MCAs will be. The incidence of CFU-S in normal murine bone marrow is very low (less than 1%; Lahiri et al., 1970; Lord and Spooncer, 1986; Van Bekkum et al., 1979) and therefore (some) purification of CFU-S in the immunization suspensions to be used, seemed necessary to enhance the likelihood to obtain haemopoietic stem cell-antigen specific MCAs.

PURIFICATION OF CFU-S

Visser et al. (1984) described a method to highly purify murine haemopoietic stem cells: Normal bone marrow cells are separated by means of discontinuous density gradient centrifugation. The low density cells are stained with the lectin WGA conjugated to the fluorochrome FITC, and the brightly fluorescent cells with medium forward light scatter and low perpendicular light scatter intensities are sorted of a FACS-II. After removal of the bound WGA-FITC conjugates, the cells are labelled with α-H-2K^k antibodies visualized with a fluorescent conjugate, and, the most brightly fluorescent cells are sorted (for details, see Chapter 2). With this method approximately 10^5 cells of a suspension that is 135-fold enriched for day-12 CFU-S and

53-fold enriched for day-8 CFU-S, can be isolated in one day from normal bone marrow.

A less complicated way to enrich for CFU-S is treatment of mice with 5-Fluorouracil (FU). Van Zant (1984) showed that treatment with FU resulted in an enrichment of late appearing CFU-S, reaching its maximum (four-fold) seven days after treatment (day-7-FU). In addition, the presence of a very primitive cell type, i.e., pre-CFU-S in FU treated bone marrow was suggested by Hodgson and Bradley (1979). Thus, day-7-FU bone marrow seemed a suitable suspension for immunization, especially when the low density cell fraction was used (see later section). The advantage of the use of the low density cell fraction in day-7-FU bone marrow for immunization over the suspension purified with the method described by Visser et al. (1984), is that the former can be obtained more easily: Approximately 10^7 day-7-FU low density cells can be isolated in about half a day.

CELL SUSPENSIONS USED FOR IMMUNIZATION

In the initial fusions (Table 3.3) rats were immunized with the low density bone marrow cell fraction of mice that were treated seven days before with FU (Chapter 2). These suspensions will be further indicated as day-7-FU-MA. It was expected that in this suspension relatively high amounts of late appearing CFU-S would be present, due to FU treatment, and because in the low density cell fraction of untreated normal bone marrow (NBM), day-8 and day-12 CFU-S are five-fold enriched (Visser et al., 1984). There are no indications that the density of CFU-S from FU-treated bone marrow is different from the density of CFU-S in normal marrow. However, it appeared that the day-7-FU-MA suspension contained an unexpected low frequency of stem cells (Table 3.4). Day-8 CFU-S were only two-fold enriched which was not unexpected since it is known that FU preferentially kills early appearing CFU-S, but the poor (four-fold) enrichment of day-12 CFU-S could not be explained.

The antibodies produced by the hybridomas obtained from the fusions in which day-7-FU-MA was used as immunogen all reacted with most of the nucleated bone marrow cells, which might be explained by the low enrichment factor of CFU-S in this suspension. Based on these results it was decided to use immunization suspensions that were more enriched for CFU-S.

Selection of the cells present in day-7-FU-MA that bound the highest amounts of WGA (day-7-FU-MA-WGA$^+$) resulted in a ten-fold enrichment of day-12 CFU-S and a four-fold enrichment of day-8 CFU-S (Table 3.4). Although the frequency of CFU-S in this fraction was 2-3 times higher than in the day-7-FU-MA fraction, the enrichments were still unsatisfactory low. Because of the low enrichment factors obtained with FU treated bone marrow, and because in the method to highly purify CFU-S, untreated NBM is used, it was decided to use only untreated NBM derived suspensions for immunizations.

Selection of the cells in the low density cell fraction which bound the highest amounts of WGA (NBM-MA-WGA$^+$) resulted in a six-fold enrichment of day-8 CFU-S and an eighteen-fold enrichment of day-12 CFU-S (Table 3.4). Although the enrichment factors were higher than in the comparable fraction

Table 3.4

BONE MARROW SUSPENSIONS USED FOR IMMUNIZATION OF RATS

suspension	enrichment factor day-8 CFU-S	day-12 CFU-S
day-7 FU-MA [1]	2	4
day-7 FU-MA-WGA$^+$ [2]	4 (3-5)	10 (9.8-10.2)
NBM-MA-WGA$^+$ [3]	6	18
NBM-MA-WGA$^+$/α-H-2K^{k+} [4]	35 (11-108)	71 (39-157)

Figures represent the mean enrichment factor (and the range of enrichment) of CFU-S with regard to the number of CFU-S in unseparated normal bone marrow suspensions. The average number of day-8 CFU-S and day-12 CFU-S in unseparated normal bone marrow was 28 ± 3, respectively 31 ± 4.

[1]day-7 FU-MA: low density cell fraction after density gradient centrifugation (MA) from bone marrow of mice treated 7 days before with 5-fluorouracil (day-7 FU).

[2]day-7 FU-MA-WGA$^+$: WGA positive blast cell fraction of day-7 FU-MA bone marrow sorted on a FACS-II as described in Chapter 2.

[3]NBM-MA-WGA$^+$: WGA positive low density blast cell fraction from normal bone marrow (NBM).

[4]NBM-MA-WGA$^+$/α-H-2K^{k+}: α-H-2K^k positive, WGA positive low density blast cell fraction from NBM.

of FU treated bone marrow, the enrichment factors were still not as high as those reported (Visser and Bol, 1981; Visser et al., 1984). Therefore, further purification of the NBM-MA-WGA$^+$ suspensions was performed. Selection of the cells that bound the highest amounts of α-H-2K^k antibodies (NBM-MA-WGA$^+$/α-H-2K^{k+}) resulted in a mean enrichment factor of 71 for day-12 CFU-S and 35 for day-8 CFU-S with regard to NBM (Table 3.4).

Based on the assumption that the seeding efficiency of CFU-S in this purified suspension was 10% for day-12 CFU-S and 5% for day-8 CFU-S (Visser et al., 1984), it was calculated that 35% of the injected cells were day-12 CFU-S and 47% were day-8 CFU-S. Data by Wolf and Priestly (1984) and Magli et al. (1982) indicate that at least half of the number of CFU-S present at twelve days after transplantation are already present at eight

days after transplantation. Thus, the mean frequency of CFU-S in the immunization suspension was about 50 percent. In some experiments the frequency was higher, in other experiments lower, due to the variability in the enrichment factors of CFU-S between different experiments as was also found by Visser et al. (1984).

IMMUNIZATION REGIMENS

There exists no standard immunization protocol for rats or mice. A typical immunization regimen for rats consists of an intraperitoneal (i.p.) priming dose of 1-5 x 10^7 cells, followed by an i.v. boost with the same or somewhat higher cell dose (Goding, 1980; Spitz et al., 1984).

According to this immunization regimen, highly purified bone marrow suspensions could not be used for immunization, because not enough stem cells can be purified for conventional i.p. or i.v. immunization of rats (see previous section). In the comparative fusions (Table 3.3) rats were primed (i.p.) and boosted (i.v.) with approximately 10^7 day-7-FU-MA cells. Because of the importance to use highly purified immunization suspensions, alternative ways for immunization had to be found, which allow to use less cells.

In vitro immunization had been reported to be very successful for soluble antigens (Raymond and Suh, 1986; Van Ness et al., 1984; Reading, 1982). An advantage of in vitro over in vivo immunization is that it is possible to produce MCAs against highly conserved or weakly immunogenic determinants and comparatively little antigen is required. A disadvantage, however, is that complex culture conditions are needed and that it is difficult to introduce insoluble antigens (like cell surfaces) into the in vitro stimulation reaction. For our purpose in vitro immunization has no advantage because the same number of cells, i.e. 10^7 cells are needed as for in vivo immunization. This high amount of cells excludes the possibility of in vitro immunization with highly purified stem cell suspensions.

Another way of immunization is immunization into the mesenterial lymph nodes (Raymond and Suh, 1986; Rits et al., 1986). This method was reported to be successful for the production of MCAs against soluble antigens available in only limited amounts.

A third method of immunization suitable for the production of MCAs against limited amounts of both soluble and particulate immunogens is direct intrasplenic (i.s.) injection (Nilson et al., 1983, 1987; Spitz et al., 1984; Svalander et al., 1987). For i.s. immunization of mice and rats about 2.5 x 10^5 cells are sufficient to evoke a strong immune response. Thus, by means of i.s. immunization, it might be possible to immunize rats with highly enriched bone marrow suspensions.

It was first tested in BN rats if i.s. immunization indeed resulted in a stronger immune response in comparison with conventional i.p. and i.v. injection. Rats were immunized with 10^5 unseparated normal bone marrow cells of BC3 mice. Ten days after immunization sera were collected and analyzed in the same way as described earlier. It can be seen in Fig. 3.3 that i.s. immunization indeed resulted in a stronger immune response than i.p. or i.v. immunization. Therefore, it was decided to use i.s. immunization in further experiments.

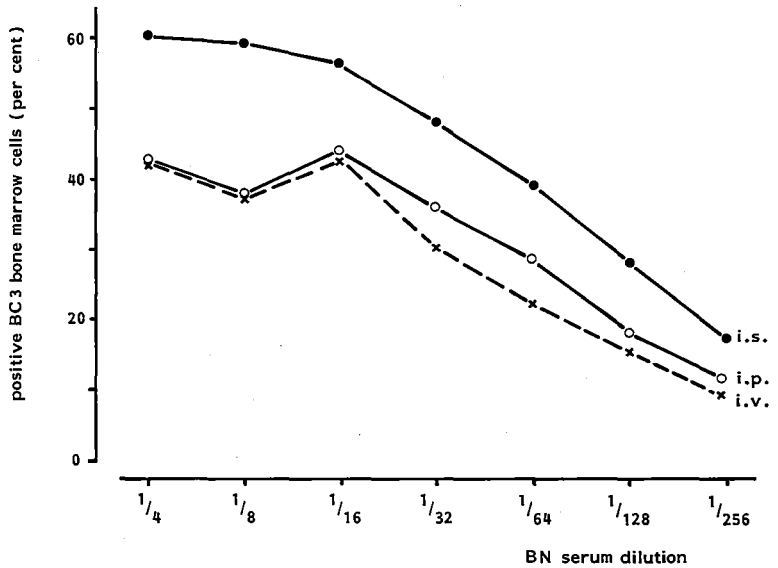


Figure 3.3:
Comparison of the strength of the immune response of BN rats after injection of 10^5 BC3 bone marrow cells via different routes. Rats were immunized by i.s., i.p. and i.v. injection. After 10 days the sera were collected (orbital puncture) and assayed on a FACS as described in Chapter 2.

FUSIONS IN WHICH THE ANIMALS WERE IMMUNIZED AND BOOSTED BY INTRASPLENIC INJECTION

Since it was shown that for i.s. immunization approximately 10^5 cells are sufficient to evoke a strong immune response (Figs. 3.3 and 3.4), it was decided to immunize rats via this route with as many cells of as highly as possible enriched bone marrow suspensions (Table 3.4). It was not always possible to immunize rats with the highly purified NBM-MA-WGA$^+$/α-H-2K^{k+} cells (Table 3.4). In each step of the purification method described by Visser et al. (1984) cells are lost. When the yield of WGA positive cells was high enough, the suspension was further purified with α-H-2K^k antibodies. If the amount of WGA positive cells was not high enough, this cell suspension was used as such for immunization.

After i.s. immunization and boosting, growing hybridomas were obtained in a relative high frequency (Table 3.5). The mean fusion frequency was at least 60%, which is in agreement with frequencies in rat x mouse fusions obtained by others (Lachmann et al., 1980; Misra et al., 1985). This high fusion frequency indicated the suitability of the employed immunization

conditions.

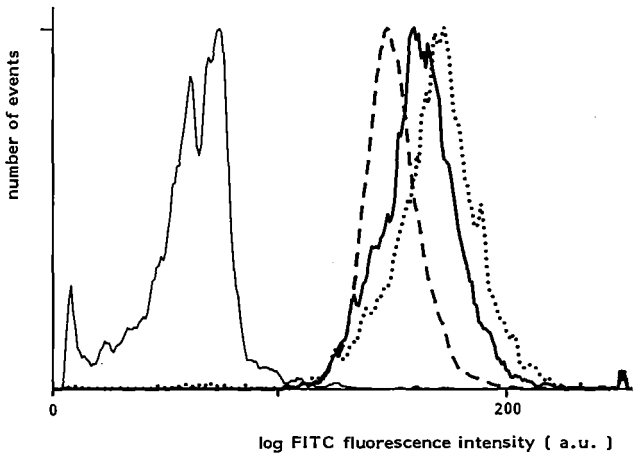


Figure 3.4:
Immune response of a BN rat after i.s. immunization with 50,000 NBM-MA-WGA^{+}/α-H-$2K^{k+}$ cells. After 4 days the spleen was removed and used in a fusion experiment. At the same time serum was collected and assayed for reactivity with BC3 NBM cells on a FACS. The fluorescence distribution of bone marrow samples treated with serum dilutions (dotted line, 0x; thick line, 20x; broken line, 80x) was compared with that of bone marrow cells incubated with GARA/FITC only (thin line).

The frequency of initially antibody producing hybridomas was rather low (one exception) and the frequency of stable antibody producing hybridomas showed a large variability. In two of the four fusions listed in Table 3.5 the frequency of stable producing clones was relatively high, while in the two other experiments it was about ten times lower. As stated earlier, the immune state of the animals can make all the difference between success and failure of a fusion. In this study, i.s. immunization resulted in a mean higher frequency of stable antibody producing hybridomas than i.p. or i.v. immunization (compare Table 3.3 and Table 3.5).

Although every fusion in Table 3.5 resulted in stable antibody producing hybridoma clones, many initially producing clones were lost upon further growth. The mean frequency of stable antibody producing hybridomas in these four experiments was 25% of the number of initially producing hybridomas. This percentage is in agreement with that found by others (Van Snick et al., 1985; Clark et al., 1983; Springer et al., 1978b). According to Goding (1980) it is realistic to expect 50-70% of all initially producing hybridomas will be lost.

Table 3.5

RESULTS OF FUSIONS IN WHICH THE RATS WERE I.S. IMMUNIZED AND BOOSTED

rat strain	immunization/boost suspension and cell number	wells containing hybrids/ total wells seeded number	%	initial positive hybrids/ total hybrids number	%	stable positive hybrids/ total positive hybrids number	%
SD	i. day-7 FU-MA-WGA$^+$ 2.5×10^5 b. day-7 FU-MA-WGA$^+$ 7.5×10^5	648/1080	60	45/648	7	1/45	2
BN	i. day-7 FU-MA 4.5×10^6 b. day-7 FU-MA-WGA$^+$ 9.6×10^5	1350/1800	75	288/288[c]	100	5/10[d]	50
BN	i. day-7 FU-MA-WGA$^+$ 6×10^5 b. NBM-MA-WGA$^+$ 2.5×10^5	356/1380[b]	26	29/356	8	2/29	7
BN	i. NBM-MA-WGA$^+$/α-H2K^{k+} [a] 5×10^4	864/1080	80	27/864	3	12/27	44

[a] in this fusion the rat was only primed and not boosted.
[b] only the fastest growing hybrids were tested.
[c] 288/1350 growing hybrids were tested.
[d] 10/288 initially positive hybrids were further grown, 5/10 appeared to be stable antibody producing hybridomas.

See for number of spleen and myeloma cells seeded per well legend Table 3.3. Spleen cells of immune SD or BN rats were fused with SP2 myeloma cells as described in Chapter 2. Rats were immunized (i) or boosted (b) with the indicated cell suspensions and numbers.

DISCUSSION

Pluripotent haemopoietic stem cells can either renew themselves (proliferate) or give rise to all mature blood cell types (differentiate) under the influence of specific growth factors and according to the haemopoietic situation. PHSC specific antigens are most likely involved in the control of these two important processes. It has been reported that only a minority of cell surface antigenic determinants are subject to polymorphic variation (Springer, 1980; Springer et al., 1978b). Therefore, it seems reasonable to assume that these processes in different mouse strains are regulated by monomorphic, or a combination of both monomorphic and polymorphic antigenic molecules, rather than by just polymorphic molecules. Therefore, rats were used for immunization, because xenogeneic immunization leads to strong immune responses against both polymorphic and monomorphic antigens.

Of the available and tested inbred rat strains (Table 3.1), BN rats gave the strongest immune response upon challenging with 10^7 normal murine bone marrow cells (Fig. 3.2). It was also planned to use rats belonging to the Lou strain, because the two available rat myeloma cell lines (Table 3.2) were derived from this rat strain, and fusion between spleen and myeloma cells of the same strain would favour the number and stability of antibody producing hybridomas (Galfre and Milstein, 1982; Hokland et al., 1986). However, Lou rats were not sufficiently available throughout this study. The lack of Lou rats seemed not insurmountable, because successful fusion between myeloma cells of Lou origin and spleen cells of other rat strains has been reported by a number of investigators (Leong et al., 1986; Chan and Takei, 1986; Campbell et al., 1986; Misra et al., 1981; Hemmi et al., 1985).

Although many investigators obtained very good results with xenogeneic fusions between rat spleen cells and mouse myeloma cells, it was surprising that fusions with Y3 and Y0 were less successful than with SP2 (Table 3.3). It has been reported that Y3 and Y0 myeloma cells are very suitable for the production of rat x rat hybridomas (Clark et al., 1983; Kilmartin et al., 1982) and that rat x rat hybridomas are characterized by a great stability early after fusion, whereas loss of antibody expression is hardly observed (Hemmi et al., 1985; Lachmann et al., 1980; Hale et al., 1985; Galfre and Milstein, 1982; Pierres et al., 1982). It was expected that fusion with Y0 would result in a somewhat higher percentage of stable antibody producing hybridomas than fusion with Y3, because the former is a non-producer myeloma line, while Y3 produces kappa-light chains. In fusions with Y3 also irrelevant antibodies will be produced, while in fusions with Y0 the hybrids will only make antibodies encoded by the parental spleen cell.

Intrasplenic (i.s.) immunization resulted in stronger immune responses than i.p. or i.v. immunization (Fig. 3.3). This might be explained by the fact that upon i.s. immunization a maximalization of the number of specific B cell blasts is achieved by a prolonged presence of relatively high concentrations of antigen. Uptake and elimination of antigen in other parts of the body as is the case with other routes of injection, is minimized by this procedure (Nilson et al., 1987; Spitz et al., 1984). Direct i.s. immunization therefore is likely to enhance the antibody response. Furthermore, i.s. immunization made it possible to immunize rats with only 10^5 cells of highly purified bone marrow suspensions (Table 3.4), that otherwise would not be

sufficient and thus enhanced the likelihood to obtain CFU-S specific MCAs.

Theoretically, all immunization suspensions listed in Table 3.4 could have resulted in CFU-S specific MCAs, because the purity of the immunogen per se is irrelevant in hybridoma technology. However, immunization with rather impure day-7-FU-MA bone marrow suspensions resulted in unsatisfactory reactivity of the obtained MCAs. A stem cell line as described by Dexter et al. (1980) would probably have been the best immunogen because of the unlimited amount of cells available and because of its reproducible purity. A number of laboratories have established immortalized cell lines derived from murine haemopoietic cells, by means of infection with a recombinant retrovirus (Dexter et al., 1980; Greenberger et al., 1983). All these lines are characterized by absolute dependency on exogenous colony stimulating factor (CSF), usually multi-CSF. A stem cell line was not available in this study.

In Table 3.5 only one fusion is shown in which the rat was immunized with the highly purified NBM-MA-WGA^{+}/α-H-2K^{k+} bone marrow suspension. However, more rats were immunized and boosted with this type of bone marrow suspension. A number of fusions between spleen cells of these rats and myeloma cells was lost at a stage before growth of hybridoma clones could be observed. The fusion cultures were lost due to contamination, which was probably introduced during the booster operation four days before fusion. Consequently, in later experiments the immunization and booster operation was performed with sterilized instruments, in a sterile environment. Under these conditions infections did no longer occur and a number of stable antibody producing hybridomas was obtained (Table 3.5).

According to (Dresser, 1986) the nature of the antigen introduced into an animal is important, but the amount of antigen is of even greater significance. Too little or too much antigen can be counter productive due to tolerization of the animal. The low frequency of initially antibody producing hybrids in the fusion experiments listed in Table 3.5 might have been due to the induction of tolerance (Thalhamer and Freund, 1985). However, this seems not very likely. It can be seen in Fig. 3.4, that i.s. injection of 5 x 10^4 cells resulted in a good immune response which suggests that no tolerization occurred. The low frequency of antibody producing hybrids might also been due to the close relationship between rats and mice. According to Brüggemann et al. (1986) rats and mice are closely related to each other and diverged some 10^7 years ago. This could explain the success of fusions between rat spleen cells and mouse myeloma cells. On the other hand, this close relationship might also be the cause of the low frequency of antibody producing hybrids (Table 3.5), as the xenogeneic immune response will be weaker the smaller the evolutionary distance between immunogen and host. Rabbits and hamsters might have been more suited for immunization than rats, but it has been reported that the spleen cells of the former two species do not produce stable hybridomas upon fusion with a murine myeloma line (NS-1) (Ralph et al., 1982).

The instability of the initially positive hybrids is most likely caused by loss of chromosomes. Hybridomas have a tendency to lose variable numbers of chromosomes of both fusion partners, especially during the early stages of proliferation after fusion. This loss of chromosomes may lead to the loss of their antibody secretion capacity, or may cause cell death when the

chromosome containing the HGPRT gene is lost (Oi and Herzenberg, 1979; Goding, 1980; Galfre and Milstein, 1982; Clark et al., 1983; Abrams et al., 1983; Yelton et al., 1978; and many others). It has been described that during selection in HAT medium segregation of the X-chromosome on which the gene for HGPRT is located causes the loss of a large number of hybridomas, whereas this occurs less frequently during selection in azaserine (Taggart amd Samloff, 1982). Another cause of the loss of antibody producing hybridomas is overgrowth by a non-antibody secreting clone present in the same culture well. Non-antibody secreting clones have a competitive growth advantage and will overgrow the slower proliferating antibody producing clones. To diminish the chance of overgrowth by non-antibody producers, antibody secreting clones should be subcloned as early as possible (Galfre et al., 1977; Springer et al., 1978a; Goding, 1980; Galfre and Milstein, 1982; Clark et al., 1983; Hokland et al., 1986; and many others).

To enhance the probability to obtain at least one stable antibody producing hybridoma, in this study, numerous wells were seeded and positive hybridomas were cloned as soon as possible. This approach has led to the production of a number of stable antibody producing hybridomas. Characteristics of the produced MCAs will be discussed in Chapter 4.

CHAPTER 4

CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES

INTRODUCTION

The availability of a number of screening assays is crucial for the production of MCAs. Some MCAs have been reported to behave unpredictably in different screening assays, i.e., the same MCAs have been shown to react in one assay, but not in another (Dangl and Herzenberg, 1982; Drover and Marchall, 1986; Fitchen et al., 1981; Haaijman et al., 1984; Hessian et al., 1986; Hough et al., 1986; Ledbetter and Herzenberg, 1979; Liebert et al., 1987; Milstein, 1986; Sieff et al., 1982; Swirsky et al., 1983). To reduce erroneous interpretations, MCAs should be screened in different ways in different assays (Swirsky et al., 1983). Furthermore, the selection of the desired MCAs is dependent on the efficiency of the screening procedures, which need to be rapid, sensitive and specific and capable of handling many samples at a time (Dangl and Herzenberg, 1982; Liebert et al., 1987; Milstein, 1986).

Often, hundreds of growing hybridomas appear at the same time after fusion. The initial screening must be done as soon as possible after these growing hybridomas appear and the results must be known soon to reduce the chance of loosing clones of interest (Chapter 3) and to avoid the unnecessary expension of uninteresting (nonproducing) clones. Production of MCAs directed against cell surface antigens on rare cells like PHSC in particular, requires highly sensitive screening assays.

Several screening procedures for MCAs against cell surface antigens have been developed. The enzyme linked immunosorbent assay (ELISA), complement mediated cell lysis and immunofluorescence assays by means of a fluorescence activated cell sorter (FACS) are commonly used ones (Dangl and Herzenberg, 1982; Drover and Marchall, 1986; Falkenburg et al., 1984; Fitchen et al., 1981; Herzenberg and Herzenberg, 1978; Parks et al., 1986). FACS analysis can be used for rapid screening of large numbers of hybridoma supernatants (Dangl and Herzenberg, 1982; Hale et al., 1985; Ledbetter and Herzenberg, 1979; Perrot et al., 1986; Springer, 1980). By combination of light scatter properties and cell surface immunofluorescence it can simultaneously be determined if antibody is present and, with what subpopulation(s) of cells in murine bone marrow it is reactive (Civin et al., 1987; Parks et al., 1986). Another assay used in this study for the initial screening of hybridoma cultures was an immunoasorbent assay (ISA) on NBM-MA-WGA$^+$ cells (Chapter 3). The ISA in contrast with FACS analysis, provides no indication of selective cell binding, but only gives information about the presence of antibody (Hough et al., 1986).

Once clones were established they were analyzed for reactivity with CFU-S and/or in vitro clonogenic progenitor cells (CFU-C) of various differentiation lineages. In this study, reactivity of MCAs with CFU-S was determined by means of a CFU-S assay after bone marrow cells were

incubated with the MCAs of interest with or without guinea pig complement. A reduction in the number of recovered CFU-S with respect to control samples, is an indication for the presence of the determinant recognized by the MCAs on CFU-S (Basch et al., 1977; Berman and Basch, 1985; Berridge, 1979; 1983; Boswell et al., 1984; Fitchen et al., 1982; Fitchen and Ferrone, 1981; Van den Engh et al., 1981). Reactivity with CFU-S or CFU-C was also determined by separating cells with increasing fluorescence intensity, i.e., antibody binding capacity (Springer, 1980) by means of a FACS, followed by a CFU-S or CFU-C assay to determine in which fraction(s) these cells were recovered (Bertoncello et al., 1986; Harris et al., 1985; Russell and Van den Engh, 1979; Van den Engh et al., 1983).

To further characterize the produced MCAs, they were screened against a panel of tumour cell lines and cell suspensions prepared from different organs and tissues. Screening against a panel of tumour cell lines and organs could give more information about presumptive stage specificity and differentiation lineage restriction of the MCAs (Civin et al., 1984; 1987; Schmidt et al., 1986; Springer, 1980; Springer et al., 1979; Strauss et al., 1986a; 1986b). In addition, tumour cells are often characterized by over-expression of markers that are expressed at an early stage during normal development and therefore could be very useful in the screening of MCAs putatively directed against CFU-S or CFU-C.

In this chapter, the strategies and tactics followed to screen and characterize the MCAs that were produced in this study are discussed.

INITIAL SCREENING OF HYBRIDOMA CULTURE SUPERNATANTS BY MEANS OF A FLUORESCENCE ACTIVATED CELL SORTER

It has been described by Visser et al. (1978) and Van den Engh et al. (1979) and many others that the different cell populations (based on morphological criteria) present in murine bone marrow can be recognized and distinguished from each other on the basis of light scatter characteristics using a FACS. Based on forward light scatter (FLS) and perpendicular light scatter (PLS), erythrocytes, lymphocytes, granulocytes and blast cells can be distinguished as major clusters. In figure 4.1 the FLS and PLS characteristics of the different cell populations present in murine bone marrow are shown.

With the FACS it is possible to analyze and separate (sort) cell populations of interest (Herzenberg and Herzenberg, 1978; Dangl and Herzenberg, 1982; Nicola, 1982). This can be achieved by gating (window setting) on either FLS, PLS or fluorescence, or a combination of these parameters. In this way, only the cells of interest are analyzed or sorted, while all other cells are excluded from the analysis or sort procedure (Dangl and Herzenberg, 1982; Janossy et al., 1978; Swirsky et al., 1983). Cells are sorted in a viable state which allows for subsequent functional assays. In this way, it was determined that most of the CFU-S are recovered from the blast cell population (Van den Engh et al., 1979), while the CFU-C from different lineages are characterized by the same (intermediate) FLS but mostly a somewhat higher PLS (Figure 4.1). CFU-C are recovered from the cells falling within the blast and granulocyte (blagra) population.

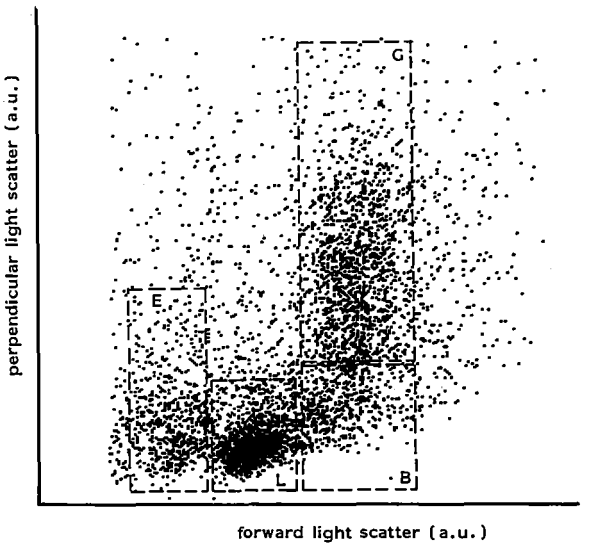


Figure 4.1:
Forward versus perpendicular light scatter intensity of normal mouse bone marrow cells. Each dot represents one cell. E = erythrocytes; L = lymphocytes; B = blast cells; G = granulocytes.

The initial screening of the supernatants of the hybridoma cultures was performed with normal mouse bone marrow cells. The supernatants were added to the cells followed by GARA/FITC and the fluorescence intensity and distribution was determined by means of FACS analysis (for details, see Chapter 2). The presence of antibody in the hybridoma culture supernatants was reflected by a higher fluorescence intensity with regard to the control sample that was incubated with GARA/FITC only.

During the initial screening procedure, windows were set around the blast cell population and the blagra population, but also around the erythrocyte, lymphocyte and granulocyte population. Antibodies that react only with cells belonging to the latter populations are obviously not CFU-S or CFU-C specific, but might be used to deplete bone marrow of mature cells and thus, for the negative selection and purification of CFU-S and CFU-C (Andrews et al., 1986; Edelman et al., 1986; Fitchen et al., 1981; Hoang et al., 1983; Mouchiroud et al., 1985; Müller-Sieburg et al., 1986). Screening in this way allows for simultaneous determination of the presence of antibody and its reactivity with subpopulations of bone marrow cells. The range of staining intensities and the percentage of positive (fluorescent) cells can also be determined.

The reactivity of the produced MCAs, expressed as the percentage of positive cells, with the different bone marrow subpopulations is presented in Table 4.1. These percentages are determined using an arbitrary threshold

for fluorescence intensity, above which one percent of the cells in the negative control sample (GARA/FITC only) was detected as positive (Davis et al., 1983; Gupta et al., 1984; Parks et al., 1986). This table shows that for most MCAs the range of percentage positive cells is rather wide. For MCAs that react with large percentages of cells (group I, Table 4.1) this was not crucial, the variation would not give rise to erroneous interpretation, but for MCAs that react with small subpopulations of bone marrow cells this could be a problem (group II, Table 4.1). These latter MCAs seemed negative in one test, while on another occasion they seemed to be reactive with a small subpopulation of cells and therefore could be putative CFU-S or CFU-C specific. The wide range in the percentages of positive cells was also observed by Davis et al. (1983). They described that staining with their MCA MBM-1 always resulted in three distinguishable subpopulations, but the exact percentage of cells falling within each of these populations varied from one experiment to another and between different batches of mice. In the present study it was observed that particularly the blast cell population could differ in size (percentage of total nucleated cells) from one experiment

Table 4.1

REACTIVITY OF NEWLY PRODUCED MCAs WITH DIFFERENT BONE MARROW SUBPOPULATIONS

		% positive			
	MCA	all cells	erythro-cytes	lympho-cytes	blast cells	granulo-cytes
	PV5.6	65 (44-85)	8 (7- 9)	68 (52-78)	64 (32-94)	79 (38-94)
	10-2.2	20 (11-25)	24 (11-33)	11 (4-15)	12 (3-29)	15 (7-24)
	10-2.3	16 (6-26)	6 (6- 6)	4 (3- 4)	16 (6-39)	12 (6-20)
I	10-9.6	56 (47-69)	11 (6-19)	21 (10-29)	73 (60-89)	87 (68-93)
	10-7.3	50 (40-61)	7 (4-14)	11 (7-17)	66 (50-74)	83 (58-93)
	7-15.1	18 (3-42)	4 (0- 6)	3 (0- 6)	29 (18-46)	28 (6-72)
	15-1.1	46 (36-60)	9 (5-12)	6 (5- 7)	44 (35-52)	82 (79-85)
	13-18.2	5 (5- 5)	12 (7-23)	3 (2- 4)	3 (2- 5)	6 (5- 7)
	13-23.1	11 (5-14)	26 (17-31)	5 (3- 8)	5 (2- 7)	8 (7- 9)
	13-19.4	10 (5-22)	12 (6-24)	11 (2-27)	10 (2-25)	11 (3-27)
	9-8.2	4 (1- 8)	7 (2-12)	1 (0- 3)	3 (0- 3)	3 (0- 5)
	15-2.4	8 (5-13)	8 (6-12)	4 (3- 4)	14 (9-16)	14 (6-25)
II	9-2.2	9 (1-15)	2 (0- 6)	1 (0- 2)	9 (3-20)	7 (0-13)
	13-5.1	4 (2- 7)	6 (3-10)	1 (1- 2)	7 (0-12)	2 (1- 4)
	13-2.5	3 (1- 5)	2 (1- 3)	2 (1- 3)	3 (1- 6)	3 (1- 5)
	13-7.1	3 (0- 5)	1 (0- 1)	1 (0- 4)	3 (0- 8)	6 (0-12)
	13-12.1	1 (0- 4)	0	0	1 (0- 1)	1 (0- 2)
	13-3.4	7 (6- 8)	7	3	7 (4- 9)	6

Cells were labelled with MCAs and GARA/FITC as described in Chapter 2. The percentage positive cells in each subpopulation was determined with regard to the negative control (GARA/FITC only). The figures represent the mean percentage of positive cells determined in 2-13 separate experiments. The numbers in parentheses represent the range of the % positive cells observed in the different experiments.

to another. The use of supernatants with different concentrations of antibody and the subclass, i.e., the stability of the antibody (Bethke et al., 1987) could also have attributed to the observed variation in the percentages of positive cells.

Since the frequency of CFU-S and of CFU-C is around 1% or less of all murine bone marrow cells (Fitchen et al., 1981; Lord and Spooncer, 1986; Van Bekkum et al., 1979) a MCA that reacted with more than 1% of all bone marrow cells could not be CFU-S or CFU-C specific. Since the blast cell population contains almost all CFU-S and comprises about 25% of all nucleated bone marrow cells a CFU-S specific MCA would react with 2-4% of all blast cells. Taking into account the non-specific binding of free antibody conjugate by dead cells and cell clumps and the non-specific binding of antibodies by cells expressing Fc-receptors (Dangl and Herzenberg, 1982; Fitchen et al., 1981; Parks et al., 1986; Sasaki et al., 1987) both giving rise to enhanced levels of background staining, MCAs that were reactive with more than 5% of all bone marrow cells and all blast cells were considered as not CFU-S specific, while MCAs that were reactive with less than 5% of all the bone marrow and blast cells were considered as putative CFU-S specific. For example, My-10, a MCA reactive with and specific for all human progenitor cell types stains 1-4% of all human bone marrow cells (Civin et al., 1984; 1987; Strauss et al., 1986a).

Based on this criterion only the MCAs 13-18.2, 9-8.2, 13-5.1, 13-2.5, 13-7.1 and 13-12.1 could be CFU-S or CFU-C specific, while the MCAs 13-23.1, 13-19.4, 15-2.4, 9-2.2 and 13-3.4 are questionable in this respect. The other MCA listed in Table 4.1 are definitely not CFU-S or CFU-C specific.

A classification of the MCAs listed in Table 4.1 can be made more profitably on the basis of the fluorescence distribution histograms. In Figure 4.2 the characteristic fluorescence distribution due to binding of representative MCAs that could not be CFU-S or CFU-C specific, because of the percentages of cells recognized by them (group I, Table 4.1) is shown. PV5.6 reacted strongly with large percentages of cells within the lymphocyte, blast and granulocyte population but not or only weakly with a very small subpopulation of erythrocytes. Thus, this MCA seems to be directed against a determinant present on leukocytes only.

MCA 10-2.2 reacted less strongly and with smaller subpopulations of all the leukocyte subpopulations. It reacted most clearly with cells of the erythrocyte population. MCA 10-9.6 was strongly reactive with large percentages of blast cells and granulocytes and weakly with probably most lymphocytes. It reacted only weakly with a small subpopulation of erythrocytes. MCA 10-7.3 (Table 4.1) showed an almost identical pattern of reactivity as MCA 10-9.6 and like MCA 10-2.3 (Table 4.1) which showed an almost identical pattern of reactivity as 10-2.2 was therefore not shown.

MCA 7-15.1 and 15-1.1 reacted in a very similar way. This was not clear from the percentages positive cells in Table 4.1. Both MCAs were only reactive with granulocytes and blast cells and not or very weakly with lymphocytes and erythrocytes. The fluorescence intensity after staining with 15-1.1 was higher than with 7-15.1.

In experiments in which the fluorescence of all bone marrow cells (including erythrocytes) and all nucleated bone marrow cells were compared,

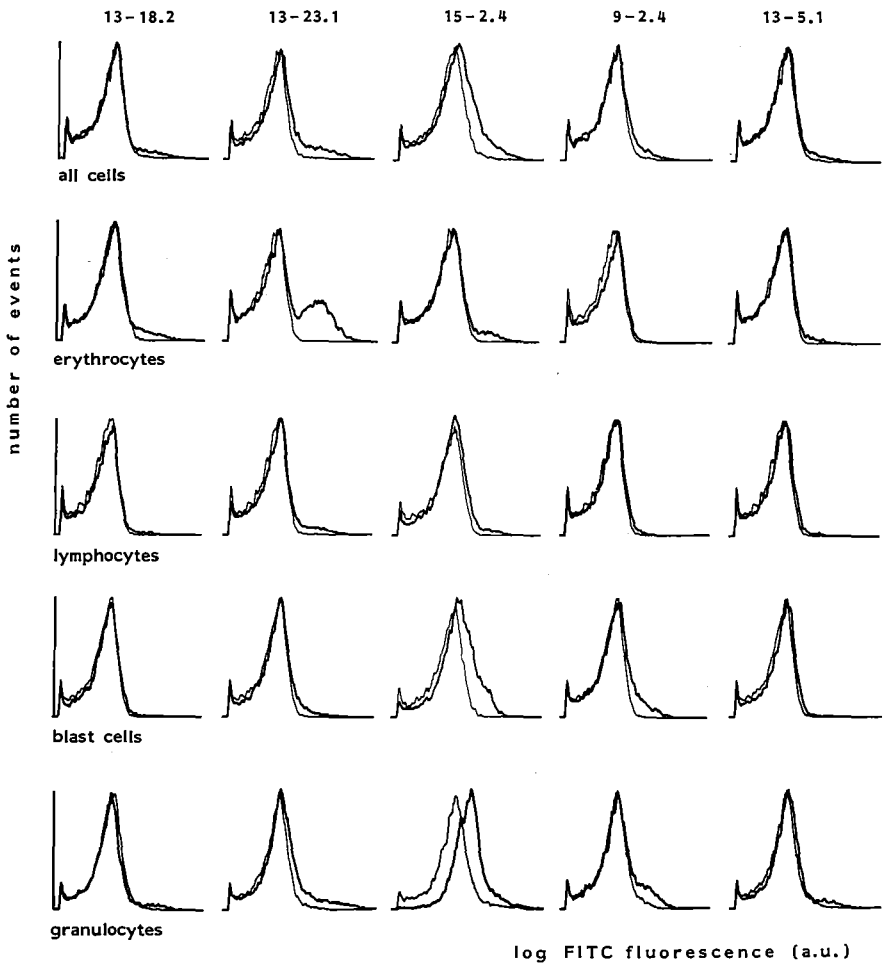


Figure 4.2:
Fluorescence of different bone marrow subpopulations treated with MCAs that could not be CFU-S or CFU-C specific. Bone marrow samples were incubated with MCAs and GARA/FITC as described in Chapter 2. The thin lines show the fluorescence of bone marrow cells incubated with GARA/FITC only.

it was shown that all MCAs except 10-2.2 were not reactive with cells within the erythrocyte population (data not shown).

In Figure 4.3 the fluorescence distribution histograms of the putative CFU-S or CFU-C specific MCAs is shown (group II, Table 4.1). MCA 13-18.2

and 13-23.1 had a very similar staining pattern as MCA 9-8.2 and 13-9.4 which are not shown. These MCAs all recognized a small percentage of total bone marrow cells and are most clearly reactive with a subpopulation of cells falling within the erythrocyte window, but also recognized a subpopulation of granulocytes and possibly a subpopulation of blast cells and lymphocytes.

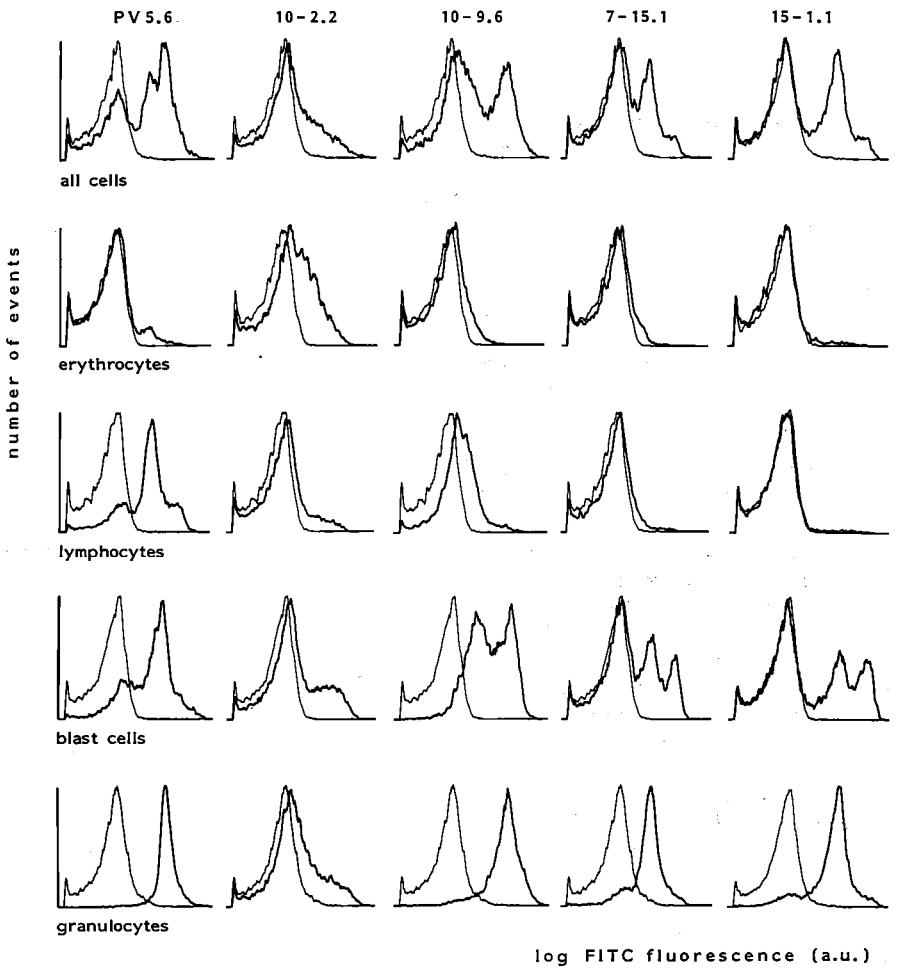


Figure 4.3:
Fluorescence of different bone marrow subpopulations treated with MCAs that could be CFU-S or CFU-C specific. Bone marrow samples were incubated with MCAs and GARA/FITC as described in Chapter 2. The thin lines show the fluorescence of bone marrow cells incubated with GARA/FITC only.

MCA 15-2.4 is mainly reactive with cells within the blast cell and granulocyte population. The same is true for MCA 9-2.2 although this MCA recognized smaller subpopulations. MCA 13-5.1 which is a representative of all the other MCAs of Table 4.1 that are not shown in Figure 4.2 and 4.3 was reactive with only a very small percentage of bone marrow cells and therefore at this stage of characterization seemed most promising.

It is clear that the fluorescence distribution histograms give more information about the reactivity of the MCAs than the percentage of positive cells (Table 4.1). For instance, staining with MCA 15-1.1 resulted in a strongly positive blast cell population and a moderately positive blast cell population. This could not be determined from Table 4.1. Interpretation of the fluorescence distribution of the MCAs shown in Figure 4.2 is unequivocal. In most of the cases there is a clear-cut difference between stained (positive) and unstained (negative) cells, which resulted in bi- or trimodal curves. Interpretation of the staining patterns of the MCAs shown in Figure 4.3 is more difficult. With these MCAs no clear distinction between negative and positive cells is observed. In many cases, for instance, in the granulocyte population after staining with MCA 15-2.4, the histogram is shifted somewhat to the right in comparison with the curve of the negative control sample. According to Parks et al. (1986) it is likely that in such cases all cells expressed the recognized antigen, but only a few of these cells expressed high enough quantities of antigen to be detected as positive.

SCREENING FOR THE PRESENCE OF ANTIBODY BY MEANS OF A GOLD-LINKED IMMUNOSORBENT ASSAY (GLISA)

Enzyme-linked immunosorbent assays (ISAs) are currently employed techniques for the screening of MCAs directed against cell surface antigens (Hough et al., 1986). Using an ISA many samples can be handled at once, in a relatively short time (Springer et al., 1979). It is often the case that hundreds of growing hybridoma cultures appear almost simultaneously after fusion, but the interval between fusion and the appearance of hybridoma cells is not always predictable. They all need to be rapidly screened, cloned and characterized to avoid the loss of interesting clones (Chapter 3). This cannot always be done by means of FACS analysis, e.g. when the machine is occupied for other experiments, and therefore a gold-linked ISA (GLISA) was used occasionally in this study.

Screening by means of a GLISA was performed against normal bone marrow cells or low density WGA positive blast cells (NBM-MA-WGA^+) cells (Chapter 3). NBM-MA-WGA^+ cells were sorted by means of a FACS into poly-L-lysin treated wells of Terasaki tissue culture trays at a density of 1000 cells per well. Subsequently, the cells were fixed to the bottom of the wells with glutaraldehyde (Chapter 2). Plates with fixed cells could be stored in the cold before use for a period of at least two weeks and up to several months (Lansdorp et al., 1980). In one day, large numbers of plates could be prepared allowing for the screening of very large numbers of hybridoma cultures (60 cultures/plate) at any time.

The screening was performed as described in Chapter 2. The result of screening of MCA 15-1.1 by means of the GLISA is shown in Figure 4.4. In

this figure, dark, intermediate and unstained cells are present, indicating strong, intermediate and no expression of the antigen recognized by the antibody. Although the GLISA gives clear answers about the presence of this antibody, this was not always the case for other MCAs (results not shown).

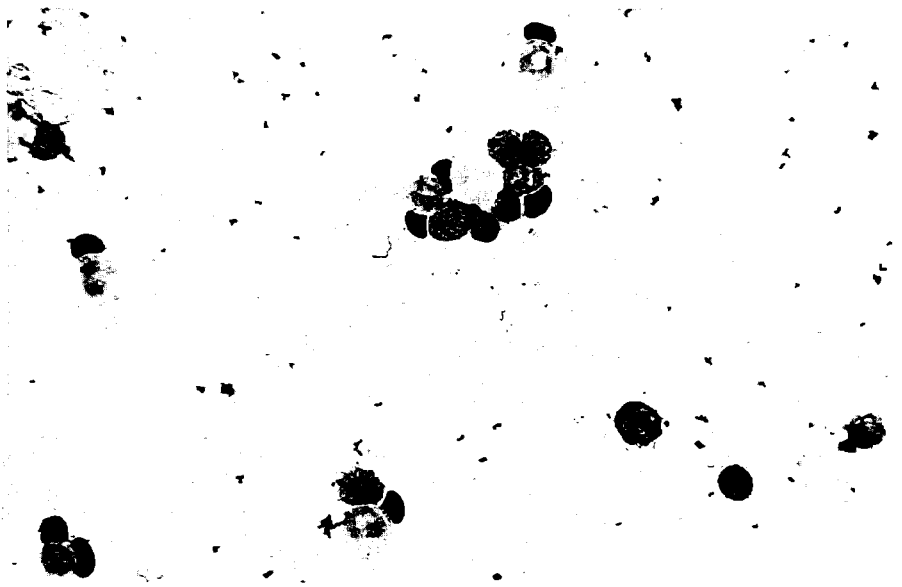


Figure 4.4:
Staining of normal bone marrow cells observed with the GLISA. Cells were fixed, incubated with MCA 15-1.1, GARA/gold and silver solution as described in Chapter 2.

An advantage of the GLISA is that it can be performed with NBM-MA-WGA$^+$ cells which were 18-fold enriched for day-12 CFU-S (Chapter 3). In contrast, the initial screening of hundreds of hybridoma supernatants by means of the FACS can only be performed with unseparated bone marrow in view of the time needed to isolate enough NBM-MA-WGA$^+$ cells.

A disadvantage of the GLISA is that the cells have to be chemically fixed to immobilize them. The fixation may affect the binding of antibody by altering the target antigen, which may lead to false positive results (especially when the antibody is of the IgM subclass) and false negative results (destruction of the determinant) (Drover and Marshall, 1986; Hough et al., 1986). By means of the GLISA it cannot be determined if and with what other cell population(s) the antibody is reactive. A negative result in the GLISA does not exclude the possibility that the antibody is present on other subpopulations of bone marrow cells and therefore could be of use for the negative selection of CFU-S or CFU-C.

DETERMINATION OF THE REACTIVITY OF THE PRODUCED MCAs WITH CFU-S AND CFU-C BY FACS SORTING

As described in a previous section a number of putative CFU-S or CFU-C specific MCAs was produced (group II, Table 4.1; Figure 4.3). In addition, a number of MCAs was produced that reacted with large percentages of bone marrow cells and therefore could not be CFU-S or CFU-C specific (group I, Table 4.1, Figure 4.2). However, these latter MCAs could be useful for the negative selection of these cells. To investigate the usefulness of the produced MCAs, the reactivity of the MCAs from both groups with CFU-S and CFU-C was determined.

Normal bone marrow cells were incubated with the MCAs of interest and stained with a fluorescent second antibody. Stained cells were subsequently run through a FACS. To investigate if the antigens that are recognized by the produced MCAs are expressed on CFU-S and in vitro clonogenic progenitors of different lineages, the colony forming ability of sorted cell fractions with increasing antibody binding capacity, i.e., fluorescence intensity (Springer, 1980) was investigated. The sorted fractions were tested for their content of day-8 and day-12 CFU-S, CFU-M, CFU-C2, CFU-GM and BFU-E.

It has been reported that spleen colonies that appear early (day-7 to -8) and late (day-12 to -14) after bone marrow transplantation (Magli et al., 1982) are derived from CFU-S that differ in the density of MHC class-I antigens expressed on their surfaces (Harris, 1984a; 1984b; Mulder, 1986; Visser et al., 1984). It cannot be excluded that a similar difference occurs for surface antigens recognized by certain of the new MCAs. Therefore, the sorted cell fractions were tested for their content of both day-8 CFU-S and day-12 CFU-S. Occasionally day-10 CFU-S only were examined.

CFU-C2 and CFU-M, which are both committed macrophage progenitors (Bol and Williams, 1980; Bradley et al., 1971; Burgess and Nicola, 1983; Van den Engh, 1975), give rise to colonies mainly consisting of mature macrophages. They were cultured in the presence of pregnant mouse uterus extract (PMUE) and a combination of crude mouse spleen cell conditioned medium (MSCM) and purified CSF-1 (colony stimulating factor-1) respectively. CFU-GM (colony forming unit-granulocyte/macrophage) were cultured in the presence of recombinant GM-CSF (granulocyte/macrophage-CSF). CFU-GM give rise to colonies consisting of either pure granulocytes, pure macrophages, or mixed colonies consisting of both granulocytes and macrophages. It has been reported that CFU-GM are ancestral to CFU-G which in turn are believed to be ancestral to CFU-M (Koizumi et al., 1982). BFU-E (burst forming unit-erythrocyte) were grown in the presence of MSCM and purified erythropoietin. These cells give rise to large colonies (bursts) containing (hemoglobinilized) erythroid cells (Gregory, 1976). The exact culture conditions for the different clonogenic progenitor cell types are described in Chapter 2.

Fractions of the blast cell population, blagra population or all nucleated cells from bone marrow suspensions labelled with MCAs were sorted in two or more fractions, depending on the fluorescence distribution. All fractions were subsequently assayed for their CFU-S and progenitor cell content.

In this study the reactivity of the MCAs with the different CFU-S and in vitro clonogenic progenitor cell types was divided in three categories.

When more than 50% of the cells under investigation was recovered from the positive fraction(s), the recognized determinant was regarded to be present (at high density) on all cells. When less than 25% of the cells was recovered from the positive fraction(s), the recognized determinant was regarded to be absent. In cases where 25-50% of the cells under investigation were recovered from the positive fraction(s) the recognized determinant was regarded to be expressed on a subpopulation of the tested cell type. In most cases high percentages (80-100%) of the investigated cell types were found in either the negative or positive fraction(s). In only a few cases (see below) 25-50% of a certain investigated cell type was found in the positive fraction(s).

In Figure 4.5 it is shown that the determinants recognized by the MCAs PV5.6 and 10-9.6 are expressed on almost all day-8 and day-12 CFU-S and all progenitor cell types tested. Between 90-100% of all the cell types assayed were recovered from the positive blagra fraction. The same was observed for MCA 10-7.3 (Table 4.2). However, because 10-7.3 had the same pattern of reactivity as 10-9.6, the former was not further investigated. The MCAs PV5.6 and 10-9.6 (10-7.3) recognized large amounts of bone marrow cells, which at first made them likely candidates for the depletion of bone marrow samples from mature cells. However, since these MCAs recognized all CFU-S and progenitor cell types tested, they could not be used for this purpose.

MCA 10-2.2 was reactive with all bone marrow subpopulations including erythrocytes (Fig. 4.2), but it was not reactive with any of the CFU-S or progenitor cell types tested (less than 10% of any tested cell type was recovered from the positive fraction). Thus, 10-2.2 could be of use for the negative selection of CFU-S and the different progenitor cell types tested. MCA 7-15.1 and 15-1.1 did not react with day-8 and day-12 CFU-S, nor with BFU-E and CFU-M. However, 67% of the CFU-GM were recovered from the 7-15.1 positive blagra cell fractions, while in the 15-1.1 positive blagra cell fractions 35% of the CFU-GM were found (mean results of two experiments). In addition, 33% of the CFU-C2 were recovered from the 15-1.1 positive cell fractions (7-15.1 not tested for expression on CFU-C2). Based on the criteria used in this study, it can be concluded that 7-15.1 and 15-1.1 were reactive with at least a subpopulation of CFU-GM and CFU-C2 (15-1.1 only), indicating the heterogeneity of these progenitor cell populations with respect to the expression of the antigens recognized by these two MCAs. MCA 7-15.1 and 15-1.1 could be used for the negative selection of CFU-S, BFU-E and CFU-M. In addition, both MCAs could be used in studies concerning the heterogeneity of granulocyte/macrophage progenitors.

In Fig. 4.6, the distribution of the different CFU-S and progenitor cell types after labelling with the MCAs of group II (Table 4.1) is shown. It is observed that none of these MCAs was reactive with any CFU-S type or any progenitor cell type, except MCA 15-2.4. MCA 15-2.4 was unreactive with day-8 and day-12 CFU-S, CFU-M and CFU-GM. However, 15-2.4 was reactive with about 50% of the BFU-E, suggesting heterogeneity of this erythroid progenitor cell population. Gregory (1976), first described heterogeneity of the BFU-E and demonstrated the existence of primitive BFU-E and mature BFU-E according to the time of appearance of colonies in culture.

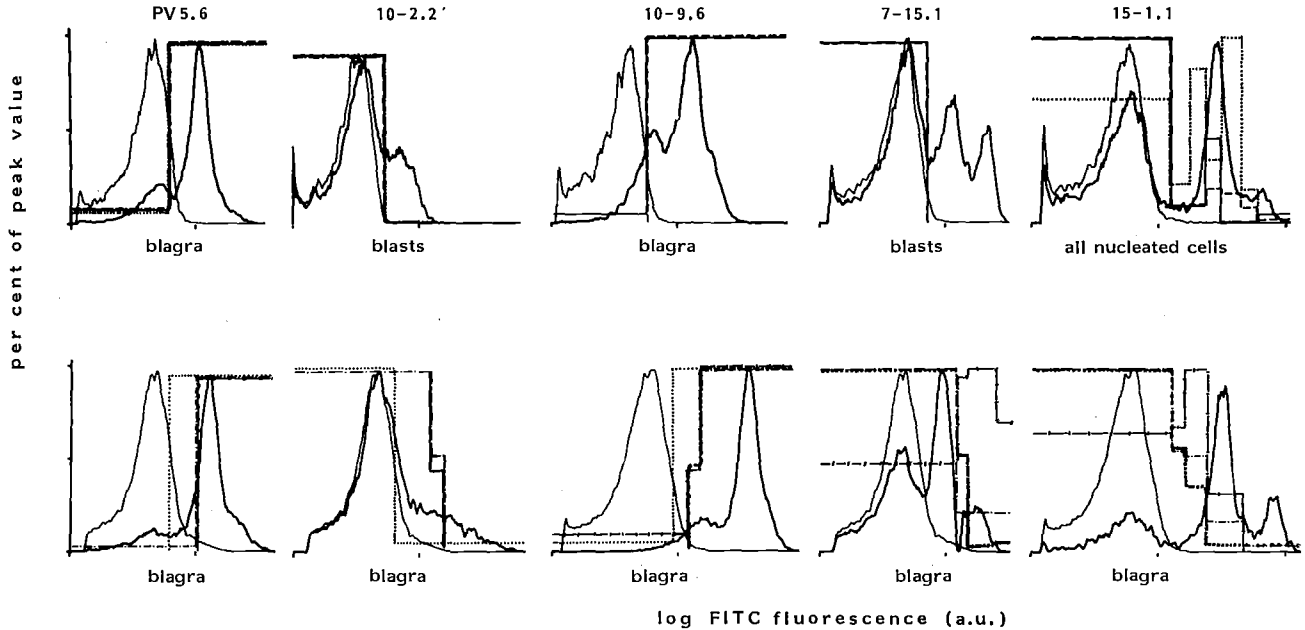


Figure 4.5:
Distribution of CFU-S and different types of progenitor cells in bone marrow subpopulations treated with MCAs that could not be CFU-S or CFU-C specific. Bone marrow cells were labelled as described in Chapter 2. The cells were sorted on a FACS-II in the fractions indicated. The sorted fractions were assayed for CFU-S and CFU-C content as described in Chapter 2. The straight lines and rectangular boxes show the number of CFU-S and CFU-C recovered in the sorted fractions as the percentage peak value of the CFU-S and CFU-C recovered per 10^5 sorted cells (——, day-8 CFU-S; - - - - -, day-12 CFU-S; -·-·-·-, CFU-C1; ·····, CFU-C2; -+++++-, CFU-GM; ××××××, BFU-E). The thin lines show the fluorescence of bone marrow incubated with GARA/FITC only.

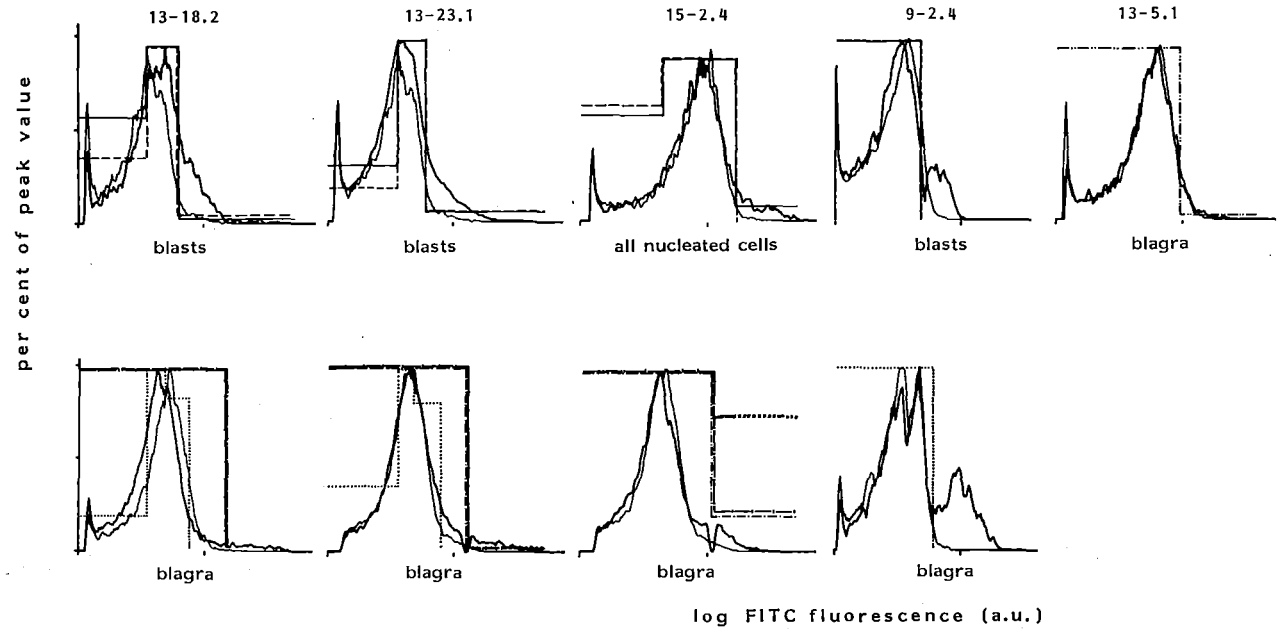


Figure 4.6:
Distribution of CFU-S and different types of progenitor cells in bone marrow subpopulations treated with the produced putative CFU-S or CFU-C specific MCAs (——, day-8 CFU-S; -·-·-·- , day-10 CFU-S; - - - - -, day-12 CFU-S; -·-·-·-, CFU-C1; ·····, CFU-C2; +++++, CFU-GM; ******* , BFU-E). See legend Figure 4.5 for details.

TABLE 4.2

REACTIVITY OF MCAs WITH CFU-S AND CFU-C DETERMINED BY CELL SORTING

	MCA	CFU-S day-8	CFU-S day-10	CFU-S day-12	CFU-GM	CFU-M	CFU-C2	BFU-E
	PV5.6	++		++	++	++	++	++
	10-2.2	-		-	-	-	-	-
	10-9.6	++		++	++	++	++	++
I	10-7.3	++		++			++	
	7-15.1	-		-	+	-		-
	15-1.1	-		-	+	-	+	-
	13-18.2	-	-	-	-	-	-	-
	13-23.1	-	-	-	-	-	-	-
	13-19.4		-					
	9-8.2	-		-			-	
II	15-2.4	-		-	-	-		+
	9-2.2	-		-			-	
	13-5.1		-					
	13-2.5		-					
	13-3.4	-	-	-	-	-	-	-
	13-7.1		-					
	13-12.1	-		-			-	

Bone marrow samples were incubated with MCAs and GARA/FITC as described in Chapter 2. Fractions were sorted on a FACS-II and tested for their CFU-S or CFU-C content (for details see subscript Fig. 4.5).
++: more than 50% of all CFU-S or CFU-C recovered from the positive cell fraction(s);
+ : 25-50% of all CFU-S or CFU-C recovered from the positive fraction(s);
- : less than 25% of all CFU-S or CFU-C recovered from the positive fraction(s).
The recovery of CFU-S and the different progenitor cell types tested from bone marrow samples labelled with the listed MCAs and GARA/FITC was 90-120% of that of bone marrow samples incubated with GARA/FITC only. Except for bone marrow cells labelled with the MCAs PV5.6, 10-9.6 and 10-7.3. The recovery of CFU-S after sorting of fractions incubated with these MCAs was 50-70%.

Although none of the MCAs shown in Figure 4.6 were reactive with CFU-S and the different in vitro clonogenic cell types tested (except 15-2.4), these MCAs did not seem very useful for the negative selection of CFU-S and in vitro clonogenic progenitor cell types, because all of them recognized only small numbers of bone marrow cells (Fig. 4.2).

In Table 4.2 the reactivity with the different CFU-S and progenitor cell

types of all the produced MCAs that were tested is summarized. Only three of the seventeen MCAs tested were reactive with CFU-S. These MCAs (PV5.6, 10-9.6 and 10-7.3) were also reactive with all progenitor cell types tested. Three other MCAs were not reactive with CFU-S, but were reactive with (a subpopulation of) BFU-E (15-2.4), CFU-GM (7-15.1 and 15-1.1) and CFU-C2 (15-1.1).

DETERMINATION OF THE REACTIVITY OF MCAs WITH CFU-S BY MEANS OF ANTIBODY AND COMPLEMENT DEPENDENT CFU-S REDUCTION

By far the most used methods to investigate the expression of certain cell surface antigens on CFU-S and in vitro clonogenic progenitors are the antibody dependent cytotoxicity assay and the complement dependent cytotoxicity assay (Falkenburg et al., 1984; Fitchen et al., 1981). In this study, both assays were used to investigate if the results obtained by the cell sorting experiments could be confirmed.

Lethally irradiated recipient mice were injected with bone marrow suspensions that were incubated with MCA alone, or with bone marrow suspensions that were incubated with MCA as well as an appropriate dilution of guinea pig complement. The reduction in the number of CFU-S recovered from these treated bone marrow suspensions was a measure for the reactivity of the MCAs with CFU-S. It was assumed that the spleen seeding efficiency of the labelled and injected cells was not different from that of untreated cells, and that an altered number of CFU-S was only due to incubation with the MCAs.

It has been described that there was no significant difference between the number of CFU-S that was recovered from samples treated with (monoclonal) antibody alone, and samples treated with (monoclonal) antibody and complement (Berridge, 1982; Berridge and Okech, 1979; Daley et al., 1984; Ralph and Berridge, 1984; Van den Engh et al., 1981). In vivo opsonization of the antibody coated CFU-S followed by phagocytosis by macrophages in the reticuloendothelial system may lead to a reduction in the number of CFC-S that settles in the spleen and thus to a reduction of CFU-S (Berridge et al., 1984; Berridge and Okech, 1979; Russell and Van den Engh, 1979; Van den Engh et al., 1983). This hypothesis is supported by the observation that inactivation of the host macrophages by injection of carrageenan partly prevented the reduction in the number of CFU-S following incubation with antibodies (Van den Engh et al., 1983). In some cases, complement activity in vivo may contribute to a further reduction of CFU-S by the binding of complement to its receptors on phagocytic cells or by cell lysis (Berridge and Okech, 1979; Ralph and Berridge, 1984).

In most experiments described here, the reactivity of the MCAs with CFU-S was determined by preincubation of bone marrow cells with the MCA of interest without the addition of complement (C'). This was not only more rapid than subsequent incubation with complement, but it also avoided problems arising about the quality and the availability of complement and about the isotypes of the MCAs, which were not always known at the time the experiments were done. Complement mediated cytotoxicity in contrast with antibody dependent reduction of CFU-S has been shown to be

dependent on the isotype of the MCAs (Ralph and Berridge, 1984; Sieff et al., 1982). Rat antibodies of the IgG1 and IgG2a isotype are incapable to fix complement, while IgG2b, IgG2c and IgM antibodies can fix complement and thus can cause lysis of antibody coated cells, when the density of the recognized antigen is sufficiently high (Ledbetter and Herzenberg, 1979; Springer, 1980).

In Table 4.3 the reactivity of the MCAs with CFU-S as determined by means of the antibody and complement dependent cytotoxicity assay is summarized. The mean results of 2-3 experiments are shown. It was observed that the percentage reduction of CFU-S for the same MCA varied considerably from one experiment to another. This large variation was not only observed after treatment with MCA alone, but also after treatment with MCA and complement. According to Berridge and Okech (1979) the large variation in the percentages of CFU-S reduction is inherent to bone marrow and could have been caused by differential efficiency of opsonization (content of macrophages) and complement lysis (same batch, stored for different intervals) in different experiments (Ralph and Berridge, 1984). It can be seen that none of the MCAs that were binding to a large percentage of bone marrow cells (group I, Table 4.1), except PV5.6, were reactive with CFU-S. In two experiments, PV5.6 caused a 70% reduction in the number of recovered day-12 CFU-S. In one of these experiments the number of day-8 CFU-S was also reduced by 70%, while in the other experiment the number of recovered day-8 CFU-S was higher than that recovered from the negative control. A hundred percent reduction of CFU-S was not observed, although all CFU-S were gained from the positive fraction in the cell sorting experiments.

MCA 10-9.6 and 10-7.3 which were strongly reactive with day-8 and day-12 CFU-S in the cell sorting experiments were not reactive with both types of CFU-S in the antibody dependent cytotoxicity assay. It has been described that the degree of reduction of CFU-S in the latter assay, was dependent on the amount of antibody bound. The more antibody bound, the higher the reduction in the number of CFU-S (Berridge and Okech, 1979; Cannistra et al., 1985; Koizumi et al., 1982; Ledbetter et al., 1980; Pillai et al., 1986) The lack of reactivity of 10-9.6 and 10-7.3 in the antibody dependent cytotoxicity assay, might indicate that the amount of 10-9.6 and 10-7.3 bound to CFU-S was too low. This suggests that determination of the reactivity of MCAs with CFU-S by means of cell sorting is more sensitive and thus more reliable than the antibody dependent cytotoxicity assay.

None of the MCAs from group II (Table 4.1) were reactive with day-8 and day-12 CFU-S. In one experiment in which the reactivity with day-10 CFU-S was determined, incubation with MCA 13-18.2 and 13-23.1 caused a more than 50% reduction of day-10 CFU-S. However, these 2 MCAs lacked reactivity with day-10 CFU-S in the cell sorting experiments. Because of the higher sensitivity of the FACS assay, particularly for low density antigens (Berridge and Okech, 1979; Cannistra et al., 1985; Falkenburg et al., 1984; Fitchen et al., 1981; Pillai et al., 1986) it is concluded that 13-18.2 and 13-23.1 are not reactive with day-10 CFU-S. For the same reason it seems also unlikely that 9-2.2 is reactive with day-8 CFU-S and that 13-3.4 is reactive with day-12 CFU-S (compare Tables 4.2 and 4.3).

Table 4.3

REACTIVITY WITH CFU-S DETERMINED BY ANTIBODY DEPENDENT CYTOTOXICITY AND COMPLEMENT MEDIATED CYTOTOXICITY

	MCA	Ig-(sub)class	day-8 CFU-S	day-10 CFU-S	day-12 CFU-S
	PV5.6	IgG2b	+/-*		+
	10-2.2	IgG2b	-		-
	10-2.2 + C'		-		-
	10-9.6	IgG2b	-		-
I	10-7.3	IgG2a	-		-
	7-15.1	IgM	-		-
	15-1.1	IgG2a	-		-
	15-1.1 + C'		-		-
	13-18.2	IgM	-	+?	-
	13-18.2 + C'		-		-
	13-23.1	IgM	-	+?	-
	13-23.1 + C'		-		-
	13-19.4	IgM	-	-	-
	13-19.4 + C'		-		-
	9-8.2	?	-		
	15-2.4	IgM	-		-
	15-2.4 + C'		-		-
II	9-2.2	IgG2b	+/-*		-
	13-5.1	IgM	-		-
	13-2.5	?	-		-
	13-3.4	IgM	-	-	+?
	13-7.1	IgG2c	-		-
	13-12.1	?	-	-	-
	13-12.1 + C'		-		-

Bone marrow samples were incubated with either MCA alone, or with MCA and guinea pig complement (C') as described in Chapter 2. The CFU-S content of the treated samples was compared with that in control samples; untreated bone marrow, respectively bone marrow incubated with C' only. Lethally irradiated mice (7-8 per group) were injected with 5×10^4 control cells or with equivalents thereof from the treated samples.
- = less than 50% reduction in the number of CFU-S in comparison with control values; + = more than 50% reduction in the number of CFU-S in comparison with control values. * +/- = results of two different experiments; +? = inconclusive/ disputed. Results of 2 to 3 experiments.
The Ig (sub)class of the MCAs was determined by an Ouchterlony immunodiffusion reaction (Chapter 2). N.B.? Subclass not known.

In a number of cases the antibody dependent and the complement mediated cytotoxicity assay was performed with the same MCAs (Table 4.3). In agreement with data in the literature no differences were observed, showing that the cytotoxic effect of the MCAs on CFU-S is independent of complement treatment in vitro (Berridge, 1979; 1982; Daley et al., 1984; Ralph et al., 1982; Ralph and Berridge, 1984; Van den Engh et al., 1981).

SCREENING OF MCAs AGAINST A PANEL OF TUMOUR CELL LINES

Tumour cell lines are made up of one particular cell type which is adapted to in vitro replication and is arrested in a particular stage of differentiation during normal development (Ledbetter and Herzenberg, 1979; Schmidt et al., 1986; Springer, 1980). Leukaemic cells do not express aberrant "new leukaemia" antigens, but are strikingly similar to normal precursor cells. Often, however, they differ from the latter by over-expression of certain antigens (Schmidt et al., 1986). They then may re-express antigens which were only (weakly) expressed at an early stage of normal differentiation, but were suppressed during the progress of differentiation (Fukada, 1985; Ledbetter and Herzenberg, 1979; Schmidt et al., 1986).

Screening against tumour cells has proven to be a useful and sensitive method for the detection of (monoclonal) antibodies reactive with cell surface antigens that are only rarely expressed on normal precursor cells (Civin et al., 1987; Delwel et al., 1987; Fukada, 1985; Milstein et al., 1979; Schmidt et al., 1986; Sela, 1986; Springer, 1980; Springer et al., 1979). An example of the usefulness of screening against tumour cells is the detection of a MCA named My-10. My-10 was raised and screened against a human immature myeloid leukaemic cell line (KG1a). This My-10 antibody appeared to be strongly reactive with these KG1a cells, but in normal human bone marrow it only stained about 1% of the cells (Civin et al., 1984; 1987; Strauss et al., 1986a; b). Among the cells recognized by the My-10 MCA are the human equivalents of the murine CFU-S (CFU-GEMM) and progenitors of all differentiation lineages, including lymphoid progenitors (Strauss et al., 1986b). Thus, My-10 is a stage specific rather than a lineage specific MCA (Civin et al., 1984). According to Fukada (1985) it should be possible to identify presumptive stage specific and cell lineage specific antigens, when MCAs are screened against a panel of tumour cell lines.

To investigate if any of the produced MCAs was differentiation-stage or -lineage specific, they were screened against a panel of murine tumour cell lines of which the characteristics are shown in Table 4.4. P388 is a lymphoid leukaemia. It is not known if this is a T- or B-lymphoid leukaemia, EL-4 is a T-cell lymphoma. DA-1 is an Interleukin-3 (IL-3) dependent myeloid leukaemia and WEHI-3B is either a myelomonocytic leukaemia, a T-lymphocytic leukaemia (Thy-1 expression) or a "mixed lymphoid-myeloid" leukaemia, indicating the transformation of a bipotent progenitor cell (Lee et al., 1982; Umiel et al., 1987). WEHI-3B cells, among other growth factors, produce IL-3.

In Fig. 4.7 the reactivity is shown of the MCAs from group I (Table 4.1). Again, the binding was determined by means of FACS analysis. In this

Table 4.4

MURINE TUMOUR CELL LINES USED FOR SCREENING

cell line	origin	nature	cell surface antigens
P388	DBA/2	lymphocytic leukaemia, with lymphoblast like morphology	Fc-receptor$^+$, complement receptor$^+$
DA-1		myeloid leukaemia, IL-3 dependent	Thy-1$^+$, Ly-5$^-$, Ia$^-$, IL-4 receptor$^+$
EL-4	C57Bl/6	T-lymphoma, secretes IFN-γ, IL-2 and MAF after PMA stimulation	Thy-1$^+$, Ly-1$^-$, Lyt-2$^-$, L3T4$^+$, TL$^-$, sIg$^-$, 14.8$^-$, Ia$^-$, H-2K/D$^+$
WEHI-3B	BALB/c	myelomonocytic, macrophage like leukaemia, or lymphocytic, or mixed lymphocytic-myelocytic leukaemia, IL-3 producing	Thy-1$^+$, Lyt-1$^-$, Lyt-2$^-$, sIg$^-$, 14.8$^+$, Ia$^-$, MAC-1$^+$ (78%), MBM-1$^+$

All cell lines were grown in the same medium as hybridomas were cultured (Chapter 2). MAF = macrophage activating factor; PMA = phorbol myristate acetate.

figure, the cell lines are ranked according to increasing autofluorescence intensities (increasing from top to bottom).

MCA PV5.6 is strongly reactive with all cell lines tested. EL-4 lymphoma cells were most brightly stained by PV5.6. This strong reactivity of PV5.6 with the panel of leukaemic cell lines of different origins was expected on basis of the staining profiles of the different bone marrow subpopulations. MCA 10-2.2 that was reactive with small percentages of all bone marrow subpopulations distinguishable by means of light scatter characteristics (Figs. 4.1 and 4.2), was also reactive with all tumour cell lines tested. Since these tumour cells are actively proliferating this could indicate that MCA 10-2.2 is directed against an antigenic determinant that is expressed on actively proliferating cells.

MCA 10-9.6 reacted with all tumour cell lines tested. It strongly stained the lymphocytic leukaemias P388 and EL-4 whereas this MCA only weakly stained lymphocytes in normal murine bone marrow (Fig. 4.2). This suggests that the determinant recognized by MCA 10-9.6 is expressed in a lower density on normal bone marrow lymphocytes than on the lymphocytic leukaemic cell lines.

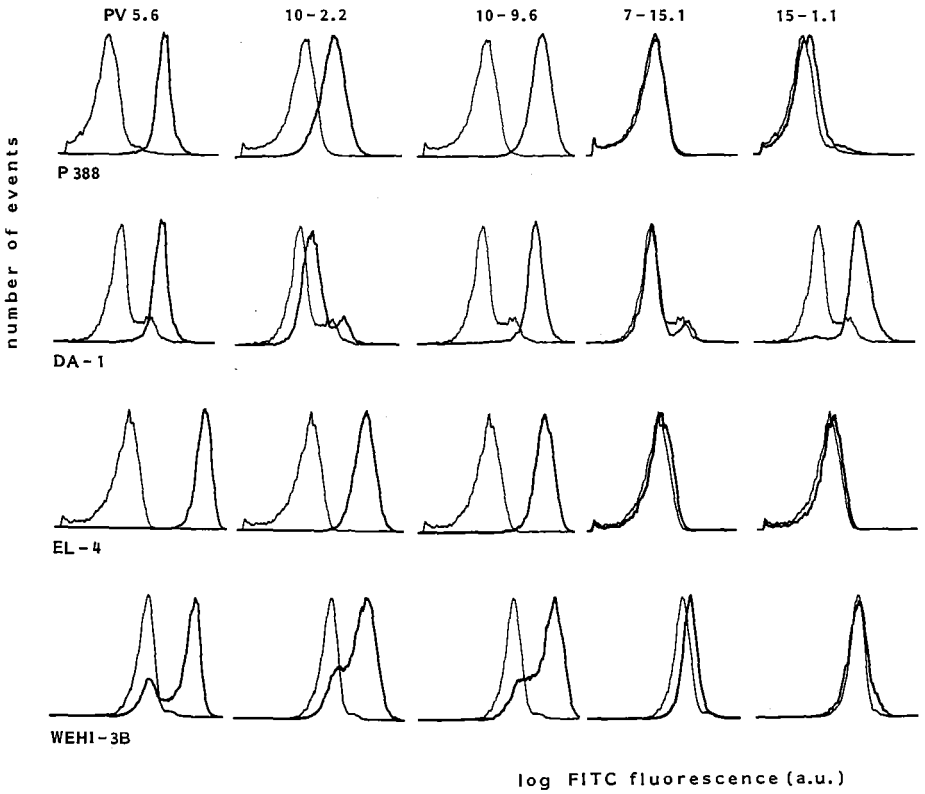


Figure 4.7:
Fluorescence of different tumour lines labelled with the MCAs that could not be CFU-S or CFU-C specific. Tumour cells were incubated with MCAs and GARA/FITC as described in Chapter 2. The thin lines show the fluorescence of tumour cells incubated with GARA/FITC only.

When WEHI-3B cells are stained with PV5.6, 10-2.2 and 10-9.6 and analyzed on the FACS, a small negative and a large positive population was observed. An explanation for this could be the mixed lymphoid myelocytic nature of WEHI-3B cells. It could also indicate that the cell line was not homogeneous (and should be subcloned). These possibilities were not further investigated in this study.

MCA 7-15.1 and 15-1.1 reacted very similarly on bone marrow cells (Fig. 4.2). Both MCAs hardly stained lymphocytes. The lack of reactivity of both MCAs with lymphocytes was confirmed by the analysis of the tumour cell lines. MCA 7-15.1 and 15-1.1 did not react with the two lymphocytic leukaemia cell lines, EL-4 and P388. A difference not earlier observed

between MCA 7-15.1 and 15-1.1 was noticed after screening both MCAs against the DA-1 cell line. MCA 7-15.1 did not react with this myeloid leukaemia, while 15-1.1 reacted strongly with DA-1 cells. Because DA-1 cells are dependent on IL-3 for their growth it could be that 15-1.1 was directed against the IL-3 receptor. This was examined by culturing the same amount of viable DA-1 cells in the presence of IL-3 (WEHI-3B conditioned medium) and in the absence or presence of different concentrations of MCA 15-1.1. After four days the cultures were terminated and the total number and the number of viable cells in each culture were counted. There was no difference observed between the cell numbers in cultures expanded in the presence or absence of 15-1.1. Thus, 15-1.1 was not reactive with the IL-3 receptor or at least did not inhibit the binding of IL-3 to its receptor.

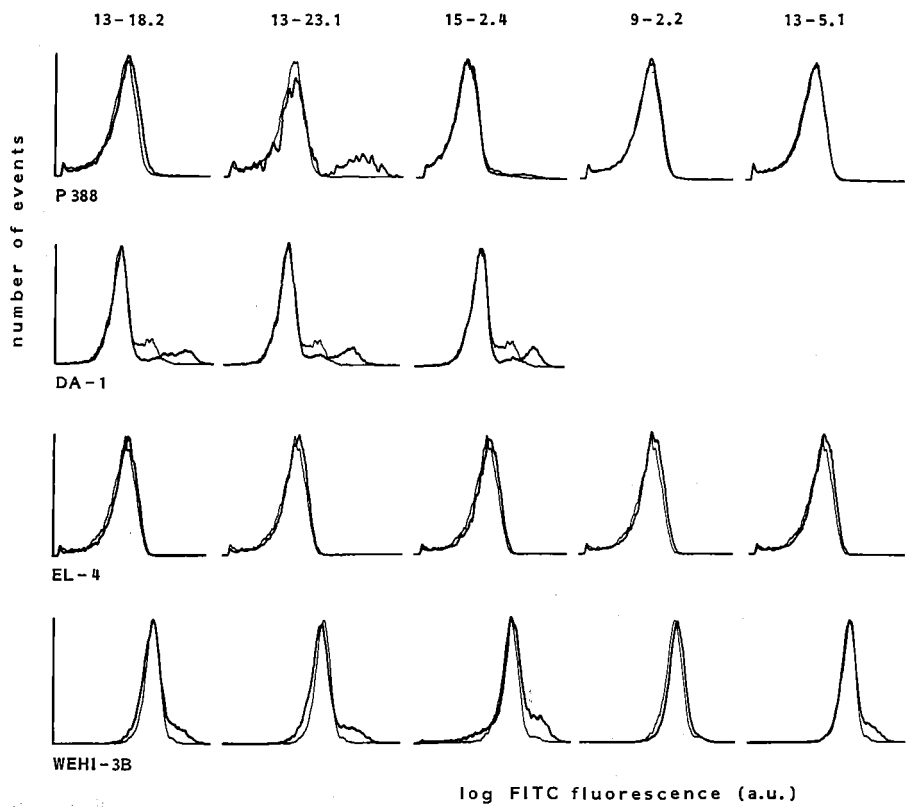


Figure 4.8:
Fluorescence of different tumour lines labelled with the MCAs that were reactive with only small amounts of bone marrow cells. Tumour cells were incubated with MCAs and GARA/FITC as described in Chapter 2. The thin lines show the fluorescence of tumour cells incubated with GARA/FITC only.

The observed difference between the reactivity of 7-15.1 and 15-1.1 with DA-1 cells suggests, but does not necessarily mean, that these two MCAs are directed against different antigens. Because 7-15.1 is an IgM antibody and 15-1.1 an IgG2a (Table 4.3), and the latter is much smaller than the former, which is a pentameric molecule complex, it could be that the determinant on DA-1 cells is more easily accessible for 15-1.1 than for 7-15.1. This might be an alternative explantation for the observed difference in reactivity of both MCAs with DA-1 cells. In addition, the affinity of the two MCAs for their determinants might be different. It has been described that IgM antibodies in general are characterized by a high avidity but a low affinity (Gosh and Campbell, 1986). These possibilities were not further investigated in this study.

In Fig. 4.8 the reactivity of the MCAs from group II (Table 4.1) with the panel of tumour cell lines is shown. MCA 13-18.2 and 13-23.1 which reacted similarly with normal bone marrow subpopulations (Fig. 4.3), both reacted with only a small subpopulation of DA-1 and WEHI-3B cells. They did not stain EL-4 cells. MCA 13-23.1 clearly stained a subpopulation of P388 cells, while MCA 13-18.2 was not reactive with these cells. Thus, MCA 13-18.2 did not react with lymphocytic cells as was already shown in Fig. 4.3, while MCA 13-23.1 seemed to be reactive with a subpopulation of lymphocytic P388 cells. MCA 9-8.2 and 13-19.4 that reacted similarly to MCA 13-18.2 and 13-23.1 with bone marrow subpopulations reacted similar to MCA 13-18.2. i.e. not with P388 cells.

MCA 15-2.4 reacted very much like 13-18.2 with the tumour cell line panel, while the reactivity pattern of both MCAs with normal bone marrow subpopulations was different (Fig. 4.3).

MCA 9-2.2 was not reactive with any of the tested leukaemic cell lines, whereas MCA 13-5.1 was only weakly reactive with a small number of WEHI-3B cells.

SCREENING OF MCAs AGAINST DIFFERENT ORGANS AND TISSUES

Each organ or tissue is characterized by a typical composition with regard to the cell types present, the amount of each cell type and the differentiation stage(s) of the cells (Table 4.5).

During embryogenesis the fetal liver is the major site of erythropoiesis, a function which is later taken over by the bone marrow and spleen (Metcalf and Moore, 1971; Löwenberg, 1975; Moore and Owen, 1965). In the fetal liver 70-90% of all cells belong to the erythroid series, including reticulocytes and erythrocytes. The other cells present mainly belong to the myeloid (granulocytic) series.

The thymus is a primary lymphoid organ which can microscopically be devided in a cortex and a medulla. The cortex is very densily packed with thymocytes, while the medulla has a lower density of lymphoid cells with many epithelial cells in between (Mathieson and Fowlkes, 1984). However, thymus cell suspensions contain a low number of nonlymphoid cells (Papiernik et al., 1987). According to data in the literature the murine thymus consists for more than 95% of T-lymphocytes of different maturation stages (Hämmerling et al., 1979; Hogarth et al., 1985; Oliveira and Thomas, 1985).

Table 4.5

DIFFERENTIAL COUNTS OF MURINE ORGANS AND TISSUES

organ/ tissue	% of total				
	immature myeloid	mature myeloid	lymphoid	monocytic	nucleated erythroid
fetal liver*	10				90
thymus	0	0	99	1	0
PBL	0	16	77	2	5
spleen	0	0	85	2	13
PEC	0	11	24	57**	8

*data from Löwenberg, 1975.
**mainly macrophages.

PBL (peripheral blood leukocytes) and PEC (peritoneal exudate cells) were isolated as described in Chapter 2. Unlabelled cell suspensions were run through a FACS-II and only nucleated cells were sorted. Cytocentrifuge preparations were made and stained as described in Chapter 2. BC3 female mice of 7-8 weeks of age were used. Figures represent mean of 2 experiments.
immature myeloid: myeloblasts, promyeloblasts and myelocytes.
mature myeloid: metamyelocytes, banded-, segmented-, and eosinophilic granulocytes.
monocytic: mainly monocytes, hardly any monoblasts and macrophages.
nucleated erythroid: mainly normoblasts, some erythroblasts.

In most cases, this was based on the percentage of α-Thy-1 (Reif and Allen, 1963) positive thymocytes. Erythroid and granulocytic cells are normally absent from the thymus (Metcalf and Moore, 1971). The thymus suspension analyzed in the present study consisted exclusively of lymphocytes. The major cell populations present in blood (peripheral blood leukocytes; PBL) are lymphocytes and to a lesser extent granulocytes, while virtually no normoblasts are present (Austyn and Gordon, 1981; Hirsch and Gordon, 1983). In the blood suspensions analyzed in this study the number of lymphocytes was about 5 times higher than that of granulocytes (mature myeloid cells).

The spleen is a secondary lymphoid organ and an important site of erythropoiesis in abnormal situations (Lord, 1967; Fruhman, 1966a; 1966b). The major cell type constituting the spleen is lymphocytes, while erythroid cells, which are present in scattered aggregates throughout the red pulp, form a minor subpopulation of spleen cells, like monocytes and granulocytes do (Hirsch and Gordon, 1983; Metcalf and Moore, 1971; Ralph et al., 1987). The data presented here are in agreement with these findings. By the use of (monoclonal) antisera it was determined that of all lymphocytes present in

the spleen, about 60% were B-lymphocytes and 40% were T-lymphocytes (Fultz et al., 1982; Hämmerling et al., 1979; Hogarth et al., 1985; McKearn et al., 1984; Shen et al., 1982).

Peritoneal exudate cells (PEC) were studied because the peritoneal cavity is the major source of mature macrophages. In PEC suspensions also a considerable amount of lymphocytes are present, while granulocytes and nucleated erythroid cells are minor constituents (Austyn and Gordon, 1981; Hirsch and Gordon, 1983). It was thought that screening of MCAs against the above discussed panel of organs and tissues might reveal possible lineage restriction of the MCAs.

In Fig. 4.9 the reactivity with the different organs and tissues of the MCAs that could not be CFU-S or progenitor cell specific is shown. PV5.6 strongly reacted with large populations of all organs and tissues except fetal liver. In fetal liver only 11% of the cells were stained which coincides well with the amount of myeloid cells in this organ. Its lack of reactivity with the majority of fetal liver cells and erythrocytes in normal bone marrow (Fig. 4.2) suggests that PV5.6 is not reactive with maturing and mature erythroid cells. The strong reactivity with large numbers of cells present in the other organs and tissues supports the notion that PV5.6 may be reactive with an antigen common to all leukocyte types.

MCA 10-2.2 reacted with most fetal liver cells. After staining with this MCA two subpopulations of fetal liver cells could be recognized. One large strongly positive population and a smaller weakly positive population. The latter consisted of more than 10% of all fetal liver cells, and therefore, must contain erythroid cells (Table 4.5). This suggests a heterogeneity of the erythroid cells in fetal liver with respect to the expression of the determinant recognized by MCA 10-2.2. This MCA also recognized a considerable proportion of spleen cells and PEC and only weakly stained PBL, but was not reactive with thymocytes.

MCA 10-9.6 weakly stained probably all fetal liver cells, while a small subpopulation is more intensely stained. MCA 10-9.6 also reacted (weakly) with large percentages of cells present in the thymus and PEC suspension, whereas in the spleen and in PBL distinct positive and negative subpopulations were observed. MCA 10-9.6 must therefore recognize a determinant shared between erythroid cells (fetal liver), T-lymphocytes (thymus, EL-4), granulocytes (bone marrow) and macrophages (PEC).

MCA 7-15.1 and 15-1.1 both reacted weakly with a small subpopulation of fetal liver cells. MCA 7-15.1 weakly stained cells present in peripheral blood, whereas after labelling with 15-1.1 a clear distinct positive subpopulation could be distinguished. Both MCAs did not stain thymus cells but they weakly stained a subpopulation of cells present in the spleen and PEC.

In Fig. 4.10 the reactivity with the different organs and tissues of the initially putative CFU-S or progenitor cell specific MCAs (group II, Table 4.1) is shown. MCA 13-18.2 stained a subpopulation of fetal liver cells, that, on basis of the reported percentage of myeloid cells present in fetal liver, must consist for at least a part of erythroid cells. MCA 13-23.1 was not screened against fetal liver cells, but reacted in the same way as MCA 13-18.2 with the other organs and tissues, except with PBL. MCA 13-18.2 did not stain PBL, whereas 13-23.1 reacted weakly with these cells. Both MCAs clearly reacted with a subpopulation of spleen cells and a proportion of PEC.

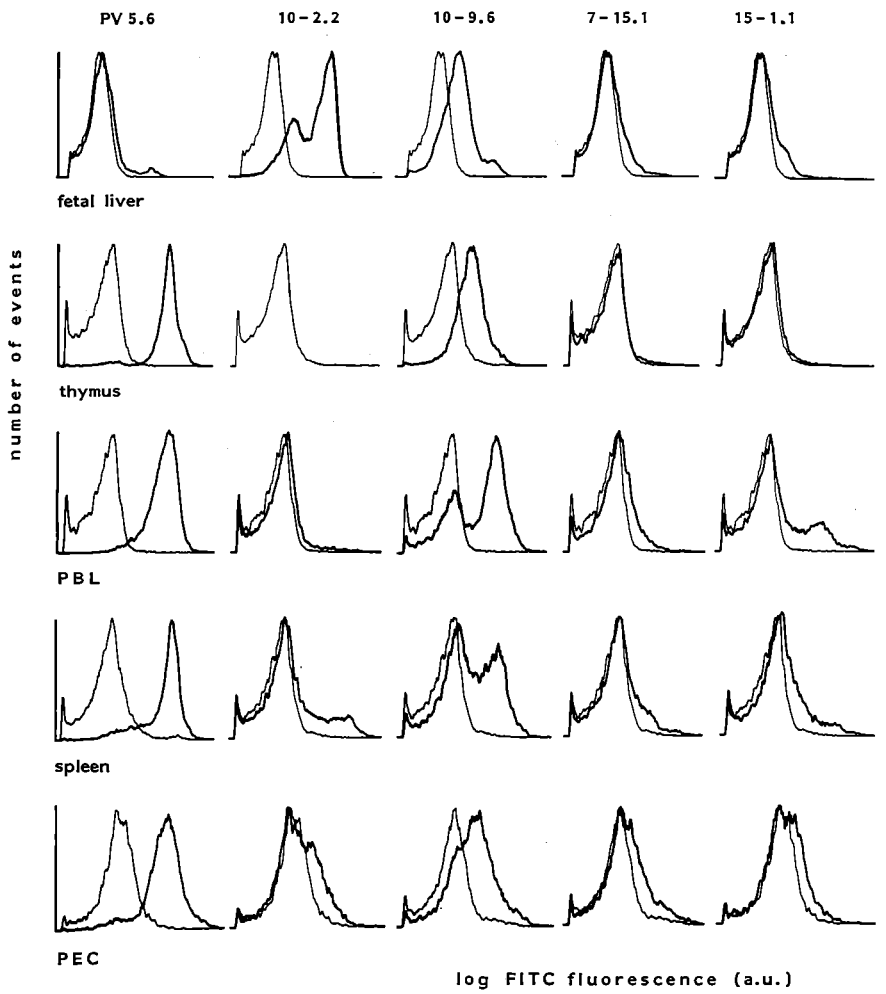


Figure 4.9:
Fluorescence of different organs and tissues labelled with the MCAs that could not be CFU-S or CFU-C specific. Organ and tissue suspensions were incubated with MCAs and GARA/FITC as described in Chapter 2. Organs and tissues were obtained from adult mice (8 weeks old). Fetal liver cells were obtained from fetuses at the 17th day of gestation. Only nucleated cells were analyzed except for fetal liver. All fetal liver cells were analyzed. The thin line shows the fluorescence of organ and tissue samples incubated with GARA/FITC only.

MCA 15-2.4 reacted with a small subpopulation of fetal liver cells, spleen cells, and PEC, but not with thymocytes and only very weakly with PBL. MCA 9-2.2 only weakly stained PBL and a small population of spleen cells, while cells from the other organs and tissues were not stained. MCA 13-5.1 recognized a very small percentage of PBL and spleen cells and a higher number of PEC.

Screening against a panel of different organs and tissues did not result in a clear picture about the reactivity of the MCAs from group II (Table 4.1) with regard to the lineage and differentiation stage restriction.

DIFFERENTIAL COUNTS OF BONE MARROW SUBPOPULATIONS AND SORTED FRACTIONS OF TOTAL BONE MARROW AND BLAST CELLS AFTER LABELLING WITH MCAs

Murine bone marrow is a very heterogenous organ with respect to the number of different cell types and maturation stages of the cell types that are present. In Table 4.6 the results of the differential counts of all nucleated bone marrow cells and those of the four different subpopulations, that can be distinguished on basis of light scatter characteristics (Fig. 4.1), are shown. The cells in each subpopulation were sorted on a cell sorter. Of the sorted cell populations cytocentrifuge slides were made which were subsequently stained for morphological analysis (Chapter 2).

Table 4.6

DIFFERENTIAL COUNTS OF BONE MARROW SUBPOPULATIONS

	cytological differentiation (%)				
subpopulation	immature myeloid	mature myeloid	lymphoid	monocytic	nucleated erythroid
all nucleated	5	40	35	8	12
erythrocytes*	2	35	41	3	19
lymphocytes	0	1	93	1	5
blast cells	5	27	35	12	21
granulocytes	4	61	11	12	12

Bone marrow subpopulations were sorted on a FACS-II, cytocentrifuged and stained as described in Chapter 2. For details see subscript Table 4.5. Values represent mean result of 5 experiments.
*nucleated cells represent only a small contamination (less than 2%) of the cells in the erythrocyte cluster.

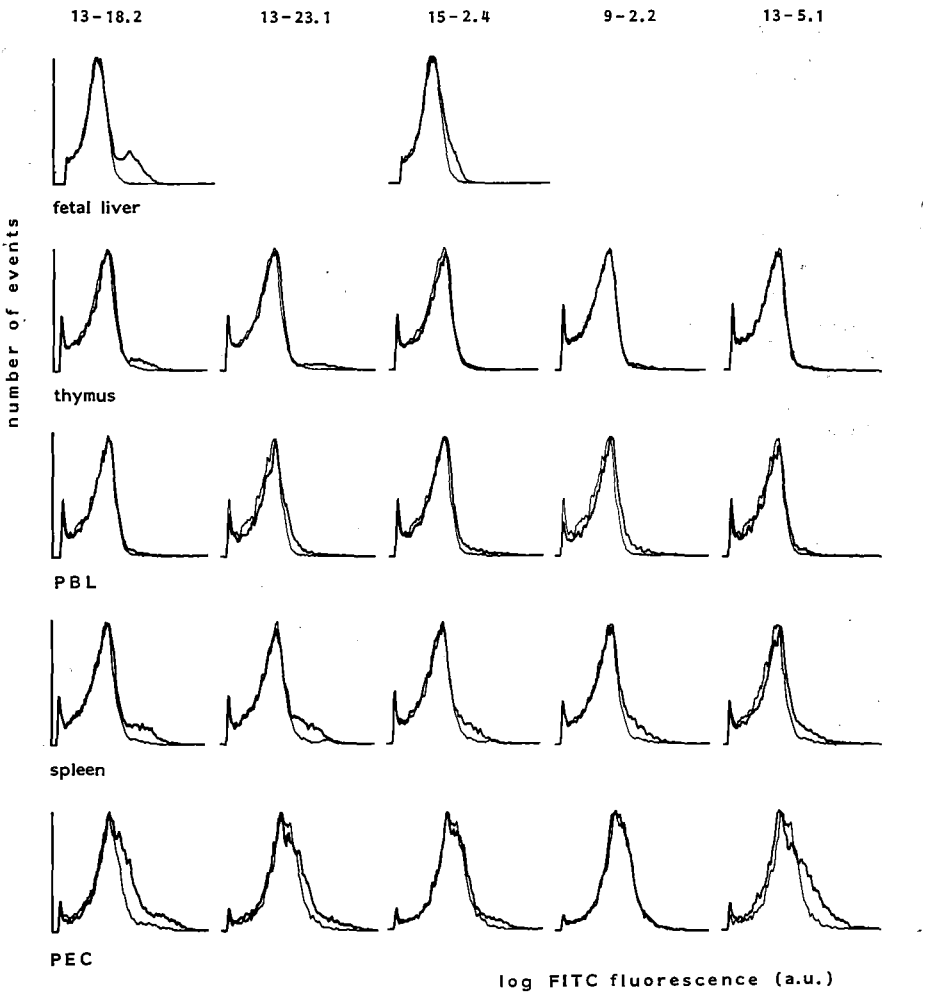


Figure 4.10:
Fluorescence of different organs and tissues labelled with the MCAs reactive with only small amounts of bone marrow cells. See legend Figure 4.9 for details.

It is clear that murine bone marrow is a major site for myelopoiesis. Of all nucleated bone marrow cells (erythrocytes were excluded from the analysis) 40% are maturing and mature myeloid cells, i.e. metamyelocytes, banded-, segmented-, and eosinophilic-granulocytes. In addition, 5% of the bone marrow cells are immature myeloid cells, i.e., myeloblasts, promyelocytes and myelocytes. This percentage of myeloid cells is in agreement with data in the literature (Austyn and Gordon, 1981; Bertoncello et al., 1986; Hirsch and Gordon, 1983; Hoang et al., 1983). In these studies, estimations on the number of cells of a certain lineage were either done by morphological analysis (Bertoncello et al., 1986; Hoang et al., 1983), or by labelling with MCAs specific for the myeloid series (Austyn and Gordon, 1981; Hirsch and Gordon, 1983). Another major constituent of murine bone marrow is formed by lymphocytes. Thirty-five percent of all bone marrow cells belong to the lymphoid lineage. This percentage of lymphocytes falls in the range (20-40%) reported by others (Opstelten et al., 1986; Shen et al., 1982). By means of MCAs that were reported to be specific for cells of either the B-lymphocytic or the T-lymphocytic series, it was determined that most lymphoid cells in the bone marrow belong to the former class. The percentage T-lymphoid cells (determined by the percentage α-Thy-1 positive cells) varied from 0% (Oliveira and Thomas, 1985) to 10% (Hogart et al., 1985), while the percentage of B-lymphoid cells varied from 19% to 40% (Hogart et al., 1985) depending on the MCA used. Eight percent of the bone marrow cells belonged to the monocytic series, which is in agreement with data found by others (Austyn and Gordon, 1981; Hirsch and Gordon, 1983). Most cells of the monocytic series were monocytes. It was observed in the present study, but also by others, that in bone marrow very few monoblasts, promonocytes and macrophages were present (Nibbering et al., 1987). Twelve percent of the bone marrow cells belonged to the erythroid lineage. Most of them were normoblasts, the latest stage during erythrocyte differentiation in which the cells are nucleated, while some erythroblasts were also present. The observed percentage of erythroid cells was in agreement with that found by others (Bertoncello et al., 1986; Hoang et al., 1983).

Thusfar, it was assumed that the subpopulations in murine bone marrow that could be distinguished on a cell sorter on basis of FLS and PLS characteristics (Fig. 4.1) were homogeneous. However, it is shown in Table 4.6 that most of the subpopulations were heterogeneous in this respect. The cells falling in the erythrocyte population contained cells of all differentiation lineages, especially lymphoid and mature myeloid cells and to a lesser extent nucleated red cells. However, these cells were greatly outnumbered by erythrocytes, but also reticulocytes, which were not counted. The lymphocyte population consisted almost entirely of lymphocytes. It could be that the number of erythroid cells (normoblasts) in this lymphocyte cluster was underestimated. It has been described that normoblasts are present in the lymphocyte population that can be distinguished on a cell sorter (Civin et al., 1987; Hoang et al., 1983). The major cell types present in the blast cell population are lymphoid cells (large lymphocytes, plasma cells) and mature myeloid cells, while erythroid cells and monocytic cells were present in somewhat smaller numbers. Immature myeloid cells comprise only 5% of all blast cells.

In the granulocyte cluster more than 60% of the cells are maturing and mature myeloid cells, while cells of the lymphoid, monocytic and erythroid series each account for about 12% of the cells present in this cluster, whereas immature myeloid cells form only about 5% of the cells.

Knowing the heterogeneity of the different bone marrow subpopulations, it was of interest to study with what cell types the MCAs that could not be CFU-S or progenitor cell specific were reactive. Only those MCAs were studied because some of them could be applied for the negative selection of these cell types, in contrast with the MCAs that were reactive with only small amounts of bone marrow cells.

The percentage of each cell type present in the negative or positive cell fraction(s) of all nucleated cells and the blast cell population are shown in Table 4.7. More than 90% of all immature and more mature myeloid cells and monocytic cells, and 80% of all the lymphoid cells present in total bone marrow were recovered from the PV5.6 positive fraction. In addition, 19% of all erythroid cells were recovered from the PV5.6 positive fraction. When PV5.6 labelled blast cell fractions were sorted, the majority of the myeloid cells, both immature and mature, and the monocytic cells was recovered from the positive fraction. In addition, about 50% of the lymphoid cells and the erythroid cells (1% of all cells present in bone marrow) were found in the positive fraction.

Virtually, 100% of the cells of all lineages except erythroid cells were recovered from the 10-2.2 negative fraction of all nucleated cells and of the blast cells. In both cell populations, 40-50% of the erythroid cells were recovered from the positive fractions. The absence of other cell types except normoblasts and erythroblasts in the 10-2.2 positive cell fractions suggests that this MCA is specific for these developmentally late erythrocyte precursor cells. The fact that about 50% of the erythroblasts and normoblasts are recovered from the 10-2.2 positive fraction indicates that these cell types are heterogeneous with respect to the expression of the antigen recognized by 10-2.2.

In total nucleated bone marrow samples MCA 10-9.6 stained almost hundred percent of the immature myeloid cells, more mature myeloid cells and monocytic cells. The number of immature myeloid cells in the 10-9.6 stained sample was very low in comparison with the mean value of these cells found in unstained bone marrow (Table 4.6) which makes the percentage of stained immature myeloid cells not very reliable. Only 10% of the lymphocytes were found in the 10-9.6 positive fraction and about 50% of the erythroid cells were present in this fraction. Analysis of the sorted blast cell fractions learned that about 90% of the mature myeloid and monocytic cells were recovered from the 10-9.6 positive fraction, whereas 56% of the immature myeloid cells and 40% of the lymphoid cells were recovered from this blast cell fraction.

Of all mature myeloid cells 97% were found in the MCA 7-15.1 negative blast cell fraction, and also about 80% of the lymphoid and erythroid cells were recovered from this fraction. In contrast, 90% of the monocytic cells and 42% of the immature myeloid cells were recovered from the 7-15.1 positive blast cell fraction.

Table 4.7

DIFFERENTIAL COUNTS OF FRACTIONS SORTED FROM ALL NUCLEATED BONE MARROW CELLS AND BLAST CELLS AFTER LABELLING WITH MCAs

			cytological differentiation (%)				
MCA	population	sorted fraction	immature myeloid	mature myeloid	lym-phoid	mono-cytic	nucl. ery-throid
PV5.6	all nucl.	-	0.3	0.5	6.8	0.3	19.2
		+	4.4	32.1	27.0	5.1	4.4
	blasts	-	1.3	11.7	20.4	1.3	8.7
		+	4.0	20.9	18.1	6.2	7.3
10-2.2	all nucl.	-	5.3	40.1	17.8	15.1	10.7
		+	0	0.9	0.1	0.4	9.6
	blasts	-	2.6	28.7	23.5	14.8	17.4
		+	0	0	0.8	0.5	11.7
10-9.6	all nucl.	-	0	0.5	37.6	0.5	8.5
		+	0.5	32.9	3.7	8.5	7.4
	blasts	-	2.2	1.5	20.4	1.1	11.3
		+	3.2	24.8	14.0	10.2	11.5
7-15.1	blasts	-	3.3	44.2	24.5	0.8	9.0
		+	2.4	1.4	5.1	6.9	2.4
15-1.1	all nucl.	-	3.6	1.0	36.7	0	9.7
		+	2.1	34.4	3.8	0.4	1.3
		++	0.1	1.1	2.0	1.1	2.7
	blasts	-	3.0	1.0	24.0	1.0	21.0
		+	0	4.1	6.0	20.2	7.1
		++	0.1	0.5	3.6	5.5	2.8

Bone marrow cells were incubated with MCAs and GARA/FITC and were sorted (-, negative; +, positive; ++, strongly positive) as described in Chapter 2. For details, see subscript Fig. 4.5 and Table 4.5.
The percentage of each cell type in each fraction was calculated with regard to the total amount of cells in either all nucleated cells or all the blast cells. Of each fraction hundred cells were counted.
Figures represent mean results of 2 experiments.

In all nucleated cell preparations, 15-1.1 did not react with the majority of lymphoid, erythroid and immature myeloid cells. However, it did react with almost all mature myeloid and monocytic cells. MCA 15-1.1 that reacted similar to 7-15.1 except with DA-1 cells and perhaps PBL, appeared to be different with respect to the reactivity with immature and mature myeloid cells in the blast cell fractions. MCA 15-1.1 did not recognize immature myeloid cells, whereas 7-15.1 reacted with about 40% of these cells. In addition, 15-1.1 reacted with about 80% of the mature myeloid cells, while 7-15.1 does not seem to be reactive with mature myeloid cells. In the blast cell fraction the majority of the monocytic cells were gained from the moderately 15-1.1 positive fraction, whereas in preparations of all nucleated cells the majority of the monocytes were recovered from the strongly positive fraction.

DISCUSSION

Murine pluripotent haemopoietic stem cells (CFU-S) and early committed progenitor cells (CFU-C) cannot be distinguished on basis of their morphological characteristics and physical properties (Van den Engh et al., 1981). CFU-S and the different types of CFU-C can be identified only by colony forming unit assays (CFU assays) (Civin et al., 1984). It is therefore of interest to identify specific cell surface markers on these cells. Such markers would make it possible to study CFU-S and CFU-C more directly. In addition, knowledge of the antigenic structure of CFU-S and CFU-C might help to understand the process of cell differentiation, the function of specific cell surface antigens in this process, and the molecular mechanisms controlling differentiation (Fitchen et al., 1980; 1981; Fukada, 1985; Koizumi et al., 1982; Till and McCulloch, 1980).

In spite of many attempts by a number of investigators no CFU-S or CFU-C specific antigens have been discovered (Heyman, 1985; Micklem, 1986; Watt et al., in press). A number of cell surface markers expressed on CFU-S and CFU-C have been described, but none of them were specific for these cell types. In fact, very few MCAs have been described that are differentiation stage or cell lineage restricted (Watt et al., in press). Perhaps the only known stage specific MCA is My-10 which is reactive with and specific for human haemopoietic progenitor cells of most, if not all, differentiation lineages (Civin et al., 1984; 1987; Strauss et al., 1986a; b).

Two diametrically opposed hypotheses have been put forth concerning the existence of CFU-S or CFU-C specific cell surface markers. Davis (1975) proposed that the pluripotent stem cell expresses none of the surface antigens of mature cells and therefore is a null cell. During the progress of differentiation maturing cells acquire differentiation antigens. In contrast, Till (1976) proposed that because of the multipotentiality of CFU-S, they must express antigens of all haemopoietic lineages. In this view the CFU-S are characterized by an abundance of surface markers, rather than a paucity of markers. None of these markers need necessarily be unique, nor need they be expressed at other than (very) low levels and with limited functional capacity. According to Watt et al. (in press) it is possible that neither of the two views is correct.

If the hypothesis proposed by Davis (1975) is correct then it is comprehensible that no CFU-S (or CFU-C) specific MCAs were identified. However, a number of antigens expressed on subpopulations of mature cells was also shown to be present on CFU-S (Fitchen et al., 1981; 1982; Harris et al., 1984a; Miller et al., 1985b; Ralph and Berridge, 1984; Russel and Van den Engh, 1979; Van den Engh et al., 1983). The determinants recognized by the in this study produced MCAs PV5.6, 10-9.6 and 10-7.3 were expressed on both CFU-S, various types of in vitro clonogenic progenitor cells and mature cells (Fig. 4.2). This indicates that the hypothesis proposed by Davis is most likely incorrect.

According to Till (1976) there might exist CFU-S specific antigens. Since no CFU-S specific antigens have been described this could mean that the hypothesis of Till was not correct, although the fact that no CFU-S specific MCAs have been produced does not necessarily mean that CFU-S specific antigens do not exist. Technical imperfections might be the reason for the fact that no CFU-S or CFU-C specific antigens have been identified and no CFU-S or CFU-C specific MCAs have been described.

A major obstacle in the production of CFU-S or CFU-C specific MCAs is that these cells are rare cells in the very heterogeneous bone marrow, and that antigens specific for these cells, like for instance certain growth factor receptors, will be expressed at very low densities (Berridge, 1979; Berridge and Okech, 1979; Metcalf, 1985c; Till 1976). Therefore, it is difficult to obtain enough of these antigens (CFU-S) to evoke strong immune responses against them.

In this study, animals were immunized with bone marrow suspensions up to 80-fold enriched for CFU-S to enhance the chance to obtain MCAs specifically directed against a determinant on these cells (Chapter 3). It could be that the degree of enrichment was not high enough to evoke immune responses against CFU-S specific antigens, assuming that such antigens exist. In addition, since all membranes consist of a large number of antigenic structures the probability to raise an immune response against immunodominant antigens expressed on the injected cells existed (Thalhamer and Freund, 1985). Immunodominant antigens mask the existence of weakly or non-immunogenic antigens, especially when the amount of these latter antigens is low (Dresser, 1986). The result of the presence of immunodominant antigens will be that no MCAs are produced against the other antigens which may be the CFU-S or CFU-C specific ones (O'Connor et al., 1987; Springer, 1980).

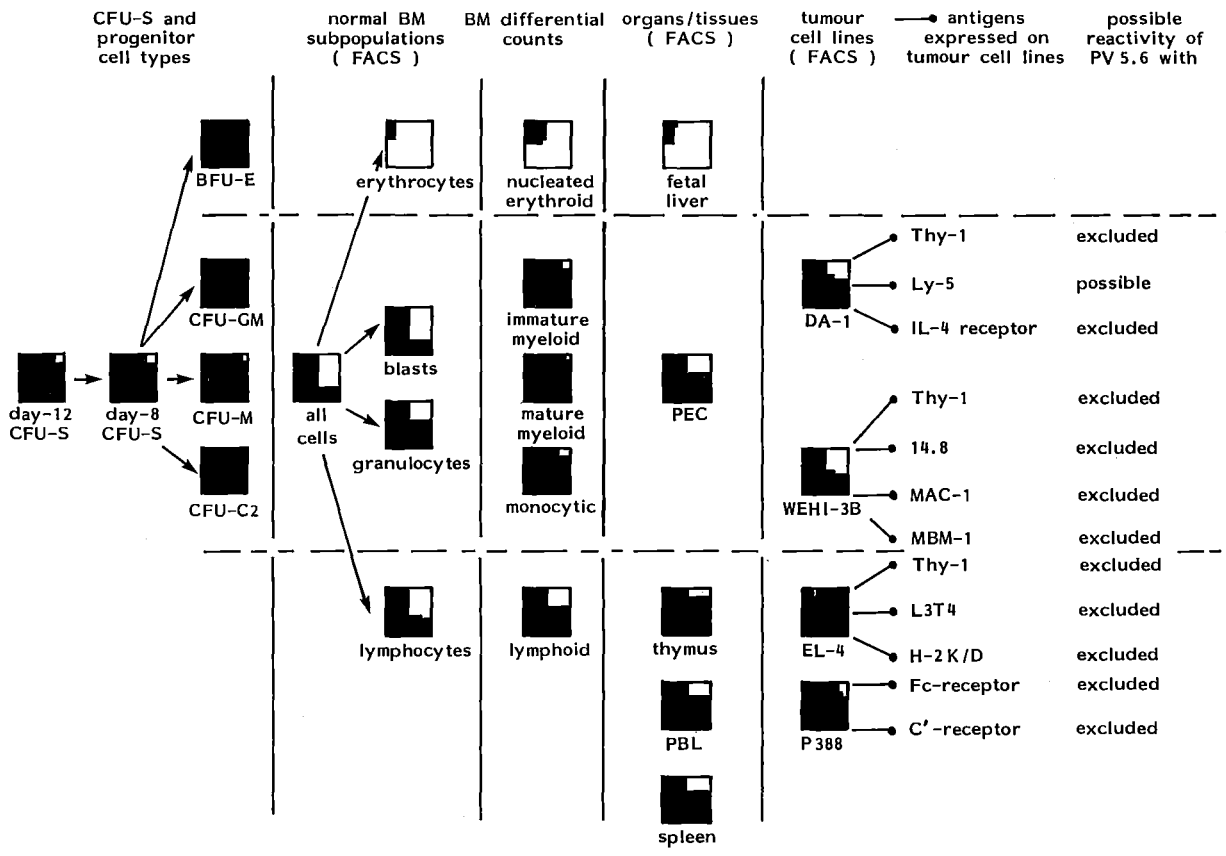
Another major factor that could have been the reason why no CFU-S or CFU-C specific MCAs were detected is the lack of sufficient sensitivity of the screening techniques. In this study a number of screening assays has been used, many of which have also been used by others. FACS analysis and sorting are in general considered to be the most sensitive and most objective ways of screening (Civin et al., 1987; Hessian et al., 1986; Hough et al., 1986; Ledbetter and Herzenberg, 1979). Still there are some disadvantages of this way of screening. Nonspecific fluorescence of dead cells and cells which express Fc-receptors on their membranes, especially monocytes, macrophages and granulocytes, but also B-cells, NK-cells and a minor population of T-lymphocytes, might give false positive results (Dangl and Herzenberg, 1982; Fitchen et al., 1981; Parks et al., 1986; Sasaki et al.,

1987). On the other hand, the arbitrary set threshold to discriminate between the presence or absence of antibodies might have given rise to false negative results in cases where supernatants gave only minimal fluorescence as was observed for the MCAs from group II (Table 4.1).

Although specific markers for CFU-S and CFU-C have not been identified, quantitative differences in expression of cell surface markers have been used to define different stages of maturation and for the enrichment of CFU-S and different types of CFU-C (Bertoncello et al., 1986; Harris et al., 1984a; Hasthorpe et al., 1985a; Hoang et al., 1983; Micklem, 1986; Miller et al., 1985a; Muller-Sieburg, 1986; Nibbering et al., 1987; Nicola, 1982; Nicola et al., 1981; Springer et al., 1979). It was therefore of interest to characterize the 5 MCAs that reacted with large percentages of bone marrow cells (Fig. 4.2) because these MCAs essentially could be of use for the depletion of mature cells from bone marrow samples and for the negative selection of CFU-S or different in vitro clonogeneic progenitor cell types. The pattern of reactivity of one such MCA, PV5.6, is schematically summarized in Fig. 4.11. This scheme is designed to allow a visual comparison of all data obtained with this MCA. Similar schemes were designed for the other four MCAs that reacted with large percentages of bone marrow cells (Figs. 4.12-4.15).

MCA PV5.6 reacted strongly with large percentages of T-lymphocytes (thymus, EL-4, PBL), B-lymphocytes (bone marrow, spleen, PBL), granulocytes (bone marrow, PEC, PBL) and monocytes/macrophages (bone marrow, PEC, WEHI-3B?). PV5.6 was not reactive with erythrocytes in bone marrow and most likely not with erythroid cells present in fetal liver. This pattern of reactivity is very similar to that described for antibodies directed against a heterogeneous group of high molecular weight glycoprotein antigens known as leukocyte common antigens (LCAs). These antigens were first described by Kumoro et al. (1975) and are encoded by the Ly-5 locus on chromosome 1 (LeCorre et al., 1987). Originally, it was thought that Ly-5 encoded antigens were expressed on thymocytes and T-lymphocytes only (Omary et al., 1980; Shen et al., 1982), but it has been proven that these antigens are also expressed on B-lymphocytes, granulocytes and monocytes/macrophages, but not on mature erythrocytes and their immediate precursors (LeFrancois et al., 1986; Ralph et al., 1987; Springer, 1980). In addition, it has been reported that LCAs are expressed on CFU-S, CFU-GM and BFU-E (Delwel et al., 1987; Miller, 1985a; Omary et al., 1980; Ralph et al., 1982; Ralph and Berridge, 1984; Van den Engh et al., 1983), which was also observed for the determinant recognized by PV5.6 and supports the notion that PV5.6 is directed against a LCA. The only observation which does not support this view is that 19% of the nucleated erythroid cells present in bone marrow were recovered from the PV5.6 positive fraction.

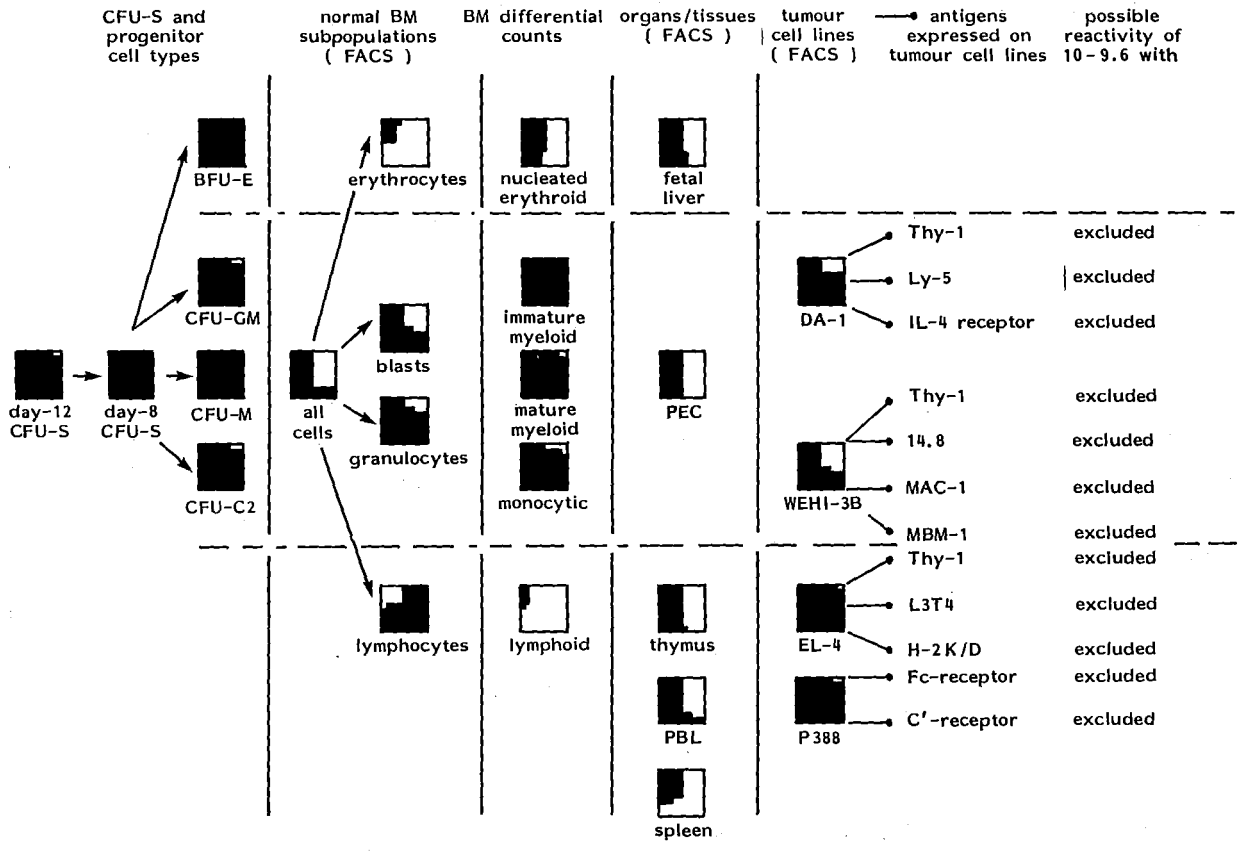
It has been described that LCAs are not expressed on the late erythroid progenitor cell; CFU-E (colony forming unit-erythroid) (Miller et al., 1985a; Van den Engh et al., 1983; Watt et al., 1983). Thus, if PV5.6 was directed against a LCA it could be of use for the negative selection of these progenitor cells. CFU-E were not assayed in the present study, because they have been reported to be selectively lost in cell sorting experiments (Lesley et al., 1984). Definite prove for the assumption that PV5.6 is directed against a LCA would have been obtained by means of immunoprecipitation, which was not done in the present study.


CFU-S and progenitor cell types
normal BM subpopulations (FACS)
BM differential counts
organs/tissues (FACS)
tumour cell lines (FACS)
antigens expressed on tumour cell lines
possible reactivity of PV 5.6 with
BFU-E
CFU-GM
CFU-M
CFU-C2
day-12 CFU-S
day-8 CFU-S
erythrocytes
blasts
granulocytes
all cells
lymphocytes
nucleated erythroid
immature myeloid
mature myeloid
monocytic
lymphoid
fetal liver
PEC
thymus
PBL
spleen
DA-1
Thy-1
Ly-5
IL-4 receptor
excluded
possible
excluded
WEHI-3B
Thy-1
14.8
MAC-1
MBM-1
excluded
excluded
excluded
excluded
EL-4
Thy-1
L3T4
H-2K/D
excluded
excluded
excluded
P388
Fc-receptor
C'-receptor
excluded
excluded

It can be excluded on basis of the known reactivity of the antigens expressed on the tumour cell panel that PV5.6 is directed against any of these antigens, except Ly-5: Thy-1 is expressed by only 0-10% of murine bone marrow cells (Hogarth et al., 1985; Oliveira and Thomas, 1985; Tidmarch et al., 1985); The Interleukin-4 receptor, like all other growth factor receptors, will only be present in very low densities on IL-4 responsive cells, e.g., (pre-)B and (pre-)T-lymphocytes, mast cells and macrophages (Metcalf, 1985c; Nicola, 1987a; O'Garra et al., 1988; Whetton and Dexter, 1986); MCA 14.8 specifically stains all B-lineage cells, including pre-B cells (Fulz et al., 1982); MAC-1 is identical to the complement receptor type 3 (CR3b) (Beller et al., 1982) and is specifically expressed on granulocytes and macrophages (Springer et al., 1979; Springer, 1980), but is absent from CFU-GM (Miller et al., 1985a) and erythroid and lymphoid cells (Springer, 1980); MBM-1 is absent from CFU-GM and BFU-E (Davis et al., 1983); L3T4 (CD4) is expressed on 90% of thymus cells (Reichert et al., 1986), T-helper cells and on some macrophages, but not on B-lymphocytes and granulocytes (Williams et al., 1985); MHC class I-antigens (H-2K and H-2D) are found on almost all cell types (Mellor, 1986). However, their density varies considerably. CFU-S and CFU-C2 express high amounts of H-2K antigens, whereas thymocytes, macrophages and granulocytes express considerably lower amounts of these antigens (Fitchen et al., 1982; Harris et al., 1984a; Russel and Van den Engh, 1979; Van den Engh et al., 1983; Williams et al., 1985); Nineteen percent of all bone marrow cells, but almost all PEC express Fc-receptors (Metcalf et al., 1975).

The pattern of reactivity of MCA 10-2.2 is depicted in Fig. 4.12. This MCA seems to be specific for erythroid cells and a determinant expressed on the panel of leukaemic cell lines. MCA 10-2.2 on the average stains 25% of the cells in the erythrocyte cluster in normal bone marrow (Table 4.1). However, probably most of the cells present in this cluster are positive for this MCA (Fig. 4.2), but only a part of them bound high enough quantities of 10-2.2 to be detected as positive (Parks et al., 1986). Almost 50% of the

Figure 4.11:
Schematic representation of the reactivity pattern of MCA PV5.6. The square boxes represent 100% of each indicated cell type. The black area in each box represents the percentage reactivity of the MCA. The reactivity of the MCA with CFU-S and progenitor cell types was determined by FACS sorting experiments. The percentage positive cells in the four bone marrow cell subpopulations are the mean percentages listed in Table 4.1. In the differential counts, the black areas represent the percentage of each cell type present in the positive fraction of all nucleated bone marrow cells labelled with MCA. The percentages were calculated from the figures in Table 4.7. The percentages positive cells in each organ and tissue and the percentage positive tumour cells were determined by FACS analysis. The antigens expressed on each tumour cell line are listed in Table 4.4


CFU-S and progenitor cell types
normal BM subpopulations (FACS)
BM differential counts
organs/tissues (FACS)
tumour cell lines (FACS)
antigens expressed on tumour cell lines
possible reactivity of 10-9.6 with
BFU-E
erythrocytes
nucleated erythroid
fetal liver
CFU-GM
immature myeloid
DA-1
Thy-1
excluded
Ly-5
excluded
IL-4 receptor
excluded
day-12 CFU-S
day-8 CFU-S
CFU-M
blasts
all cells
mature myeloid
PEC
granulocytes
WEHI-3B
Thy-1
excluded
14.8
excluded
MAC-1
excluded
MBM-1
excluded
CFU-C2
monocytic
lymphocytes
lymphoid
thymus
EL-4
Thy-1
excluded
L3T4
excluded
H-2K/D
excluded
Fc-receptor
excluded
PBL
P388
C'-receptor
excluded
spleen

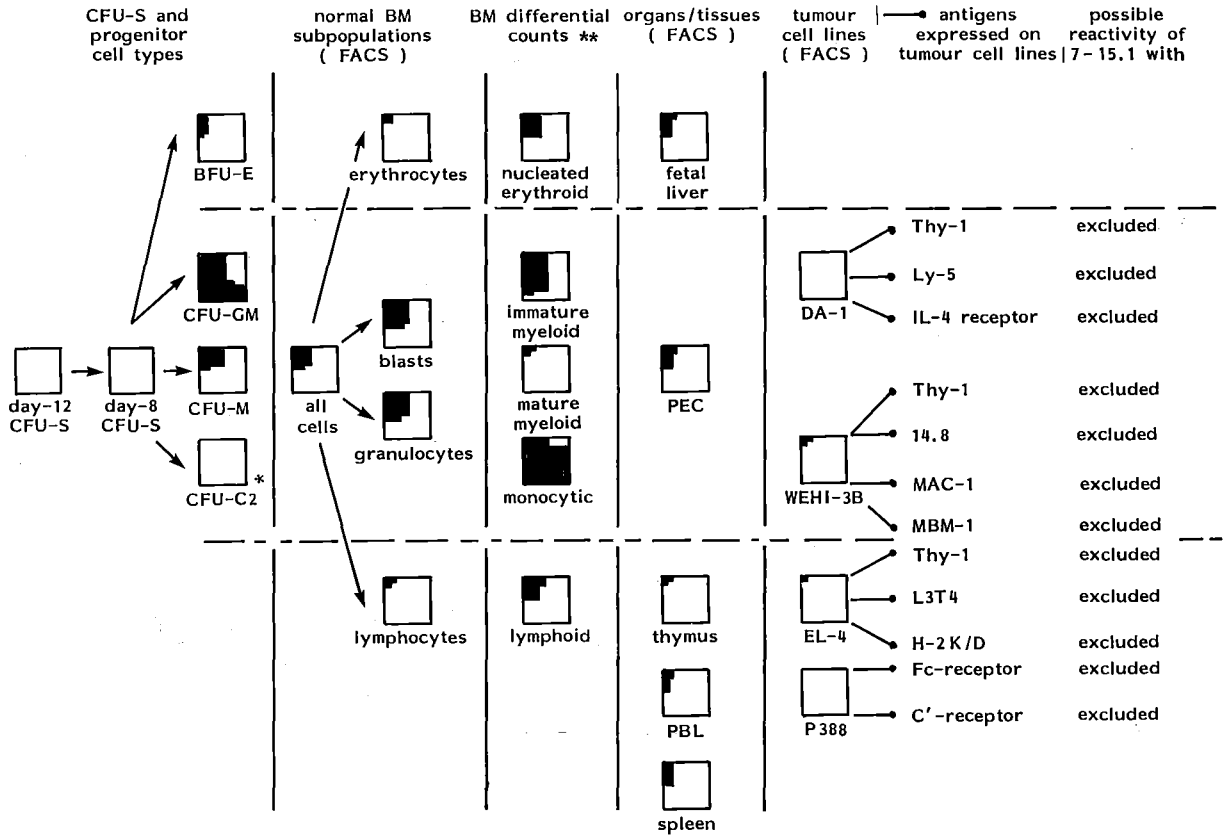
(H129.121, Van Agthoven et al., 1984) known to be directed against the TFR. The positive cells in the erythrocyte cluster are most likely reticulocytes present in this population and capable to take up iron, a process in which the TFR is involved (Van Agthoven et al., 1984).

Because 10-2.2 is not expressed on BFU-E it could be of use for the negative selection of these primitive erythroid progenitor cells. In addition, it could be of use in the study of the growth control of leukaemic cells. Sauvage et al. (1987) showed that an anti-TFR MCA had an inhibitory effect on the growth of a leukaemic cell line (SL-2). Immunoprecipitation would have proven if 10-2.2 is indeed directed against the TFR. However, this was not done in this study.

On basis of the reactivity of 10-2.2 and the expression of the antigens on the 10-2.2 positive tumour cell lines it can be excluded that 10-2.2 is directed against one of these antigens. Thy-1 is expressed on almost all thymus cells (Ledbetter et al., 1980; Ledbetter and Herzenberg, 1979; Sarmiento et al., 1982); Ly-5 is expressed on all leukocytes, but not on erythroid cells (Williams et al., 1985; Fig. 4.11); the IL-4 receptor is not expressed on erythroid cells (O'Garra et al., 1988); 14.8 is reactive with all B lineage cells (Fultz et al., 1982); MAC-1 is not expressed on erythroid cells (Springer, 1980); MBM-1 is expressed on 58% of bone marrow cells and 30% of spleen cells (Davis et al., 1983); L3T4 is expressed on thymocytes (Williams et al., 1985); H-2K and H-2D antigens are not or only weakly expressed on red blood cells (Fitchen et al., 1982; Harris and Gill III, 1985) and FcRs are not expressed on erythroid cells (Parks et al., 1986).

The reactivity of MCA 10-9.6 is shown in Fig. 4.13. This MCA did not or only weakly stained a very small percentage of cells in the erythrocyte cluster present in bone marrow. It did react with about 50% of the nucleated erythroid cells present in bone marrow and with probably most nucleated erythroid cells in fetal liver suspensions. This suggests a difference between these cells with respect to the the expression of the determinant recognized by 10-9.6 in these different organs. Nine percent of the lymphocytic cells present in bone marrow were recovered from the 10-9.6 positive fraction. This percentage coincides well with the % T-lymphocytes present in bone marrow as determined by means of a MCA directed against Thy-1 antigens (Hogarth et al., 1985; Tidmarch et al., 1985). However, 10-9.6 weakly stained most cells present in the lymphocyte cluster as distinguished on the FACS (Fig. 4.2). This indicates that other cells than lymphocytic cells must be present in this cluster, which are weakly 10-9.6 positive. It has been reported for human bone marrow that in the lymphocyte cluster also normoblasts were present (Civin et al., 1987). This also implies that the percentage nucleated erythroid cells in the lymphocyte population was underestimated (Table 4.6). The reactivity of 10-9.6 with most thymus cells and all T-lymphocytic leukaemic EL-4 cells is in favour of the assumption that 10-9.6 was directed against a determinant expressed on cells of the T-cell

Figure 4.13:
Schematic representation of the reactivity pattern of MCA 10-9.6. See legend, Fig. 4.11 for details.

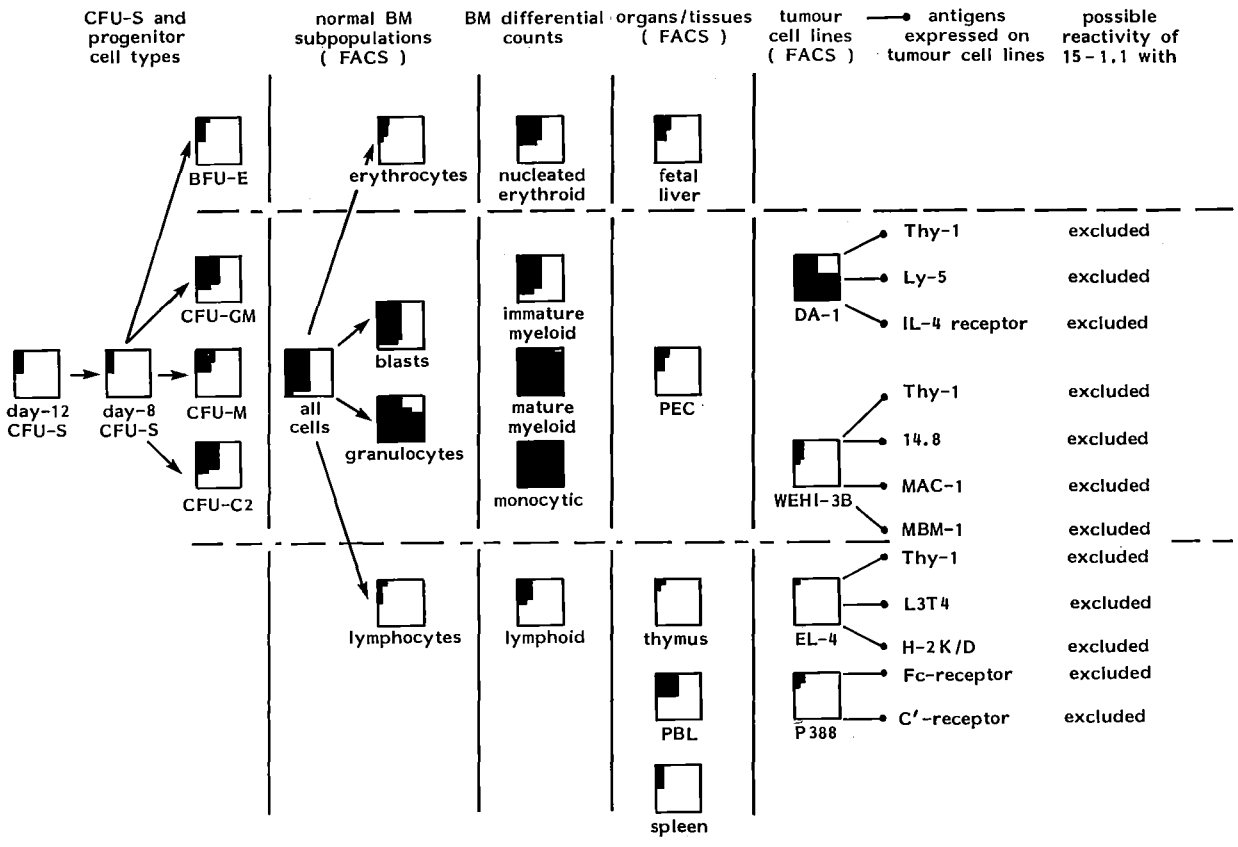

CFU-S and progenitor cell types
normal BM subpopulations (FACS)
BM differential counts **
organs/tissues (FACS)
tumour cell lines (FACS)
antigens expressed on tumour cell lines
possible reactivity of 7-15.1 with
BFU-E
erythrocytes
nucleated erythroid
fetal liver
CFU-GM
immature myeloid
blasts
day-12 CFU-S
day-8 CFU-S
CFU-M
all cells
mature myeloid
PEC
granulocytes
monocytic
CFU-C2 *
lymphocytes
lymphoid
thymus
PBL
spleen
DA-1
Thy-1
Ly-5
IL-4 receptor
excluded
WEHI-3B
Thy-1
14.8
MAC-1
MBM-1
excluded
EL-4
Thy-1
L3T4
H-2K/D
P388
Fc-receptor
C'-receptor

lineage. In addition, about 60% of the lymphocytes in peripheral blood and 40% of the lymphocytes in the spleen are α-Thy-1 positive (Ledbetter and Herzenberg, 1979). Together with the percentages of 10-9.6 positive cell types present in these organs, these percentages support the notion that 10-9.6 was directed against a determinant expressed on T-lineage cells but not on B-lineage cells. Because 10-9.6 is reactive with all CFU-S and in vitro clonogenic progenitor types tested and the determinant remained expressed on most of the mature cell types, this MCA seems not of much use for the purification of these cells.

It can be excluded that 10-9.6 is directed against the antigens present on the tumour cell lines, because Thy-1 is not expressed on fetal liver cells (Habu and Okumura, 1984); Ly-5 is not reactive with erythroid cells (Williams et al., 1985); the IL-4 receptor is expressed on low percentages of cells including B cells (Palacios et al., 1986; Ohara and Paul, 1987); 14.8 is B cell lineage specific; MAC-1 is absent from CFU-GM (Miller et al., 1985a) and lymphoid cells (Springer et al., 1979); MBM-1 is not expressed on BFU-E and CFU-GM (Davis et al., 1983); L3T4 is not expresssed on granulocytes (Williams et al., 1985); H-2K and H-2D are weakly expressed on granulocytes (Van den Engh et al., 1983) and because FcRs are present on only 19% of the bone marrow cells (Metcalf et al., 1975).

The pattern of reactivity of MCA 7-15.1 is schematically presented in Fig. 4.14. This MCA stained a large proportion of the cells present in the granulocyte cluster, and two clusters (together about 40%) of the cells present in the blast cell population, but did not stain, or very weakly stained cells in the erythrocyte and lymphocyte cluster (Fig. 4.2). Lack of reactivity with lymphocytes was also suggested by the fact that only very small numbers of thymus cells, spleen cells and PBL were stained, and the EL-4 and P388 cells were not stained. In Fig. 4.14 the differential counts of blast cells are shown. Of all nucleated erythroid cells present in the blast cell population, 21% was gained from the 7-15.1 positive blast cell fraction. Therefore, it cannot be excluded that 7-15.1 recognized a determinant expressed on these cells, although this does not seem very likely on basis of the small percentage of 7-15.1 positive fetal liver cells. This implies that this MCA could be reactive with both immature and mature myeloid cells and monocytes (Table 4.6). It is clear from the results of the differential counts on the blast cell fraction that 7-15.1 is reactive with monocytes. However, the determinant recognized by 7-15.1 seems to be lost from mature macrophages present in PEC suspensions, because 57% of the cells in these suspensions are macrophages (Table 4.5) and 7-15,1 only weakly stains a small population

Figure 4.14:
Schematic representation of the reactivity pattern of MCA 7-15.1. See legend, Fig. 4.11 for details.
* CFU-C2 not determined.
** blast cells instead of all nucleated cell fraction.


CFU-S and progenitor cell types
normal BM subpopulations (FACS)
BM differential counts
organs/tissues (FACS)
tumour cell lines (FACS)
antigens expressed on tumour cell lines
possible reactivity of 15-1.1 with
BFU-E
CFU-GM
CFU-M
CFU-C2
day-12 CFU-S
day-8 CFU-S
erythrocytes
blasts
all cells
granulocytes
lymphocytes
nucleated erythroid
immature myeloid
mature myeloid
monocytic
lymphoid
fetal liver
PEC
thymus
PBL
spleen
DA-1
Thy-1
Ly-5
IL-4 receptor
WEHI-3B
Thy-1
14.8
MAC-1
MBM-1
EL-4
Thy-1
L3T4
H-2K/D
P388
Fc-receptor
C'-receptor
excluded
excluded
excluded
excluded
excluded
excluded
excluded
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excluded
excluded

of PEC. From the constitution of PEC suspensions it follows that 7-15.1 must react with mature granulocytes which was also suggested by the weak reactivity with PBL, but is in contrast with the results of the differential counts. It could be that the determinant recognized by this MCA becomes earlier expressed on granulocytic progenitor cells than on monocyte progenitor cells, since a larger subpopulations of the CFU-GM, that can give rise to both granulocytes and macrophages, is stained by 7-15.1 than of the less primitive CFU-M, which exclusively differentiates along the monocytic lineage. It could be, although no evidence for this was obtained, that the CFU-GM stained by 7-15.1 are capable to give rise to granulocytes only. MCA 7-15.1 could be of use to study the hierarchical organisation of granulocyte and macrophage progenitors and to study the divergence of the granulocyte and macrophage lineages. In addition, because this MCA is not reactive with CFU-S, BFU-E and most CFU-M, it can be used for the negative selection and purification of these cells.

The determinant recognized by MCA 15-1,1 appears at the CFU-GM stage during granulocytic development (Fig. 4.15). During the progress of differentiation along the granulocytic pathway the density of the determinant recognized by 15-1.1 increases (Table 4.7): it is expressed on 38% of the immature granulocytic cells, whereas the more mature granulocytic cells in bone marrow are all positive for 15-1.1. It can be deduced from the reactivity with PBL and PEC and the percentage of granulocytic cells in these suspensions, that 15-1.1 is also reactive with mature granulocytes in these tissues. The expression of the determinant recognized by 15-1.1 in the monocytic lineage first appears at the CFU-C2/CFU-M stage of development. Monocytes in bone marrow express this determinant in higher densities than granulocytes do (Table 4.7). It can be deduced from the weak reactivity of 15-1.1 with a small percentage of PEC that the 15-1.1 determinant, abundantly present on bone marrow monocytes, is lost from mature macrophages.

Thus, 15-1.1 seems to be a monocytic and granulocytic marker but it cannot be excluded that 15-1.1 is also weakly reactive with a very small subpopulation of nucleated erythroid cells and/or lymphocytes present in bone marrow. This MCA can be used for the depletion of mature myeloid cells and monocytes, and for the purification by means of negative selection of BFU-E and CFU-S. The latter application will be extensively discussed in Chapters 5 and 6. It could also be useful for studies on the heterogeneity of CFU-GM and CFU-C2/CFU-M in combination with 7-15.1.

It can be excluded that 15-1.1 is directed against the Thy-1 antigen because it is not reactive with thymus cells (Lanier, 1984). For the same reason, it can be concluded that 15-1.1 is not directed against an antigen encoded by the Ly-5 locus (Williams et al., 1985). Because 15-1.1 is absent from bone marrow (B) lymphocytes, but is reactive with a large percentage of other bone marrow cells it can be excluded that this MCA is directed against the IL-4 receptor.

Figure 4.15:
Schematic representation of the reactivity pattern of MCA 15-1.1. See legend, Fig. 4.11 for details.

MCA 15-2.4 reacted with only small percentages of bone marrow cells and cells from other organs and tissues (Table 4.1; Fig. 4.10). It is the only MCA of group II (Table 4.1) that is reactive with an in vitro clonogenic progenitor cell type tested in this study (Table 4.2). MCA 15-4.2 was reactive with half of the BFU-E population assayed after 10-14 days culture and therefore, could be used for studies concerning the heterogeneity of this progenitor cell type. The determinant recognized by 15-2.4 is probably not expressed on nucleated erythroid cells in fetal liver and on cells in the erythrocyte cluster present in bone marrow, and therefore, seems directed against an antigen present on only part of the immature erythroid cells.

The other MCAs that reacted with only small percentages of bone marrow cells did not react with CFU-S or any of the progenitor cell types tested (Table 4.2). Because of the low percentages of cells recognized by these MCAs they are not of much use for the negative selection and enrichment of these cell types. Due to the small percentages of positive cells present in the various bone marrow subpopulations and organs and tissues after staining with this group of MCAs it was not possible to determine with what type(s) of cells these MCAs were reactive.

MCA PV5.6 and 10-2.2 are probably the only MCAs obtained in this study that are directed against previously described antigens, e.g. leukocyte common antigen and transferrin receptor, respectively. All other MCAs produced here (Table 4.1) are directed against unknown and thusfar undescribed antigens.

No CFU-S or in vitro clonigenic cell type specific MCAs were developed in this study. This could have been due to technical imperfections and insufficient sensitivity of the procedures employed to screen the MCAs, the not very high enrichment factor of CFU-S in the suspensions used for immunization (Chapter 3), but it could also be that in contrast with the human system, where a progenitor cell specific antigen seems to exist (Civin et al., 1984; 1987; Strauss et al., 1986a; 1986b) there exists no such antigen for murine haemopoietic stem cells. Such a situation may be analogous to that observed for tumour antigens. Those are often more tumour selective than tumour specific (Schmidt et al., 1986; Sela, 1986), i.e., antigens present on tumour cells are also expressed on normal healthy cells, but in different density allowing the identification and separation of tumour cells from normal cells. Similarly, alterations in cell surface antigens expressed on haemopoietic stem cells, that might be involved in the process of differentiation, may represent quantitative changes or structural changes of an antigenic array at the cell surface, rather than an absolute loss or gain of stem cell specific (types of) antigen(s) (Fitchen et al., 1981; Milstein et al., 1979).

CHAPTER 5

PURIFICATION AND ANALYSIS OF CFU-S BY BY MULTI-PARAMETER AND MULTI-COLOUR IMMUNOFLUORESCENCE FLOW CYTOMETRY

INTRODUCTION

The combination of immunofluorescence and flow cytometry has become an integral tool in the study of biological systems, especially in the discrimination between cells in heterogeneous populations like bone marrow (Civin et al., 1987; Lanier and Loken, 1984). This combination not only allows different subpopulations of cells to be distinguished in normal bone marrow on the basis of forward and right angle light scattering (Van den Engh and Visser, 1979), but at the same time, the enumeration of selected cells expressing a certain cell surface antigen and the determination of the quantity of the antigen expressed (Loken et al., 1987a; Civin and Loken, 1987). Quantitation of immunofluorescence is very important because a (sub)population of cells is often identified on the basis of the amount of a particular antigen, rather than simply on its presence or absence (Parks et al., 1986; Chapter 4).

A number of investigators exploited quantitative differences in the level of cell surface expression of lectin binding sites, or antigens, or a combination of both fluorescently labelled lectins and MCAs to purify CFU-S or CFU-C by means of flow cytometry (Nicola et al., 1981; Nicola, 1982; Watt et al., 1983; Visser and Bol, 1981; Hoang et al., 1983; Harris et al., 1984a; 1984b; Springer et al., 1979; Micklem, 1986; Mouchiroud et al., 1985; Bauman et al., 1986; Bauman and Chen, 1987; Lord and Spooner, 1986; Bertoncello et al., 1986; 1987).

Most methods to enrich CFU-S or CFU-C subsets consist of several steps and are of long duration and result in a low recovery of selected cells (Mouchiroud et al., 1985; Lord and Spooner, 1986). A MCA specific for CFU-S or different subsets of CFU-C, would be an ideal reagent for the identification and purification of CFU-S or CFU-C. It would also reduce the number of steps in enrichment procedures and thus would speed up such procedures. However, despite many extensive studies no such MCAs have been described (see Chapter 4), at least for murine stem cells.

Multicolour immunofluorescence and multiparameter flow cytometric analysis, in combination with measurements of forward and perpendicular light scatter intensities, allows for direct examination of the correlated expression of multiple antigens on the surface of individual cells (Lanier et al., 1986; Lanier and Loken, 1984; Loken and Lanier, 1984). Multicolour and multiparameter flow cytometry has proven very valuable for the detection of previously unidentified subpopulations of B- and T-lymphocytes in blood and bone marrow (Ledbetter et al., 1980; Hardy et al., 1982; Mathieson and Folwkes, 1984; Reichert et al., 1986; Crispe et al., 1987; Herzenberg et al., 1987; Lanier and Loken, 1984), and for the determination of the maturational sequence of antigen expression of erythroid cells and B-lymphocytic cells

present in unfractionated bone marrow (Parks et al., 1984; Loken et al., 1987a; 1987b). In addition, multicolour and multiparameter analysis has proven to be an alternative approach to identify CFC-S, making use of list mode data aquisition and processing which obviates the need for sorting of cells, and to purify CFU-S or CFU-C more rapidly than is possible with the sequential multistep procedures (Pallavicini, 1985a; 1985b, 1987; McCarthy et al., 1987; Civin et al., 1987).

In this chapter, a multicolour and multiparameter flow cytometric procedure is described that enables the in vitro identification of CFC-S present in unseparated bone marrow suspensions. This method makes it possible to directly determine the presence of certain cell surface antigens on CFC-S, and precludes the need to sort labelled cell fractions and to do a CFU-S assay. This approach represents a considerable saving of mice, and was exploited to rapidly and accurately screen newly produced MCAs (Chapter 4). Finally, a widely applicable procedure for the negative selection of CFU-S employing MCA 15-1.1 will be described.

TWO-COLOUR IMMUNOFLUORESCENCE MULTIPARAMETER FLOW CYTOMETRIC IDENTIFICATION AND ISOLATION OF CFU-S

The number of murine pluripotent haemopoietic stem cells is traditionally determined by means of the spleen colony forming assay (CFU-S assay; Till and McCulloch, 1961). This assay does not allow a direct enumeration of the stem cells (CFC-S), since the colonies that appear after 6 to 14 days in the spleens of lethally irradiated recipients are the progeny of individual CFU-S (Wu et al., 1967) and only a fraction of the injected stem cells in known to home to the spleen and form a colony there (Lahiri et al., 1970).

Direct measurements on CFC-S are only possible when highly purified CFC-S suspensions are available (Bodger et al., 1984; Hoang et al., 1983; Strife et al., 1987; Van Bekkum et al., 1971). In 1984, Visser and colleagues described such a procedure. This method consists of three subsequent separation steps. First, bone marrow cells are separated on a discontinuous density gradient of metrizamide and simultaneously labelled with the FITC conjugated sialic-acid binding lectin wheat germ agglutinin (WGA/FITC). Secondly, the low density cells (1.078 g/cm^3) are analysed on a FACS-II and the WGA/FITC positive cells with medium forward and low perpendicular light scatter intensities are sorted. The WGA/FITC is removed from the cells by incubation with the competing sugar N-acetyl-D-glucosamine. Finally, the sorted cells are incubated with a biotinylated MCA directed against H-2K^k antigens (α-H-2K^k-biotin) and FITC conjugated avidin (Av/FITC) and sorted a second time to select the strongly α-H-2K^k positive cells. In the second sorting run, the same light scatter windows as in the first sort were used (for details, see Chapter 2).

Based on the fact that multicolour immunofluorescence and multiparameter flow cytometric analysis enables the direct examination of the correlated expression of multiple antigens on the surface of individual cells (Lanier and Loken, 1984; Loken and Lanier, 1984; Lanier et al., 1986) and the high degree of purity of CFC-S that was obtained with the procedure described above, it was investigated if CFC-S could be identified and

isolated on the basis of simultaneous expression measurements of WGA binding sites and $H\text{-}2K^k$ antigens. Therefore, WGA and $\alpha\text{-}H\text{-}2K^k$-biotin must be labelled with different fluorophores. Low density bone marrow cells were incubated with $\alpha\text{-}H\text{-}2K^k$-biotin and phycoerythrin conjugated avidin (Av/PE) and with WGA/FITC (see Chapter 2). The labelled cells were analyzed and sorted on the RELACS, a dual laser flow cytometer built and developed at the Radiobiological Institute TNO (Stokdijk et al., 1985).

For the simultaneous measurement of the green FITC fluorescence and the orange PE fluorescence only one laser is needed. Both fluorescent dyes can efficiently be excited by light of the same wavelength, i.e. 488 nm (Fig. 5.1A). FITC and PE, a photosynthetic pigment isolated from red algae (Glazer, 1982; Oi et al., 1982) emit light at different wavelengths. This makes it possible to use both dyes simultaneously when the total emission spectrum is separated into its two components through the use of optical filters (Dean and Pinkel, 1978). In Fig. 5.1B it is shown that the two fluorescence signals can be separated when the right filter combinations are used. In this study, the FITC and PE fluorescence were separated with a 570 nm dichroic mirror and selectively collected using a 530 nm BP filter for FITC fluorescence and a 577 nm BP filter for PE fluorescence.

As can be seen in Fig. 5.1B, a part of the FITC fluorescence will be detected by the detector for the PE fluorescence. To yield "true" FITC and PE signals on each detector, the spill over of the FITC fluorescence into the detector of the PE fluorescence must be electronically corrected (Lanier et al., 1986; Parks et al., 1986, 1983; Preffer et al., 1986). The signals from FITC into the detector used to measure PE fluorescence were electronically compensated to background levels using samples that were stained with WGA/FITC only (Loken and Lanier, 1984; Parks et al., 1986; Wognum et al., 1987).

Forward and perpendicular light scatter intensities (FLS and PLS) were measured through 488 nm BP filters and were linearly amplified. The FLS signal was used to generate a light scatter pulse width or time of flight (TOF) signal. TOF can be used to distinguish single cells from coincident arrivals of clumps of two cells or more (Civin and Loken, 1987; Sharpless et al., 1975). FITC and PE fluorescence signals were processed using logarithmic amplifiers. The correction of the FITC spill over into the PE detector was done before logarithmic conversion (Parks et al., 1986).

Of each cell five parameters, i.e., TOF, FLS, PLS, FITC- and PE fluorescence were measured and stored in a list mode file by a HP9000-200 computer. Subsequential reanalysis of the data was performed with the ELDAS software package written in the Radiobiological Institute by R. Jonker. Analysis of the list mode data was done using window settings which were derived from the FACS analysis and sorting experiments on the individual markers separately (Visser et al., 1984).

In Fig. 5.2A the bivariate contour plot of the FLS versus the PLS intensities of all nucleated low density bone marrow cells is shown. This contour plot was computer generated from list mode data of 10^5 cells after exclusion of cell clumps by means of TOF (7.2% of all events in this case). Erythrocytes were gated out electronically before data storage to prevent the storage of irrelevant events in the list mode file. In this figure also the FLS and PLS windows that were set to select the blast cells, indicated by the

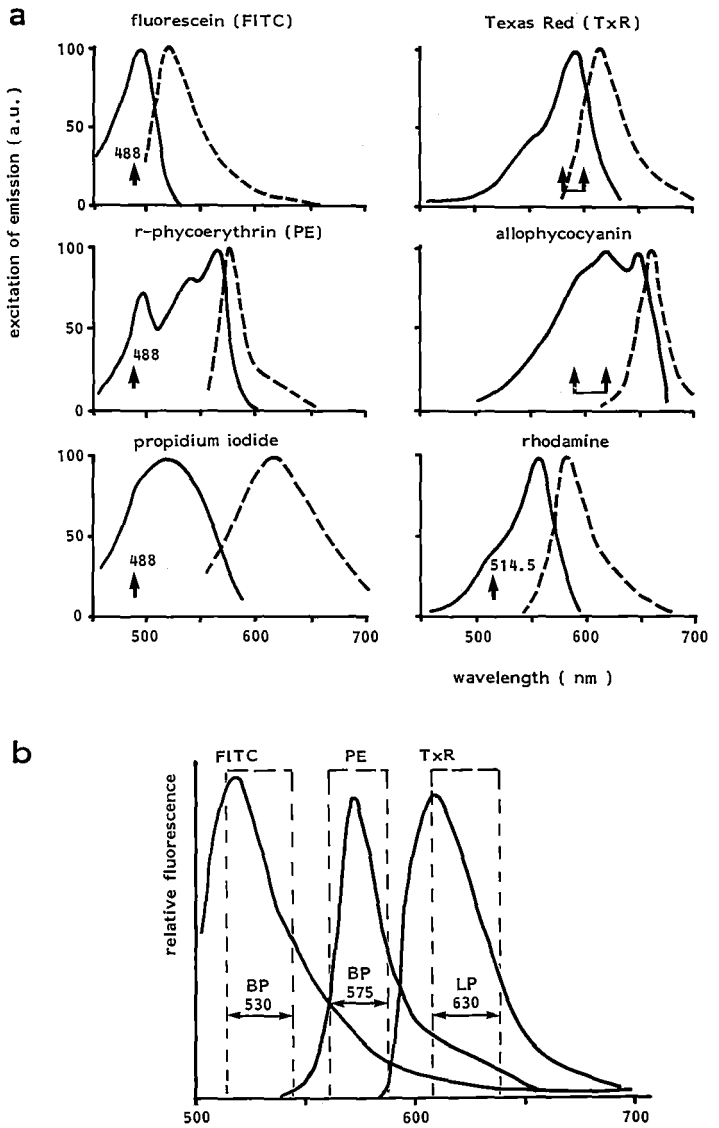


Figure 5.1:

a. Excitation spectra (solid lines) and emission spectra (broken lines) of dyes used in flow cytometric immunofluorescence studies. The arrows mark the argon-ion laser lines normally used to excite the dyes. The arrowed ranges mark the best range for excitation using a tunable dye-laser. Adapted from Parks et al. (1986).

b. The emission curves of FITC, PE and TxR. The rectangles approximate the transmission windows of the filters used to separate the emission from the three dyes. Adapted from Loken and Lanier (1984).

(shaded) rectangle, are shown. The windows shown were the same for computer reanalysis of the data and for the one sort procedure (see below). The fluorescence of these selected cells was further analyzed. In Fig. 5.2B the bivariate contour plot of the FITC fluorescence (due to WGA binding) versus the PE fluorescence (due to α-H-2K^k binding) distribution of the selected low density blast cells from Fig. 5.2A is shown. Fig. 5.2B also shows the FITC and PE fluorescence windows that were set to select the CFU-S from all the blast cells. First, the brightly WGA/FITC positive blast cells were selected; about 4 to 8 percent of the total low density cells. Subsequently, the PE fluorescence distribution of the selected WGA positive low density blast cells was analysed. A PE threshold was set so that 20 to 30 percent of the remaining WGA positive low density blast cells with high PE fluorescence intensities were selected. In this way, between 0.8 and 2.4% of the low density cells were selected. In the present study the mean yield of low density cells in three experiments was 15% of all bone marrow cells. This indicates that between 0.1 and 0.4% of all nucleated bone marrow cells were selected by the density separation and the light scatter and fluorescence windows. This percentage selected cells is the same as that selected by two sequential sorting runs as described earlier (Visser et al., 1984).

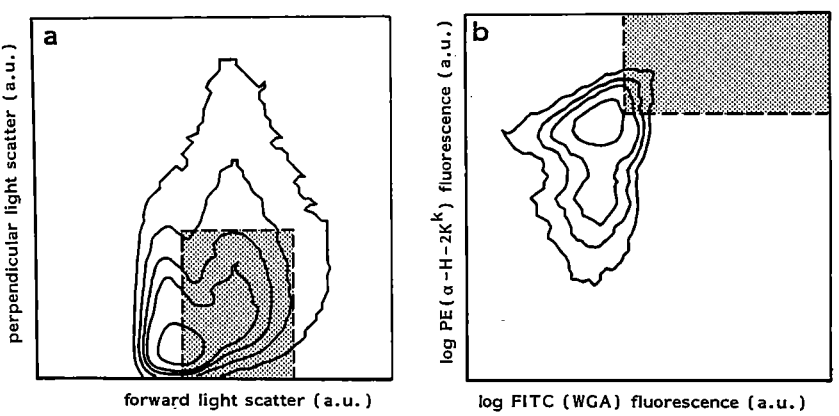


Figure 5.2:

a. Bivariate distribution of the forward versus the perpendicular light scatter of nucleated low density mouse bone marrow cells. This plot was generated from listmode data of 10^5 cells. The rectangle indicates the selected blast cell population. Contour lines were drawn at 2.6, 10.0, 21.0, 31.0 and 63% of peak value.

b. Bivariate logarithmic distribution of the FITC(WGA) versus the PE(α-H-2K^k) fluorescence distribution of the blast cells, selected as indicated in A. The rectangle indicates the fluorescence windows to select for CFC-S. Contour lines were drawn at 2.7, 8.2, 16.0 and 44.0 percent of peak value. Cells were stained and analysed on the RELACS as described in Chapter 2.

The CFU-S specificity of the staining and analysis procedure was tested by sorting the cells on the RELACS according to FLS, PLS, FITC (WGA) and PE (α-H-2K^k) intensities as shown in Fig. 5.2. In Table 5.1 the results of three different experiments, in which the cells were sorted in a single run, are presented. The sorted cells with medium FLS, low PLS, high FITC- and high PE-fluorescence intensities contained about 42 times more day-12 CFU-S and 10-20 times more day-8 CFU-S than control bone marrow samples (NBM). Similar to the sequential two sort procedure described by Visser et al. (1984), the one sort procedure resulted in 2-3 times more day-12 CFU-S than day-8 CFU-S. However, the enrichment of CFU-S obtained with the latter procedure is a factor 3 to 4 lower. The same holds for the recovery of

Table 5.1

ENRICHMENT AND RECOVERY OF CFU-S USING THE ONE SORT PROCEDURE

mouse strain	sorted fraction	% nucleated cells	CFU-S/10^5 nucleated cells		enrichment (% recovery) CFU-S	
			day-8	day-12	day-8	day-12
C3H	NBM	100	26.4±3.5	25.7±3.7	1 (100)	1 (100)
	WGA$^+$/α-H-2K^{k+}	0.3	550 ±183	1100 ±305	21 (6.3)	43 (12.8)
BC3	NBM	100	24.4±3.4	28.9±3.5	1 (100)	1 (100)
	WGA$^+$/α-H-2K^{k+}	0.4	250 ±125	1188 ±272	10 (4.5)	41 (18.1)
BC3	NBM	100	n.d.	25.6±2.2		1 (100)
	WGA$^+$/α-H-2K^{k+}	0.1	n.d.	1000 ±354		39 (3.1)
	WGA$^+$/α-H-2K^{k+}*	0.1	n.d.	1200 ±275		47 (4.7)

Low density bone marrow cells were labelled with WGA/FITC, α-H-2K^k-biotin and Av/PE as described in Chapter 2. Cells indicated by an asterisk (*) were labelled with WGA conjugated to the red fluorescent dye Texas Red (WGA/TxR) instead of WGA/FITC. Cells were analysed and sorted on the RELACS as described in the text. For enumeration of the number of CFU-S in normal (unseparated, unstained) bone marrow (NBM) and the sorted fractions, irradiated mice (6-8 per group) were injected with 3 x 10^4 respectively 150 cells. Spleen colonies were counted at 8 and 12 days post transplantation. Recovery of CFU-S was calculated as follows:

$$\left(\frac{\%\text{ nucleated cells in fraction} \times \text{CFU-S/}10^5\text{ nucleated cells in fraction}}{\text{CFU-S/}10^5\text{ nucleated cells in NBM}}\right)$$

n.d.: not done

CFU-S. The recovery of day-8 CFU-S and day-12 CFU-S on the average was 5.4% and 10%, respectively. Assuming that the day-12 CFU-S isolated by the one sorting run procedure have the same spleen seeding efficiency (10%) upon transplantation as those isolated by the procedure of Visser et al. (1984), it can be calculated that the isolated cell suspension consisted for 10% of day-12 CFU-S.

An explanation for the low enrichment factors and recoveries of CFU-S obtained with the one sort procedure could be that the recovery of low density cells after the density cut was rather high (15%), so that no optimal pre-enrichment of CFU-S was obtained. In three experiments, the enrichment factor of day-8 and day-12 CFU-S after a density cut was 2 and 2.5, respectively, which is 2-2.5 times lower than that obtained by Visser et al. (1984). Another explanation for the observed differences between the two methods could be the use of different avidin conjugates to detect bound α-H-2K^k-biotin molecules. This possibility was tested in more detail (see below).

THE INFLUENCE OF DIFFERENT AVIDIN- AND WGA-CONJUGATES ON THE RECOVERY OF CFU-S

The main differences between the one sort procedure described above and the two sequential sort procedure described by Visser et al. (1984) is that in the former Av/PE was used instead of Av/FITC and that WGA/FITC was not removed from the sorted cells.

Results of an experiment in which both avidin conjugates were compared in a two sorts experiment on the FACS are presented in Table 5.2. Both samples were treated identically except for the avidin conjugate. Selection with Av/PE resulted in a four-fold lower enrichment of day-12 CFU-S and a 1.5-fold lower enrichment of day-8 CFU-S. The lower enrichment of CFU-S after isolation with Av/PE compared with selection by means of Av/FITC indicates that the reported protection of CFU-S by biotin and avidin against opsonisation by macrophages (Van den Engh et al., 1983; Bauman et al., 1985) does not apply to biotin-Av/PE. A possible explanation for the observed phenomenon could be that after incubation with Av/PE not all biotin moieties on the α-H-2K^k molecule are occupied by Av/PE due to steric hindrance, because Av/PE conjugates are much bigger than Av/FITC conjugates. This could explain why the reduction of day-12 CFU-S was larger than that of day-8 CFU-S, since Mulder (1986) found that using the same batch of α-H-2K^k-biotin, day-12 CFU-S expressed more H-2K^k antigens than day-8 CFU-S. Incomplete shielding of the Fc-part of the α-H-2K^k molecule by avidin conjugates would result in opsonization by macrophages (Adler et al., 1978; Van den Engh et al., 1983; Bauman et al., 1985). According to Bauman et al. (1985) the degree of protection against opsonization is dependent on the amount of biotin per antibody molecule. High amounts give better protection.

The effect of different avidin conjugates was further investigated on NBM and the low density bone marrow fraction obtained after a density cut (Table 5.3). Incubation of NBM cells with Av/PE or Av/FITC resulted in 24 respectively 20 percent lower numbers of day-12 CFU-S in comparison with

unstained bone marrow, while no reduction in day-12 CFU-S was observed after incubation with Avidin/Texas Red (Av/TxR) (see later section). Addition of free avidin molecules (Av) resulted in an increased recovery of day-12 CFU-S for all the conjugates, above control values. This indicates that with Av/FITC and Av/PE in particular, not all biotin moieties on the α-H-2K^k molecules were occupied, resulting in opsonization of coated cells by macrophages and a loss of day-12 CFU-S.

Table 5.2

DIFFERENT AVIDIN CONJUGATES INFLUENCE THE ENRICHMENT OF CFU-S

sorted fraction	CFU-S/10^5 nucleated cells		enrichment CFU-S	
	day-8	day-12	day-8	day-12
NBM	26.7±3.5	31.0±3.8	1	1
-WGA/α-H-2K^k-biotin + Av/FITC	700 ±313	2500±646	26.2	80.6
-WGA/α-H-2K^k-biotin + Av/PE	467 ±226	600±226	17.5	19.4
+WGA/α-H-2K^k-biotin + Av/PE	267 ±189	533±267	10.0	17.2

Low density bone marrow cells were stained and sorted with the two sorts procedure on a FACS-II as described by Visser et al. (1984). WGA/FITC conjugates were either removed (-WGA) by incubation with N-acetyl-D-glucosamine, or were not removed (+WGA), before incubation with α-H-2K^k-biotin and Av/FITC or Av/PE.
For the spleen colony assay lethally irradiated mice (5-8 per group) were injected with 3 x 10^4 NBM cells or 100 cells of the sorted fractions. Colonies were counted after 8 and 12 days.

Labelling of low density cells with α-H-2K^k-biotin alone, resulted in a 33% reduction of day-12 CFU-S numbers in comparison with the unlabeled control. After addition of Av/PE or Av/FITC to cells treated with α-H-2K^k-biotin only a slightly higher number of day-12 CFU-S was found. As was observed in NBM the least reduction was observed when Av/TxR was used. Addition of free avidin resulted in higher recoveries, in particular in case Av/PE was used. It is clear that Av/PE and Av/FITC did not completely protect coated day-12 CFU-S against opsonization. It may be that the concentration of Av/PE and Av/FITC, in contrast with the concentration of Av/TxR, was not high enough to shield all biotin moieties on the α-H-2K^k molecules.

Table 5.3

THE EFFECT OF DIFFERENT AVIDIN CONJUGATES AND WGA CONJUGATES ON THE NUMBER OF CFU-S

incubation	day-12 CFU-S/10^5 nucleated cells	% recovery
NBM	16.7 ± 5.2	100
WGA/FITC	16.7 ± 3.3	100
WGA/PYR	23.3 ± 5.0	140
WGA/TxR	24.7 ± 3.2	148
α-H-2K^k-biotin + Av/PE	12.7 ± 2.3	76
α-H-2K^k-biotin + Av/PE + Av	19.3 ± 2.8	116
α-H-2K^k-biotin + Av/FITC	13.3 ± 5.3	80
α-H-2K^k-biotin + Av/FITC + Av	19.3 ± 2.8	116
α-H-2K^k-biotin + Av/TxR	16.0 ± 2.5	96
α-H-2K^k-biotin + Av/TxR + Av	22.0 ± 3.0	132
low density bone marrow fraction	36.7 ± 3.9	100
α-H-2K^k-biotin	24.5 ± 3.4	67
α-H-2K^k-biotin + Av/PE	26.9 ± 3.8	73
α-H-2K^k-biotin + Av/PE + Av	36.3 ± 3.9	99
α-H-2K^k-biotin + Av/FITC	28.3 ± 3.7	77
α-H-2K^k-biotin + Av/FITC + Av	32.2 ± 3.6	88
α-H-2K^k-biotin + Av/TxR	33.0 ± 3.9	90
α-H-2K^k-biotin + Av/TxR + Av	39.2 ± 4.0	107

Bone marrow cells were labelled with WGA/FITC, WGA/1-pyrenebutyryl (WGA/PYR) or WGA/TxR: 5 x 10^4 labelled cells were injected per irradiated mouse (8 per group). NBM cells and low density fraction bone marrow cells were incubated with α-H-2K^k-biotin alone (low density fraction), and 1 of 3 different avidin conjugates, with or without extra avidin. The concentrations used were the same as used for CFC-S identification and sorting experiments (Chapter 2) 2.5 x 10^4 low density fraction cells were injected per mouse (8 mice per group). Spleen colonies were counted 12 days post transplantation.

Hardly any difference was observed between the recovery of day-12 CFU-S from unseparated bone marrow cells or low density bone marrow cells when these suspensions were labelled with α-H-2K^k-biotin and Av/FITC or Av/PE (Table 5.3). In contrast, however, in experiments where low density cells were sorted employing either Av/FITC or Av/PE, a clear difference was observed between the recovery of CFU-S (Table 5.2). At present, no obvious explanation for this exists. The removal of accessory cells during the sort procedure has been proposed to cause a decrease in the recovery of

CFU-S (Bauman et al., 1985; Bertoncello et al., 1985; McCarthy et al., 1987; Visser and Eliason, 1983; Wickman et al., 1983). To achieve the highest recovery of CFU-S after sorting either the use of Av/TxR or the addition of free avidin after sorting of the cells seems advisable.

In Table 5.1, it is shown that selection of CFU-S with WGA/TxR or WGA/FITC resulted in about the same enrichment factor for day-12 CFU-S when the lectins were not removed from the cells. The enrichment with WGA/TxR was 1.2 times higher than with WGA/FITC. In Table 5.2 it is shown that removal of WGA/FITC from cells isolated by two sequential sorts did not result in a higher number of day-12 CFU-S, while the number of day-8 CFU-S was 1.7 times higher when the fluorescent lectin was removed.

No reduction of day-12 CFU-S was observed after incubation of NBM cells with WGA/FITC, WGA/TxR and WGA/PYR (see later section), although the recovery of CFU-S with the latter two conjugates was 1.4 times higher than with WGA/FITC (Table 5.3). Lord and Spooncer (1986) obtained very high enrichments of CFU-S by two sequential sorts using WGA/FITC. They did not observe any influence on the recovery of CFU-S when the lectin was not removed from the sorted cells. Others, however, did report a significant reduction of CFU-S numbers when the WGA conjugates were not removed from the cells (Ploemacher and Brons, 1988a).

Purification of CFU-S by the two-colour immunofluorescence one sort procedure with α-H-2K^k-biotin, Av/PE and WGA/FITC did not result in the highest possible enrichment and recovery of CFU-S (Table 5.1, 5.2). In this respect, selection with Av/TxR and WGA/FITC using two lasers (see later section) would have been a better option. However, for double or triple labelling procedures Av/PE may be a useful conjugate if the homing and survival of the selected CFU-S is not of interest. The purity of the CFU-S selected with the method described above does not seem to be very high. However, in reality it is probably 4 times higher than shown, because the calculations did not take into account the loss of spleen seeding capacity of Av/PE labeled cells. The selection procedure described here can be used for the in vitro identification of CFC-S and would allow direct analysis of the cell surface characteristics of these cells, when probes conjugated to a third fluorochrome are used.

THREE-COLOUR IMMUNOFLUORESCENCE MULTIPARAMETER FLOW CYTOMETRIC SCREENING OF MCAs FOR REACTIVITY WITH CFU-S

In a previous section it was shown that at least part of the CFC-S could be identified on the basis of light scatter characteristics and the correlated expression of WGA binding sites and H-2K^k antigens. This procedure can be used for the screening of MCAs for reactivity with CFU-S without performing a CFU-S assay when MCAs are detected with a different fluorochrome.

Although it is technically possible to excite three different fluorochromes with one laser (Sasaki et al., 1987) the use of a dual laser system is recommended because it reduces the dependency on (only) optical filters to discriminate the different fluorescence signals. With the dual laser system

the different dyes are excited sequentially so that their emission occurs at different times and their spectra may overlap without causing any difficulty in analysis (Dean and Pinkel, 1978; Loken and Lanier, 1984).

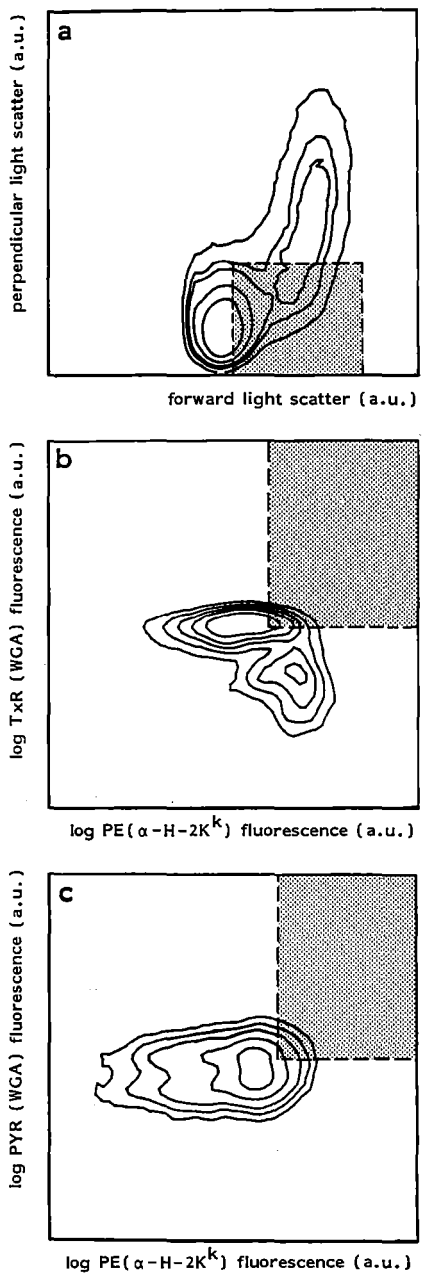
In this study the second (argon) laser of the RELACS, which allows for 8 parameter measurements, was used to excite either the red fluorescent dye Texas Red (TxR) (Titus et al., 1982), or the blue fluorescent dye 1-pyrenebutyryl (PYR) (Molecular Probes). Because most MCAs have been inactivated by the conjugation process with TxR (Parks et al., 1983) and no procedure was known to conjugate PYR to MCAs, it was decided to use WGA conjugates of these dyes which are readily available. TxR was excited by the 590 nm line emitted from a tunable dye laser circulating rhodamine 6G, which in turn was excited by the second laser operating in all lines mode (Arndt-Jovin et al., 1980; Lanier and Loken, 1984), TxR fluorescence was collected using two RG 630 nm LP filters (Fig. 5.1). PYR was excited by the second laser tuned to emit UV light with lines at 351 nm and 364 nm.

The first laser, tuned at 488 nm, was used for the generation of TOF, FLS and PLS signals, for the excitation of PE fluorescence due to bound α-H-2K^k-biotin, and for the excitation of GARA-FITC fluorescence due to the binding of the MCA to be screened. By digital delaying of the signals from the first laser, all 6 parameters of a single cell were read and stored simultaneously in a list mode file. Fluorescence signals from both lasers were processed using logarithmic amplifiers, TOF and scatter signals were processed linearly. FITC fluorescence falling into the detector used to measure PE fluorescence was corrected before logarithmic conversion as described in a previous section.

Screening of MCAs for reactivity with CFU-S was done basically in the same way as described in a previous section. Now, NBM was used, while WGA/TxR or WGA/PYR was used to select the brightly WGA positive blast cells instead of WGA/FITC. Results of this selection are shown in Fig. 5.3. First the blast cells were selected from all nucleated bone marrow cells by means of FLS and PLS, after exclusion of erythrocytes and cell clumps (TOF) (Fig. 5.3A). Subsequently, the strongly WGA positive blast cells were selected and finally the most strongly WGA/α-H-2K^k positive blast cells. In Figs. 5.3B and 5.3C the bivariate fluorescence distribution of the selected blast cells from Fig. 5.3A are shown. The fluorescence windows set to select the WGA/α-H-2K^k positive cells from all blast cells are indicated. In this way, 0.8 to 1.2% of the total amount of nucleated cells were selected.

In the contour plot of Fig. 5.3B a clear WGA negative, α-H-2K^k positive subpopulation of blast cells was present, while this population was absent in the contour plot of Fig. 5.3C. This difference can be caused either by a not completely identical blast cell selection (the plots were made in two different experiments), or by a less effective concentration and/or excitation of one dye or the other. In most experiments WGA/TxR was used (Table 5.1).

Screening of MCAs was done by measuring the FITC fluorescence of the selected WGA/α-H-2K^k positive blast cells. In Fig. 5.4, the results of the screening for 5 MCAs are presented. Histograms from control samples (incubated with GARA/FITC only) were superimposed over histograms from MCA stained samples. The characteristics of the MCAs PV5.6, 10-2.2 and 10-7.3 were discussed in Chapter 4. In brief, MCA PV 5.6 was shown to be


a
perpendicular light scatter (a.u.)
forward light scatter (a.u.)
b
log TxR (WGA) fluorescence (a.u.)
log PE(α-H-2K^k) fluorescence (a.u.)
c
log PYR (WGA) fluorescence (a.u.)
log PE(α-H-2K^k) fluorescence (a.u.)

directed against a leukocyte common antigen and to be reactive with day-8 and day-12 CFU-S. MCA 10-2.2 did not react with day-8 or day-12 CFU-S, but was mainly reactive against normoblasts and erythroblasts. MCA 10-7.3 (identical with MCA 10-9.6) was directed against day-8 CFU-S and day-12 CFU-S and, against mature myelomonocytic cells. In Fig. 5.4 it is shown that PV 5.6 was reactive with all WGA/α-H-2K^k positive blast cells (0.9% of all nucleated cells), while in the total bone marrow sample and in the blast cell population some unstained cells were present. MCA 10-2.2 did very weakly react with the WGA/α-H-2K^k positive blast cells, in contrast with the total blast cells and total bone marrow sample where a clearly distinct subpopulation of erythroid cells was stained. This could indicate that in the selected WGA/α-H-2K^k positive blast cells some erythroid cells were present. However, since only weakly stained cells were found, the presence of other cell types with a less specific binding of 10-2.2 (e.g., monocytes) cannot be excluded. MCA 10-7.3 reacted with 98% of the selected WGA/α-H-2K^k positive blast cells (1.2% of all nucleated cells), which was expected on ground of the data in Chapter 4. In the total bone marrow sample and in the blast cell population a larger proportion of the cells were unstained.

In multicolour analysis each stain must give the same fluorescence profile as when the reagent is employed individually (Parks et al., 1984; Lanier and Loken, 1984). Comparison of the fluorescence profiles of the MCAs in Fig. 5.4 with those in Fig. 4.2, where instead of all nucleated cells, all cells are presented, shows that this was the case. The three examples show that the procedure described here to screen MCAs for reactivity with CFU-S leads to similar conclusions about the reactivity of the MCAs as obtained by cell sorting and a CFU-S assay (Chapter 4). Screening in the former way can substantially reduce the number of CFU-S assays and is therefore much faster. About 20 MCAs can be screened in one day, which by means of the CFU-S assay would be hardly possible due to the time needed to sort and transplant the cells. In addition, the outcome of the tests is known the same day, whereas this takes 8-12 days when a CFU-S assay was performed.

Figure 5.3:

A. Bivariate distribution of the FLS versus the PLS intensities of nucleated mouse bone marrow cells. This plot was generated from listmode data of 10^5 cells. The rectangle indicates the selected blast cell population. Contour lines were drawn at 4.7, 9.3, 17.0, 37.0 and 70% of peak value.
B. Bivariate logarithmic distribution of the PE(α-H-2K^k) versus the TxR(WGA) fluorescence distribution of the blast cells as indicated in A. The rectangle indicates the fluorescence windows to select CFC-S. Contour lines were drawn at 11, 22, 36,60 and 75% of peak value.
C. Bivariate distribution of the PE(α-H-2K^k) versus the PYR(WGA) fluorescence distribution of the blast cells as indicated in A. The rectangle indicates the fluorescence windows to select CFC-S. Contour lines were drawn at 4.7, 9.3, 17.0, 37.0 and 70% of peak value. Cells were stained and analyzed on the RELACS as described in Chapter 2.

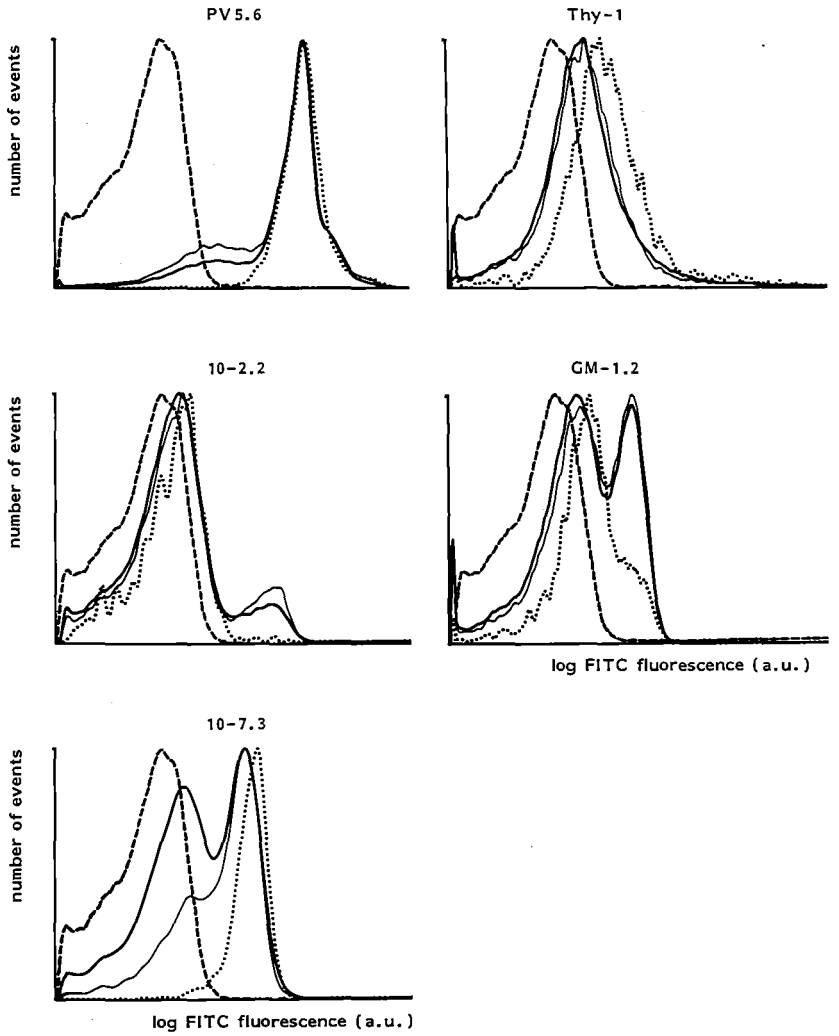


Figure 5.4:
FITC fluorescence distribution due to binding of MCA PV5.6, 10-2.2, 10-7.3, α-Thy-1 and α-GM1.2 to all nucleated cells (thick solid line), blast cells (thin solid line) and WGA/α-H-2K^{k} positive blast cells (dotted lines) from normal bone marrow. Cells were stained analysed and selected as described in the text and in Chapter 2. The control sample (broken line) was incubated without MCA.

The validity of the in vitro screening assay was also demonstrated with two commercially available MCAs: α-Thy-1 and α-GM1.2 (Fig. 5.4). It has long been controversial if the mouse CFU-S do express the Thy-1 antigen

(Watt et al., in press). By means of the cell sorter it was shown that CFU-S express low levels of Thy-1 antigen (Müller-Sieburg et al., 1986; Williams et al., 1985). It has been reported that day-12 CFU-S are weakly Thy-1 positive, while both Thy-1 positive and negative day-8 CFU-S exist (Berman and Bash, 1985; Mulder, 1986). Using three-colour analysis it was shown that most of the WGA/α-H-2K^k positive blast cells are weakly stained by the α-Thy-1 antibody. It can be seen that the selected putative CFU-S population was stained brighter than the unseparated blasts and total bone marrow. Since the histograms were generated by listmode the differences in peak channel number were true differences in fluorescence intensities and not due to staining variation from one sample to another (Pallavicini et al., 1985b). Miller et al. (1985b) showed that BFU-E and CFU-GM also express the Thy-1 antigen. Although not tested for progenitors, it is likely that the selected WGA/α-H-2K^k positive cells also contain CFU-C, because some types of CFU-C are copurified with CFU-S (Visser et al., 1984; Lord and Spooncer, 1986). The presence of CFU-C in the selected WGA/α-H-2K^k positive blast cells could have been responsible for the staining by α-Thy-1.

Bauman et al. (1986) described a two sorting run procedure to highly purify CFU-S, BFU-E and CFU-GM on basis of light scatter characteristics, WGA/FITC staining and selection of the α-GM1.2/FITC negative cells in the second sort. MCA GM1.2 is not reactive with CFU-S or CFU-C, but it is only reactive with mature myelomonocytic cells (Hibbs et al., 1984) and thus could be used for negative selection of CFU-S. In Fig. 5.4 it is shown that most WGA/α-H-2K^k positive blast cells are weakly stained with this antibody, while a small portion of these cells are somewhat more intensly stained. This indicates that a part of the selected WGA/α-H-2K^k positive blast cells must be mature myelomonocytic cells, which is in agreement with data observed by others. The main contaminants in the highly purified CFU-S population obtained by Lord and Spooncer (1986) were granulocytic cells which have a wide density range and variable light scattering ability. Visser et al. (1984) also reported the presence of a small population (15% of all sorted cells) of more mature cells in their highly purified CFU-S suspensions. In the next section, it will be shown that the WGA/α-H-2K^k positive blast cells are contaminated with monocytes.

The data of Bauman et al. (1986) are not necessarily in contradiction with the results presented here. Firstly, in FACS screening, the border set between negative and positive events is arbitrary chosen. Secondly, the simultaneous measurement of the α-GM1.2 fluorescence of the WGA/α-H-2K^k positive blast cells is probably more sensitive than the subsequent analysis. The latter proposition is supported by the data of Civin and Loken (1987), Parks et al. (1984), Lanier and Loken (1984), Herzenberg et al. (1987) and others.

SELECTION OF CFU-S BY MEANS OF WGA AND MCA 15-1.1 FLUORESCENCE

In Fig. 5.5 the result of the screening of MCA 15-1.1 (Chapter 4) against the WGA/α-H-2K^k positive blast cells is shown. It can be seen that the intermediately positive population present in the total bone marrow sample and in the selected blast cells disappeared by selection of the WGA/α-H-2K^k positive blast cells. It was shown in Chapter 4 that this

intermediately 15-1.1 positive blast cell population consisted predominantly of monocytes and some mature granulocytes. It was also shown (see Chapter 6) that the monocytes are lost upon repeated manipulation of cell suspensions if no precautions are taken. This could explain why Lord and Spooncer (1986) obtained a higher enrichment of CFU-S after a second WGA sort.

Labelling with MCA 15-1.1 separated the selected WGA/α-H-2K^k positive blast cell population into two subpopulations. Since it was shown (Chapter 4) that this MCA did not react with CFU-S it is clear that the selected cells in addition to CFU-S contain more mature cells, as was also suggested by the results of the three-colour screening with MCA GM1.2. The strongly positive population in the WGA/α-H-2K^k positive blast cells predominantly consisted of monocytes, but also some erythroid, lymphoid and more mature granulocytic cells were present (results not shown). It was expected that sorting the 15-1.1 negative, WGA/α-H-2K^k positive blast cells would result in a higher enrichment of CFU-S than selection with WGA and α-H-2K^k only. The result of 3 experiments in which CFU-S were selected according to 15-1.1/FITC fluorescence are listed in Table 5.4. In the first experiment it was shown that selection of the 15-1.1 negative, WGA/α-H-2K^k positive blast cells from unseparated bone marrow, resulted in a considerable enrichment of day-12 CFU-S. In this fraction, 8 times more CFU-S were present than in the 15-1.1 strongly positive, WGA/α-H-2K^k positive blast cells. In the second experiment, it was demonstrated that selection of the low density fraction, 15-1.1 negative, WGA/α-H-2K^k positive blast cells resulted in a 2-fold enrichment of day-12 CFU-S compared to selection of the WGA/α-H-2K^k positive blast cells only as shown in Table 5.1. Since it was shown that the 15-1.1 negative blast cells were α-H-2K^k positive (Fig. 5.6) it was investigated if selection by means of 15-1.1 could replace selection by means of α-H-2K^k. This would have several advantages. First, since 15-1.1 was directly conjugated with FITC (Chapter 2) the use of 15-1.1/FITC would speed up the labelling procedure. Second, 15-1.1 is not reactive with CFU-S, and therefore injection of 15-1.1 selected CFC-S would not lead to a reduction in the number of CFU-S, in contrast with α-H-2K^k labelled CFC-S (Tables 5.2 and 5.3). Thirdly, the α-H-2K^k MCA used in this study (clone 11-4.1; Oi et al., 1978) is only reactive against cells of mice with a MHC k phenotype (H-2^k) or with cells of mice with a partially H-2^k phenotype like mice of the BC3 strain, used in this study, which have an H-2$^{k/b}$ phenotype. In contrast, it was determined that 15-1.1 was not H-2 phenotype restricted. MCA 15-1,1 reacted similar, i.e., same percentage of positive cells and same staining profile, with bone marrow cells from mice with H-2^k, H-2$^{k/b}$, H-2^b, H-2^d, H-2^f and H-2^q phenotype (data not shown). From this it is clear that selection of CFU-S with 15-1.1 would be more widely applicable than with α-H-2K^k.

It is shown in the second experiment of Table 5.4 that selection with 15-1.1 and WGA only, resulted in the same enrichment, approximately 100-fold, as selection with 15-1.1, WGA and α-H-2K^k, when low density bone marrow cells were used. It should be noted that in the former case the cells were also incubated with α-H-2K^k-biotin and Av/PE, but were not selected for PE fluorescence. In the third experiment shown in Table 5.4 normal unseparated bone marrow cells were incubated with WGA/TxR and 15-1.1/FITC, but not with α-H-2K^k-biotin + Av/PE. Selection of the 15-1.1 negative, WGA positive blast cells resulted in a 42-fold enrichment for day-8

Table 5.4

SELECTION OF CFU-S WITH MCA 15-1.1

exp	sorted fraction	% nucleated cells	CFU-S/10^5 nucleated cells		enrichment (% recovery) CFU-S	
			day-8	day-12	day-8	day-12
	NBM	100	n.d.	24.8±2.9		1 (100)
I	$WGA^+/\alpha\text{-H-}2K^{k+}/15\text{-}1.1^+$		0.65	n.d.	175 ± 66	7 (4.6)
	$WGA^+/\alpha\text{-H-}2K^{k+}/15\text{-}1.1^-$		0.40	n.d.	1450 ± 66	58 (23.4)
	NBM	100	n.d.	24.2±3.0		1 (100)
II	$WGA^+/\alpha\text{-H-}2K^{k+}/15\text{-}1.1^-$		0.13	n.d.	2450 ±350	101 (13.2)
	$WGA^+/15\text{-}1.1^-$	0.17	n.d.	2300 ±339		95 (16.2)
	NBM	100	18.3±2.3	18.2±2.1	1 (100)	1 (100)
III	$WGA^+/15\text{-}1.1^-$	0.38	760 ±269	1583 ±363	42 (16)	87 (33.1)
	$WGA^+/15\text{-}1.1^+$	13.8	9.3±5.4	10.9±6.5	0.5 (6.6)	0.6 (7.8)

In experiment 1 and 2, NBM cells and the low density cell fraction respectively, were labelled with WGA/TxR, α-H-$2K^k$-biotin + Av/PE, and 15-1.1/FITC. In experiment 3, NBM cells were labelled with WGA/TxR and 15-1.1/FITC only. The cells were analyzed and sorted on the RELACS as described in the text. For the spleen colony forming assay, irradiated mice (6-10 per group) were injected with 3 x 10^4 NBM cells, and 150 sorted cells, respectively, except for mice receiving $WGA^+/15\text{-}1.1^+$ cells (experiment 3). These were injected with 450 cells. Colonies were counted 8 and 12 days post transplantation.
n.d.: not done.

CFU-S and an 87-fold enrichment of day-12 CFU-S. Selection of the 15-1.1 strongly positive, WGA positive blast cells shows that these cells contain fewer day-8 and day-12 CFU-S than unseparated bone marrow.

DISCUSSION

The regulation of haemopoiesis is dependent on complex interactions among haemopoietic cells, growth factors and stromal elements (Metcalf, 1984; McCulloch, 1983; Dexter, 1982). Cell surface molecules on haemopoietic cells must form an integral part of this system, and must be involved in the mechanisms controlling self-renewal and commitment of stem cells (Watt et al., in press). Knowledge of the cell surface make-up of CFU-S could therefore attribute to a better understanding of the complex processes taking place during the initial stages of haemopoiesis.

The very low incidence of CFU-S in the extremely heterogeneous bone marrow population has prevented direct analysis of their cell surface characteristics (Van Bekkum, 1977; Van Bekkum et al., 1979; Visser and Bol, 1981; Lord and Spooncer, 1986; Watt et al., in press). Nevertheless, many antigens have been found to be present on CFU-S. The presence of these antigens could only be determined indirectly by sorting labelled cell fractions with different fluorescence intensities on a cell sorter, or by treatment of bone marrow samples with antibody, with or without complement, followed by a CFU-S assay (Chapter 4).

It is known, from subsequent separation and analysis procedures, that CFU-S are characterized by a low density, a medium forward and a low perpendicular light scatter intensity and high densities of WGA binding sites and H-2K^k antigens on their surfaces (Visser et al., 1984). With the use of multicolour and multiparameter flow cytometry and listmode data processing it is possible to determine these characteristics (except density) simultaneously (Figs. 5.2 and 5.3). This procedure allowed for the rapid identification of CFC-S in vitro, without the necessity of performing a CFU-S assay. In this study, parallel CFU-S assays were necessary to show that the selected cells were indeed CFC-S. It is evident from the results in Tables 5.1, 5.2 and 5.4 that the selected cell population indeed contained CFU-S.

The lower enrichment and recovery of CFU-S obtained in the one sort procedure in comparison with the enrichment and recovery obtained by Visser et al. (1984) in the two sequential sort procedure, appeared to be mainly due to the used avidin conjugate to detect bound α-H-2K^k-biotin (Table 5.2). It was shown (Table 5.3) that Av/FITC and particularly Av/PE hardly protected CFU-S against opsonization, as was suggested by others (Van den Engh et al., 1983; Bauman et al., 1985). The use of higher concentrations of Av/PE and Av/FITC, or the addition of extra avidin to cover up the biotin moieties on the α-H-2K^k molecules, after the cells were sorted, or the use of Av/TxR would probably have resulted in higher enrichments and recoveries of CFU-S with the one sort procedure.

The lower enrichment of CFU-S does not detract from the fact that by the addition of an extra fluorescence parameter, the CFC-S identification procedure allows for the direct analysis of the presence of certain antigens on, at least a part of, the CFC-S. From the results shown in Fig. 5.4 it can

be concluded that the multicolour and multiparameter flow cytometric screening of the five MCAs for reactivity with CFU-S leads to similar results as obtained in Chapter 4 and those obtained by others who employed sorting of α-Thy-1 labelled (Berman and Basch, 1985; Mulder, 1986; Müller-Sieburg et al., 1986) and α-GM1.2 labelled cell fractions (Bauman et al., 1986). Thus, this new screening procedure can substantially reduce the number of CFU-S assays and can be a powerful means for the rapid screening of MCAs. It is estimated that with the multicolour screening procedure the reactivity of about 20 MCAs with CFU-S can accurately be determined in one day. This way of screening can prevent the culture of many hybridomas of which only a few appear to be interesting by the time the results of the CFU-S assay are known.

Screening of MCA 15-1.1 with the multicolour and multiparameter procedure learned that the selected CFC-S population did not entirely consist of CFU-S, as was already evident from the obtained enrichments (Table 5.1) and the screening of α-GM1.2 (Fig. 5.4). MCA 15-1.1 separated the selected CFC-S population into two subpopulations (Fig. 5.5). CFU-S are mainly present in the 15-1.1 negative subpopulation, while the 15-1.1 strongly positive subpopulation mainly consisted of monocytes and mature granulocytes, which is in agreement with the result of the screening and the known reactivity of α-GM1.2 (Table 5.4; Fig. 5.4; Hibbs et al., 1984).

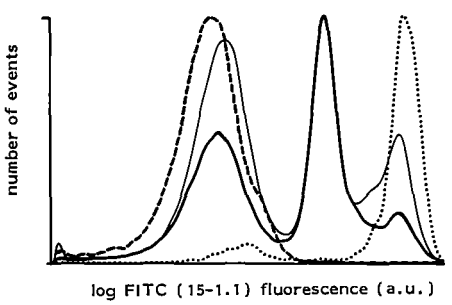


Figure 5.5:
FITC fluorescence distribution due to binding of MCA 15-1.1 to all nucleated bone marrow cells (thick solid line), blast cells (thin solid line) and WGA/α-H-$2K^k$ positive blast cells (dotted line). Cells were stained, analysed and selected as described in the text and in Chapter 2. The control sample (broken line) was incubated without MCA.

Based on listmode data analysis (Fig. 5.6) a two-colour immunofluorescence procedure was developed to rapidly identify and purify CFC-S on the basis of 15-1.1 fluorescence and WGA fluorescence. This procedure has the advantage over selection by means of α-H-$2K^k$ that it is faster due to fewer labelling steps, it excludes any influence of remaining labels on the sorted CFC-S, which might influence the homing and recovery of CFU-S, and that

it is widely applicable, because it is not restricted to cells of mice with specific H-2 haplotypes.

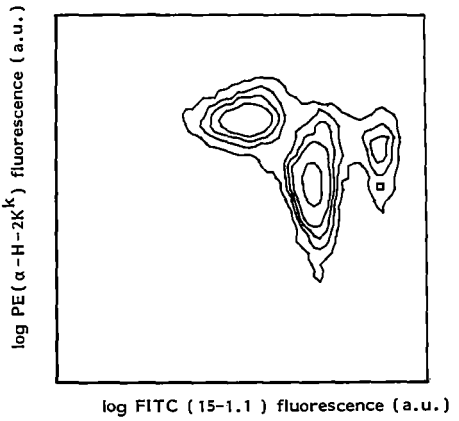


Figure 5.6:
Bivariate presentation of the FITC (15-1.1) fluorescence versus the PE(α-H-2K^k) fluorescence distribution of normal bone marrow blast cells. Cells were selected as described in the text. Contour lines were drawn at 6.5, 13.0, 19.0, 29.0 and 58% peak value.

Selection of CFU-S using two lasers to excite 15-1.1/FITC fluorescence and WGA/TxR fluorescence allows for the addition of other probes detected with other dyes, like for instance allophycocyanin coupled to another MCA and propidium iodide to exclude dead cells (Fig. 5.1; Chen et al., 1987; Parks et al., 1983; Müller-Sieburg et al., 1986). It was shown by others that certain CFU-C types copurify with CFU-S (Bauman et al., 1986; Bauman and Chen, 1987; Visser et al., 1984; Lord and Spooncer, 1986; Pallavicini et al., 1985a; 1987). This was also suggested by the reported reactivity of α-Thy-1 with CFU-GM and BFU-E (Miller et al., 1985) and the observed reactivity of α-Thy-1 with the selected WGA/α-H-2K^k positive blast cells (Fig. 5.4). Although it was not tested in the present study, it should be possible, when the right probes are used, to very accurately discriminate between subsets of CFC-C and CFC-S and to isolate these cell types by means of multicolour immunofluorescence and multiparameter flow cytometry.

Highly purified CFC-S and subsets of CFC-C could be used to study the effects of purified growth factors on these cells (Hoang et al., 1983; Strife et al., 1987). On the other hand, multicolour immunofluorescence multiparameter flow cytometry could be used to examine the effect of growth factors on the cell surface antigen densities and types of CFC-S without doing a CFU-S assay.

CHAPTER 6

HETEROGENEITY OF MURINE HAEMOPOIETIC STEM CELLS DETECTED BY RHODAMINE-123 LABELLING

INTRODUCTION

Since its description, the spleen colony forming assay (CFU-S assay; Till and McCulloch, 1961) has long been regarded as a means to identify and enumerate murine pluripotent haemopoietic stem cells (PHSC). However, data have accumulated indicating that the spleen colony forming cells (CFC-S) are a heterogeneous population of haemopoietic stem cells, the properties of which depend on the time the spleen colonies are counted (Magli et al., 1982). Spleen colonies counted at 8 days after transplantation are formed by other cells (day-8 CFU-S) than those observed at 12 days after transplantation (formed by day-12 CFU-S) (Magli et al., 1982; Wolf and Priestley, 1986).

Day-8 and day-12 CFU-S have been shown to differ in a number of properties, such as their capacity to generate secondary CFU-S upon transplantation (self-renewal) (Worton et al., 1969b; Siminovitch et al., 1963; Hellman et al., 1978; Ross et al., 1982; Micklem and Odgen, 1976; Botnick et al., 1979; Micklem et al., 1975; Micklem, 1983; Hodgson and Bradley, 1979; Rosendaal et al., 1979), and their capacity to differentiate into the various haemopoietic lineages (Hodgson and Bradley, 1979; Schofield, 1978; Dexter et al., 1979; Jones et al., 1980; Mulder et al., 1985). Differences between day-8 and day-12 CFU-S regarding their proliferative status (Micklem et al., 1975; Visser et al., 1981; 1984; Baines and Visser, 1983; Hodgson and Bradley, 1984), their cell surface characteristics (Harris et al., 1984a; 1984b; 1985; Visser et al., 1984; Mulder, 1986; Ploemacher and Brons, 1988a) and their sensitivity to a variety of cytostatic drugs (Schofield and Lajtha, 1973; Rosendaal et al., 1979; Hodgson and Bradley, 1979; Hodgson et al., 1982; Van Zant, 1984) have also been reported. It is now generally held that day-12 CFU-S have a lower proliferative index and, a higher self- replicative capacity than day-8 CFU-S and therefore are more primitive cells (Hodgson and Bradley, 1979; Rosendaal et al., 1979; Siminovitch et al., 1963; Micklem, 1983; Johnson and Nicola, 1984; Metcalf, 1984).

Several different procedures have been described to purify murine haemopoietic stem cells, employing density gradient centrifugation, counter flow centrifugal elutriation, labelling with (fluorescent) lectins, antibodies or intracellular dyes and subsequent separation using light-activated cell sorters, complement lysis or immunoadherence (panning) (Nicola et al., 1981; Visser and Bol, 1981; Hoang et al., 1983; Johnson and Nicola, 1984; Visser et al., 1984; Muller-Sieburg et al., 1986; Bauman et al., 1986; Lord and Spooncer, 1986; Pallavicini et al., 1985a; 1984b; Baines et al., 1984; Ploemacher et al., 1987; Ploemacher and Brons, 1988a). The various separa-

tion procedures have also contributed to the notion that day-8 CFU-S and day-12 CFU-S are different cells.

Recent observations have indicated that day-12 CFU-S are a heterogeneous population (Bertoncello et al., 1985; Mulder et al., 1985; Mulder, 1986; Mulder and Visser, 1987; Visser and De Vries, in press; Ploemacher and Brons, 1988b). On the basis of the uptake of the supravital, fluorescent dye rhodamine 123 (Rh123) two subpopulations of day-12 CFU-S could be distinguished (Bertoncello et al., 1985; Mulder and Visser, 1987). Day-12 CFU-S present in the dull Rh123 fluorescent fraction had a significantly higher marrow repopulating ability (MRA) than day-12 CFU-S in the Rh123 bright fraction (Bertoncello et al., 1985) and were also significantly more effective for 30-day radioprotection (Mulder and Visser, 1987). Day-8 CFU-S were almost exclusively recovered from the Rh123 bright fraction. In addition, Mulder (1986) reported that day-12 CFU-S from a four day suspension culture in the presence of IL-3 had a reduced radioprotective ability and a reduced capacity to repopulate the thymus in comparison with day-12 CFU-S in uncultured bone marrow, which also indicated that day-12 CFU-S were heterogeneous. The newly discovered heterogeneity of day-12 CFU-S made it of interest to study what type(s) of day-12 CFU-S are isolated by several procedures to highly purify haemopoietic stem cells, since the endpoint of these procedures had been spleen colony formation at day-12.

In this chapter the Rh123 fluorescence of highly purified CFU-S suspensions has been investigated. The CFU-S were purified employing fluorescence activated cell sorting and WGA and α-H-2K^k fluorescence (Visser et al., 1984) (see also Chapter 5), two sequential sorts based on WGA fluorescence (Lord and Spooncer, 1986) and on basis of WGA and 15-1.1 fluorescence as described in Chapter 5. Fractions differing in Rh123 fluorescence, which were purified by the latter two procedures were assayed for their content of in vitro clonogenic progenitors and for their capacity to generate colonies when stimulated with a variety of haemopoietic growth factors.

RHODAMINE-123 FLUORESCENCE DISTRIBUTION OF HIGHLY PURIFIED CFU-S SUSPENSIONS

Rhodamine-123 (Rh123) is a cationic lipophilic fluorescent dye that accumulates specifically in the mitochondria of living cells (Johnson et al., 1980; James and Bohman, 1981). The Rh123 fluorescence intensity is not only determined by the mitochondrial content of a cell, but also by its metabolic state and the progression through the cell cycle (Chen et al., 1982; Darzynkiewicz et al., 1981; 1982). It has been shown that the Rh123 uptake and the state of the cell cycle are correlated: actively cycling cells fluoresce more intensely than quiescent cells (Cohen et al., 1981; Darzynkiewicz et al., 1981).

Recently, Bertoncello et al. (1985) using low density bone marrow cells, and Mulder and Visser (1987) using unseparated bone marrow, described that day-12 CFU-S are heterogeneous with respect to the uptake of Rh123 and their marrow repopulating ability (MRA) and radioprotective ability (RPA). These observations lead us to investigate if highly purified day-12 CFU-S are also heterogeneous.

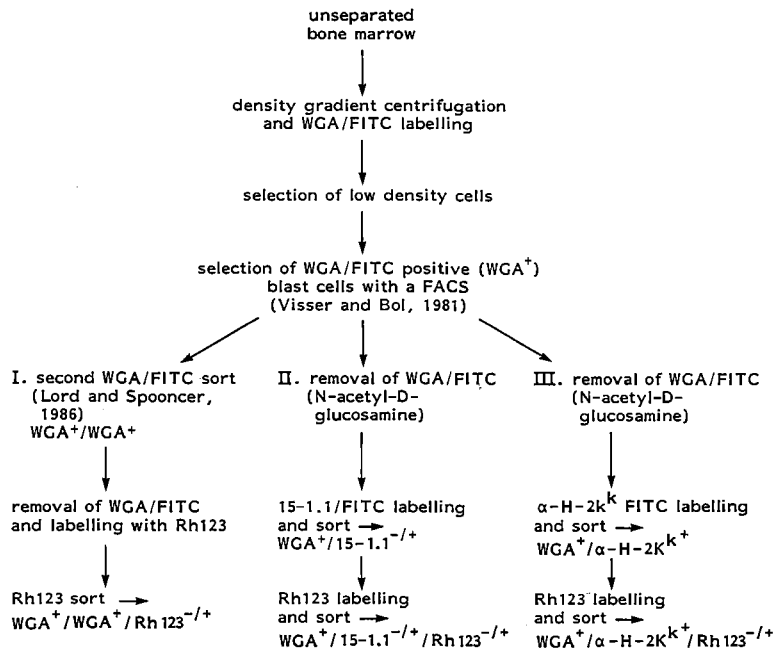
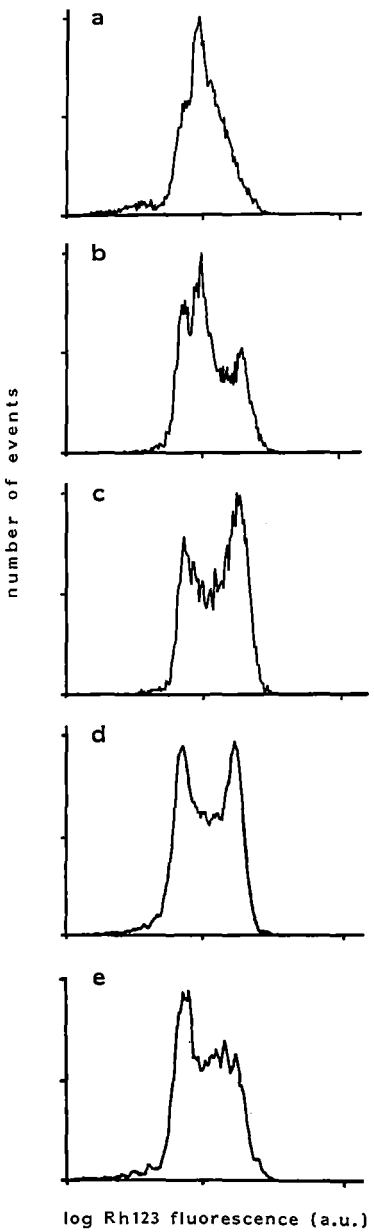


Figure 6.1:
Schematic representation of the various separation steps in the three purification procedures for CFU-S used in the present study (for details, see Chapter 2 and text).
15-1.1$^{-/+}$; 15-1.1 negative and moderately positive cells.
Rh123$^{-/+}$; Rh123 dull (-) or bright (+) fraction.

In the present study, CFU-S were purified in three different ways. The different steps in each procedure are schematically shown in Fig. 6.1. The initial separation steps in the three procedures were the same and were based on the purification procedure described by Visser and Bol (1981). They obtained an 80-fold enrichment of day-8 and day-10 CFU-S by sorting low density bone marrow cells on basis of WGA/FITC fluorescence, within a predefined light scatter blast cell window (Chapters 4 and 5). CFU-S were further purified by means of: 1) a second WGA/FITC sort using the same electronic windows as during the first sort (WGA^{+}/WGA^{+}; I, Fig. 6.1) (Lord and Spooncer, 1986); 2) a second sort on basis of MCA 15-1.1 fluorescence (Chapter 5) (WGA^{+}/15-1.1$^{-/+}$; II, Fig. 6.1); 3) a second sort on basis of α-H-2K^{k} fluorescence as described by Visser et al. (1984) (see also previous chapters) (WGA^{+}/α-H-2K^{k+}; III, Fig. 6.1). In the latter two procedures bound WGA/FITC conjugates were removed from the cells before they were


a
b
c
d
e
number of events
log Rh123 fluorescence (a.u.)

labelled with MCA (chapter 2). As the last step in each of these three procedures, the different purified CFU-S suspensions were labelled with Rh123, and their Rh123 fluorescence distribution was analyzed on a FACS-II. Results of the Rh123 staining are shown in Figure 6.2. The low density blast cell fraction showed a homogeneous Rh123 fluorescence distribution (Fig. 6.2.A), except for a small negative population (5% of all cells in the blast cell window), which was not further investigated in the present study. A similar distribution was observed by Mulder and Visser (1987) using unseparated bone marrow, except that the small negative population was absent. In the WGA/FITC positive cell fraction three subpopulations could be distinguished (Fig. 6.2.B). No Rh123 negative cells were present, but a Rh123 dull, an intermediate and a bright subpopulation could be distinguished. In the WGA^+/WGA^+ cell fraction (I, Fig. 6.1), the intermediate Rh123 subpopulation had disappeared and only a Rh123 dull and Rh123 bright subpopulation were left (Figure 6.2.C). In this suspension most of the sorted cells belong to the Rh123 bright subpopulation. If after the first WGA/FITC sort the suspension was further purified on the basis of MCA 15-1.1 fluorescence (II, Fig. 6.1) and subsequently analyzed for Rh123 fluorescence, two subpopulations of equal size were observed (Fig. 6.2.D). In the $WGA^+/\alpha\text{-H-}2K^{k+}$ suspension (III, Fig. 6.1) also two Rh123 subpopulations were observed. In this suspension, the Rh123 dull fraction was larger than the Rh123 bright fraction (Fig. 6.2.E). From the Rh123 fluorescence histograms shown in Figure 6.2, it is clear that as a result of the sorting procedures a much better distinction could be made between Rh123 dull and bright cells than was possible without extra purification.

It was shown by Mulder and Visser (1987) that, in contrast with day-12 CFU-S, day-8 CFU-S were homogeneous with respect to Rh123 staining and were observed in the bright fraction only. To investigate if the heterogeneity in Rh123 fluorescence intensity of day-12 CFU-S was also observed in purified day-12 CFU-S suspensions, the different Rh123 fractions were sorted and assayed for their CFU-S content (Table 6.1). It can be seen that day-12 CFU-S purified with all three procedures were heterogeneous with respect to their Rh123 uptake. After two sequential WGA/FITC sorts almost equal numbers of day-12 CFU-S could be recovered from the Rh123 dull and bright fraction, whereas after selection by means of MCA 15-1.1 or $\alpha\text{-H-}2K^k$

Figure 6.2:
Rh123 fluorescence distribution of bone marrow cell fractions.

a. Low density bone marrow fraction.
b. WGA positive low density blast cells, selected as described by Visser and Bol (1981).
c. Double WGA sorted low density blast cells (WGA^+/WGA^+) (Lord and Spooncer, 1986).
d. Low density blast cells sorted on basis of WGA and 15-1.1 fluorescence ($WGA^+/15\text{-}1.1^-$) (Chapter 5).
e. Low density blast cells sorted on basis of WGA and $\alpha\text{-H-}2K^k$ fluorescence ($WGA^+/\alpha\text{-H-}2K^{k+}$) (Visser et al., 1984).

Cells were labelled, analyzed and sorted on a FACS-II as described in Chapter 2.

fluorescence, the enrichment of Rh123 bright day-12 CFU-S was almost two-fold that of Rh123 dull day-12 CFU-S.

Table 6.1

ENRICHMENT OF CFU-S AFTER Rh123 STAINING

suspension sorted	enrichment factor day-8 CFU-S	day-12 CFU-S
unfractionated bone marrow	1	1
$WGA^+/WGA^+/Rh123^-$	8 (0- 13)	130 (39-208)
$WGA^+/WGA^+/Rh123^+$	158 (108-220)	123 (86-179)
$WGA^+/15\text{-}1.1^{-/+}/Rh123^-$	9 (8- 10)	139 (96-231)
$WGA^+/15\text{-}1.1^{-/+}/Rh123^+$	159 (106-217)	245 (235-269)
$WGA^+/\alpha\text{-}H\text{-}2K^{k+}/Rh123^-$	12 (7- 18)	87
$WGA^+/\alpha\text{-}H\text{-}2K^{k+}/Rh123^+$	88 (64-117)	143

Low density bone marrow cells were labelled, analyzed and sorted as described in the text and Chapter 2 (see also Fig. 6.1). The figures represent the mean enrichment factor and range of enrichment factors (within parenthesis) of 6 (WGA^+/WGA^+), 3 ($WGA^+/15\text{-}1.1^{-/+}$) and 2 (day-8 CFU-S) respectively 1 (day-12 CFU-S) ($WGA^+/\alpha\text{-}H\text{-}2K^{k+}$) experiments. The enrichment factors were calculated with regard to the number of CFU-S per 10^5 unfractionated bone marrow cells, which were 28.3 ± 4.9 and 30.6 ± 5.3 for day 8 and day 12 CFU-S, respectively in BCBA mice (WGA^+/WGA^+ and $WGA^+/15\text{-}1.1^{-/+}$) and 27.3 ± 4.1 and 29.8 ± 5.0 for day 8 and day 12 CFU-S, respectively in C3H mice ($WGA^+/\alpha\text{-}H\text{-}2K^{k+}$). For each determination groups of 7-10 lethally irradiated mice were used.

In addition, and in agreement with the results obtained with unseparated bone marrow (Mulder and Visser, 1987) in all three purified CFU-S suspensions, day-8 CFU-S were also highly purified but these are predominantly recovered from the Rh123 bright fractions. This is most clearly visible after two sequential WGA/FITC sorts (WGA^+/WGA^+) and after the selection of the $WGA^+/15\text{-}1.1^-$ cells and is less pronounced after an $\alpha\text{-}H\text{-}2K^k$ sort ($WGA^+/\alpha\text{-}H\text{-}2K^{k+}$). The enrichment of day-8 CFU-S in the Rh123 bright fractions were respectively 20, 18 and 7 times higher than in the Rh123 dull fractions.

Bertoncello et al. (1985) using low density bone marrow cells have studied the distribution of marrow repopulating ability (MRA) and day-13

CFU-S over the dull and bright Rh123 fractions. They showed that 92% of the total MRA content and 38% of the day-13 CFU-S were recovered from the Rh123 dull fraction, while the brightly stained fraction contained 62% of the day-13 CFU-S, but only 8% of the total MRA content. It can be calculated from the observed enrichment factors of day-12 CFU-S in the Rh123 dull fractions after selection by means of MCA 15-1.1 or α-H-2K^k (Table 6.1) that the percentage of dull day-12 CFU-S in these suspensions are in agreement with those observed by Bertoncello et al. (1985), while the percentage of Rh123 dull day-12 CFU-S after a double WGA/FITC sort was somewhat higher.

In the present study the radioprotective ability (RPA), measured as the number of cells needed to rescue 50% of lethally irradiated animals from death 30 days after transplantation (30-day survival), of the Rh123 dull and bright cells present in the CFU-S suspension obtained after a double WGA/FITC sort was determined (Table 6.2). Although the number of day-12 CFU-S needed for 50% survival in the two experiments was different, it is obvious that in agreement with data from unseparated bone marrow described by Mulder and Visser (1987), Rh123 dull day-12 CFU-S were more effective in radioprotection than Rh123 bright day-12 CFU-S. Thus, in combination with the previously described purification procedures, Rh123 staining allows for the purification of a subpopulation of day-12 CFU-S which resembles the genuine pluripotent haemopoietic stem cell in MRA and RPA. It also indicates that the content of day-12 CFU-S is not the most reliable indicator of pluripotent haemopoietic stem cells.

Table 6.2

RADIOPROTECTION BY SORTED STEM CELLS

	number of cells needed for 50% survival	number of day-12 CFU-S needed for 50% survival
experiment 1		
unfractionated bone marrow	$5 \times 10^4 - 10^5$	10-21
WGA$^+$/WGA$^+$/Rh123$^-$	500-1000	7-14
WGA$^+$/WGA$^+$/Rh123$^+$	> 1200	> 32
experiment 2		
unfractionated bone marrow	2.5×10^4	7.4
WGA$^+$/WGA$^+$/Rh123$^-$	250	4
WGA$^+$/WGA$^+$/Rh123$^+$	$< 250-750$	$< 9-27$

Low density blast cells from BCBA mice were sorted on basis of two subsequential WGA sorts followed by Rh123 staining and sorting as described in the text (see also Fig. 6.1). Sorted Rh123$^-$ (dull) and Rh123$^+$ (bright) fractions were transplanted into groups of ten lethally irradiated recipient mice and the percentage of mice surviving after transplantation of serial dilutions of cells was determined after 30 days. Results of two independent experiments are shown.

SELECTION BY MEANS OF MCA 15-1.1 IS USEFUL FOR OBTAINING HIGHLY PURIFIED POPULATIONS OF Rh123 DULL FLUORESCENT DAY-12 CFU-S

As was already suggested in Chapter 5, and is now further described in Table 6.3, selection of the $WGA^+/15\text{-}1.1^-$ low density blast cells resulted in a high purification of CFU-S. Selection of the $WGA^+/15\text{-}1.1^+$ (moderately positive) low density blast cells resulted in a relatively small enrichment of day-12 CFU-S, while no day-8 CFU-S were gained from this fraction. No or very few (day-8) CFU-S were obtained from the $WGA^+/15\text{-}1.1^{++}$ (strongly positive) low density blast cell subpopulation (Table 6.3). In Table 6.3 it is shown that purification of CFU-S using MCA 15-1.1 results in an almost equal enrichment of day-8 and day-12 CFU-S. Employing α-H-$2K^k$ fluorescence, Visser and colleagues (1984) obtained an 135-fold and a 53-fold enrichment for day-12 and day-8 CFU-S, respectively. Although, the enrichment for day-12 CFU-S was similar with both procedures, the enrichment of day-8 CFU-S using MCA 15-1.1 was 2 to 3 times higher. Using two sequential WGA/FITC sorts to select CFU-S, Lord and Spooncer (1986) obtained a 25% higher enrichment of day-12 CFU-S than of day-8 CFU-S. In this respect, selection of CFU-S on the basis of 15-1.1/FITC fluorescence resembles the double WGA selection procedure more than the selection procedure employing α-H-$2K^k$ fluorescence. The similarity between the former two selection procedures was also demonstrated in Table 6.1, except for the enrichment of day-12 CFU-S in the Rh123 bright fractions.

Table 6.3

SELECTION OF CFU-S BY MEANS OF MCA 15-1.1

suspension sorted	enrichment factor day-8 CFU-S	day-12 CFU-S
unseparated bone marrow	1	1
$WGA^+/15\text{-}1.1^-$	130 (91-172)	140 (88-225)
$WGA^+/15\text{-}1.1^+$	0	16 (0- 29)
$WGA^+/15\text{-}1.1^{++}$	2 (0- 3)	0

BCBA low density bone marrow cells were labelled with WGA/FITC and sorted on a FACS-II as described in Chapter 2. After removal of the WGA conjugates the sorted cells were labelled with 15-1.1/FITC and sorted again. Three subpopulations were sorted: $15\text{-}1.1^-$ (negative), $15\text{-}1.1^+$ (moderately positive) and $15\text{-}1.1^{++}$ (strongly positive) (see Fig. 6.3). Groups of 8 mice were injected with 2.5×10^4 unseparated bone marrow cells and 100-200 cells of the sorted fractions. The figures represent the mean enrichment factor of 2 experiments (day-8 CFU-S) and 3 experiments (day-12 CFU-S), respectively. The figures in parenthesis represent the range of enrichments. The enrichment was calculated with regard to the number of CFU-S/10^5 unfractionated bone marrow cells which was 28.3 ± 4.9 and 30.6 ± 5.3 for day 8 and day 12 CFU-S, respectively.

When the enrichment factors were compared before and after staining with Rh123 of the sorted $WGA^+/(15\text{-}1.1^- + 15\text{-}1.1^+)$ cells (Tables 6.3 and 6.1), it is observed that selection of the Rh123 dull fraction did not lead to a further enrichment of day-12 CFU-S. In contrast it lead to a considerable (14-fold) depletion of day-8 CFU-S, resulting in a population almost completely devoid of day-8 CFU-S. On the other hand, selection of the Rh123 bright $WGA^+/(15\text{-}1.1^- + 15\text{-}1.1^+)$ subpopulation resulted in an almost two-fold extra enrichment of day-12 CFU-S, while day-8 CFU-S were only slightly higher enriched in comparison with the results obtained without the Rh123 staining. The separation of the day-12 CFU-S populations illustrates the usefulness of Rh123 based sorting for the purification of very primitive day-12 CFU-S.

Selection by means of 15-1.1/FITC fluorescence in a second sort will result in a better defined and more reproducible selection of CFU-S than selection by means of a second WGA/FITC sort. It is shown in Fig. 6.3A and B that as a result of the density gradient centrifugation and subsequent sorting of the WGA/FITC positive blast cells, the number of $15\text{-}1.1^{++}$ cells was markedly increased, while both the $15\text{-}1.1^-$ and $15\text{-}1.1^+$ blast cell subpopulations were decreased in size when compared to 15-1.1 stained unseparated blast cells. This phenomenon was only observed when the (glass) tubes used for collection of the WGA/FITC positive blast cells were pretreated. This pretreatment consisted of the addition of a sterile salt solution containing 10-15% fetal calf serum to the tubes one hour before use. The filled tubes were kept at room temperature. Immediately before use most of the serum containing salt solution was removed leaving about 0.5 ml behind. When the tubes were not pretreated, both the $15\text{-}1.1^{++}$ and $15\text{-}1.1^+$ subpopulations were significantly reduced in size, while in comparison with pretreated tubes, the $15\text{-}1.1^-$ subpopulation was increased in size (Fig. 6.3C). The loss of $15\text{-}1.1^+$ and $15\text{-}1.1^{++}$ cells must have been caused by adherence of a great part of the cells present in these subpopulations to the wall of the untreated tubes. Morphological analysis of the different 15-1.1 subpopulations that can be distinguished after a WGA/FITC sort revealed that the 15-1.1 negative population consisted predominantly of immature myeloid cells. Forty percent of the cells were myeloblasts and 20% promyelocytes. In contrast, the $15\text{-}1.1^+$ and $15\text{-}1.1^{++}$ subpopulations consisted primarily of monocytes and mature granulocytic cells (data not shown, see also chapter 4). It is known that monocytes (and macrophages), but also banded and segmented granulocytes (Ploemacher and Brons, 1988a) are adherent cells, which bind WGA conjugates. Therefore it was not surprising that many of these cells were lost when no precautions were taken. The fact that the $15\text{-}1.1^+$ and $15\text{-}1.1^{++}$ subpopulations hardly contained any CFU-S in comparison with the $15\text{-}1.1^-$ subpopulation (Table 6.3) and that cells present in the former two subpopulations are selectively lost when the tubes used to collect the WGA/FITC positive blast cells were not pretreated, might be an explanation for the higher enrichment of CFU-S after a second WGA/FITC sort using the same sort criteria (Lord and Spooncer, 1986). Since it cannot be predicted what percentage of the adherent WGA positive low density blast cells will be lost during the washing, incubation and sorting steps it is obvious that selection on basis of 15-1.1/FITC fluorescence will result in a more defined and repro-

ducible selection of CFU-S, since this MCA is specifically directed against these adherent, mature cells.

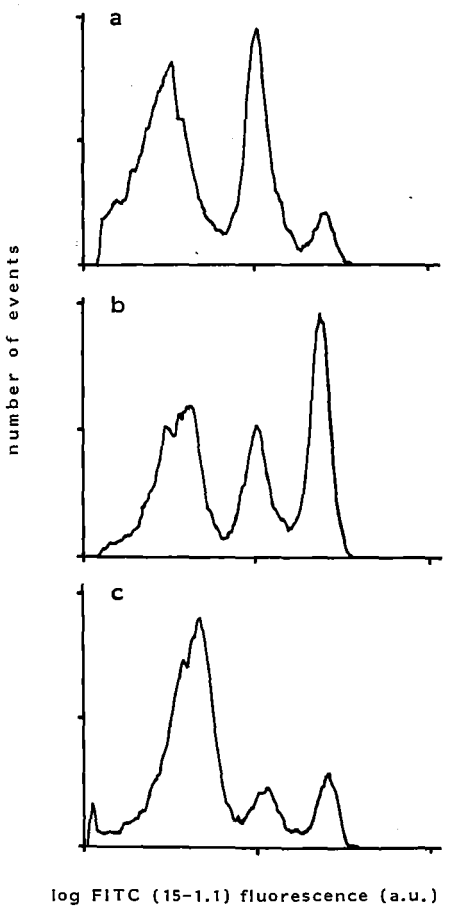


Figure 6.3:
Frequency distributions of the fluorescence intensities of fractionated mouse bone marrow cells labelled with 15-1.1/FITC.

a. Blast cell population in unseparated bone marrow.
b. Blast cell population after a density cut and selection of the brightly WGA fluorescent cells. The collection tubes were pretreated with a salt solution containing serum as described in the text.
c. Same population as in B) except for the fact that in this case the collection tubes were not pretreated.

Cells were labelled, analyzed and sorted as described in Chapter 2.

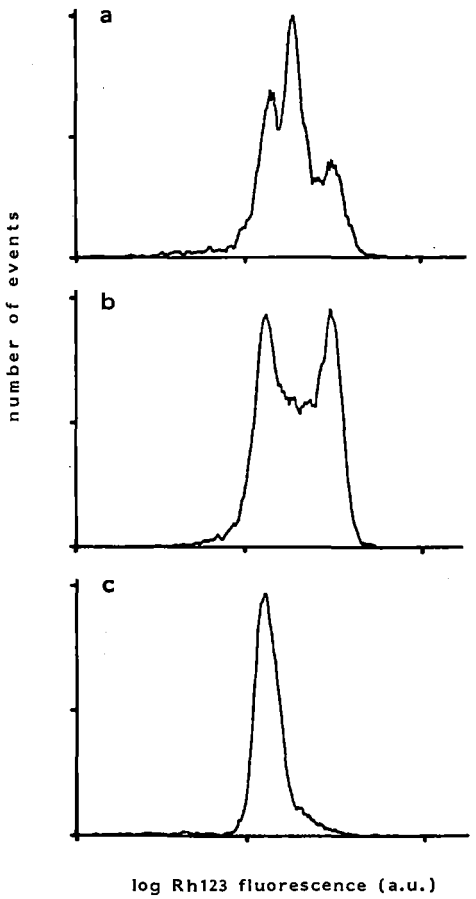


Figure 6.4:
Frequency distributions of the Rh123 fluorescence intensities of fractionated mouse bone marrow cells.
a. Low density WGA positive blast cells.
b. $WGA^+/15\text{-}1.1^-$ low density blast cells.
c. $WGA^+/15\text{-}1.1^+$ low density blast cells.
Cells were labelled, analyzed and sorted as described in Chapter 2.

Selection of CFU-S on basis of 15-1.1 fluorescence is needed to obtain pure populations of Rh123 dull day-12 CFU-S. In Figure 6.4A the Rh123 distribution of the low density WGA/FITC positive blast cells is shown. The

three subpopulations observed are not always present. It was noticed in this study that sometimes no dull and intermediate peaks were discernable. This could be due to the quality of the separation in the density gradient, the staining intensity and the variation in the bone marrow constitution from one experiment to another (see also chapter 4). However, when the WGA/FITC low density blast cells are sorted on basis of 15-1.1/FITC fluorescence, selecting the 15-1.1$^-$ or 15-1.1$^-$ plus 15-1.1$^+$ subpopulation(s), a Rh123 dull and bright fraction were always observed. In Fig. 6.4B this is shown for the 15-1.1$^-$ subpopulation. When the 15-1.1$^+$ subpopulation was stained with Rh123 and subsequently analyzed, only one Rh123 dull fraction was observed (Fig. 6.4C). Although it was shown in Table 6.3 that this fraction contained about 10% of the day-12 CFU-S (which might have caused the Rh123 dull peak), it is more likely, that this Rh123 dull subpopulation consisted predominantly of monocytes. This means that when CFU-S are purified by means of two subsequent WGA/FITC sorts or a WGA/FITC sort followed by an α-H-2K^k sort and the sorted cells are subsequently stained with Rh123 and sorted again, it is most likely that an unpredictable amount of monocytes will be sorted in the Rh123 negative fraction, especially when pretreated tubes are used. Employing 15-1.1/FITC fluorescence, selecting WGA$^+$/15-1.1$^-$ blast cells only, will avoid the isolation of monocytes and therefore will result in purer populations of Rh123 dull day-12 CFU-S.

IN VITRO COLONY FORMATION OF THE Rh123 DULL AND BRIGHT SUB-POPULATIONS PRESENT IN THE WGA$^+$/15-1.1$^-$ BONE MARROW SUSPENSION

The development in the mid 1960's of a series of techniques for the clonal culture of haemopoietic cells in semisolid culture medium (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) has greatly attributed to the present understanding of haemopoietic development. Using these techniques, the formation of discrete colonies consisting of neutrophilic granulocytes or macrophages or both was observed. It was determined that each colony was derived from a single progenitor cell designated as CFU-C (colony forming unit-culture) (Wu et al., 1968). The presence of colonies consisting only of granulocytes or macrophages implied the existence of unipotent or lineage restricted progenitor cells (CFU-G and CFU-M, respectively), whereas the presence of both cell types within one colony implied the existence of bipotent progenitor cells (CFU-GM). Modifications of these original techniques now permit the clonal proliferation of progenitors of almost all haemopoietic differentiation lineages (Metcalf, 1985b) and have led to the identification of multipotent, oligopotent, tripotent and other bipotent progenitor cells, which differ in their capacities for self-renewal and lineage specific differentiation (Johnson and Metcalf, 1977; Nakahata and Ogawa, 1982a; 1982b; Fausner and Messner, 1979; Keller and Phillips, 1982; Dexter et al., 1984a; McCulloch, 1983; Metcalf, 1984; 1985b; 1986a; Spivak et al., 1985; Whetton and Dexter, 1986; Suda et al., 1983; 1984; Ogawa et al., 1985).

It has become apparent from the in vitro clonal culture techniques that the survival, proliferation and differentiation of progenitor cells is strictly dependent on and under the absolute control of a family of glycoprotein

haemopoietic growth factors called colony stimulating factors (CSFs) (Nicola, 1987a; 1987b; Stanley et al., 1986; Metcalf, 1985a; 1985b; 1986a; Nicolas and Vardas, 1984; Walker and Burgess, 1985; Whetton and Dexter, 1986). These CSFs are necessary at each level of commitment and need to be present to stimulate cell divisions. They influence the duration of the cell cycle of responding cells and the ultimate number of progeny produced (Nicola, 1987a; Metcalf, 1985a; 1985c). Analysis of the cell types contained within a colony grown in vitro provides a minimal estimate for the differentiation capacity of a progenitor cell. This has led to the characterization of several CSFs (Nicola, 1987a). In the murine system a number of CSFs that control the formation and function of granulocytes and macrophages have been purified to homogeneity and the genes have been cloned, sequenced and brought to expression (reviewed by Metcalf, 1985b; 1985c; 1986b; Broxmeyer, 1986).

On the basis of in vitro clonal studies, haemopoietic growth factors may be classified into multilineage growth factors, which stimulate the proliferation and differentiation of primitive multipotent progenitor cells into more than one fully differentiated blood cell type, and lineage specific growth factors which act on cells capable of forming only one differentiated cell type (Stanley et al., 1986; Warren and Moore, 1988; Walker et al., 1985; Nicola, 1987b; Spivak, 1986). An example of a multilineage growth factor is interleukin-3 (IL-3; Ihle et al., 1982), whereas CSF-1 or M-CSF (Stanley and Heard, 1977) is an example of a lineage specific growth factor. A third class of haemopoietic growth factors has been described that enhances the effect of other factors while they do not exert any growth promotion on their own. Such factors have been termed synergistic factors (SFs), although enhancing factors is probably a better term (for a recent review on SFs, see Quesenberry, 1986). Addition of SFs together with other growth factors results in increased colony numbers or sizes. In addition, SFs enable other growth factors like CSF-1 to act on more primitive cells than those stimulated to proliferate and differentiate by these growth factors alone (Quesenberry, 1986; Chen and Clark, 1986; Koike et al., 1986; Warren and Moore, 1988; Bartelmez and Stanley, 1985; Williams et al., 1987a; Bol and Williams, 1980; Stanley et al., 1986; Iscove, 1978; Iscove et al., 1982; Broxmeyer, 1986). An example of a SF is hemopoietin-1 (Bartelmez and Stanley, 1985; Jubinsky and Stanley, 1985).

In this section results of experiments are reported that were designed to characterize both Rh123 subpopulations present in the CFU-S enriched $WGA^+/15\text{-}1.1^-$ suspension (see previous sections). For this purpose both Rh123 fractions were cultured in semisolid agar medium to investigate if these two fractions did contain in vitro colony forming cells besides CFU-S and also if a difference between the two fractions with respect to the number of colony forming cells could be observed. The Rh123 subpopulations were cultured in the presence of recombinant murine IL-3 (prepared by Dorssers et al., unpublished results), recombinant human interleukin-1α (IL-1) which was recently shown to be biologically, biochemically and antigenically identical with hemopoietin-1 (H-1; Jubinsky and Stanley, 1985; Bartelmez and Stanley, 1985; Moore and Warren, in press; Mochizuki et al., 1987; Groopman, 1987), a crude preparation of PMUE which is known to be the main source of CSF-1 or M-CSF (Bradley et al., 1971; Stanley and Heard,

1977; Bradley and Hodgson, 1979), or with combinations of these factors. The results are shown in Table 6.4. It can be seen that neither in the Rh123 dull, nor in the Rh123 bright fraction, any colony growth was observed in the presence of IL-1 alone. This lack of colony formation is in agreement with data in the literature for H-1 or IL-1 (Stanley et al., 1986; Mochizuki et al., 1987; Moore and Warren, 1987; Bartelmez and Stanley, 1985; Jubinsky and Stanley, 1985; Neta et al., 1987; Quesenberry, 1986). Stimulation with (combinations of) other growth factors showed that both Rh123 fractions were enriched in colony forming cells with regard to unseparated bone marrow. The number of colonies derived from responsive progenitor cells in the Rh123 bright fraction was considerably higher than in the Rh123 dull fraction, particularly when colony formation was induced in the presence of IL-3 alone.

Table 6.4

IN VITRO CULTURE OF PURIFIED STEM CELL FRACTIONS

growth factor(s) added	colonies/10^5 nucleated cells: unseparated bone marrow	sorted fraction: $WGA^+/15\text{-}1.1^-$ $Rh123^-$	sorted fraction: $WGA^+/15\text{-}1.1^-$ $Rh123^+$
PMUE	140 ± 13	1010 ± 625 (7)	3349 ± 1807 (24)
IL-1	2 ± 1	0 (0)	0 (0)
IL-3	107 ± 12	1095 ± 623 (10)	11953 ± 2619 (112)
IL-1 + IL-3	121 ± 12	2474 ± 913 (20)	15674 ± 3156 (130)
IL-1 + PMUE	160 ± 14	1991 ± 882 (12)	4890 ± 1478 (31)
IL-3 + PMUE	174 ± 15	7100 ± 1840 (41)	20325 ± 3005 (117)
IL-1 + IL-3 + PMUE	183 ± 13	14166 ± 2790 (77)	21411 ± 5127 (117)

BCBA low density bone marrow cells were stained and sorted as described in the text and Chapter 2. Unseparated bone marrow cells and cells in the sorted fractions were cultured in semi-solid agar medium as described in Chapter 2. The figures represent the mean number of colonies per 10^5 nucleated cells of two experiments ± SE counted at day 7. Numbers of cells plated per culture dish: unseparated bone marrow 5×10^4; $WGA^+/15\text{-}1.1^-/Rh123^-$ (dull) 100 and 600 cells; $WGA^+/15\text{-}1.1^-/Rh123^+$ (bright) 100 and 500 cells in two separate experiments. Numbers within parentheses represent the mean enrichment factor of colonies in sorted fractions with regard to unseparated bone marrow cells.

After stimulation with IL-3 the number of colonies in the Rh123 bright fraction was 11 times higher than that in the Rh123 dull fraction. IL-3 is known to have a broad range of proliferative effects on murine haemopoietic

populations (Metcalf et al., 1987). IL-3 has been reported to stimulate, by direct action, the clonal proliferation in semisolid cultures of multipotent, granulocytic (neutrophilic), macrophage, eosinophilic, megakaryotic and erythroid progenitor cells to form colonies of differentiating progeny (Suda et al., 1985; Rennick et al., 1985; Ihle et al., 1982; 1983; Bazill et al., 1983; Cutler et al., 1985; Nicola and Metcalf, 1986; Clark-Lewis et al., 1984). Our present data indicate that IL-3 by itself is not sufficient to stimulate colony formation by purified pluri- or multipotent haemopoietic stem cells.

Rh123 dull cells seem equally sensitive to IL-3 and PMUE when it is assumed that this fraction is homogeneous. However, it could also indicate that two different types of progenitors are present in this fraction. The Rh123 bright cells were 3 to 4 times more sensitive to IL-3 than to PMUE. PMUE is relatively specific in stimulating the proliferation of relatively mature macrophage progenitors with low proliferative potential (CFU-M or LPP-CFC; Bradley and Hodgson, 1979; Bradley et al., 1980), resulting in the formation of small colonies that almost exclusively consist of macrophages. Another more primitive progenitor cell which generates very large macrophage colonies in agar culture has been described. These progenitor cells have been named high proliferative potential colony forming cells (HPP-CFC; Bradley and Hodgson, 1979; Bradley et al., 1980). The formation of colonies by HPP-CFC is dependent on the presence of a combined stimulus consisting of a source of CSF-1 and a source of synergistic activity; either H-1 or IL-3 (Bradley and Hodgson, 1979; Bradley et al., 1980; Bertoncello et al., 1986, 1987). Subsets of HPP-CFC differing in their regeneration rate after FU treatment and in their recloning capacity can be identified by their differing SF requirements (McNiece et al., 1986; 1987; Bertoncello et al., 1986; 1987; Bartelmez and Stanley, 1985; Bartelmez et al., 1985). HPP-CFC-1 are responsive to the combined stimulus of CSF-1 plus a source of H-1, whereas HPP-CFC-2 are responsive to the combination of CSF-1 and IL-3. It has been determined by replating experiments that HPP-CFC-1 are more primitive cells than HPP-CFC-2, since the former can generate the latter, while both types of HPP-CFC can generate LPP-CFC (McNiece et al., 1986; 1987). Recently, Bertoncello et al. (1987) described the existence of another type of HPP-CFC that strictly requires a combination of CSF-1, H-1 and IL-3 and has the characteristics of a very primitive cell type. However, the status of this subpopulation of HPP-CFC in relation to HPP-CFC-1 is as yet unknown.

In the Rh123 dull fraction the combined stimulus of PMUE and IL-1 resulted in a two-fold higher number of colonies than was expected on basis of the colony counts observed with each factor alone. In the Rh123 bright fraction the combination of PMUE and IL-1 resulted in only a slightly higher number of colonies than expected. Addition of PMUE and IL-3 to the Rh123 dull fraction showed that both factors acted synergistically; the number of colonies was 3-4 times higher than expected, whereas in the Rh123 bright fraction the number of colonies was about equal to that expected. The combination of all three factors used in this study, clearly resulted in the highest number of colonies produced by progenitor cells present in the Rh123 dull fraction. This was also true for cells present in the Rh123 bright fraction, but addition of IL-1 to cultures stimulated with IL-3 and PMUE did

not result in extra colonies in this fraction in comparison with the number of colonies observed with PMUE and IL-3. In the Rh123 dull fraction the number of colonies observed with IL-3 and IL-1 was about 2-fold higher than expected, whereas in the Rh123 bright fraction the combined stimulation with IL-3 and IL-1 did not or only weakly increased the number of colonies. It was reported that H-1 acts on the most primitive cells yet shown to proliferate and differentiate in culture and that H-1 permits CSF-1 or IL-3 to act on cells more primitive than those acted on by either factor alone (Stanley et al., 1986). The results indicate that the Rh123 dull cells are more sensitive to IL-1 in the presence of other growth factors than the Rh123 bright cells, suggesting that the former are more primitive cells.

In the present study, the diameter of the colonies was not determined routinely. However, there were clearly large HPP-CFC derived colonies present. In one experiment, the colonies could be easily counted with the naked eye. Colonies stimulated with IL-3 and PMUE or IL-1 and PMUE were generally large and colonies cultured in the presence of all three growth factors were even larger. In contrast, the great majority of the colonies grown in the presence of IL-3 or PMUE alone or in the presence of IL-3 plus IL-1 could not be counted with the naked eye. The number of macroscopic colonies in the Rh123 bright fraction was higher than the number of such colonies in the Rh123 dull fraction. The highest amount of macroscopic colonies in the Rh123 dull fraction was observed in the presence of the combined stimulus of PMUE, IL-3 and IL-1. With this combination, 24% of all colonies could be counted with the naked eye. In the Rh123 bright fraction, stimulation with PMUE plus IL-3 and PMUE + IL-1 + IL-3 resulted in the highest number of macroscopic colonies, i.e., 17% of all colonies present (data not shown).

During the course of the cell separation procedures used in the present study most of the monocytes and lymphocytes were removed (previous section). When exposed to IL-1, bone marrow accessory cells like endothelial cells, fibroblasts and macrophages, but also T-lymphocytes are capable of CSF production. In the same way exposure to one kind of CSF can induce the release of other cytokines, such as IL-1 and other kinds of CSFs by these cell types (Moore et al., 1980; Moore and Warren, 1987; Lee et al., 1987; Neta et al., 1987; Broudy et al., 1987; Broxmeyer, 1986; Oppenheim et al., 1987; Warren and Ralph, 1986). These secondary factors can influence the proliferation and differentiation of progenitor cells present in cultures where monocytes and lymphocytes are present (Williams et al., 1987a; Broxmeyer, 1986). It could be that removal of these cells adversely affects the growth of progenitor cells present in the sorted fractions. This was investigated by adding monocytes ($WGA^+/(15\text{-}1.1^+$ and $15\text{-}1.1^{++})$ low density blast cells), or lymphocytes (low density WGA negative cells) to the cultures in quantities corresponding to their frequency in unseparated bone marrow. Addition of monocytes or lymphocytes resulted in basically the same numbers of colonies as observed without the addition of these cells (data not shown). This was observed for both Rh123 fractions. This indicates that the colony formation observed by progenitors present in these fractions was a result of the direct action of the added growth factors on their target cells.

IN VITRO COLONY FORMATION OF THE Rh123 DULL AND BRIGHT SUB-POPULATIONS PRESENT IN THE WGA^+/WGA^+ BONE MARROW SUSPENSION

In the previous section it was shown that the used growth factors acted directly on the sorted and cultured cells. However, it has also been reported that serum present in the culture medium might contain SFs, CSFs as well as other growth factors and hormones that can modulate CSF activities (Eliason, 1986; Eliason and Odartchenko, 1985; Suda et al., 1986; Koike et al., 1986). It was shown by Eliason (1986) who compared serum-free and serum containing cultures, that fetal calf serum modulated the effects of 3 different growth factors in different ways and to different extents. In addition, crude sources of CSFs might also contain serum proteins that may influence CSF activities (Baines et al., 1984). Thus, to improve the specificity of the culture system and to delineate the roles of CSFs and the characteristics of the cultured cells it is desirable to use purified growth factor suspensions and serum free chemically defined culture systems.

In the present study the Rh123 dull and bright cells present in the population of cells isolated by two subsequent WGA/FITC sorts (Lord and Spooncer, 1986) were examined for their colony forming ability in vitro in serum free methylcellulose culture medium (see Chapter 2). Results of an experiment are shown in Table 6.5. In general, the Rh123 bright fraction contained much more progenitor cells than the Rh123 dull fraction, except for cultures stimulated with IL-1 alone. IL-1 on its own is unable to support any colony formation in both Rh123 fractions. Stimulation with IL-3 resulted in a high number of colonies in the Rh123 bright fraction; one out of 5 plated cells was capable to develop a colony. The Rh123 dull fraction was much less efficiently stimulated by IL-3 alone.

In the experiment presented here (Table 6.5) a purified, serum free preparation of CSF-1 was used (Chapter 2). CSF-1 stimulated the formation of macrophage colonies only, over a wide range of concentrations (Wagemaker, personal communication). The Rh123 bright fraction contained 14 times more CSF-1 responsive progenitor cells than the Rh123 dull fraction. The same ratio between the number of colonies from the Rh123 bright and dull fractions was observed after stimulation by IL-3. However, the number of colonies stimulated by CSF-1 was 2-3 times lower than that stimulated by IL-3. Although both CSFs stimulate macrophage progenitors the difference in colony numbers indicates that this might be different progenitors.

The number of CSF-1 responsive cells in the Rh123 bright fraction of the WGA^+/WGA^+ suspension was 2.5 times higher in the experiments described in Table 6.5 than that in the Rh123 bright fraction of the $WGA^+/15\text{-}1.1^-$ cells, stimulated with PMUE (Table 6.4). PMUE is the main source of CSF-1 and both primarily support the formation of macrophage colonies (Bradley et al., 1971; McNiece et al., 1986; Stanley and Heard, 1977). The number of colonies developed after stimulation with CSF-1 in the Rh123 dull fraction of the WGA^+/WGA^+ population was lower than that observed after stimulation with PMUE in the corresponding Rh123 dull fraction from Table 6.4 (compare Tables 6.5 and 6.4). This either indicates that the two sorted suspensions are different, or that the presence or absence of serum in the culture media or growth factor preparations or the

semisolid supportive medium caused the differences. This was not further investigated.

Table 6.5

IN VITRO CULTURE OF PURIFIED STEM CELL FRACTIONS

growth factor(s) added	colonies per 10^5 nucleated cells: sorted fraction WGA^+/WGA^+ $Rh123^-$	WGA^+/WGA^+ $Rh123^+$
IL-1	0	0
IL-3	1497 ± 669	21150 ± 2744
CSF-1	599 ± 423	8383 ± 1506
Epo + hemin	0	1198 ± 874
IL-1 + IL-3	299 ± 299	38623 ± 3401
IL-1 + CSF-1	898 ± 519	13174 ± 1986
IL-1 + Epo + hemin	299 ± 299	898 ± 519
IL-3 + CSF-1	12874 ± 1963	39222 ± 3427
IL-3 + Epo + hemin	5389 ± 1270	36826 ± 3321
	0*	5988 ± 1339*
IL-1 + IL-3 + CSF-1	9880 ± 1720	49401 ± 3846
IL-1 + IL-3 + Epo + hemin	1198 ± 599	41317 ± 3053
	0*	6886 ± 1436*

BCBA low density blast cells were sorted on basis of WGA/FITC fluorescence and Rh123 fluorescence as described in the text and Chapter 2. Sorted cell fractions were cultured in serum-free methylcellulose medium (Chapter 2). Colonies were scored after 14 days. Cells were plated in duplicate at a density of 170 cells per dish. Figures indicated with an asterisk (*) represent the number of colonies containing erythroid cells. The figures represent the mean number of colonies per 10^5 sorted cells ± SE.

Erythropoietin (Epo) was the first humoral factor shown to regulate haemopoietic differentiation. Epo is obligatory for the proliferation and differentiation of committed erythroid progenitor cells (Suda et al., 1986; Spivak, 1986). Epo increases the number of developing erythroid progenitor cells which are usually defined in vitro by their ability to form colonies of cells containing hemoglobulin. Epo does not interact with either pluripotent or multipotent stem cells and, on its own is hardly able to stimulate the formation of colonies (Goodman et al., 1985). For a recent review on the actions of Epo, see Spivak (1986).

The protoporphyrin hemin was shown to enhance colony formation by mouse erythroid progenitors and multilineage stem cells in the presence of Epo (Monette and Sigounas, 1984; 1987; Holden et al., 1983; Lu and Broxmeyer, 1983) and therefore was used in the present study. It can be seen that only a few Epo plus hemin responsive cells were present in the Rh123 bright fraction, whereas in the Rh123 dull fraction no such cells were present. The observed colonies did not contain any hemoglobinized cells.

Schooley et al. (1987) reported that IL-1 antagonizes the capacity of Epo to stimulate the proliferation of erythroid progenitor cells in vitro. A similar observation was made in the present study when the Rh123 cell fractions were stimulated with IL-1 and Epo plus hemin. There were no hemoglobinized cells present in the formed colonies.

Simultaneous addition of IL-1 and IL-3 to Rh123 bright cells resulted in an almost two-fold higher number of colonies than was expected on basis of the colony counts after stimulation with each factor individually. In the Rh123 dull fraction stimulation with IL-1 and IL-3 resulted in a lower colony number than was expected. The different effects on the colony formation after the combined stimulus with IL-1 and IL-3 on both Rh123 fractions illustrates that the progenitor cells present in each fraction must be different.

Stimulation of the Rh123 bright fraction with IL-1 and CSF-1, the combination that stimulates colony formation by HPP-CFC-1 (McNiece et al., 1986; 1987; Bartelmez and Stanley, 1985), resulted in a slightly higher than expected number of colonies. The same result is observed for the Rh123 dull subpopulation. The observed lack of synergism between IL-1 + CSF-1 with respect to extra colony formation is in contrast with data by others using FU treated bone marrow or partly purified untreated bone marrow (Baines et al., 1984; Bartelmez and Stanley, 1985; Quesenbery, 1986; Stanley et al., 1986; McNiece et al., 1986; 1987) and could have been due to the purification procedures.

Stimulation with IL-3 and CSF-1 resulted in a six-fold higher than expected colony number in the Rh123 dull fraction which clearly demonstrates that these factors can synergize in this fraction, which was also observed in Table 6.4. Synergism between these two factors was also observed by Williams et al. (1987a), who used highly purified CFU-GM progenitors as targets. In the Rh123 bright fraction the combined stimulus with IL-3 and CSF-1 also resulted in more than the expected number of colonies with respect to the colony numbers observed with each factor separately. In the Rh123 dull fraction a 3- to 4-fold higher number of colonies was observed than expected after stimulation with IL-3 plus Epo and hemin, while in the Rh123 bright fraction the number of colonies was only slightly higher than expected. Synergism between IL-3 and Epo was observed by a number of investigators (Iscove, 1978; Iscove et al., 1982; Bartelmez et al., 1985; Koike et al., 1986). In contrast with the Rh123 dull fraction where no hemoglobinized cells were detected after stimulation with IL-3 and Epo plus hemin in the Rh123 bright fraction about 16% of all colonies contained hemoglobinized cells. Although not further determined in the present study, the latter colonies could have been derived from the most primitive erythroid progenitor cell (BFU-E) (Axelrad et al., 1974) or from CFU-GEMM which are known to require both IL-3 and Epo at some time point in their differentia-

tion (Iscove, 1978; Prystowsky et al., 1984; Suda et al., 1985; Whetton and Dexter, 1986).

The combined stimulus of IL-3, IL-1 and CSF-1 in the Rh123 bright fraction resulted in a very high number of colonies. Almost half of the plated cells gave rise to a colony. In the Rh123 dull fraction, this combination of growth factors did not result in the best colony formation. The number of colonies with IL-3 plus IL-1 and CSF-1 was somewhat lower than the colony number observed after stimulation with IL-3 plus CSF-1. This may be due to antagonism between IL-3 and IL-1. An antagonism between IL-1 and IL-3 could also be found when the Rh123 dull fraction was stimulated with IL-1 plus IL-3 and Epo plus hemin. The number of colonies observed with this combination of stimuli was 4 to 5 times lower than that observed with IL-3 plus Epo plus hemin. In that case, CSF-1 seems to be better capable to overcome the antagonistic behaviour of IL-3 and IL-1 than Epo plus hemin. Finally, in the Rh123 bright fraction, addition of IL-1 plus IL-3 and Epo plus hemin resulted in a slightly higher total number of colonies and number of haemoglobinized colonies than addition of IL-3 plus Epo and hemin. In this fraction no antagonistic effect is observed.

SUSPENSION CULTURE OF THE Rh123 SUBPOPULATIONS PRESENT IN THE DOUBLE WGA SORTED CELL FRACTION

The Rh123 subpopulations that can be distinguished in the suspension isolated on the basis of two sequential WGA/FITC sorts (Fig. 6.2C) were cultured under serum-free conditions in suspension cultures in order to investigate the capacity of both subpopulations to generate day-8 and day-12 CFU-S and different types of in vitro clonogenic progenitor cells. Comparison of the numbers and types of CFU-S and progenitor cells, before and after the culture in the presence of added growth factors, could give information about the properties of the cells present in the cultures with respect to their self-renewal and differentiation capacities.

In this experiment the number of spleen colonies detectable after injection of cultured Rh123 dull cells was too low to draw any reliable conclusions about the effect of the different growth factors or combination of growth factors. In addition, the day-12 CFU-S content of the Rh123 dull fraction cultures could not be accurately determined because most of the transplanted recipient mice died before the twelfth day post-transplantation. For these reasons, the results of the culture of the Rh123 dull cell fraction are not shown in Table 6.6. In this table only the numbers of day-8 and day-12 CFU-S present in the Rh123 bright fraction before and after a four day culture period are shown.

At the onset of the cultures, high numbers of day-8 and day-12 CFU-S were present. Both types of CFU-S were (almost) completely lost when cultured in the presence of IL-1 alone. In the presence of IL-3 the day-8 CFU-S number was almost two-fold increased, whereas at the same time the number of day-12 CFU-S was almost two-fold decreased. Migliaccio and Visser (1986) observed an almost 3-fold increase in day-7 CFU-S and a 5-fold decrease in day-12 CFU-S numbers when they cultured WGA^{+}/α-H-$2K^{k+}$ sorted cells for four days with IL-3 under exactly the same

culture conditions. These investigators concluded that the increase in day-7 CFU-S was due to IL-3 induced proliferation of resting day-12 CFU-S. However, they did not use Rh123 to further separate the sorted cells before culturing them. Stimulation with a combination of IL-3 plus IL-1 resulted in a synergistic production of day-8 CFU-S, while the number of day-12 CFU-S was maintained at the same level as with IL-3 alone. The extra production of day-8 CFU-S in the presence of IL-3 plus IL-1 suggests that there are two cell types present in the Rh123 bright fraction that can form day-8 CFU-S. One type is sensitive to IL-3 alone, whereas the other is sensitive to a combination of IL-3 and IL-1. Alternatively, IL-3 could be more effective in the stimulation of cells that can form day-8 CFU-S when IL-1 is also present.

Table 6.6

SUSPENSION CULTURE OF PURIFIED STEM CELLS

	number of CFU-S per 10^5 nucleated cells $WGA^+/WGA^+/Rh123^+$	
	day-7 CFU-S	day-12 CFU-S
starting material	3800 ± 330	2600 ± 255
cultured with		
IL-1	107 ± 107	0
IL-3	6767 ± 853	1504 ± 614
IL-1 + IL-3	18582 ± 1413	1654 ± 499

At day 0 lethally irradiated BCBA mice were injected with 500 sorted cells for determination of the CFU-S content (chapter 2). A part of the sorted cells were put into serum-free suspension cultures (170 cells/culture). After 4 days the cultures were terminated and their CFU-S content determined. Groups of 5 to 8 recipient mice were injected with the equivalent of 133 initially seeded cells. The number of CFU-S after suspension culture is expressed per 10^5 initially seeded sorted bone marrow cells.

In Table 6.7 the progenitor cell content of both Rh123 subpopulations before and after a four day culture period in the presence of (combinations of) 3 different growth factors are shown. Both Rh123 fractions were tested for their content of progenitor cells responsive to CSF-1 alone, recombinant murine GM-CSF alone and a combination of purified Epo and Con-A stimulated mouse spleen cell conditioned medium (MSCM). In the experiments described in Table 6.7 CSF-1 responsive progenitor cells are designated as CFU-M. Recombinant GM-CSF responsive progenitors are designated as CFU-GM. It

Table 6.7

SUSPENSION CULTURE OF PURIFIED STEM CELL FRACTIONS

number of progenitor cells/10^5 initially seeded nucleated cells

	WGA^+/WGA^+/Rh123 dull			WGA^+/WGA^+/Rh123 bright		
	CFU-M	CFU-GM	BFU-E	CFU-M	CFU-GM	BFU-E
starting material	599± 423	3593± 1037	0	8383± 1584	39820± 3453	8982±1640
cultured with						
IL-1	0	0	0	0	0	0
IL-3	0	6000± 3464	0	344000±26230	416000±28844	22000±6633
CSF-1	0	4000± 2828	0	72000±12000	42000± 9165	0
IL-1+IL-3	2000± 2000	20000± 6325	0	402000±28355	418000±28914	106000±14560
IL-1+CSF-1	12000± 4899	2000± 2000	0	96000±13856	40000± 8944	0
IL-3+CSF-1	16000± 5637	50000±10000	2000±2000	566000±41840	640000±35777	62000±11136
IL-1+IL-3+CSF-1	128000±16000	144000±16971	6000±3464	644000±39786	760000±38987	52000±10198

BCBA bone marrow cells were sorted as described in the text and Chapter 2. Sorted cells were cultured for 4 days in serum-free suspension culture (167 cells/culture) or immediately plated (167/culture dish) in serum-free methylcellulose medium as described in chapter 2. After 4 days the suspension cultures were terminated and the equivalent of 25 originally seeded cells plated in methylcellulose. Progenitor numbers are expressed as numbers per equivalent of 10^5 originally seeded cells. Colonies were counted after 14 days. Figures represent mean numbers of colonies from duplicate plates ± SE.

has been reported that the combination of Epo and MSCM (providing IL-3) (Gearing et al., 1985; Metcalf, 1985a; Kelvin et al., 1986) not only stimulates the formation of pure erythroid bursts, but also the formation of colonies containing granulocytes, erythrocytes, megakaryocytes and macrophages. These latter colonies are derived from multilineage stem cells indicated by the abbreviation CFU-GEMM (Iscove, 1978; Fauser and Messner, 1979; Johnson and Metcalf, 1977; Prystowsky et al., 1984; Suda et al., 1985; Whetton and Dexter, 1986). In the present study no distinction was made between the two types of colonies. All colonies generated in the presence of Epo + MSCM are designated as BFU-E, although in unseparated bone marrow this combination results in about one half mixed colonies and one half pure erythroid colonies (Wagemaker, personal communication).

At the onset of the cultures a marked difference between the progenitor cell content of both fractions was observed. The Rh123 bright fraction contained much more progenitor cells of each type than the Rh123 dull fraction. In both fractions CFU-GM were predominant. In both fractions 5-6 times fewer CFU-M were present. The Rh123 dull fraction did not contain any BFU-E, while these cells were abundantly present in the Rh123 bright fraction. It has been described that in general most of the progenitor cells, including CFU-GEMM are rapidly proliferating and possess little or no self-renewal capacity and act as an amplification compartment for the production of mature cells (Metcalf, 1985a; 1985b; Moore and Williams, 1974; Dexter et al., 1984a; Metcalf et al., 1979; Nakahata and Ogawa, 1982a; Ogawa et al., 1983). This implies that any increase or maintenance of the progenitor cells after liquid culture must have been caused by the generation of these progenitor cells by more primitive cells.

After 4 days in liquid culture in the presence of IL-1 alone, no CFU-M, CFU-GM and BFU-E could be recovered from both Rh123 fractions. Obviously, IL-1 alone was not capable to maintain or stimulate precursor cells for the tested progenitors. Stimulation of the Rh123 dull cell fraction with IL-3 or CSF-1 alone did not result in the generation of CFU-M and BFU-E, whereas CFU-GM were generated in the presence of IL-3 or CSF-1. This indicates that in the Rh123 dull fraction IL-3 and CSF-1 responsive cells must be present that were able to form CFU-GM but not CFU-M and BFU-E during a four day culture period.

The combined stimulus of IL-1 and IL-3 of Rh123 dull cells resulted in the generation of CFU-GM in a synergistic fashion. Again no BFU-E were generated and it is doubtful if CFU-M were generated. According to Stanley et al. (1986) H-1 permits IL-3 to act on more primitive progenitors than those acted upon by IL-3 alone. If this was true for the Rh123 dull cells it means that this more primitive precursor was not oligopotent since it did not generate CFU-M and BFU-E or that the culture period was too short to allow the differentiation of this precursor into CFU-M and BFU-E. Culturing with IL-1 plus CSF-1 resulted in a high production of CFU-M, but CFU-GM and BFU-E were not generated. These CFU-M are most likely formed by HPP-CFC-1 (Bradley and Hodgson, 1979; Bradley et al., 1980; McNiece et al., 1986; 1987; Bartelmez and Stanley, 1985). This is supported by data of McNiece et al. (1987) who reported that HPP-CFC-1 could generate CFU-M but not BFU-E. IL-3 + CSF-1, the combination which is known to stimulate HPP-CFC-2 to form colonies (McNiece et al., 1986; 1987) induces the

generation of CFU-M and CFU-GM and perhaps BFU-E in a synergistic fashion. The most effective stimulation of colony formation by Rh123 dull cells is the combined addition of IL-3, IL-1 and CSF-1. Bertoncello et al. (1987) described the existence of a subpopulation of HPP-CFC that strictly requires these three growth factors for colony development. It is clear from the presented data that the IL-3 + IL-1 + CSF-1 responsive progenitors must be at least oligopotent since they very efficiently generated CFU-M, CFU-GM and BFU-E. However, it is not known to what extent the assayed progenitor cells overlap.

From the numbers of progenitor cells it is clear that during the culture a production of total nucleated cells must have occurred. It is not known how many cells are produced during the culture period, because the cell content of the culture tubes was not determined in this study.

Stimulation of the Rh123 bright cells with IL-3 resulted in a significant generation of CFU-M (41 times the input number), CFU-GM (10 times the input number) and BFU-E (2-3 times the input number). The generation of BFU-E in the presence of IL-3 was not observed in the Rh123 dull fraction. This indicates that in the Rh123 bright fraction a tripotent or even a multipotent IL-3 responsive precursor cell was present, which was not detected in the Rh123 dull fraction. Stimulation of the Rh123 bright cells with CSF-1 alone resulted in the generation of CFU-M and CFU-GM but not BFU-E. This indicates that a CSF-1 responsive precursor for CFU-M and CFU-GM must have been present. This was unexpected since thusfar the generation of CFU-M was demonstrated by HPP-CFC only, which require two or more growth factors (Bradley et al., 1980; Bradley and Hodgson, 1979). Simultaneous addition of IL-1 plus IL-3 to the cultures did not lead to an increased generation of CFU-M and CFU-GM in comparison with IL-3 alone, whereas both factors synergistically induced the generation of BFU-E. A similar effect was observed for day-8 CFU-S (Table 6.6) and it has been reported that BFU-E and day-8 CFU-S might be developmentally closely related (Chertkov and Drize, 1984; Gregory, 1976; Magli et al., 1982). No synergistic effect of IL-1 and CSF-1 was observed concerning the generation of CFU-M, CFU-GM and BFU-E. This could indicate that no HPP-CFC-1 were present in the Rh123 bright fraction.

Only a slightly more than additive generation of CFU-M and CFU-GM was observed when IL-3 plus CSF-1 were added to the cultures. In contrast, the number of BFU-E was almost three times higher than expected and is hard to explain. Stimulation of the Rh123 bright cells with all three growth factors did not result in an extra increase in progenitor numbers in comparison with cultures stimulated with IL-3 + CSF-1 which was clearly the case in the Rh123 dull fraction.

Thus, the suspension cultures followed by methylcellulose culture revealed a heterogeneity of both Rh123 fractions in response to growth factors. Enhancement by IL-1 is more clearly observed in cultures of Rh123 dull sorted cells than in that of the Rh123 bright cells.

DISCUSSION

For a better understanding of the mechanisms controlling the process of

proliferation (self-renewal) and commitment to differentiation of pluripotent haemopoietic stem cells (PHSC), pure populations of these cells are desirable. Several procedures have been developed for the purification of haemopoietic stem cell subpopulations (Bauman et al., 1986; Hoang et al., 1983; Lord and Spooncer, 1986; Müller-Sieburg et al., 1986; Nicola et al., 1981; Ploemacher et al., 1987; Visser and Bol, 1981; Visser et al., 1984). In many of these procedures the CFU-S assay was used to identify and enumerate PHSC during the purification steps. However, the validity of the CFU-S assay as a means to quantitate PHSC came up for debate since it was shown that stem cells that give rise to spleen colonies eight days post-transplantation (day-8 CFU-S) are not pluripotent but committed stem cells and are different from cells that generate colonies twelve days post-transplantation (day-12 CFU-S) (Magli et al., 1982; Wolf and Priestley, 1986; Mulder et al., 1985; Harris et al., 1984a). In addition, it has been demonstrated that part of the spleen colonies visible at day-8, remain throughout day-12 post-transplantation (persistent colonies) (Magli et al., 1982; Wolf and Priestley, 1986; Priestley and Wolf, 1985).

It is generally held that day-12 CFU-S are more primitive cells than day-8 CFU-S (Micklem, 1983; Hodgson and Bradley, 1979; Rosendaal et al., 1979; Mulder et al., 1985; Metcalf, 1984). However, at least a part of day-12 CFU-S are not pluripotent. A discrepancy has been reported between the content of day-12 CFU-S and that of cells that could reconstitute the bone marrow of lethally irradiated recipients and protect these animals against radiation induced death (Mulder et al., 1985; Boggs et al., 1982; Ploemacher and Brons, 1988b; Hodgson and Bradley, 1979; 1984; Hodgson et al., 1982; Van Zant, 1984). Marrow repopulating ability (MRA) and radioprotective ability (for 30 days) are considered to be properties of real PHSC and might be better ways to determine the presence of PHSC in a bone marrow suspension than the CFU-S assay (Rosendaal et al., 1979; Hodgson and Bradley, 1979; 1984; Watt et al., in press; Van Bekkum et al., 1981; Bertoncello et al., 1988; Metcalf, 1984).

In the present study it was demonstrated that day-12 CFU-S purified from untreated bone marrow using three different procedures were heterogeneous with respect to the amount of uptake of the fluorescent dye Rh123, whereas day-8 CFU-S were uniformly stained with Rh123 (Table 6.1; Fig. 6.2). Furthermore, it was shown that subpopulations of highly purified day-12 CFU-S that could be distinguished on the basis of Rh123 staining were qualitatively different cells. Day-12 CFU-S in the Rh123 dull fraction were much more effective in the 30-day radioprotection of lethally irradiated mice than day-12 CFU-S from the Rh123 bright fraction (Table 6.2). In spite of this the majority of the day-12 CFU-S were recovered from the Rh123 bright fraction as were almost all day-8 CFU-S (Table 6.1). Similar observations were made by Mulder and Visser (1987) using unseparated bone marrow. In addition, it was shown by Bertoncello et al. (1985) that Rh123 dull cells had a much better MRA than Rh123 bright cells. In the present study the MRA of the sorted fraction was not determined. However, according to Van Bekkum et al. (1981) and Bertoncello et al. (1988) determination of the radioprotective ability is probably the most accurate way to determine the presence of PHSC. Thus, day-12 CFU-S from the Rh123 dull fractions may be closely similar to true PHSC, while day-12 CFU-S from the

Rh123 bright fraction seem to have lost pluripotency. The presence of almost all day-8 CFU-S and the majority of day-12 CFU-S in the Rh123 bright fraction of the sorted suspensions, and the relatively poor radioprotective capacity of cells in this fraction compared with the Rh123 dull cells, indicate that day-8 CFU-S and most of the day-12 CFU-S are incapable to protect lethally irradiated recipients against radiation induced death. Although it seems that Rh123 dull and bright day-12 CFU-S are qualitatively different cells it cannot be excluded that an even more primitive cell, the pre-CFU-S is copurified in the Rh123 dull fraction and that this cell is also responsible for the better RPA of the Rh123 dull cells (Hodgson and Bradley, 1979; 1984; Hodgson et al., 1982; Van Zant, 1984; Mulder and Visser, 1987).

The heterogeneity of day-12 CFU-S revealed by Rh123 staining might be an explanation for the observed discrepancy between the day-12 CFU-S content and the marrow repopulating or radioprotective ability of a graft (Mulder et al., 1985; Bertoncello et al., 1985; Boggs et al., 1982; Hodgson et al., 1982; Hodgson and Bradley, 1979; 1984; Van Zant, 1984). It might also explain the defective ability of day-12 CFU-S isolated by two sequential WGA/FITC sorts to reconstitute haemopoiesis in vitro (Spooncer et al., 1985) because it can be calculated from the enrichment factors for CFU-S (Table 6.1) that only 33% of the isolated CFU-S are Rh123 dull, using the procedure described by Lord and Spooncer (1986).

Actively cycling cells are more intensely stained by Rh123 than quiescent cells (Cohen et al., 1981; Darzynkiewicz et al., 1981). This should then indicate that almost all day-8 CFU-S and the majority of day-12 CFU-S are in active stages of the cell cycle (Fig. 6.1). This is in contrast with data reported by others showing that less than 10% of both day-8 and day-12 CFU-S are killed by S-phase specific agents, like tritium thymidine, cytosine arabinoside and hydroxyurea (Cherkov and Drize, 1984; Van Zant, 1984; Johnson and Nicola, 1984). In addition, it was reported that CFU-S isolated on basis of WGA and α-H-2K^{k} fluorescence were all quiescent (G_0/G_1) cells (Visser et al., 1984; Migliaccio and Visser, 1986). Since there does not seem to be much difference between the three sort procedures used in the present study (Table 6.1; Fig. 6.2), it is assumed that the isolated cells using all three procedures are quiescent cells. Thus, the heterogeneity in Rh123 staining intensities cannot be caused by the presence of actively cycling and quiescent cells. It seems more likely that the Rh123 staining reveals the existence of two stages of quiescence. The Rh123 dull cells may then be deep dormant (G_0) cells, whereas the Rh123 bright cells may be (early) G_1 cells (Baines and Visser, 1983). In addition, it has been suggested that Rh123 uptake depends on differentiation processes which might cause the presence of many or very active mitochondria (Mulder and Visser, 1987). In this respect, it was not surprising that almost all day-8 CFU-S were brightly stained because it was demonstrated that day-8 CFU-S are differentiating cells (Mulder et al., 1985). The few day-8 CFU-S recovered from the Rh123 dull fractions are probably quiescent cells or persistant day-8 colonies that remain in the spleen throughout day-12 post-transplantation (Wolf and Priestley, 1986).

Rh123 selectively stains mitochondria (Johnson et al., 1980; James and Bohman, 1981) and the intensity of the staining is a measure for their activity. The observed heterogeneity of day-12 CFU-S with respect to the

staining intensity could indicate that mitochondria of day-12 CFU-S can switch rapidly from an inactive (Rh123 dull) to an active (Rh123 bright) state. Since it has been reported that stem cells depend on glycolysis which takes place in the cytoplasm, and not on respiration which process is located in the mitochondria (Dello Sharba et al., 1987; Whetton and Dexter, 1986; Whetton et al., 1984; Dexter et al., 1984b), the Rh123 staining seems to be able to distinguish between stem cells that depend on glycolysis only and stem cells that depend on both glycolysis and respiration. Alternatively, it could be that the Rh123 dull cells have much less mitochondria than the Rh123 bright cells which meets the proposition that putative haemopoietic stem cells have very few mitochondria (Visser et al., 1984).

It can be seen in Table 6.1 that employing any of the three procedures to purify CFU-S in combination with Rh123 staining, resulted in a high enrichment of Rh123 dull day-12 CFU-S. No big differences are observed between the three procedures, although it seems that selection by means of WGA and α-H-2K^k fluorescence resulted in the lowest enrichment of Rh123 dull day-12 CFU-S. In the present study the spleen seeding efficiency (f-factor; Lahiri and Van Putten, 1970) of the different Rh123 fractions was not determined, so that it is not known what the purity of the fractions with respect to the different CFU-S types was. In this respect, it should be noted that Lord and Spooncer (1986) did not observe a change in the f-factor of CFU-S present in the suspension obtained by two sequential WGA/FITC sorts and CFU-S present in unseparated bone marrow. However, it has been proposed that cells which exhibit MRA preferentially home to the bone marrow rather than the spleen (Hodgson and Bradley, 1979; Van Zant, 1984), thus it could be that Rh123 dull cells which contain cells with MRA (Bertoncello et al., 1985) have a different (lower) spleen seeding efficiency as Rh123 bright cells. It should be noticed than, however, that each stem cell in the bone marrow may give rise to several spleen colony forming cells and, therefore, to more than one late appearing spleen colony (Micklem et al., 1975).

It can be deduced from Figs. 6.3 and 6.4 that selection by means of 15-1.1 and WGA fluorescence is likely to result in the highest purity of Rh123 dull cells which exhibit repopulating ability. Selection with MCA 15-1.1 selectively removes granulocytes and monocytes, which appear to be Rh123 dull (Fig. 6.3) and bind WGA/FITC conjugates (Ploemacher and Brons, 1988a). This removal of mature monocytes and granulocytes will be less reproducible and specific when a second WGA/FITC sort or an α-H-2K^k sort are used. Thus, purification of cells exhibiting RPA employing WGA and 15-1.1 fluorescence and Rh123 fluorescence as a last step, at present, seems the most effective and reproducible way. When 15-1.1 and WGA are conjugated with different fluorochromes this could be done more rapidly (Chapter 5).

Haemopoietic growth factor requirements can be used to define subsets of early haemopoietic progenitor cells (Bertoncello et al., 1987; Metcalf, 1984; Watt et al., in press). When the Rh123 fractions present in the WGA$^+$/15-1.1$^-$ and WGA$^+$/WGA$^+$ sorted suspensions were cultured in semisolid media, a striking difference between the Rh123 dull and bright fractions was observed. The Rh123 bright fractions contained (much) more colony forming cells than the Rh123 dull fractions, whereas both the Rh123 dull and bright

fractions contained more in vitro clonogenic progenitors than unseparated bone marrow (Tables 6.4 and 6.5). The Rh123 bright cells are very responsive to IL-3, resulting in high cloning efficiencies. Addition of other growth factors in combination with IL-3, roughly resulted in the expected (additive) numbers of colonies. It has been reported that IL-3 does not act on cells in G_0, but that other factors, e.g. IL-1 are required to make the cells leave G_0 and start cycling (Quesenberry, 1986; Kelvin et al., 1986; Koike et al., 1987; Suda et al., 1985) for which IL-3 is needed. In addition, according to Moore and Warren (1987), IL-3 is not capable to act directly on the most primitive stem cells. These findings support the notion that Rh123 bright cells are in G_1 (active mitochondria) and are not the most primitive stem cells.

Addition of two or three growth factors to cultures of $WGA^+/15\text{-}1.1^-$ Rh123 dull cells, resulted in a higher than additive number of colonies (Table 6.4). This synergism between growth factors indicated that Rh123 dull cells are more primitive than Rh123 bright cells (where no synergism was observed) because it has been reported that synergistic factors enable growth factors to act on more primitive cells (Quesenberry, 1986). The observation that Rh123 dull cells are sensitive to IL-1 in the presence of IL-3 and PMUE, while Rh123 bright cells are not, also suggested that Rh123 dull cells are more primitive than Rh123 bright cells. It has been reported that IL-1 target cells are more primitive than IL-3 sensitive cells (Stanley et al., 1986; Bartelmez and Stanley, 1985; Jubinsky and Stanley, 1985). According to Pike and Nossal (1985) the action of IL-1 is related primarily, if not exclusively, to early events in the activation of G_0 cells, which is in favour of the notion that Rh123 dull cells are G_0 cells.

When the Rh123 dull fraction of the double WGA sorted suspension was cultured, it was observed that IL-1 antagonized the action of IL-3 (Table 6.5). This is hard to explain in view of the reported data showing that haemopoietic growth factors do not cross react with other growth factor binding sites (Nicola, 1987b), although Walker et al. (1985b) reported that some growth factors can down modulate receptors for other growth factors. The pattern of down modulation parallels the pattern of potency of its biological activity.

The observed difference between the Rh123 dull cell fractions of the $WGA^+/15\text{-}1.1^-$ and WGA^+/WGA^+ suspensions with respect to their sensitivity to IL-1 in combination with other growth factors is hard to explain, because no differences were observed for the Rh123 bright fractions and the staining profiles after Rh123 labelling (Fig. 6.2) and the enrichments of CFU-S types also seemed very similar (Table 6.1). However, the different culture conditions, i.e., agar and methylcellulose respectively in the presence or absence of serum might have caused the observed differences. In addition, it might be that PMUE besides CSF-1 contains other factors that might have influenced the results.

It was observed that the Rh123 dull cells in the double WGA sorted suspension could not be stimulated to produce erythroid cells, whereas the Rh123 bright cells could generate erythroid cells in the presence of IL-3 plus Epo and hemin. This lack of erythroid colony formation by the Rh123 dull cells is in agreement with the observation by Bertoncello et al. (1988) that in unseparated bone marrow, the Rh123 bright fraction was predomi-

nantly responsible for the erythroid repopulation.

A four-day period in suspension culture of the Rh123 bright fraction from the WGA^+/WGA^+ sorted cell population in the presence of IL-3 or IL-3 + IL-1 resulted in a production of day-8 CFU-S whereas day-12 CFU-S were maintained at about 60% of the input level (Table 6.6). The production of day-8 CFU-S is most likely caused by day-12 CFU-S present in the Rh123 bright fraction. These Rh123 bright day-12 CFU-S strongly resemble day-12 CFU-S that were present in cultures of purified CFU-S suspensions selected on basis of WGA and α-H-$2K^k$ fluorescence (Migliaccio and Visser, 1986; Mulder et al., 1985). It was shown that these day-12 CFU-S did not repopulate the thymus and did not exhibit RPA (Mulder et al., 1985; Mulder, 1986). Thus, Rh123 bright day-12 CFU-S obviously lost pluripotency which coincides with the production of day-8 CFU-S and with the dependency on respiration.

In vitro clonogenic progenitors, were generated by the Rh123 dull cells during a four day suspension culture period (Table 6.7). This is in agreement with the observation by Ploemacher et al. (1987) who reported that highly purified day-12 CFU-S suspensions which exhibited MRA and RPA had a high ability to proliferate and differentiate into more mature progeny. Rh123 dull cells were effectively stimulated by the combination of IL-1 plus IL-3 plus CSF-1 to generate clonogenic progenitor cells and seemed to be very sensitive to IL-1 in combination with CSF-1 and IL-3 (Table 6.7). In contrast, Rh123 bright cells did not seem to be sensitive to IL-1 in combination with CSF-1 or IL-3, but responded to IL-3 only. The Rh123 bright fraction generated much more progenitors than the Rh123 dull fraction. Although some BFU-E were generated by the Rh123 dull cells, much larger numbers of BFU-E were generated by Rh123 bright cells. This might be explained by the close relationship between day-8 CFU-S which were almost exclusively recovered from the Rh123 bright fraction (Table 6.1) and BFU-E (Magli et al., 1982; Gregory, 1976). It has been reported that the most primitive stem cells are absolutely dependent on IL-1 for survival, proliferation and up regulation of growth factor receptors (Moore and Warren, 1987). Since it has been proposed that IL-1 acts primarily if not exclusively on G_0 cells (Pike and Nossal, 1985) it may be that IL-1 acts as a competent factor inducing the Rh123 dull cells to leave G_0 and enter the active stages of the cell cycle (Quesenberry, 1986; Kelvin et al., 1986).

In conclusion, the Rh123 staining in combination with (previous) described purification procedures for day-12 CFU-S, allows for the purification of a subpopulation of day-12 CFU-S which resemble the ultimate PHSC in MRA and RPA. Rh123 staining also reveals heterogeneity of in vitro clonogenic progenitor cells with respect to their sensitivity for haemopoietic growth factors.

CHAPTER 7

GENERAL DISCUSSION

The haemopoietic system is a very complex system consisting of a large variety of cell types belonging to a number of differentiation lineages at different stages of maturation and with different functional capacities. The haemopoietic system can be subdivided into three compartments of increasing size and maturity: 1) the pluripotential (PHSC) and multipotential (MHSC) haemopoietic stem cells; 2) the lineage restricted committed progenitor cells; and 3) the mature blood cell types (chapter 1, Fig. 1.1). It is generally believed that all haemopoietic cells are derived from a small pool of PHSC which have the ability to self-renew and to generate the lineage restricted stem cells (CFU-C). CFU-C have lost the ability to self-renew but proliferate and differentiate into the mature blood cell types which exhibit highly specialized functions but have only a limited lifespan. Thus, haemopoiesis provides a unique opportunity to study the mechanisms controlling the generation of a great diversity of cell types from a common ancestor cell.

Relatively much is known about the mature cell types with regard to their function, cell surface characteristics and morphology. In contrast, relatively little is known about the antigenic and functional properties of the various stem cell subpopulations and the changes they undergo during commitment to a particular differentiation lineage and subsequent terminal differentiation. This has generally been due to the very low frequency of stem cells in haemopoietic tissues and the fact that the various stem cell subsets cannot be distinguished from each other on the basis on morphological criteria (Ferrero et al., 1985; Van den Engh et al., 1981). Indeed, most stem cell types are identified solely by their ability to reconstitute the haemopoietic system of lethally irradiated recipients or to form colonies in vivo or in vitro. One of the problems in the study of haemopoiesis is that it is difficult to obtain various stem cell subpopulations in quantities sufficient and homogeneous enough for analysis at the molecular level. Another still unresolved problem is the lack of knowledge of the events leading to self-renewal or differentiation commitment of haemopoietic stem cells.

Resolution of these problems would be aided by the availability of probes to distinguish and isolate multi-lineage and single-lineage restricted stem cells at the time of commitment. Since commitment takes place before typical morphological characteristics are developed (Van den Engh et al., 1980) and since the cell membrane is intimately involved in receiving and transmitting molecular information (Berridge, 1982), the cell surface properties of a cell at a specific stage of commitment might be directly related to its maturational state and functional capacity. Therefore, methods depending upon the recognition of cell surface determinants specifically expressed by stem cells of different potentiality could be valuable for studies concerning the self-renewal and differentiation commitment of stem cells. Monoclonal antibodies (MCAs), especially in combination with flow cytometry,

have been shown to be powerful tools for the detection of cell surface determinants.

MCAs which recognize cell surface determinants unique to different types of haemopoietic stem cells would be ideal reagents to analyse the earliest differentiation steps in the stem cell compartment including the order of restriction of potentialities, to elucidate the relationships between the various stem cell subsets and to identify, purify and characterize subpopulations of stem cells. Such MCAs might identify receptors involved in interactions between stem cells and humoral or cellular regulatory elements. Comparison of the antigenic profile of stem cells and their progeny provides a method of analyzing cell surface changes that occur as cells differentiate and mature. This knowledge may help to elucidate the molecular mechanisms controlling differentiation and the function of certain specific surface antigens in this process.

On basis of the above described considerations, in this study attempts were undertaken to produce MCAs that would specifically react with antigenic determinants uniquely expressed on subpopulations of haemopoietic stem cells (chapter 3). Despite an efficient (intrasplenic) immunization protocol (Fig. 3.4) which allowed the use of bone marrow suspensions significantly enriched for day-12 CFU-S, no MCAs were obtained which were exclusively directed against determinants uniquely expressed on these stem cells. In addition, no MCAs specific for any in vitro clonogenic progenitor cell type or for cells of a single differentiation pathway were produced (chapter 4).

These results are in agreement with data from the literature and may most simply be explained by the assumption that stem cell specific antigens do not exist, which is in contrast with the hypothesis proposed by Till (1976; Fig. 1.1). On the other hand, stem cell specific antigenic determinants may exist but were not detected in the present studies due to a number of reasons. For instance, it is well known that polyethylene glycol (PEG) induced fusions have a low efficiency (fusion frequency under the most optimal conditions 10^{-5}) and lack of specificity (Reason et al., 1987; Lo et al., 1984). Several considerable improvements of the fusion efficiency including "bridging" of specific immune B-cells to the antigen and simultaneously to the myeloma cells by means of the avidin-biotin system prior to fusion, whether or not in combination with electrofusion, have been described (Reason et al., 1987; Wojchowski and Sytkowski, 1986; Lo et al., 1984). However, these improvements are described for soluble antigens only and do not seem feasible for cellular immunogens. Circumvention of this problem would be to use solubilized cell membranes obtained from purified stem cell suspensions. However, to solubilize membranes detergents are needed which may destroy the antigenic sites.

A number of techniques that would improve the immunogenic response against weakly immunogenic antigens in a mixture of antigens or on a complex antigen (like cells are) have been described. They include neonatal tolerization with common unwanted antigens, cascade immunization and treatment with cytostatic drugs (French et al., 1986; Golumbeski and Dimond, 1986; Thalhamer and Freund, 1985). A disadvantage of most of these procedures is that they are complicated and long-lasting and therefore they were not used in the present study.

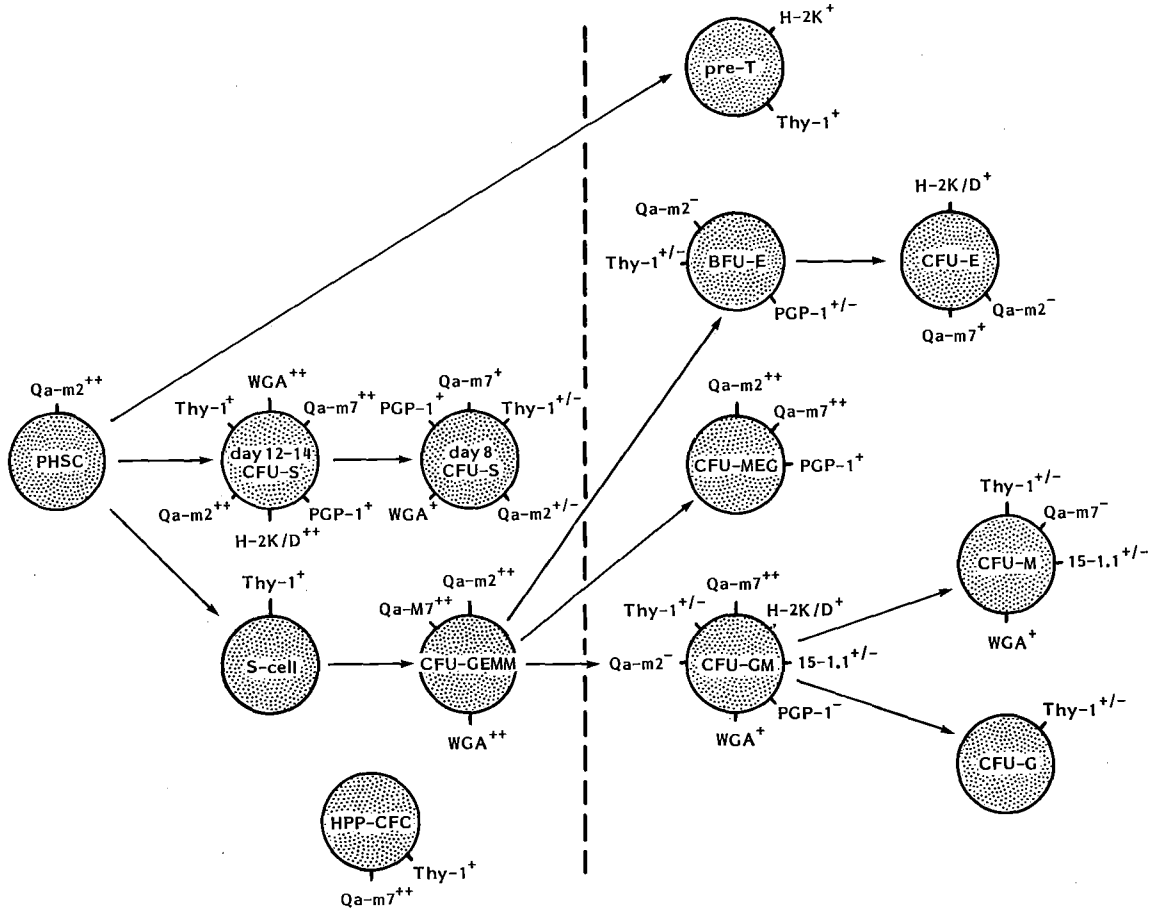
Apart from the possibly insufficient purity of the immunization sus-

pensions used, another reason why no stem cell specific antigens were detected might be a low sensitivity of the screening assays used. It is assumed that stem cell specific markers are related to the functional capacity of a cell, this could for instance be a growth factor receptor. It is known that growth factor receptors on haemopoietic cells are expressed in very low densities (Whetton and Dexter, 1986; Nicola, 1987a; 1987b; Metcalf, 1985c). The limit for cellular fluorescence detection in a flow cytometer is approximately 1000 molecules (Civin and Loken, 1987). This indicates that a distinction between a cell expressing for instance 100 growth factor receptors and a cell expressing no growth factor receptors at all can barely be made or not at all. Thus, stem cell specific MCAs may have been missed. This would have been circumvented by sorting the MCA labelled cells on a FACS followed by an assay of colony formation. Such an assay, however, would be impractical as a screening procedure for large numbers of newly formed hybridomas.

On basis of the data described in chapter 4 of this thesis and the above mentioned considerations, the hypothesis of Till (1976) which does not exclude the existence of stem cell specific antigenic determinants can neither be accepted nor rejected. The hypothesis of Davis (1975) that PHSC are "null" cells can be rejected on the basis of the reactivity of some of the produced MCAs with CFU-S.

Haemopoietic differentiation is a process of continuous changes rather than a series of discrete steps and involves the control of many different genes some of which are coding for cell surface determinants (Till, 1976; Fitchen et al., 1981; Civin and Loken, 1987). As a consequence it can be expected that cell surface antigens expressed on closely related cell types do not abruptly appear and disappear during the differentiation process and that changes in cell surface marker expression are observed rather as smooth quantitative increases or decreases. Indeed, most procedures for enriching haemopoietic stem cell subsets have relied on quantitative differences in the level of expression of cell surface determinants. Early use of the FACS dealt primarily with sorting for cells expressing a single antigenic determinant. These studies did not result in very high enrichments and purifications of stem cell subsets. Better enrichments and purities were obtained by sequential multiparameter procedures employing more than one fluorescently labeled MCA or a combination of a fluorescently labelled MCA and lectins. In Fig. 7.1 the distribution and quantitative expression of certain cell surface determinants that appeared useful for purification and isolation of stem cell subsets is shown. From this figure it is clear that a particular differentiation state is characterized by the presence of a particular array of surface determinants and their quantitative expression, rather than the absence or presence of a single determinant.

It can therefore be envisaged that multiparameter analysis in which the expression of a number of different determinants is measured at the same time might improve the speed and sensitivity for the identification and purification of a particular stem cell subpopulation. In this thesis (chapter 5) the use of such an approach has resulted in a new widely applicable procedure for the enrichment of murine CFU-S. In addition, it was shown that using simultaneous multiparameter analysis the expression of determinants on CFC-S could be determined without the need for CFU-S assays.


pre-T
H-2K+
Thy-1+
BFU-E
Qa-m2−
Thy-1+/−
PGP-1+/−
CFU-E
H-2K/D+
Qa-m2−
Qa-m7+
PHSC
Qa-m2++
day 12-14 CFU-S
WGA++
Thy-1+
Qa-m7++
Qa-m2++
PGP-1+
H-2K/D++
day 8 CFU-S
Qa-m7+
PGP-1+
Thy-1+/−
WGA+
Qa-m2+/−
CFU-MEG
Qa-m2++
Qa-m7++
PGP-1+
S-cell
Thy-1+
CFU-GEMM
Qa-m2++
Qa-M7++
WGA++
CFU-GM
Thy-1+/−
Qa-m7++
H-2K/D+
Qa-m2−
15-1.1+/−
PGP-1−
WGA+
CFU-M
Thy-1+/−
Qa-m7−
15-1.1+/−
WGA+
CFU-G
Thy-1+/−
HPP-CFC
Thy-1+
Qa-m7++

It may be expected that investigation of more MCAs, either available ones, e.g., Thy-1, Qa-m7, or MCAs to be generated, might result in an improvement in the isolation and purification of particular haemopoietic stem cell populations.

Besides the use of MCAs which appeared to be very powerful tools for the resolution and enrichment of subsets of haemopoietic stem cells it has been demonstrated that haemopoietic stem cell subpopulations could also be separated on basis of differences in their DNA content and metabolic state by using a cell sorter (Pallavicni et al., 1985a; 1985b; 1987; Baines and Visser, 1983; Bertoncello et al., 1985; Mulder and Visser, 1987; Visser et al., 1981). With this approach (chapter 6), it was demonstrated that day-12 CFU-S suspensions highly enriched and purified on basis of light scatter characteristics and cell surface properties, were heterogeneous with respect to the amount of uptake of the supravital mitochondrial stain rhodamine 123 (Rh123). This not only suggested a difference between stem cells in these purified populations with respect to the state of activation of the mitochondria but also revealed a difference in reconstituting capacities of these cells. The Rh123 dull cell fraction appeared to have a higher capacity to protect lethally irradiated recipients against death, at least for 30 days, than the cells in the Rh123 bright fraction. Thus, a combination of multiple cell surface determinant expression measurements and Rh123 staining might result in the enrichment and purification of true PHSC. To confirm that the Rh123 dull day-12 CFU-S suspension contains PHSC, the long-term (> 3 months) reconstitution capacity of this cell fraction should be tested. The observed heterogeneity in highly purified day-12 CFU-S suspension indicates that the interpretation of results obtained with highly purified day-12 CFU-S suspensions should be reevaluated. The heterogeneity might also explain the observed discrepancy between the radioprotective capacity of a graft and the day-12 CFU-S content and the lack of self-renewal capacity in vitro of purified day-12 CFU-S suspensions (Boggs et al., 1982; Van Zant, 1984; Hodgson and Bradley, 1979; Spooncer et al., 1985).

The results presented in this thesis clearly demonstrate that a combination of measurements of physical, cell surface and metabolic properties are indispensable for the purification of murine haemopoietic stem cell populations to high degrees. The availability of such highly purified stem cell suspensions is very important for a better understanding of the contribution of specific cell surface molecules (e.g., growth factor receptors)

Figure 7.1:
Schematic representation of the distribution of a number of cell surface determinants expressed on cells of the primitive stem cell compartment.

++	:	high density or all cells positive for this determinant.
+	:	intermediate density.
+/-	:	low(er) density or subpopulation of cells positive for this determinant.
-	:	expression of determinant not demonstrable.

Data obtained from the literature cited in this thesis and from the present studies (MCA 15-1.1).

to cellular function during sequential stages of development. In addition, these highly purified stem cell suspensions are of importance in the search for growth factors which induce self-renewal which is necessary for the introduction of cloned genes to correct genetic defects by bone marrow transplantation. Culture of stem cells in unseparated bone marrow for this purpose leads to unwanted differentiation due to the production of growth factors by mature cells. Finally, purified pluripotent haemopoietic stem cells are not only an interesting alternative for T-cell depletion to obviate the often life-threatening acute Graft-versus-Host disease of allogeneic transplants, the use of purified stem cells also has considerable advantages to study the role of committed precursor cells or specific subpopulations of lymphocytes in graft-rejection.

SUMMARY

Haemopoiesis is a complex ongoing process in which many interactions between a large number of different cell types of various maturation stages and humoral factors are involved. All the different cell types that can be found in the peripheral blood are derived from a small pool of common ancestor cells: the pluripotential haemopoietic stem cells (PHSC). PHSC are capable to reconstitute cell compartments of the haemopoietic system of lethally irradiated recipients. The fraction of cells that gives rise to discrete macroscopic colonies on the spleen of lethally irradiated recipients (CFU-S) are generally equated with PHSC. Besides the PHSC a number of haemopoietic stem cells with more restricted potentialities have been described by the use of in vitro clonal assays. Some of these stem cells can give rise to descendants in a number of lineages: the multipotential haemopoietic stem cells (MHSC), whereas others can give rise to mature cells belonging to only one (or two) differentiation pathway(s): the lineage restricted stem cells, or committed progenitor cells (CFU-C). The various stem cell subsets are present in only very low frequencies in the complex heterogeneous bone marrow and cannot be distinguished from each other on the basis of morphological criteria. Indeed, most stem cell subpopulations are still solely identified on the basis of the type of colonies they produce in in vivo or in vitro clonal assays.

It is generally assumed that cell surface molecules on haemopoietic stem cell subsets must be intimately involved in the mechanisms controlling the selfrenewal, proliferation and commitment of these cells. Therefore, a detailed knowledge of the cell surface make-up of haemopoietic stem cell subsets might contribute to a better understanding of the function of these cells and the complex processes taking place during the earliest steps in haemopoietic stem cell differentiation. Monoclonal antibodies (MCAs) that would exclusively react with cell surface determinants uniquely expressed on (subsets of) haemopoietic stem cells, together with flow cytometry and cell sorting, would be ideal reagents for the identification, purification and characterization of these cells. Highly purified stem cell suspensions would make it possible to directly analyse these cells and to further elucidate the relationships between the various haemopoietic stem cell subpopulations and the processes that control self renewal, proliferation and differentiation commitment of these cells.

The experiments described in this thesis were aimed at the production of haemopoietic stem cell (subset) specific monoclonal antibodies and the application of these for the purification and characterization of the haemopoietic stem cell subpopulations.

In chapter 1 some of the basic principles of haemopoiesis and the various haemopoietic stem cell subpopulations and the assays for their detection are introduced. In addition. different techniques for the segregation and purification of stem cells are introduced. Special attention is payed to the role cell surface structures may play in the control of haemopoiesis and the usefulness of the knowledge of these structures for the purification of stem cell subsets.

In chapter 2, the animals, equipment and technical procedures used in this thesis are described in detail.

Chapter 3 deals with the production of MCAs that would specifically be directed against cell surface determinants uniquely expressed on CFU-S and/or various types of CFU-C. It was demonstrated, by comparison of three different rat strains and myeloma cell lines of rat and mouse origin, that fusions between immune BN-rat spleen cells and mouse myeloma cells (SP2) resulted in the highest number of stable MCA producing hybridomas. In addition, it was found that the frequency of MCA producing hybridomas obtained was higher when rats were immunized by intrasplenic (i.s.) injection of relatively small numbers of cells (approximately 10^5) than after i.p. and/or i.v. immunization with approximately 100-fold higher numbers of cells. This injection made it possible to use significantly for day-12 CFU-S enriched cell suspensions for immunization.

In chapter 4, ten of the in this way produced MCAs are characterized. It appeared that none of the MCAs was specifically directed against CFU-S or any of the tested committed progenitor cell types (CFU-GM, CFU-M, CFU-C2, BFU-E). The reactivity of the MCAs could be roughly divided into two groups. One group of MCAs reacted with only small percentages of bone marrow cells. None of the MCAs from this group, except one (15-2.4) was reactive with CFU-S or any of the tested committed progenitor cells. MCA 15-2.4 reacted with 50% of the BFU-E. The MCAs of the other group all reacted with larger percentages of bone marrow cells and some of them reacted with CFU-S or (subpopulations of) committed progenitor cells. Two MCAs from this second group were most likely directed against antigenic determinants on previously described cell surface structures: the leukocyte common antigen (MCA PV5.6) and the transferrin receptor (MCA 10-2.2), and could be of use for the negative selection of CFU-E and BFU-E, respectively. Two other MCAs of this group were directed against a determinant expressed on a subpopulation of CFU-M (MCA 15-1.1) and/or CFU-GM (MCAs 15-1.1 and 7-15.1) and could therefore be potentially of use in studies concerning the heterogeneity of granulocyte-macrophage progenitors. In addition, MCA 7-15.1 and MCA 15-1.1 in particular, could be of use for the negative selection and purification of CFU-S, CFU-C2 and BFU-E.

In chapter 5, a two-colour immunofluorescence, multiparameter single sort procedure is described for the purification of CFU-S. Based on this procedure, making use of list mode data analysis and processing, a three-colour immunofluorescence, multiparameter flow cytometric screening assay was developed to determine the reactivity of MCAs with CFU-S. The use of this procedure resulted in similar conclusions about the reactivity of the MCAs with CFU-S as cell sorting experiments followed by a CFU-S assay (Chapter 4) and thus can drastically reduce the number of CFU-S assays needed. With this in vitro screening procedure approximately 20 MCAs could be accurately screened for reactivity with CFU-S in one day. In addition, it has the advantage that the results are known the same day.

Screening of MCA 15-1.1 in this way resulted in the development of a new procedure for the negative selection and purification of CFU-S. It was demonstrated that selection with MCA 15-1.1 could replace selection with α-H-2K^k-biotin and a fluorescent avidin conjugate as was used previously. This is not only faster but also more widely applicable since the expression

of the determinant recognized by MCA 15-1.1 is not mouse strain (H-2 haplotype) restricted. In addition, it was shown (chapters 5 and 6) that selection by means of MCA 15-1.1 results in purer CFU-S suspensions than can be obtained with previously applied purification procedures, because in the latter also an unpredictable amount of monocytes and granulocytes are selected, which is avoided by selection with MCA 15-1.1.

In chapter 6, CFU-S were purified in three different ways. It was demonstrated by staining of the purified suspensions with the supravital mitochondrial dye Rh123 that the day-12 CFU-S in these suspensions were heterogeneous with respect to the amount of Rh123 uptake. The Rh123 dull fractions contained almost exclusively day-12 CFU-S whereas the Rh123 bright fractions contained the majority of day-12 CFU-S and almost all day-8 CFU-S. The day-12 CFU-S in both Rh123 fractions appeared to be qualitatively different cells. The Rh123 dull day-12 CFU-S were significantly better in 30-day radioprotection experiments than the Rh123 bright day-12 CFU-S. The former day-12 CFU-S might therefore resemble PHSC, whereas the latter seem to have lost pluripotency. This observed heterogeneity of purified day-12 CFU-S after Rh123 staining necessitates a reevaluation of the results obtained with highly purified day-12 CFU-S suspensions and challenges the validity of the day-12 CFU-S assay as a measure for PHSC.

Differences between both Rh123 fractions were also demonstrated after in vitro culture. Rh123 bright cells produced more colonies in agar and methylcellulose cultures than Rh123 dull cells. Rh123 bright cells could be very well stimulated by IL-3 alone, whereas the Rh123 dull cells were best stimulated in the presence of more than one growth factor and seemed more susceptible to the enhancing effect of IL-1 than the Rh123 bright cells. It was also demonstrated that in a four day suspension culture Rh123 dull cells could not or hardly be stimulated to generate BFU-E, whereas the Rh123 bright cells could produce much larger numbers of these progenitors.

Finally, in chapter 7 (General Discussion) the most important results and their possible implications for future research are discussed and placed in broader perspective.

SAMENVATTING

Hemopoëse is een ingewikkeld continu proces waarin vele interakties tussen een groot aantal verschillende celtypen van verschillende rijpingsstadia en humorale factoren zijn betrokken. Al de verschillende celtypen die in het bloed voorkomen zijn afkomstig van een klein aantal gemeenschappelijke voorlopercellen: de pluripotente hemopoëtische stamcellen (PHSC). PHSC zijn in staat om al de compartimenten van het hemopoëtische systeem van letaal bestraalde ontvangers te reconstitueren. De cellen die in staat zijn discrete macroscopische knobbels (kolonies) op de milt van letaal bestraalde ontvangers te vormen (CFU-S) worden veelal gelijk gesteld met PHSC. Behalve de PHSC is er, door gebruik te maken van in vitro klonogene testen, een aantal hemopoëtische stamcellen met beperktere potenties beschreven. Sommige van deze stamcellen kunnen nakomelingen geven in een aantal differentiatielijnen: de multipotente hemopoëtische stamcellen (MHSC), terwijl anderen volwassen cellen kunnen vormen die slechts tot één (of twee) differentiatielijn(en) behoren: de differentiatielijn-gebonden stamcellen of gecommiteerde voorlopercellen (CFU-C). De verschillende typen stamcellen komen slechts in zeer lage frequentie voor in het bijzonder heterogene beenmerg en kunnen niet van elkaar onderscheiden worden op grond van morfologische criteria. De meeste stamcelsubpopulaties zijn tot op heden uitsluitend te identificeren op grond van het type kolonie dat ze kunnen vormen in in vitro of in vivo klonogene testen.

Het wordt algemeen verondersteld dat molekulen op het oppervlak van hemopoëtische stamcelsubpopulaties nauw betrokken moeten zijn bij de mechanismen die de produktie van nieuwe stamcellen ("self-renewal"), de proliferatie en de differentiatielijn "vastlegging" ("commitment") van deze cellen reguleren. Daarom zou een gedetailleerde kennis van de samenstelling van het celoppervlak van hemopoëtische stamcelsubpopulaties kunnen bijdragen tot een beter begrip van de functie van deze cellen en de complexe processen die plaats vinden tijdens de vroegste gebeurtenissen in de differentiatie van hemopoëtische stamcellen. Monoklonale antilichamen (in het Engels "monoclonal antibodies", MCAs) die uitsluitend zouden reageren met celoppervlaktedeterminanten die specifiek aanwezig zijn op (subpopulaties van) hemopoëtische stamcellen zouden, samen met "flow" cytometrie en celsortering, ideale reagentia zijn voor de identificatie, zuivering en het karakteriseren van deze cellen. In hoge mate gezuiverde stamcelsuspensies zouden de direkte analyse van deze cellen mogelijk maken, alsmede het ophelderen van de relaties tussen de verschillende hemopoëtische stamcelsubpopulaties en de processen die de "self-renewal", proliferatie en differentiatie "commitment" van deze cellen reguleren.

De in dit proefschrift beschreven experimenten waren gericht op de produktie van hemopëtische stamcel (subpopulatie) specifieke monoklonale antilichamen en de toepassing van deze voor het zuiveren en karakteriseren van hemopoëtische stamcelsubpopulaties.

In hoofdstuk 1 worden enkele basisprincipes van hemopoëse en de verschillende hemopoëtische stamcelsubpopulaties en de technieken voor de

detektie van deze cellen geïntroduceerd. Verder worden verschillende technieken voor de scheiding en zuivering van stamcellen besproken. Speciale aandacht wordt besteed aan de rol die celoppervlaktestructuren kunnen spelen in de regulatie van hemopoëse en het nut van kennis van deze structuren voor het zuiveren van stamcelsubpopulaties.

In hoofdstuk 2 worden de in dit proefschrift gebruikte dieren, apparaten en technische procedures in detail beschreven.

Hoofdstuk 3 handelt over de produktie van MCAs die specifiek gericht zouden zijn tegen celoppervlaktedeterminanten die uitsluitend op CFU-S en/of verschillende CFU-C-typen zouden voorkomen. Het werd aangetoond door vergelijking van drie verschillende rattestammen en myelomacellijnen van rat- en muis-origine, dat fusies tussen miltcellen van BN ratten en muizemyelomacellen (SP2) in de hoogste frequentie van MCA- producerende hybridomas resulteerde. Bovendien bleek dat de frequentie van MCA-producerende hybridomas hoger was wanneer ratten werden geïmmuniseerd door het direkt in de milt ("intrasplenic") injecteren van relatief kleine aantallen cellen (circa 10^5) dan na i.p. en/of i.v. immunizatie met ongeveer 100x grotere celaantallen. De "intrasplenic" injectie maakte het mogelijk om met significant voor dag-12 CFU-S verrijkte celsuspensies te immuniseren.

In hoofdstuk 4 worden 10 van de op deze manier geproduceerde MCAs gekarakterizeerd. Het bleek dat geen enkel MCA specifiek gericht was tegen CFU-S of een van de geteste gecommiteerde voorloperceltypen (CFU-GM, CFU-M, CFU-C2, BFU-E). De reaktiviteit van de MCAs kon in twee groepen verdeeld worden. Eén groep MCAs reageerde met slechts kleine percentages beenmergcellen. Geen enkel MCA van deze groep, op één na (MCA 15-2.4) reageerde met CFU-S of een van de geteste gecommiteerde voorlopercellen. MCA 15-2.4 reageerde met 50% van de BFU-E. De MCAs van de andere groep reageerden allemaal met grote(re) percentages beenmergcellen en sommige reageerden met CFU-S en/of (subpopulaties van) gecommiteerde voorlopercellen. Twee MCAs uit deze groep waren hoogst waarschijnlijk gericht tegen antigene determinanten van eerder beschreven celoppervlaktestructuren: de "leukocyte common antigen" (MCA PV5.6) en de transferrine receptor (MCA 10-2.2). Deze MCAs zouden nuttig kunnen zijn voor de negatieve selektie van respectievelijk CFU-E en BFU-E. Twee andere MCAs uit deze groep waren gericht tegen een determinant aanwezig op een subpopulatie van de CFU-M (MCA 15-1.1) en CFU-GM (MCA 15-1.1 en MCA 7-15.1) en zouden daarom mogelijk van nut kunnen zijn voor studies naar de heterogeniteit van granulocyt-macrophaag voorlopers. MCA 7-15.1 en MCA 15-1.1 in het bijzonder, zouden bovendien van nut kunnen zijn voor de negatieve selektie en zuivering van CFU-S, CFU-C2 en BFU-E.

In hoofdstuk 5 wordt een twee-kleuren immunofluorescentie, multiparameter, éénstaps "sort" procedure voor de zuivering van CFU-S beschreven. Gebaseerd op deze methode en gebruik makend van "list mode" data analyse en verwerking, werd een drie-kleuren immunofluorescentie, multiparameter "flow" cytometrische "screening" methode ontwikkeld om de reactiviteit van MCAs met CFU-S te bepalen. Het gebruik van deze methode resulteerde in dezelfde conclusies wat betreft de reaktiviteit van MCAs met CFU-S als celsorteringsexperimenten gevolgd door een CFU-S test (hoofdstuk 4) en kan daarom het aantal benodigde CFU-S testen drastisch verminderen. Met deze in vitro "screening" methode konden in één dag ongeveer 20 MCAs nauw-

keurig getest worden op reaktiviteit met CFU-S met als voordeel dat de resultaten van de testen dezelfde dag bekend waren.

Het op deze manier "screenen" van MCA 15-1.1 resulteerde in de ontwikkeling van een nieuwe methode voor de negatieve selektie en zuivering van CFU-S. Het werd aangetoond dat selektie met MCA 15-1.1 selektie met α-H-2K^k- biotine en een fluorescerend avidine conjugaat, zoals eerder werd gebruikt, kon vervangen. Dit is niet alleen sneller, maar ook algemener toepasbaar omdat de expressie van de determinant die door MCA 15-1.1 herkend wordt niet beperkt is tot bepaalde muizestammen (H-2 haplotype). Het werd bovendien aangetoond (hoofdstukken 5 en 6) dat selektie van CFU-S door middel van MCA 15-1.1 in in hogere mate gezuiverde CFU-S suspensies resulteerde dan mogelijk was met de eerdere gebruikte zuiveringsprocedures. Het bleek dat met de laatsten ook een onvoorspelbaar aantal monocyten en granulocyten wordt geselekteerd, hetgeen vermeden wordt door selektie met MCA 15-1.1.

In hoofdstuk 6 werden CFU-S op drie verschillende manieren gezuiverd. Het werd aangetoond, door de gezuiverde suspensies te "kleuren" met de supravitale mitochondriale kleurstof Rh123, dat de dag-12 CFU-S in deze suspensies heterogeen waren wat betreft de hoeveelheid opgenomen Rh123. De fracties die weinig Rh123 hadden opgenomen (Rh123 "dull" fracties) bevatten bijna uitsluitend dag-12 CFU-S, terwijl de fracties die veel Rh123 hadden opgenomen (Rh123 "bright" fracties) de meerderheid van de dag-12 CFU-S en bijna alle dag-8 CFU-S bevatten. De dag-12 CFU-S in beide Rh123 fracties bleken kwalitatief te verschillen. De Rh123 "dull" dag-12 CFU-S bleken significant beter te zijn dan de Rh123 "bright" dag-12 CFU-S in 30-dagen radioprotectie experimenten. De Rh123 "dull" dag-12 CFU-S zouden daarom PHSC kunnen zijn, terwijl de Rh123 "bright" day-12 CFU-S het pluripotente karakter lijken te hebben verloren. De waargenomen heterogeniteit van gezuiverde dag-12 CFU-S maakt het noodzakelijk de verkregen resultaten met gezuiverde dag-12 CFU-S suspensies opnieuw te beschouwen en stelt bovendien vraagtekens bij de waarde van de dag-12 CFU-S test als maat voor PHSC.

Verschillen tussen beide Rh123 fracties werden ook aangetoond na in vitro kweken. Rh123 "bright" cellen produceerden meer kolonies in agar en methylcellulosekweken dan Rh123 "dull" cellen. De Rh123 "bright" cellen bleken zeer goed gestimuleerd te kunnen worden door IL-3 alleen, terwijl de Rh123 "dull" cellen het best gestimuleerd werden in de aanwezigheid van meer dan één groeifactor en bovendien gevoeliger bleken voor het kolonie-aantal-verhogende ("enhancing") effect van IL-1 dan de Rh123 "bright" cellen. Het werd ook aangetoond dat in een 4 dagen suspensiekweek Rh123 "dull" cellen niet of nauwelijks gestimuleerd konden worden om BFU-E te genereren, terwijl de Rh123 "bright" cellen veel grotere aantallen van deze voorlopercellen konden produceren.

Tenslotte worden in hoofdstuk 7 (Algemene Discussie) de belangrijkste resultaten en hun mogelijke toepasbaarheid voor verder onderzoek bediscussieerd en in een breder perspectief geplaatst.

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REFERENCES

Abrams, P.G., Knost, J.A., Clarke, G., Wilburn, S., Oldham, R.K. and Foon, K.A. (1983). Determinations of the optimal human cell lines for development of human hybridomas. J. Immunol. 131: 1201-1204.

Abramson, S., Miller, R.G. and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. exp. Med. 145: 1567-1579.

Adler, S.S., Kuznetsky, R.D. and Trobaugh, F.E. Jr. (1978). Anti-CFU-S activity of rabbit anti-mouse brain serum: mechanisms of action. In: Experimental Hematology Today (eds.: Baum, S.J. and Ledney, G.D.), Springer Verlag, New York, pp. 23-32.

Andrews, R.G., Takahashi, M., Segal, G.M., Powell, J.S., Bernstein, I.D. and Singer, J.W. (1986). The L4T3 antigen is expressed by unipotent and multipotent colony-forming cells, but not by their precursors. Blood 68: 1030-1035.

Arndt-Jovin, D.J., Grimwade, B.G. and Jovin, T.M. (1980). A dual laser flow sorter utilizing a CW pumped dye laser. Cytometry 1: 127-131.

Astaldi, G.C.B., Janssen, M.C., Lansdorp, P.M., Willems, C., Zeijlemaker, W.P. and Oosterhof, F. (1980). Human endothelial culture supernatant (HECS): A growth factor for hybridomas. J. Immunol. 125: 1411-1414.

Austyn, J.M. and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. 11: 805-815.

Axelrad, A.A., McLeod, D.L., Schreeve, M.M. and Heath, D.S. (1974). Properties of cells that produce erythrocytic colonies in vitro. In: Proceedings of the Second International Workshop on Hemopoiesis in Culture. (ed.: Robinson, W.A.). Washington, U.S. Government Printing Office, p. 226-234.

Baines, P. and Visser, J.W.M. (1983). Analysis and separation of murine bone marrow stem cells by H33342 fluorescence-activated cell sorting. Exp. Hematol. 11: 701-708.

Baines, P., Lajmanovich, A. and Holland, D. (1984). Enrichment of high proliferation potential colony forming cells from mouse marrow by selecting low-density cells expressing receptors for wheat germ agglutinin. Leukemia Res. 8: 853-861.

Bartelmez, S.H. and Stanley, E.R. (1985). Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage specific HGF: Assay of hemopoietin-1. J. Cell. Physiol. 122: 370-378.

Bartelmez, S.H., Sacca, R. and Stanley, E.R. (1985). Lineage specific receptors used to identify a growth factor for developmentally early hemopoietic cells: assay of hemopoietin-2. J. Cell. Physiol. 122: 362-369.

Basch, R.S., Janossy, G. and Greaves, M.F. (1977). Murine pluripotential stem cells lack Ia antigen. Nature 270: 520-522.

Bauman, J.G.J., Mulder, A.H., and Van den Engh, G.J. (1985). Effect of surface antigen labeling on spleen colony formation: Comparison of the indirect immunofluorescence and the biotin-avidin methods. Exp. Hematol. 13: 760-767.

Bauman, J.G.J., Wagemaker, G. and Visser, J.W.M. (1986). A fractionation procedure of mouse bone marrow cells yielding exclusively pluripotent stem cells and committed progenitors. J. Cell. Physiol. 128: 133-142.

Bauman, J.G.J. and Chen, M.G. (1987). Light scatter and cell surface properties of murine megakaryocyte progenitor cells. Exp. Hematol. 15: 1074-1079.

Bazill, H.W., Haynes, M., Garland, J. and Dexter, T.M. (1983). Characterization and partial purification of a haemopoietic growth factor in WEHI-3 cell conditioned medium. Biochem. J. 210: 747-759.

Bazin, H. (1982). Production of rat monoclonal antibodies with the Lou rat non-secreting IR983F myeloma cell line. Prot. Biol. Fluids 29: 615-618.

Bazin, H., Beckers, A., Deckers, C. and Mariamé, M. (1973). Brief communication: Transplantable immunoglobulin-secreting tumors in rats. V. Monoclonal immunoglobulins secreted by 250 ileocecal immunocytomas in Lou/Wsl rats. J. Natl. Cancer Inst. 51: 1359-1361.

Becker, A.J., McCulloch, E.A. and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197: 452-454.

Becker, A.J., McCulloch, E.A., Siminovitch, L. and Till, J.E. (1965). The effect of differing demands for blood cell production on DNA synthesis by hemopoietic colony-forming cells of mice. Blood 26: 296-308.

Beller, D.J., Springer, T.A. and Schneiber, R.D. (1982). Anti-MAC-1 selectively inhibits the mouse and human type three complement receptor. J. exp. Med. 156: 1000-1009.

Berman, J.W. and Basch, R.S. (1985). Thy-1 antigen expression by murine hematopoietic precursor cells. Exp. Hematol. 13: 1152-1156.

Berridge, M.V. (1979). A new class of cell surface antigens. J. Exp. Med. 150: 977-986.

Berridge, M.V. (1982). Surface antigens of murine hemopoietic stem cells. IV. Characteristics of antisera exhibiting cell-lineage specificity, and differentiating ability of stem cells surviving treatment with antisera against cell-lineage antigens. Stem Cells 2: 229-245.

Berridge, M.V. (1983). Surface antigens of murine hemopoietic stem cells. VIII. Antisera define lineage antigens held in common between granulocyte-macrophage cells and between lymphoid cells. Int. J. Cell Cloning 1: 360-376.

Berridge, M.V. and Okech, N. (1979). Surface antigens of murine hemopoietic stem cells. I. Cross reactivity of antisera against differentiated hemopoietic cells with bone marrow stem cells. Exp. Hematol. 7: 452-468.

Berridge, M.V. and Okech, N. (1980). Surface antigens of murine hemopoietic stem cells. II. Rosetting method used to compare the cross-reactivity of antisera against mouse brain, sperm and testis cells with bone marrow stem cells and progenitor cells. J. Immunol. 124: 732-738.

Berridge, M.V., Ralph, S.J., Seng-Tan, A. and Jeffrey, K. (1984). Changes in cell surface antigens during stem cell ontogeny. Exp. Hematol. 12: 121-129.

Bertoncello, I., Hodgson, G.S., Bradley, T.R., Hunter, S.D. and Barber, L. (1985). Multiparameter analysis of transplantable hemopoietic stem cells. I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of Rhodamine-123 fluorescence. Exp. Hematol. 13: 999-1006.

Bertoncello, I. Bartelmez, S.H., Bradley, T.R., Stanley, E.R., Harris, R.A., Sandrin, M.S., Kriegler, A.B., McNiece, I.K., Hunter, S.D. and Hodgson, G.S. (1986). Isolation and analysis of primitive hemopoietic progenitor cells on the basis of differential expression of Qa-M7 antigen. J. Immunol. 136: 3219-3224.

Bertoncello, I., Bartelmez, S.H., Bradley, T.R. and Hodgson, G.S. (1987). Increased Qa-M7 antigen expression is characteristic of primitive hemopoietic progenitors in regenerating bone marrow. J. Immunol. 139:1096-1103.

Bertoncello, I., Hodgson, G.S and Bradley, T.R. (1988). Multiparameter analysis of transplantable hemopoietic stem cells. II. Stem cells of long-term bone marrow reconstituted recipients. Exp. Hematol. 16: 245-249.

Bessis, M. (1977). Blood smears reinterpreted. Springer Verlag, New York,

Bethke, U., Kniep, B. and Mühlradt, P.F. (1987). Forssman glycolipid, an antigenic marker for a major subpopulation of macrophages from murine spleen and peripheral lymph nodes. J. Immunol. 138: 4329- 4335.

Bodger, M.P., Hann, I.M., Maclean, R.F. and Beard, M.E.J. (1984). Enrichment of pluripotent hemopoietic progenitor cells from human bone marrow. Blood 64: 774-779.

Boggs, D.R., Boggs, S.S., Saxe, D.F., Gress, R.A. and Confield, D.R. (1982). Hematopoietic stem cells with high proliferative potential. Assay of their concentration in marrow by the frequency and duration of cure of W/W^V mice. J. Am. Soc. Clin. Inv. 70: 242-253.

Bol, S.J.L. (1980). The recognition of early developmental stages in haemopoiesis. Thesis, State University Leiden.

Bol, S.J.L. and Williams, N. (1980). The maturation state of three types of granulocyte/macrophage progenitor cells from mouse bone marrow. J. Cell. Physiol. 102: 233-243.

Bol, S.J.L., Visser, J.W.M. and Van den Engh, G.J. (1979). The physical separation of three subpopulations of granulocyte/macrophage progenitor cells from mouse bone marrow. Exp. Hematol. 7: 541-553.

Bol, S.J.L., Van Vliet, M. and Van Slingerland, R. (1981). Electrophoretic mobility properties of murine hemopoietic cells from mouse bone marrow. Exp. Hematol. 9: 431-443.

Bol, S.J.L., Visser, J.W.M., Williams, N. and Van den Engh, G.J. (1977). Physical characterization of haemopoietic progenitor cells by equilibrium density centrifugation. In: Cell separation methods (ed.: Bloemendal, H.,) Elsevier/North Holland, Amsterdam, pp. 39-52.

Bomford, A., Young, S.P., Nouri-Aria, K. and Williams, R. (1983). Uptake and release of transferrin and iron by mitogen-stimulated human lymphocytes. Brit. J. Haematol. 55: 93-101.

Boswell, H.S., Wade, P.M. and Quesenberry, P.J. (1984). Thy-1 antigen expression by murine high-proliferative capacity hematopoietic progenitor cells. J. Immunol. 133: 2940-2949.

Botnick, L.E., Hannon, E.C. and Hellman, S. (1979). Nature of the hemopoietic stem cell compartment and its proliferative potential. Blood Cells 5: 195-210.

Bradley, T.R. and Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 44: 287-300.

Bradley, T.R., Stanley, E.R. and Sumner, M.A. (1971). Factors from mouse tissues stimulating colony growth of mouse bone marrow cells in vitro. Aust. J. exp. Biol. med. Sci. 49: 595-603.

Bradley, T.R. and Hodgson, G.S. (1979). Detection of primitive macrophage progenitor cells in mouse bone marrow. Blood 54: 1446-1450.

Bradley, T.R., Hodgson, G.S. and Bertoncello, I. (1980). Characteristics of primitive macrophage progenitor cells with high proliferative potential. Repopulating ability in 5-Fluorouracil treated mouse bone marrow. In: Experimental Hematology Today (eds.: Baum, S.J., Ledney, G.D. and Van Bekkum, D.W.). S. Karger, New York, pp. 285-297.

Broudy, V.C., Kaushansky, K., Harlan, J.M. and Adamson, J.W. (1987). Interleukin-1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granulocyte colony stimulating factor. J. Immunol. 139: 464-468.

Broxmeyer, H.E. (1986). Biomolecule-cell interactions and the regulation of myelopoiesis. Int. J. Cell Cloning 4: 378-405.

Brüggemann, M., Free, J., Diamond, A., Howard, J., Cobbold, S. and Waldmann, H. (1986). Immunoglobulin heavy chain locus of the rat: Striking homology to mouse antibody genes. Proc. Natl. Acad. Sci. U.S.A. 83: 6975-6079.

Burgess, A. and Nicola, N. (1983). Growth factors and stem cells. Academic Press, Sydney.

Campbell, M. (1984). Monoclonal antibody technology. (eds.: Burdon, R.H., Knippenberg, P.H.), Elsevier, Amsterdam, The Netherlands.

Campbell, M., Bieber, M., Levy, R. and Teng, N.N.H. (1986). Influence of avidity and idiotype recognition on the modulation of surface immunoglobulin on malignant human B cells by rat monoclonal anti-idiotype antibodies. J. Immunol. 136: 2983-2988.

Cannistra, S.A., Daley, J.F., Larcom, P. and Griffin, J.D. (1985). Expression of Ia antigens of myeloid progenitor cells in chronic myeloid leukemia: Direct analysis using partially purified colony-forming cells. Blood 65: 414-422.

Cantor, H. and Boyse, E.A. (1975). Functional subclasses of T lymphocytes bearing different Ly antigens: I. The generation of functionally distinct T-cell subclasses in a differative process independent of antigen. J. exp. Med. 141: 1376-1389.

Cantor, H., Shen, F.W. and Boyse, E.A. (1976). Separation of helper T cells from suppressor T cells expressing different Lyt components. II. Activation by antigens after immunization, specific suppressor and helper activities are mediated by distinct T cell subclasses. J. exp. Med. 143: 1391-1405.

Castagnola, C., Visser, J.W.M., Boorsma, J.M.A. and Van Bekkum, D.W. (1981). Purification of rat pluripotent hemopoietic stem cells. Stem Cells 1: 250-260.

Ceredig, R., Lowenthal, J.W., Nabholtz, M., MacDonald, H.R. (1985). Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. Nature 314: 98-100.

Chan, P.Y. and Takei, F. (1986). Expression of a T cell receptor-like molecule on normal and malignant murine T cells detected by rat monoclonal antibodies to nonclonotypic determinants. J. Immunol. 136: 1346-1350.

Chatenoud, L. (1986). The immune response against therapeutic monoclonal antibodies. Immunology Today 7: 367-368.

Chen, B.D.-M. and Clark, C.R. (1986). Interleukin-3 (IL-3) regulates the in vitro proliferation of both blood monocytes and peritoneal exudate macrophages: synergism between a macrophage lineage-specific colony stimulating factor (CSF-1) and IL-3. J. Immunol. 137: 563-570.

Chen, L.B., Summerhayes, I.C., Johnson, L.V., Walsh, M.L., Bernal, S.D. and Lampidis (1982). Probing mitochondria in living cells with Rhodamine 123. Cold Spring Harbor Symp. Quant. Biol. 46: 141.

Chen, S., Botteri, F., Van der Putten, H., Landel, C.P. and Evans, G.A. (1987). A lymphoproliferative abnormality associated with inappropriate expression of the Thy-1 antigen in transgeneic mice. Cell 51: 7-19.

Chertkov, J.L. and Drize, N.J. (1984). Cells forming spleen colonies at 7 or 11 days after injection have different proliferation rates. Cell Tissue Kinet. 17: 247-252.
Chervenick, P.A. and Boggs, D.R. (1971). In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. Blood 37: 131-135.
Chitambar, C.R., Massey, E.J. and Seligman, P.A. (1983). Regulation of transferrin receptor expression on human leukemic cells during proliferation and induction of differentiation. Effects of gallium and dimethylsulfoxide. J. Clin. Invest. 72: 1314-1325.
Civin, C.I. and Loken, M.R. (1987). Cell surface antigens on human marrow cells: Dissection of hematopoietic development using monoclonal antibodies and multiparameter flow cytometry. Int. J. Cell. Cloning 5: 267-288.
Civin, C.I., Banquerigo, M.L., Strauss, L.C. and Loken, M.R. (1987). Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10 positive progenitor cells in normal human bone marrow. Exp. Hematol. 15: 10-17.
Civin, C.I., Strauss, L.C., Broval, C., Fackler, M.F., Schwartz, J.F., Shaper, J.H. (1984). Antigenic analysis of hematopoiesis: III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J. Immunol. 133: 157-165.
Clark, S.C. and Kamen, R. (1987). The human hemopoietic colony-stimulating factors. Science 236: 1229-1237.
Clark, M., Cobbold, S., Hale, G. and Waldmann, H. (1983). Advantages of rat monoclonal antibodies. Immunology Today 4: 100-101.
Clark-Lewis, I., Kent, S.B.H. and Schrader, J.B. (1984). Purification to apparent homogeneity of a factor stimulating the growth of multiple lineages of hemopoietic cells. J. Biol. Chem. 259: 7488-7494.
Cohen, R.L., Muirhead, K.A., Gill, J.E., Waggoner, A.S. and Horan, P.K. (1981). A cyanine dye distinguishes between cycling and non-cycling fibroblasts. Nature 290: 593-595.
Cote, R.J., Morrissey, D.M., Houghton, A.N., Beattie, E.J., Oettgen, H.F. and Old, L.J. (1983). Generation of human monoclonal antibodies reactive with cellular antigens. Proc. Natl. Acad. Sci. U.S.A. 80: 2026-2030.
Crispe, I.N., Moore, M.W., Husmann, L.A., Smith, L., Bevan, M.J. and Shimonkevitz, R.P. (1987). Differentiation potential of subsets of $CD4^-8^-$ thymocytes. Nature 329, 336-339.
Curry, J.L. and Trentin, J.J. (1967). Hemopoietic spleen colony studies. I. Growth and differentiation. Developm. Biol. 15: 395-413.
Curry, J.L., Trentin, J.J. and Wolf, N. (1967). Hematopoietic spleen colony studies. II. Erythropoiesis. J. exp. Med. 125: 703-720.
Cutler, R.L., Metcalf, D., Nicola, N.A. and Johnson, G. (1985). Purification of a multipotential colony-stimulating factor from pokeweed-mitogen-stimulated mouse spleen cell conditioned medium. J. Cell. Physiol. 260: 6579-6587.
Daley, M.J., Williams, T. and Warner, N.L. (1984). Expression of cell surface determinant(s) on murine myeloma stem cells and hematopoietic stem cells. Eur. J. Immunol. 14: 1096-1100.
Dangl, J.L. and Herzenberg, L.A. (1982). Review article: Selection of hybridomas and hybridoma variants using the fluorescence activated cell sorter. J. Immunol. Meth. 52: 1-14.
Darzynkiewicz, Z., Staiano-Coico, L. and Melamed, M.R. (1981). Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. Proc. Natl. Acad. Sci. U.S.A. 78: 2382-2387.

Darzynkiewicz, Z., Traganos, F., Staiano-Coico, L., Kapuscinski, J. and Melamed, M.R. (1982). Interactions of rhodamine 123 with living cells studied by flow cytometry. Cancer Res. 42: 799-806.

Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. and Springer, T.A. (1981). Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127: 590-595.

Davis, S. (1975). Hypothesis: Differentiation of the human lymphoid system based on cell surface markers. Blood 45: 871-880.

Davis, J.M., Kübler, A.-M., Conscience, J.-F. (1983). MBM-1, a differentiation marker of mouse hemopoietic cells defined by a rat monoclonal antibody. Exp. Hematol. 11: 332-340.

Dean, P.N. and Pinkel, D. (1978). High resolution dual laser flow cytometry. J. Hist. Cytochem. 26: 622-627.

Dello Sharba, P., Cipolleschi, M.G. and Olivotto, M. (1987). Hemopoietic progenitor cells are sensitive to the cytostatic effect of pyruvate. Exp. Hematol. 15: 137-142.

Delwel, H.R., Leenen, P.J.M., Berends, D., De Both, N.J. and Van Ewijk, W. (1987). The expression of differentiation antigens by Rauscher virus-induced erythroid, lymphoid, and myeloid cell lines. Leuk. Res. 11: 25-30.

Dexter, T.M. (1982). Stromal cell associated haemopoiesis. J. Cell. Physiol. (Suppl. 1): 87-94.

Dexter, T.M. and Spooncer, E. (1987). Growth and differentiation in the hemopoietic system. Ann. Rev. Cell. Biol. 3: 423-441.

Dexter, T.H., Allen, T.D., Scot, D. and Teich, N.M. (1979). Isolation and characterisation of a bi-potent haematopoietic cell line. Nature 277: 471-474.

Dexter, T.M., Garland, J., Scott, D., Scolnick, E. and Metcalf, D. (1980). Growth factor-dependent hemopoietic precursor cell lines. J. Exp. Med. 152: 1036-1047.

Dexter, T.M., Spooncer, E., Schofield, R., Lord, B.J. and Simmons, P. (1984a). Haemopoietic stem cells and the problem of self-renewal. Blood Cells 10: 315-339.

Dexter, T.M., Whetton, A.D. and Bazill, G.W. (1984b). Haemopoietic cell growth factor and glucose transport: its role in cell survival and the relevance of this in normal haemopoiesis and leukemia. Differentiation 27: 63.

Dick, J.E., Magli, M.C., Huszar, D., Phillips, R.A. and Berstein, A. (1985). Introduction of a selectable gene into primitive stem cells capable of longterm reconstitution of the hemopoietic system of W/W^{V} mice. Cell 42: 71-79.

Dresser, D.W. (1986). Immunization of experimental animals. In: Handbook of experimental immunology. Vol. I. Immunochemistry (Chp. 8). (eds. Weir, D.M., Herzenberg, L.A., Blackwell, C. and Herzenberg, L.A.), Blackwell, Oxford, U.K.

Drover, S. and Marshall, W.H. (1986). Glutaraldehyde fixation of target cells to plastic for ELISA assays of monoclonal anti-HLA antibodies produces artefacts. J. Immunol. Meth. 90: 275-281.

Edelman, P., Vinci, G., Villeval, J.L., Vainchenker, W., Henri, A., Mighernia, R., Rouger, P., Reviron, J., Breton-Gorius, J., Sureau, C. and Edelman, L. (1986). A monoclonal antibody against an erythrocyte ontogeneic antigen identifies fetal and adult erythroid progenitors. Blood 67: 56-63.

Eliason, J.F. (1986). Granulocyte-macrophage colony formation in serum-free culture. Effects of purified colony stimulating factors and modulation by hydrocortisone. J. Cell. Physiol. 128: 231-238.

Eliason, J.F. and Odartchenko, N. (1985). Colony formation by primitive hemopoietic progenitor cells in serum-free medium. Proc. Natl. Acad. Sci. U.S.A. 82, 775-779.

Falkenburg, J.H.F., Van der Vaart-Duinkerken, N., Veenhof, W.F.J., Goselink, H.M., Van Eeden, G., Parlevliet, J. and Jansen, J. (1984). Complement-dependent cytotoxicity in the analysis of antigenic determinants on human hematopoietic progenitor cells with HLA-DR as a model. Exp. Hematol. 12: 817-821.

Fauser, A.A. and Messner, H.A. (1979). Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophic granulocytes and erythroblasts. Blood 53: 1023-1027.

Ferrero, D., Gabbianelli, M., Peschle, C., Lange, B. and Rovera, G. (1985). Surface phenotypes of human hemopoietic progenitor cells defined by monoclonal antibodies. Blood 66: 496-502

Fitchen, J.H. and Ferrone, S. (1981). Expression of I subregion antigens on murine hematopoietic stem cells (HSC). In: J. Supramol. Struct. and Cell. Biochem. 14 (suppl. 5) p. 107 (abstract).

Fitchen, J.H., Ferrone, S., Quaranta, V., Molinaro, G.A. and Cline, M.J. (1980). Monoclonal antibodies to HLA-A.B, and Ia-like antigens inhibit colony formation by human myeloid progenitor cells. J. Immunol. 125: 2004-2008.

Fitchen, J.H., Foon, K.A. and Cline, N.J. (1981). The antigenic characteristics of hematopoietic stem cells. New Engl. J. Med. 305: 17-25.

Fitchen, J.H., Hays, E.F. and Ferrone, S. (1982). Expression of the H-2 antigenic complex on murine haematopoietic stem cells. Scand. J. Immunol. 15: 547-551.

Ford, C.E., Hamerton, J.L., Barnes, D.W.H. and Loutit, J.F. (1956). Cytological identification of radiation chimaeras. Nature 177: 452.

Fowler, J.H., Wu, A.M., Till, J.E., McCulloch, E.A. and Siminovitch, L. (1967). The cellular composition of hemopoietic spleen colonies. J. Cell. Physiol. 69: 65.

French, D., Fischberg, E., Buhl, S. and Scharff, M.D. (1986). The production of more useful monoclonal antibodies. Immunol. Today 7: 344-346.

Fruhman, G.J. (1966a). Shunting of erythropoiesis in mice following the injection of zymosan. Life Sciences 5: 1549-1556.

Fruhman, G.J. (1966b). Bacterial endotoxin: effects on erythropoiesis. Blood 27: 363-370.

Fukuda, M. (1985). Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. Biochim. Bioph. Acta 780: 119-150.

Fultz, M.J., Scher, I., Finkelman, F.D., Kincade, P. and Mond, J.J. (1982). Neonatal suppression with anti-Ia antibody. I. Suppression of murine B lymphocyte development. J. Immunol. 129, 992-995.

Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977). Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 266: 550-552.

Galfre, G. and Milstein, C. (1981). Preparation of monoclonal antibodies: Strategies and procedures. In: Methods in Enzymology - Part B, Immunochemical Techniques. (eds.: Langone, J.L., Van Vunakis, H.), Academic Press, New York, vol. 73: p. 3-46.

Galfre, G. and Milstein, C. (1982). Preparation of monoclonal antibodies: Strategies and Procedures. In: Properties of the monoclonal antibodies produced by hybridomas technology and their application to the study of diseases. Eds. Houba, V. and Chan, S.H., Geneva, Switzerland, p. 1-46.

Galfre, G., Milstein, C. and Wright, B. (1979). Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. Nature 277: 131-133.

Gaulton, G.N., Bangs, J., Maddack, S., Springer, T., Eardley, D.D. and Strom, T.B. (1985). Characterization of a monoclonal rat anti- mouse interleukin 2 (IL-2) receptor antibody and its use in the biochemical characterization of the murine IL-2 receptor. Clin. Immunol. and Immunopath. 36: 18-29.

Gearing, A.J.H., Johnstone, A.P. and Thorpe, R. (1985). Production and assay of the interleukins. J. Immun. Meth. 83: 1-27.

Ghosh, S. and Campbell, A.M. (1986). Multispecific monoclonal antibodies. Immunol. Today 7: 217-222.

Glazer, A.N. (1982). Phycobilisomes: Structure and dynamics. Annual Rev. Microbiol. 36: 173-198.

Goding, J.W. (1980). Review article: Antibody production by hybridomas. J. Immunol. Meth. 39: 285-308.

Goldschneider, I., Metcalf, D., Battije, F. and Mandel, T. (1980). Analysis of rat hemopoietic cells on the fluorescence activated cell sorter. I. Isolation of pluripotent hemopoietic stem cells and granulocyte-macrophage progenitor cells. J. exp. Med. 152: 419-437.

Golub, E.S. (1972). Brain-associated stem cell antigen; an antigen shared by brain and hemopoietic stem cells. J. exp. Med. 136: 369-374.

Golumbeski, G.S. and Dimond, R.L. (1986). The use of tolerization in the production of monoclonal antibodies against minor antigenic determinants. Anal. Biochem. 154: 373-381.

Goodman, J.W., Hall, E.A., Miller, K.L. and Shinpock, S.G. (1985). Interleukin 3 promotes erythroid burst formation in "serum-free" cultures without detectable erythropoietin. Proc. Natl. Acad. Sci. U.S.A. 82: 3291-3295.

Greenberger, J.S., Sakakeeny, M.A., Humphries, K.R., Eaves, C.J. and Eckner, R.J. (1983). Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. U.S.A. 80: 2931-2935.

Gregory, C.J. (1976). Erythropoietin sensitivity as a differentiation marker in het hemopoietic system: studies of three erythropoietic responses in culture. J. Cell. Physiol. 89: 289-302.

Griffin, J.D., Linch, D., Sabbath, K., Larcom, P. and Schlossman, S.F. (1984). A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leukemia Research 8: 521-534.

Groopman, J.E. (1987). Hematopoietic growth factors: from methylcellulose to man. Cell 50: 5-6.

Gupta, A.D., Brice, M., Yokochi, T., Papayannopoulou, T. and Stamatoyannopoulos, G. (1984). GM 58/8: a monoclonal antibody that identifies a new lineage-specific determinant expressed by myeloid progenitors (CFU-GM) and their progeny. Br. J. Haemat. 58: 147-158.

Haaijman, J.J., Deen, C., Kröse, C.J.M., Zijlstra, J.J., Coolen, J. and Radl, J. (1984). Monoclonal antibodies in immunocytology. A jungle full of pitfalls. Immunol. Today 5: 56-58.

Habu, S. and Okumura, K. (1984). Cell surface antigen marking the stages of murine T cell ontogeny and its functional subsets. Immun. Rev. 82: 117139.
Hale, G., Clark, M. and Waldmann, H. (1985). Therapeutic potential of rat monoclonal antibodies: isotype specificity of antibody-dependent cell-mediated cytotoxicity with human lymphocytes. J. Immunol. 134: 3056-3061.
Hämmerling, G.J., Hämmerling, U. and Flaherty, L. (1979). Qat-4 and Qat-5, new murine T-cell antigens governed by the TLa region and identified by monoclonal antibodies. J. Exp. Med. 150: 108-116.
Hara, H. and Ogawa, M. (1978). Murine hemopoietic colonies in culture containing normoblasts, macrophages and megakaryocytes. Am. J. Hematol. 4: 23-34.
Hardy, R.R., Hayakawa, K., Haaijman, J. and Herzenberg, L.A. (1982). B-cell subpopulations identified by two-colour fluorescence analysis. Nature 297: 589-591.
Harris, H.W. and Gill III, T.J. (1986). Expression of class I transplantation antigens. Transplantation 42: 109-117.
Harris, R.A., Hogarth, P.M., McKenzie, I.F.C. and Pennington, D.G. (1983). Differential expression of Qa-m2 alloantigen on murine hemopoietic progenitor cells. Selective enrichment for megakaryocyte progenitors. Exp. Hemat. 11: 527-541.
Harris, R.A., Hogarth, P.M., Wadeson, R.J., Collins, P., McKenzie, I.F.C. and Pennington, D.G. (1984a). An antigenic difference between cells forming early and late haematopoietic spleen cell colonies (CFU-S). Nature 307: 638-641.
Harris, R.A., Hogarth, P.M., Pennington, D.G. and McKenzie, I.F.C. (1984b). Qa antigens and their differential distribution on lymphoid, myeloid and stem cells. J. Immunogenetics 11: 265-281.
Harris, R.A., Sandrin, M.S., Sutton, U.R., Hogarth, P.M., McKenzie, I.F.C. and Pennington, D.G. (1985). Variable expression of Qa-M7, Qa-M8 and Qa-M9 antigenic determinants on primitive hemopoietic precursor cells. J. Cell. Physiol. 123: 451-458.
Haskill, J.S., McNeil, T.A. and Moore, M.A.S. (1970). Density distribution analysis of in vivo and in vitro colony forming cells in bone marrow. J. Cell. Physiol. 75: 167-180.
Hasthorpe, S., Bartlett, P.F. and Rogerson, J. (1985). Expression of tumor associated surface membrane antigens on marrow CFU-s, CFU-gm, BFU-e and brain CFU-s. Int. J. Cell. Cloning 3: 10-21.
Hellman, S., Botnick, L.E., Hannon, E.C. and Vigneulle, R.M. (1978). Proliferative capacity of murine hematopoietic stem cells. Proc. Natl. Acad. Sci. U.S.A. 75: 490-494.
Hemmi, S., Fenner, M., Binz, H., Winterhalter, K. and Wigzell, H. (1985). Studies of monoclonal antibodies specific for major histocompatibility complex products of the rat. I. Production and characterization of monoclonal antibodies. Scand. J. Immunol. 21: 549-563.
Herweijer, H. (1988). Photodamage cell selection: development of a high-speed cell selection method based on flow cytometry. Thesis, University of Leiden, Leiden, The Netherlands.
Herzenberg, L.A. and Herzenberg, L.A. (1978). Analysis and separation using the fluorescence activated cell sorter (FACS). In: Handbook of Experimental Immunology, 3rd Edition. Blackwell, London 22: pp. 1-22.
Herzenberg, L.A., Sweet, R.G. and Herzenberg, L.A. (1976). Fluorescence-activated cell sorting. Sci. Am. 234: 108-117.

Herzenberg, L.A., Stall, A.M., Braun, J., Weaver, D., Baltimore, D., Herzenberg, L.A. and Grosschedl, R. (1987). Depletion of the predominant B-cell population in immunoglobulin μ heavy chain transgenic mice. Nature 329: 71-73.

Hessian, P.A., Highton, J. and Palmer, D.G. (1986). Development of an enzyme immunoassay for the quantitation of cellular antigen expression. J. Immunol. Meth. 91: 29-34.

Heyman, R. (1985). Cell surface antigen mutants of haematopoietic cells. Biochem. J. 225: 27-40.

Hibbs, M.L., Hogarth, P.M., Scott, B.M., Harris, R.A. and McKenzie, I.F.C. (1984). Monoclonal antibody to murine neutrophils: identification of the GM-2.2 specificity. J. Immunol. 133: 2619-2623.

Hirsch, S. and Gordon, S. (1983). Polymorphic expression of a neutrophil differentiation antigen revealed by monoclonal antibody 7/4. Immunogenetics 18: 229-239.

Hoang, T., Gilmore, D., Metcalf, D., Cobbold, S., Watt, S., Clark, M., Furth, M. and Waldmann, H. (1983). Separation of hemopoietic cells from adult mouse marrow by use of monoclonal antibodies. Blood 61: 580-588.

Hodgson, G.S. and Bradley, T.R. (1979). Properties of haemopoietic stem cells surviving 5-Fluorouracil treatment: Evidence for a pre-CFU-s cell. Nature 281: 381-382.

Hodgson, G.S. and Bradley, T.R. (1984). In vivo kinetic status of hematopoietic stem and progenitor cells as inferred from labeling with bromodeoxyuridin. Exp. Hematol. 12: 683-687.

Hodgson, G.S., Bradley, T.R. and Radley, J.M. (1982). The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. Exp. Hematol. 10: 26-35.

Hogarth, P.M., Graig, J. and McKenzie, I.F.C. (1980). A monoclonal antibody detecting the ly-9.2 (lgp 100) cell-membrane alloantigen. Immunogenetics 11: 65-74.

Hogarth, P.M. and McKenzie, I.F.C. (1981). Monoclonal antibodies to lymphocyte differentiation antigens. In: Monoclonal antibodies and T-cell hybridomas. Eds. Hämmerling, G.J., Hämmerling, U. and Kearny, J.F. Elsevier/North-Holland Biomedical Press, Amsterdam, p. 32-37.

Hogarth, P.M., Walker, I.D., McKennie, I.F.C. and Springer, T.A. (1985). the Ly-15 alloantigenic system: A genetically determined polymorphism of the murine lymphocyte function-associated antigen-1 molecule. Proc. Natl. Acad. Sci. U.S.A. 82: 526-530.

Hokland, P., Kerndrup, G., Griffin, J.D. and Ellegaard, J. (1986). Analysis of leukocyte differentiation antigens in blood and bone marrow from preleukemia (refractory anemia) patients using monoclonal antibodies. Blood 67: 898-902.

Holdon, S.A., Steinberg, H.N., Matzinger, E.A. and Monette, F.C. (1983). Further characterization of the hemin-induced enhancement of primitive erythroid progenitor cell growth in vitro. Exp. Hematol. 11: 953-960.

Horenstein, A.L., Glait, H.M. and Koss, A. (1987). An improved selection procedure for the rescue of hybridomas: A comparative study of methotrexate versus aminopherin. J. Immunol. Meth. 98: 145-149.

Hough, T., Singh, M.B., Smart, I.J. and Knox, R.B. (1986), Immunofluorescent screening of monoclonal antibodies to surface antigens of animal and plant cells bound to polycarbonate membranes. J. Immunol. Meth. 92: 103-107.

Hudson, L. and Hay, F.C. (1981). Hybridoma cells and monoclonal antibody. In: Practical Immunology. Blackwell Scientific Publications, 2nd edition, p. 303-327.

Humphries, R.K., Eaves, A.C. and Eaves, C.J. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. Proc. Natl. Acad. Sci. U.S.A. 78: 3629-3633.

Humphries, R.K., Jacky, P.B., Dill, F.J. and Eaves, A.C. (1979). CFU-S in individual erythroid colonies derived in vitro from adult mouse marrow. Nature 279: 718-720.

Ihle, J.N., Keller, J., Henderson, L., Klein, F. and Palaszynski, E. (1982). Procedure for the purification of interleukin-3 to homogeneity. J. Immunol. 129: 2431-2436.

Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T.D., Fitch, F., Prystavsky, N.B., Goedwasser, E., Schrader, J.W., Palaszynski, E., Dy, M. and Lebel, B. (1983). Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell stimulating activity and histamine-producing cell-stimulating factor activity. J. Immunol. 131: 282-287.

Inoue, T., Carsten, E.P., Cronkite, E.P. and Kelley, J.E.T. (1981). Separation and concentration of murine hematopoietic stem cells (CFUs) using a combination of density gradient sedimentation and counter flow centrifugal elutriation. Exp. Hematol. 9: 563-572.

Iscove, N.N. (1978). Erythropoietin-independent stimulation of early erythropoiesis in adult bone marrow cultures by conditioned media from lectin stimulated mouse spleen cells. In: Hematopoietic cell differentiation (eds.: Golde, D.W., Cline, M.J., Metcalf, D. and Fox C.F.), Academic Press, New York, pp. 37-52.

Iscove, N.N., Roitsch, C.A., Williams, N. and Guilbert, L.J. (1982). Molecules stimulating early red cell, granulocyte, macrophage, and megakaryocyte precursors in culture. Similarity in size, hydrophobicity, and charge. J. Cell. Physiol. (suppl.) 1: 65-78.

Iscove, N.N. and Guilbert, L.J. (1978). Erythropoietin-independence of early erythropoiesis and two-regulator model of proliferative control in the hemopoietic system. In: In vitro aspects of erythropoiesis (ed. M.J.J.R. Murphy), Springer-Verlag, New York, pp. 3-7.

Jacobson, L.O., Marks, E.K., Robson, M.J., Gaston, E.A. and Zirkle, R.E. (1949): The effect of spleen protection on mortality following X-irradiation. J. Lab. Clin. Med. 34: 1538.

James, T.W. and Bohman, R. (1981). Proliferation of mitochondria during the cell cycle of the human cell line HL-60. J. Cell. Biol. 89: 256.

Janossy, G., Francis, G.E., Capellaro, D., Goldstone, A.H. and Greaves, M.F. (1978). Cell sorter analysis of leukemia-associated antigens on human myeloid precursors. Nature 276: 176-178.

Johnson, G.R. (1980). Colony formation in agar by adult bone marrow multipotential hemopoietic cells. J. Cell. Physiol. 103: 371-383.

Johnson, G.R. and Metcalf, D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Natl. Acad. Sci. U.S.A. 74, 3879-3882.

Johnson, G.R. and Nicola, N.A. (1984). Characterization of two populations of CFU-S fractionated from mouse fetal liver by fluorescence-activated cell sorting. J. Cell. Physiol. 118: 45-52.

Johnson, L.V., Walsh, M.L. and Chen, L.B. (1980). Localisation of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. U.S.A. 77: 990-994.

Jones, B.C., Radley, J.M., Bradley, T.R. and Hodgson, G.S. (1980). Enhanced megakaryocyte repopulating ability of stem cells surviving 5-fluorouracil treatment. Exp. Hematol. 8: 61-64.

Jubinsky, P.T. and Stanley, E.R. (1985). Purification of hemopoietin 1: a multilineage hemopoietic growth factor. Proc. Natl. Acad. Sci. U.S.A. 82, 2764-2768.

Kannourakis, G. and Bol, S. (1987). Fractionation of normal and β-thalassemic human hemopoietic progenitor cells by immunomagnetic beads. Exp. Hematol. 15: 1103-1108.

Kearny, J.F., Radbruck, A., Liesegang, B. and Rajewsky, K. (1979). A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J. Immunol. 123: 1548-1550.

Keller, G.M. and Phillips, R.A. (1982). Detection in vitro of a unique multipotent hemopoietic progenitor. J. Cell. Physiol. (suppl.) 1: 31-36.

Keller, G., Holmes, W. and Miller, R.A. (1984). Clonal generation of multipotent and unipotent hemopoietic blast cell colonies in vitro. J. Cell. Physiol. 120: 29-35.

Keller, G., Paige, C., Gilboa, E. and Wagner, E.F. (1985). Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature 318: 149-154.

Kelvin, D.J., Chance, S., Shreeve, M., Axelrad, A.A., Connolly, J.A. and McLeod, D. (1986). Interleukin 3 and cell cycle progression. J. Cell. Physiol. 127: 403-409.

Kilmartin, J.V., Wright, B. and Milstein, C. (1982). Rat monoclonal anti-tubulin antibodies derived by using a new nonsecreting rat cell line. J. Cell. Biology 93: 576-582.

Kincade, P.W., Flaherty, L., Lee, G., Wantanbe, T. and Michaelson, J. (1980). Qa-antigen expression on functional lymphoid, myeloid and stem cells in adult mice. J. Immunol. 124: 2879-2885.

Koestler, T.P., Rieman, D., Muirhead, K., Greig, R.G. and Poste, G. (1984). Identification and characterization of a monoclonal antibody to an antigen expressed on activated macrophages. Proc. Natl. Acad. Sci. U.S.A. 81: 4505-4509.

Köhler, G. (1986). Derivation and diversification of monoclonal antibodies. Science 233: 1281-1286.

Köhler, G. (1980). Immunoglobulin chain loss in hybridoma lines. Proc. Natl. Acad. Sci. U.S.A. 77: 2197-2199.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

Köhler, G. and Milstein, C. (1976). Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. 6: 511-519.

Köhler, G. and Shulman, M.J. (1978). Cellular and molecular restrictions of the lymphocyte fusion. Curr. Topics. Microb. Immunol. 81: 143-148.

Koike, K., Stanley, R.E., Ihle, J.N. and Ogawa, M. (1986). Macrophage colony formation supported by purified CSF-1 and/or interleukin 3 in serum-free culture: Evidence for hierarchical difference in macrophage colony-forming cells. Blood 67: 859-864.

Koike, K., Ogawa, M., Ihle, J.N., Miyaka, T., Shimizu, T., Miyajuna, A., Yokota, T. and Arai, K.I. (1987). Recombinant murine granulocyte-macrophage (GM) colony-stimulating factor supports formation of GM and multipotential blast cell colonies in culture: Comparison with the effects of interleukin-3. J. Cell. Physiol. 131: 458-464.

Koizumi, S., Fine, R.L., Curt, G.A., Griffin, J.D. and Chabner, B.A. (1985). Enrichment of myeloid progenitor cells from normal human bone marrow using an immune-rosette technique. Exp. Hematol. 13: 560-565.

Koizumi, S., Yamagami, M., Miura, M., Horita, S., Sano, M., Ikuta, N. and Tanigushi, N. (1982). Expression of Ia-like antigens defined by monoclonal OKIa1 antibody on hemopoietic progenitor cells in cord blood: a comparison with human bone marrow. Blood 60: 1046-1049.

Kumoro, K., Itakura, K., Boyse, E.A. and John, M. (1975). Ly-5: a new T-lymphocyte antigen system. Immunogenetics 1: 452-456.

Kürzinger, K., Reynolds, T., Germain, R.N., Davignon, D., Martz, E. and Springer, T.A. (1981). A novel lymphocyte function-associated antigen (LFA-1): Cellular distribution, quantitative expression and structure. J. Immunol. 127: 596-602.

Lachmann, P.J., Oldroyd, R.G., Milstein, C. and Wright, B.W. (1980). Three rat monoclonal antibodies to human C3. Immunology 41: 503-515.

Lahiri, S.K. and Van Putten, L.M. (1969). Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kinet. 2: 21-28.

Lahiri, S.K. and Van Putten, L.M. (1972). Location of the G0-phase in the cell cycle of the mouse haemopoietic spleen colony forming cells. Cell Tissue Kinet. 5: 365.

Lahiri, S.K., Keizer, H.J. and Van Putten, L.M. (1970). The efficiency of the assay for haemopoietic colony forming cells. Cell. Tissue Kinet. 3: 355-362.

Lajtha, L.G., Pozzi, L.V., Schofield, R. and Fox, M. (1969). Kinetic properties of haemopoietic stem cells. Cell Tissue Kinet. 2: 39.

Lala, P.K. and Johnson, G.R. (1978). Monoclonal origin of B-lymphocyte colony forming cells in spleen colonies formed by multipotential hemopoietic stem cells. J. exp. Med. 148: 1468-1477.

Lane, R.D. (1985). A short-duration poly ethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J. Immunol. Meth. 81: 223-228.

Lanier, L.L. (1984). Lymphocyte surface antigens. Mini-symposium. Federation Proceedings 44: 2863.

Lanier, L.L. and Loken, M.R. (1984). Human lymphocyte subpopulations identified by using three color immunofluorescence and flow cytometry analysis: correlation of Leu-2, Leu-3, Leu-7, Leu-8 and Leu-11 cell cell surface antigen expression. J. Immunol. 132: 151-156.

Lanier, L.L., Allison, J.P. and Phillips, J.H. (1986). Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. J. Immunol. 137: 2501-2507.

Lansdorp, P.M., Astaldi, G.C.B., Oosterhof, F., Janssen, M.C. and Zeijlenmaker, W.P. (1980). Immunoproxidase procedures to detect monoclonal antibodies against cell surface antigens. Quantitation of binding and staining of individual cells. J. Immunol. Meth. 39: 393-405.

Larric, J.W. and Creswell, P. (1979). J. Supramolec. Struct. 11: 579-585.

LeCorre, R., Gerlier, D., Martin, A., Le Garrec, Y., Corradin, G., Bron, C. and Toujas, L. (1987). A new epitope of the T200 molecule family defined by the 3A35 monoclonal antibody and expressed by macrophages and activated T lymphocytes. Eur. J. Immunol. 17: 327-333.

Ledbetter, J.A. and Herzenberg, L.A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunological Reviews 47: 63-90.

Ledbetter, J.A., Rouse, R.V., Micklem, H.S. and Herzenberg, L.A. (1980). T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. J. Exp. Med. 152: 280-295.

Lee, J.C., Hapel, A.J. and Ihle, J.N. (1982). Constitutive production of a unique lymphokine (IL-3) by the WEHI-3 cell line. J. Immunol. 128: 2393-2398.

Lee, M., Segal, G.M. and Bagby, G.C. (1987). Interleukin-1 induces human bone marrow derived fibroblasts to produce multilineage hematopoietic growth factors. Exp. Hematol. 15: 983-988.

Lefrancois, L., Thomas, M.L., Bevan, M.J. and Trowbridge, I.S. (1986). Different classes of T lymphocytes have different mRNAs for the leukocyte-common antigen, T200. J. Exp. Med. 163: 1337-1342.

Lemischka, I.R., Raulet, D.H. and Mulligan, R.C. (1986). Developmental potential and dynamic behaviour of hematopoietic stem cells. Cell 45: 917-927.

Lemke, H., Hämmerling, G.J. and Hämmerling, U. (1979). Fine specificity analysis with monoclonal antibodies of antigens controlled by the major histocompatibility complex and by the Qa/TL region in mice. Immunol. Rev. 47: 175-206.

Leong, M.M., Milstein, C. and Pannell, R. (1986). Luminescent detection method for immunodot, western and southern blots. J. Histochem. Cytochem. 34: 1645-1650.

Lesley, J., Hyman, R., Schulte, R. and Trotter, J. (1984). Expression of transferrin receptor on murine hematopoietic progenitors. Cell. Immunol. 83: 14-25.

Lewis, J.P. and Trobaugh, F.E. (1964). Haematopoietic stem cell. Nature 204: 589-590.

Liebert, H., Laino, L. and Wahl, R.L. (1987). A semi-automated fluorescent (SAF) assay using viable whole cells for screening hybridoma supernatants. J. Immunol. Meth. 101: 85-90.

Littlefield, J.W. (1964). Selection of hybrids from mating of fibroblasts in vitro and their presumed recombinants. Science 145: 709.

Lo, M.M.S., Tsong, T.Y., Conrad, M.K., Strittmather, S.M., Hester, L.D. and Snyder, S.H. (1984). Monoclonal antibody production by receptor mediated electrically induced cell fusion. Nature 310: 792-794.

Loken, M.R. and Lanier, L.L. (1984). Three-color immunofluorescence analysis of Leu antigens on human peripheral blood using two lasers on a fluorescence-activated cell sorter. Cytometry 5: 151-158.

Loken, M.R., Shah, V.O., Dattilio, K.L. and Civin, C.I. (1987a). Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood 69: 255-263.

Loken, M.R., Shah, V.O., Dattilco, K.L. and Civin, C.I. (1987b). Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. Blood 70: 1316-1324.

Lord, B.J. (1967). Erythropoietic cell proliferation during recovery from acute haemorrhage. Br. J. Hemat. 13: 160-167.

Lord, B.I. and Spooncer, E. (1986). Isolation of haemopoietic spleen colony forming cells. Lymphokine Research 5: 59-72.

Lorenz, E., Uphoff, D.E., Reid, T.R. and Shelto, E. (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer Inst. 12: 197-201.

Löwenberg, B. (1975). Fetal liver cell transplantation. Role and nature of the fetal haemopoietic stem cell. Thesis, Erasmus University, Rotterdam.

Lu, L. and Broxmeyer, H.E. (1983). The selective enhancing influence of hemin and products of human erythrocytes on colony formation by human multipotential (CFU-GEMM) and erythroid (BFU-E) progenitor cells in vitro. Exp. Hematol. 11: 721-729.

Magli, M.C., Iscove, N.N. and Ordartchenko, N. (1982). Transient nature of early hematopoietic spleen colonies. Nature 295: 527-529.

Mathieson, B.J. and Fowlkes, B.J. (1982). Cell surface antigen expression on thymocytes: development and phenotypic differentiation of intrathymic subsets. Immunol. Rev. 82: 141-173.

Matsson, P., Pallavicini, M.G. and Summers, L. (1987). Quantitative flow cytometric and clonogenic evaluation of glass bead affinity fractionation of antibody-labeled murine bone marrow. J. Immunological Meth. 105: 45-53.

McCarthy, K.F., Hale, M.L. and Fehnel, P.L. (1987). Purification and analysis of rat hematopoietic stem cells by flow cytometry. Cytometry 8: 296-305.

McCulloch, E.A. (1983). Stem cells in normal and leukemic hemopoiesis. Blood 63: 1-13.

McKearn, J.P., Baum, C. and Davie, J.M. (1984). Cell surface antigens expressed by subsets of pre-B-cells and B cells. J. Immunol. 132: 332-339.

McNiece, I.K., Bradley, T.R., Kriegler, A.B. and Hodgson, G.S. (1986). Subpopulations of mouse bone marrow high-proliferative-potential colony-forming cells. Exp. Hematol. 14: 856-860.

McNiece, I.K., Williams, N.T., Johnson, G.R., Kriegler, A.B., Bradley, T.R. and Hodgson, G.S. (1987). Generation of murine hematopoietic precursor cells from macrophage high-proliferative-potential colony-forming cells. Exp. Hematol. 15: 972-977.

Mellor, A. (1986). The class I MHC gene family in mice. Immunology Today 7: 19-24.

Merchav, S. (1986). The effect of Rauscher leukaemia virus on haemopoietic cell differentiation in genetically defective W/W^v and normal mice. Thesis, State University, Leiden.

Merchav, S. and Wagemaker, G. (1984). Detection of murine bone marrow granulocyte/macrophage progenitor cells (GM-CFU) in serum-free cultures stimulated with purified M-CSF or GM-CSF. Int. J. Cell. Cloning 2: 356-367.

Messner, H.A., Izaguirre, C.A. and Jamal, N. (1981). Identification of T-lymphocytes in human mixed hemopoietic colonies. Blood 58: 402-405.

Metcalf, D. (1984). The hemopoietic colony stimulating factors. Elsevier, Amsterdam.

Metcalf, D. (1985a). Multi-CSF-dependent colony formation by cells of a murine hemopoietic cell line: Specificity and action of multi-CSF. Blood 65: 357-362.

Metcalf, D. (1985b). The granulocyte-macrophage colony-stimulating factors. Science 229: 16-22.

Metcalf, D. (1985c). The granulocyte-macrophage colony-stimulating factors. Cell 43: 5-6.

Metcalf, D. (1986a). Editorial comment: How reliable are in vitro clonal cultures? Some comments based on hemopoietic cultures. Int. J. Cell. Cloning 4: 287-294.

Metcalf, D. (1986b). Annotation. Haemopoietic growth factors now cloned. Br. J. Haematology 62: 409-412.

Metcalf, D. and Moore, M.A.S. (1971). Haemopoietic cells: Frontiers of Biology. (eds.: Neuberger, A. and Tatum, E.L.), North-Holland, Amsterdam.
Metcalf, D., Moore, M.A.S. and Shortman, K. (1971). Adherence column and buoyant density separation of bone marrow stem cells and more differentiated cells. J. Cell. Physiol. 78: 441-450.
Metcalf, D., Johnson, G.R. and Mandel, T.E. (1979). Colony formation in agar by multipotential hemopoietic cells. J. Cell. Physiol. 98: 401-420.
Metcalf, D., Begley, C.G., Nicola, N.A. and Johnson, G.R. (1987). Quantitative responsiveness of murine hemopoietic populations in vitro and in vivo to recombinant multi-CSF (IL-3). Exp. Hematol. 15: 288-295.
Metcalf, D., Nossal, S.J.V., Warner, N.L., Miller, J.F.A.P., Mandel, T.E., Layton, J.E. and Gutman, G.A. (1975). Growth of B lymphocyte colonies in vitro. J. exp. Med. 142: 1534-1549.
Micklem, H.S. (1983). Factors affecting serially transplanted bone marrow: a commentary. Blood Cells 9: 527-531.
Micklem, H.S. (1986). Monoclonal antibodies to murine haemopoietic cells. In: Monoclonal antibodies. Methods in Hematology. Beverley, P.C.L. (ed.). Churchill Livingstone, U.K. 13: 182-206.
Micklem, H.S. and Ogden, D.A. (1976). Ageing of haemopoietic cell populations in the mouse. In: Stem cells of renewing cell populations (eds.: Cairnie, A.B. and Lala, P.K.), Osmond D.G. Academic Press, New York, p. 331-341.
Micklem, H.S., Anderson, N. and Ross, E. (1975). Limited potential of circulating haemopoietic stem cells. Nature 256: 41-43.
Migliaccio, A.R. and Visser, J.W.M. (1986). Proliferation of purified murine hemopoietic stem cells in serum-free cultures stimulated with purified stem-cell-activating factor. Exp. Hematol. 14: 1043-1048.
Miller, B.A., Antognetti, G. and Springer, T.A. (1985a). Identification of cell surface antigens present on murine hematopoietic stem cells. J. Immunol. 134: 3286-3290.
Miller, B.A., Lipton, J.M., Linch, D.C., Burakoff, S.J. and Nathan, D.G. (1985b). Thy-1 is a differentiation antigen that characterizes immature murine erythroid and myeloid hematopoietic progenitors. J. Cell. Physiol. 123: 25-32.
Miller, R.G. (1973). Separation of cells by velocity sedimentation. In: New techniques in biophysics and cell biology. (eds.: Pain, R.H., Smith, B.J.), John Wiley and Sons, Ltd., London, vol. 1, p. 87-112.
Miller, R.G. and Phillips, R.A. (1969). Separation of cells by velocity sedimentation. J. Cell. Physiol. 73: 191-201.
Milstein, C. (1986). Overview: Monoclonal antibodies. In: Handbook of Experimental Immunology: vol. 4. Applications of Immunilogical Methods in Biomedical Sciences (eds.: Weir, D.M., Herzenberg, L.A., Blackwell, C. and Herzenberg, L.A.), Blackwell, Oxford, U.K., Chapter 107.
Milstein, C., Galfre, G., Secher, D.S. and Springer, T.A. (1979). Monoclonal antibodies and cell surface antigens. Cell Biology International Reports 3: 1-16.
Misra, D.N., Kunz, H.W., Cortesse-Hassett, A. and Gill III, T.J. (1985). Monoclonal antibodies against specific rat class I major histocompatibility complex antigens. Transpl. Proc. 17: 1815-1817.
Misra, D.N., Noeman, S.A., Kunz, H.W. and Gill III, T.J. (1981). Production of monoclonal antibodies to rat MHC antigens using different myelomas. Transpl. Proc. 13: 1347-1355.

Mockizuki, D.Y., Eisenman, J.R., Conlon, P.J., Larsen, A.D. and Tuskinki, R.J. (1987). Interleukin 1 regulates hematopoietic activity, a role previously ascribed to hemopoietin-1. Proc. Natl. Acad. Sci. U.S.A. 84: 5267-5271.

Monette, F.C. (1979). Antibodies against pluripotent stem cells: their use in studying stem cell function. Blood Cells 5: 175-191.

Monette, F.S. and Sigounas, G. (1984). Factors affecting the proliferation and differentiation of clonogenic hematopoietic stem cells in vitro. Blood Cells 10: 261.

Monette, F.S. and Sigounas, G. (1987). Sensitivity of murine multipotential stem cell colony (CFU-GEMM) growth to interleukin-3, erythropoietin and hemin. Exp. Hematol. 15: 729-734.

Moore, M.A.S. and Owen, J.J.T. (1965). Chromosome marker studies on the development of the haemopoietic system in the chick embryo. Nature 208: 956.

Moore, M.A.S. and Williams, N. (1974). Functional, morphologic and kinetic analysis of the granulocyte-macrophage progenitor cell. In: Hemopoiesis in culture. Second International Workshop (ed.: Robinson, W.A.). Government Printing Office, Washington D.C., pp. 17-29.

Moore, M.A.S. and Warren, D.J. (1987). Synergy of interleukin 1 and granulocyte colony-stimulating factor: In vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc. Natl. Acad. Sci. U.S.A. 84: 7134-7138.

Moore, M.A.S., Williams, N. and Metcalf, D. (1972). Purification and characterization of the in vitro colony forming cell in monkey hemopoietic tissue. J. Cell. Physiol. 79: 283-293.

Moore, M.A.S., Warren, D.J. and Souza, L. (1987). In: Leukemia, recent advances in biology and treatment (eds.: Gale, R.P. and Golde, D.W.), Liss, New York, in press.

Moore, R.N., Openheim, J.J., Farrar, J.J. et al. (1980). Production of lymphocyte activating factor (interleukin 1) by macrophages activated with colony stimulating factors. J. Immunol. 125: 1302-1305.

Morse III, H.C., Shen, F.W. and Hämmerling, U. (1987). Genetic nomenclature for loci controlling mouse lymphocyte antigens. Immunogenetics 25: 71-78.

Mouchiroud, G., Berthier, R., Newton, J.A., Chapel, A. and Holland, D. (1985). Monoclonal antibodies against human hemopoietic cells and the separation of progenitor cells from bone marrow. Exp. Hematol. 13: 566-573.

Mulder, A.H. (1986). Differentiation of hemopoietic stem cells in the thymus. Thesis, Erasmus University, Rotterdam.

Mulder, A.H., Visser, J.W.M. and Van den Engh, G.J. (1985). Thymus regeneration by bone marrow cell suspensions differing in the potential to form early and late spleen colonies. Exp. Hematol. 13: 768-775.

Mulder, A.H. and Visser, J.W.M. (1987). Separation and functional analysis of bone marrow cells separated by Rhodamine-123 fluorescence. Exp. Hematol. 15: 99-104.

Muller-Sieburg, C.E., Whitlock, C.A. and Weismann, I.R. (1986). Isolation of two early B lymphocyte progenitors from mouse marrow: A committed pre-pre-B cell and a clonogenic Thy-1b hematopoietic stem cell. Cell 44: 653-662.

Nakahata, T. and Ogawa, M. (1982a). Clonal origin of murine hemopoietic colonies with apparent restriction to granulocyte-macrophage megakaryocyte (GMM) differentiation. J. Cell. Physiol. 111: 239-246.

Nakahata, T. and Ogawa, M. (1982b). Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc. Natl. Acad. Sci. U.S.A. 79: 3843-3847.

Nakahata, T., Gross, A.J. and Ogawa, M. (1982). A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. J. Cell. Physiol. 113: 455-458.

Neta, R., Sztein, M.B., Oppenheim. J.J., Gillis, S. and Douches, S.D. (1987). The in vivo effects of interleukin 1. I. Bone marrow cells are induced to cycle after administration of interleukin 1. J. Immunol. 139: 1861-1866.

Nibbering, P.H., Leij, P.C.J. and Van Furth, R. (1987). Quantitative immunocytochemical characterization of mononuclear phagocytes. Cell. Immunol. 105: 374-385.

Nicola, N.A. (1982). Electronic cell sorting of hemopoietic progenitor cells. In: Cell Separation: Methods and Selected Applications 1, Pretlow, T.G., Pretlow, T., Academic Press, New York, pp. 191-221.

Nicola, N.A. (1987a). Why do hemopoietic growth factor receptors interact with each other? Immunol. Today 8: 134-140.

Nicola, N.A. (1987b). Hemopoietic growth factors and their interactions with specific receptors. J. Cell. Physiol. (suppl.) 5: 9-14.

Nicola, N.A. and Metcalf, D. (1986). Binding of iodonated multipotential colony-stimulating factor (interleukin-3) to murine bone marrow cells. J. Cell. Physiol. 128: 180-188.

Nicola, N.A. and Vadas, M. (1984). Hemopoietic colony-stimulating factors. Immunol. Today 5, 76-80.

Nicola, N.A., Metcalf, D., Melcher, H. von and Burgess, A.W. (1981). Isolation of murine fetal hemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood 58: 376-386.

Nicola, N.A., Burgess, A.W., Staber, F.G., Johnson, G.R., Metcalf, D. and Battye, F.C. (1980). Differential expression of lectin receptors during hemopoietic differentiation: Enrichment for granulocyte-macrophage progenitor cells. J. Cell. Physiol. 103: 217-237.

Nijhoff, W. and Wierenga, P.K. (1984). Isolation of hemopoietic stem cells from spleens of thiamphenicol pretreated mice. Exp. Cell. Res. 155: 583-587.

Nilson, B.O., Grovnik, K.O. and Svalander, P.C. (1983). Experiments with immunization of mice with blastocysts by an intrasplenic route. Upsalla. J. Med. Sci. 88: 151.

Nilson, B.O., Svalander, P.C. and Larsson, A. (1987). Immunization of mice and rabbits by intrasplenic deposition of nanogram quantities of protein attached to sepharose beads or nitrocellulose paper strips. J. Immunol. Meth. 99: 67-75.

Nowell, P.C., Cole, L.J., Habermeyer, J.G. and Roan, P.L. (1956). Growth and continued function of rat marrow cells in X-irradiated mice. Cancer Res. 16: 258-261.

Nowinski, R., Berglund, C., Lane, J., Lostrom, M., Bernstein, I., Young, W., Hakomori, S.-J., Hill, L. and Cooney, M. (1980). Human monoclonal antibody against Forssman antigen. Science 210: 537-539.

O'Connor, R., Bradley, J.G., Lawlor, E. and Cottor, T.G. (1987). Production and characterization of a monoclonal antibody to a myeloid specific differentiation antigen. Leuk. Res. 11: 481-488.

O'Garra, A., Umland, S., DeFrance, T. and Christiansen, J. (1988). B cell factors are pleiotropic. Immunology Today 9: 45-54.

Ogawa, M., Porter, P.N. and Nakahata, T. (1983). Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). Blood 61: 823-829.

Ogawa, M., Pharr, P.N. and Suda, T. (1985). Stochastic nature of stem cell functions in culture. In: Hematopoietic stem cell physiology (eds.: Cronkite, E., Dainiak, N., McCaffrey, R., Palek, J. and Quesenberry, P.), Alan R. Liss, Inc., New York, pp. 11-19.

Ogden, D.A. and Micklem, H.S. (1976). The fate of serially transplanted bone marrow cell populations from young and adult donors. Transplantation 22: 287-293.

Ohara, J. and Paul, W.E. (1987). Receptors for B cell stimulatory factor-1 expressed on cells of haematopoietic lineages. Nature 325: 537-540.

Oi, V.T. and Herzenberg, L.A. (1979). Immunoglobulin-produced hybrid cell lines. In: Selected methods in cellular immunology. Eds. Mishell and Shiigi, p. 351-372.

Oi, V.T., Glazer, A.N. and Stryer, L. (1982). Fluorescent phycobiliprotein conjugates for the analysis of cells and molecules. J. Cell. Biol. 93: 981-986.

Oi, V.T., Jones, P.P., Goding, J.W., Herzenberg, L.A. and Herzenberg, L.A. (1978). Properties of monoclonal antibodies to mouse Ig allotypes H-2, and Ia antigens. Current Topics Microb. Immunol. 81: 115-129.

Oliveira, O.L.P. and Thomas, D.B. (1985). A new thymocyte membrane differentiation antigen for mouse and rat. Immunogenetics 21: 559-568.

Omary, M.B., Trowbridge, I.S. and Scheid, M.P. (1980). T200 cell surface glycoprotein of the mouse. J. Exp. Med. 151: 1311-1316.

Oppenheim, J.J., Kovacs, E.J., Matsushima, K. and Durum, S.K. (1986). There is more than one interleukin 1. Immunol. Today 7: 45-56.

Opstelten, D., Deenen, G.J., Rozing, J. and Hunt, S.V. (1986). B lymphocyte associated antigens on terminal deoxynucleotidyl transferase-positive cells and pre-B cells in bone marrow of the rat. J. Immunol. 137: 76-84.

Ozata, K. and Sachs, D.H. (1981). Monoclonal antibodies to mouse MHC antigens III. Hybridoma antibodies reacting to antigens of the $H\text{-}2^b$ haplotype reveal genetic control of isotype expression. J. Immunol. 126: 317-321.

Paige, C.J., Kincade, P.W., Moore, M.A. and Lee, G. (1979). The fate of fetal and adult B cell progenitors grafted with immunodeficient CBA/N mice. J. Exp. Med. 150: 548-563.

Palacios, R., Neri, T. and Brockhaus, M. (1986). Monoclonal antibodies specific for interleukin 3-sensitive murine cells. J. Exp. Med. 163: 369-382.

Pallavicini, M.G., Summers, L.J., Giroud, F.J., Dean, P.N. and Gray, J.W. (1985a). Enrichment of murine hemopoietic clonogenic cells by multivariate analysis and sorting. Exp. Hematol. 13: 1173-1181.

Pallavicini, M.G., Summers, L.J., Giroud, F.J., Dean, P.N. and Gray, J.W. (1985b). Multivariate analysis and list mode processing of murine hemopoietic subpopulations for cytokinetic studies. Cytometry 6: 539-549.

Pallavicini, M.G., Levin, J., Summers, L. and Levin, F. (1987). Multivariate flow cytometric characterization and enrichment of murine megakaryocyte progenitors (CFU-Meg). Exp. Hematol. 15: 705-709.

Papiernik, M., Lehuen, A. and Savino, W. (1987). Definition of a differentiation antigen on the surface of phagocytic cells of thymic reticulum which is down-regulated by interferon-γ. Cell. Immunol. 105: 208-219.
Parks, D.R., Hardy, R.R. and Herzenberg, L.A. (1983). Dual immunofluorescence - new frontiers in cell analysis and sorting. Immunol. Today 4: 145-150.
Parks, D.R., Hardy, R.R. and Herzenberg, L.A. (1984). Three-color immunofluorescence analysis of mouse B-lymphocyte subpopulations. Cytometry 5: 159-168.
Parks, D.R., Lanier, L.L. and Herzenberg, L.A. (1986). Flow cytometry and fluorescence activated cell sorting (FACS). In: Handbook of Experimental Immunology. vol. 1. Immunochemistry (eds. Weir, D.M., Herzenberg, L.A., Blackwell, C. and Herzenberg, L.A.), Chapter 29, Blackwell, Oxford, U.K.
Pierres, M., Goridis, C. and Goldstein, P. (1982). Inhibition of murine T cell-mediated cytolysis and T cell proliferation by a rat monoclonal antibody immunoprecipitating two lymphoid cell surface polypeptides of 94000 and 180000 molecular weight. Eur. J. Immunol. 12: 60-69.
Pike, B.L. and Nossal, G.J.V. (1985). Interleukin 1 can act as a B-cell growth and differentiation factor. Proc. Natl. Acad. Sci. U.S.A. 82: 8153-8157.
Pillai, P.S., Reynolds, S.D., Chase, J.H. and Scott, D.W. (1986). Differential expression of a surface antigen recognized by a monoclonal antibody, G11D, on unprimed and primed B cells. J. Immunol. 137: 791-797.
Ploemacher, R.E., Brons, N.H.C. and Leenen, P.J.M. (1987). Bulk enrichment of transplantable hemopoietic stem cell subsets from lipopolysaccharide-stimulated murine spleen. Exp. Hematol. 15: 154-162.
Ploemacher, R.E. and Brons, N.H.C. (1988a). Isolation of hemopoietic stem cell subsets from murine bone marrow: I. Radioprotective ability of purified cell suspensions differing in the proportion of day-7 and day-12 CFU-S. Exp. Hematol. 16: 21-26.
Ploemacher, R.E. and Brons, N.H.C. (1988b). Isolation of hemopoietic stem cell subsets from murine bone marrow: II. Evidence for an early precursor of day-12 CFU-S and cells associated with radioprotective ability. Exp. Hematol. 16: 27-32.
Pluznik, D.H. and Sachs, L. (1965). The cloning of normal "mast" cells in tissue culture. J. Cell. Comp. Physiol. 66: 319.
Pontecorvo, G. (1976). Production of indefinitely multiplying mammalian somatic cell hybrids by polyethyleneglycol (PEG)-treatment. Somat. Cell. Genetics 1: 397.
Porter, P.N., Meints, R.H. and Messner, K. (1979). Exp. Hematol. 7: 183-188.
Potter, M. (1972). Immunoglobulin producing tumors and myeloma proteins of mice. Physiol. Rev. 52: 631-719.
Preffer, F.S., Colvin, R.B., Leary, C.P., Boyle, L.A., Tuazon, T.V., Lazarovits, A.I., Cosimi, A.B. and Kurnick, J.T. (1986). Two-color flow cytometry and functional analysis of lymphocytes cultured from human renal allografts: identification of a leu-2^{+}3^{+} subpopulation. J. Immunol. 137: 2823-2830.
Pretlow, T.G. and Pretlow, T.P. (1979). Centrifugal elutriation (counter streaming centrifugation) of cells. Cell. Biophys. 4: 195-207.
Proce, G.B., Stewart, S. and Krogsrud, R.L. (1979). Characterization of stem cells and progenitors of hemopoiesis by cell sorting. Blood Cells 5: 161-174.

Priestly, G.V. and Wolf, N.S. (1985). A technique for the daily examination of spleen colonies in mice. Exp. Hematol. 13: 733-735.
Prystowsky, M.B., Otten, G., Naujokas, M.F., Vardiman, J., Ihle, J.N., Goldwasserm E. and Fitch, F.W. (1984). Multiple hemopoietic lineages are found after stimulation of mouse bone marrow precursor cells with interleukin 3. Am. J. Path. 117: 171-179.
Quesenberry, P.J. (1986). Synergistic hematopoietic growth factors. Int. J. Cell Cloning 4: 3-15.
Quesenberry, P. and Levitt, L. (1979). Hematopoietic stem cells. New Engl. J. Med. 301: 755-760.
Ralph, S.J. and Berridge, M.V. (1984). Expression of antigens of the "T200" family of glycoproteins on hemopoietic stem cells: evidence that thymocyte cell lineage antigens are represented on "T200". J. Immunol. 132: 2510-2514.
Ralph, S.J., An Tan, S. and Berridge, M.U. (1982). Monoclonal antibodies detect subpopulations of bone marrow stem cells. Stem Cells 2: 88-107.
Ralph, S.J., Thomas, M.L., Morton, C. and Trowbridge, I.S. (1987). Structural variants of human T200 glycoprotein (leukocyte-common antigen). EMBO J. 6: 1251-1257.
Raymond, Y. and Suh, M. (1986). Lymph node primary immunization of mice for the production of polyclonal and monoclonal antibodies. J. Immunol. Meth. 93: 103-106.
Reading, C.L. (1982). Review article: The cry and methods for immunization in culture and monoclonal antibody production. J. Immunol. Meth. 53: 261-291.
Reason, D., Carminati, J., Kimura, J. and Henry, C. (1987). Directed fusion in hybridoma production. J. Immunol. Meth. 99: 253-257.
Reichert, R.A., Weissman, I.L. and Butcher, E.C. (1986). Phenotypic analysis of thymocytes that express homing receptors for peripheral lymph nodes. J. Immunol. 136: 3521-3528.
Reif, A.E. and Allen, J.M.V. (1963). Specificity of isoantisera against leukemic and thymic lymphocytes. Nature 200: 1332-1333.
Reinherz, E.R., Kung, P.C., Goldstein, G., Levey, R.H. and Schlossman, S.F. (1980). Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc. Natl. Acad. Sci. U.S.A. 77: 1588-1592.
Rennick, D.H., Lee, F.D., Yokota, T., Arai, K.-J, Cantor, H. and Nabel, G.J. (1985). A cloned mast cell growth factor cDNA encodes a multilineage hematopoietic growth factor: Multiple activities of interleukin 3. J. Immunol. 134: 910-914.
Rickard, K.A., Shadduck, R.K., Howard, D.E. and Stohlman, F. (1970). A differential effect of hydroxyurea on hemopoietic stem cell colonies in vitro and in vivo. Proc. Soc. Exp. Biol. Med. 134: 152-156.
Risser, R. (1979). Abelson antigen is expressed on hematopoietic spleen colony forming cells from mice carrying the AV-2^5 virus sensitivity gene. Proc. Natl. Acad. Sci. U.S.A. 76: 5350-5354.
Rits, M., Cormont, F., Bazin, H., Meykens, R. and Vaerman, J.P. (1986). Rat monoclonal antibodies. VI. Production of IgA secreting hybridomas with specificity for the 2,4,-dinitrophenyl (DNP) hapten. J. Immunol. Meth. 89: 81-87.
Rosendaal, M., Hodgson, G.S. and Bradley, T.R. (1976). Haemopoietic stem cells are organized for use on the basis of their generation-age. Nature 264: 68-69.

Rosendaal, M., Hodgson, G.S. and Bradley, T.R. (1979). Organization of haemopoietic stem cells: The generation dye hypothesis. Cell Tissue Kinet. 12: 17-29.

Ross, E.A.M., Anderson, N. and Micklem, H.S. (1982). Serial depletion of the murine hematopoietic system: implication for hematopoietic organization and the study of cellular ageing. J. Exp. Med. 155: 432-444.

Rozenszajn, L.A., Shoham, D. and Kalechman, I. (1975). Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29: 1041-1055.

Russell, J.L. and Van den Engh, G.J. (1979). The expression of histocompatibility-2 antigens on hemopoietic stem cells. Tissue Antigens 13: 45-52.

Sarmiento, M., Loken, M.R., Trowbridge, I.S., Coffman, R.L. and Fitch, F.W. (1982). High molecular weight lymphocyte surface proteins are structurally related and are expressed on different cell populations at different times during lymphocytic maturation and differentiation. J. Immunol. 128: 1676-1684.

Sasaki, D.T., Dumas, S.E. and Engleman, E.G. (1987). Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. Cytometry 8: 413-420.

Sauvage, C.A., Mendelsohn, J.C., Lesley, J.F. and Trowbridge, I.S. (1987). Effects of monoclonal antibodies that block transferrin receptor function of the in vivo growth of a syngeneic murine leukemia. Cancer Research 47: 747-753.

Schmidt, J.A., Marshall, J., Hayman, M.J., Doderlein, G. and Beug, H. (1986). Monoclonal antibodies to novel erythroid differentiation antigens reveal specific effects of oncogenes on the leukaemic cell phenotype. Leukemia Research 10: 257-272.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4: 7-25.

Schofield, R. and Lajtha, L.G. (1973). Effect of isopropyl methane sulphonate (IMS) on haemopoietic colony-forming cells. Br. J. Haematol. 25: 195-202.

Schooley, J.C. (1966). The effect of erythropoietin on the growth and development of spleen colony forming cells. J. Cell. Physiol. 68: 249-262.

Schooley, J.C., Kullgren, B. and Allison, A.C. (1987). Inhibition by interleukin-1 of the action of erythropoietin on erythroid precursors and its possible role in the pathogenesis of hypoplastic anaemias. Br. J. Haematol. 67: 11-17.

Schroeder, J.W., Lewis, S.J., Clark-Lewis, I. and Culvernor, J.G. (1981). The persisting (P) cell: Histamine content, regulation by a T cell-derived factor, origin from a bone marrow precursor and relationship to mast cells. Proc. Natl. Acad. Sci. USA 78: 323-327.

Sela, M. (1986). Overview: Antigens. In: Handbook of Experimental Immunology, vol. 1. Immunochemistry (Chapter 1). (eds.: Weir, D.M., Herzenberg, L.A., Blackwell, C., Herzenberg, L.A., Blackwell, C. and Herzenberg, L.A.), Blackwell, Oxford, U.K.

Shapiro, H.M. (1985). Practical Flow Cytometry. A.L. Liss, New York.

Sharpless, T.K., Traganos, F., Darzunkiewicz, Z. and Melamed, M.R. (1975). Flow cytofluorometry: Discrimination between single cells and cell aggregates by direct size measurements. Acta Cytol. 19: 577.

Shen, F.W. (1981). Monoclonal antibodies to mouse lymphocyte differentiation alloantigens. In: Monoclonal antibodies and T-cell hybridomas. Eds. Hämmerling, G.J., Hämmerling, U. and Kearny, J.F. Elsevier/North-Holland Biomedical Press, Amsterdam, p. 25-31.

Shen, F.W., Yakura, H. and Tung, J.S. (1982). Some compartments of B cell differentiation. Imm. Rev. 69: 69-80.
Shortman, K. (1968). The separation of different cell classes from lymphoid organs. II. The purification and analysis of lymphocyte populations by equilibrium density gradient centrifugation. Aust. J. Exp. Biol. Med. Sci. 46: 375-396.
Shortman, K. (1972). Physical procedures for the separation of animal cells. Ann. Rev. Biophys. Bioenginering 1: 93-130.
Shulman, M., Wilde, C.D., Köhler, G. (1978). A better cell line for making hybridomas secreting specific antibodies. Nature 276: 269-270.
Sieff, C., Bicknell, D., Caine, G., Robinson, J., Ram, G. and Greaves, M.F. (1982). Changes in cell surface antigen expression during hemopoietic differentiation. Blood 60: 703-713.
Siminovitch, L., McCulloch, E.A. and Till, J.E. (1963). The distribution of colony-forming cells among spleen colonies. J. Cell. Comp. Physiol. 62: 327-336.
Spitz, M., Spitz, L., Thorpe, R. and Eugui, E. (1984). Intrasplenic primary immunization for the production of monoclonal antibodies. J. Immunol. Meth. 70: 39-43.
Spivak, J.L. (1986). The mechanism of action of erythropoietin. Int. J. Cell Cloning 4: 139-166.
Spivak, J.L., Smith, R.R.L. and Ihle, J.N. (1985). Interleukin 3 promotes the in vitro proliferation of murine pluripotent hematopoietic stem cells. J. Clin. Invest. 1613-1621.
Spooncer, E., Lord, B.J. and Dexter, T.M. (1985). Defective ability to self-renew in vitro of highly purified primitive haemopoietic cells. Nature 316: 62-64.
Springer, T.A. (1980). Cell-surface differentiation in the mouse. In: Monoclonal antibodies (eds.: Kenneth, R.H., McKearn, T.J. and Bechtol, K.B.). Plenum Press, New York, p. 185-217 (Chapter 12).
Springer, T., Galfre, G., Secher, D. and Milstein, C. (1978a). Monoclonal xenogeneic antibodies to mouse leukocyte antigens: identification of macrophage-specific and other differentiation antigens. Curr. Topics Microb. Immunol. 81: 45-50.
Springer, T., Galfre, G., Secher, D.S. and Milstein, C. (1978b). Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. Eur. J. Immunol. 8: 539-551.
Springer, T., Galfre, G., Secher, D.S. and Milstein, C. (1979). Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. 9: 301-306.
Staber, F.G., Schafli, E. and Moroni, C. (1978). Expression of endogenous C-type viral antigen on normal mouse haemopoietic stem cells. Nature 275: 669-771.
Stähli, C., Staehelin, T., Miggiano, V., Schmidt, J. and Haring, P. (1980). High frequencies of antigen-specific hybridomas: Dependence on immunization parameters and prediction by spleen cell analysis. J. of Immunol. Meth. 32: 297-304.
Stanley, E.R. and Heard, P.M. (1977). Factors regulating macrophage production and growth purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J. Biol. Chem. 252: 4305-4312.
Stanley, E.R., Bradley, T.R. and Sumner, M.A. (1971). Properties of the mouse embryo conditioned medium factor(s) stimulating colony formation by mouse bone marrow cells grown in vitro. J. Cell. Physiol. 78: 301-318.

Stanley, E.R., Bartocci, A., Patinkin, D., Rosendaal, M. and Bradley, T.R. (1986). Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. Cell 45: 667-674.

Stern, P.L., Willison, K.R., Lennox, E., Galfre, G., Milstein, C., Secher, D.S. and Ziegler, A. (1978). Monoclonal antibodies as probes for differentiation and tumor-associated antigens: a Forssman specificity on teratocarcinoma stem cells. Cell 14: 775-783.

Stokdijk, W., Van den Engh, G.J. and Van Dekken, H. (1985). The electronics of the RELACS: the Rijswijk Experimental light-activated cell sorter. International Conference Analytical Cytology XI. Hilton Head (SC). November 17-22, abstract 318.

Strauss, L.C., Rowley, S.D., La Russa, V.F., Sharkis, S.J., Stuart, R.K. and Civin, C.I. (1986a). Antigenic analysis of hematopoiesis. V. Characterization of My-10 antigen expression by normal lymphohematopoietic progenitor cells. Exp. Hematol. 14: 878-886.

Strauss, L.C., Brovall, C., Fachler, M.J., Schwartz, J.F., Shaper, J.H., Loken, M.R. and Civin, C.I. (1986b). Antigenic analysis of hematopoiesis. IV. The My-11 hematopoietic cell surface antigen is expressed by myelomonocytic and lymphoid, but not erythroid, progenitor cells. Exp. Hematol. 14: 935-945.

Strife, A., Lambek, C., Wisniewski, D., Gulati, S., Gasson, J.C., Golde, D.W., Welte, K., Gabrilove, J.L. and Clarkson, B. (1987). Activities of four purified growth factors on highly enriched human hematopoietic progenitor cells. Blood 69: 1508-1523.

Suda, J., Suda, T. and Ogawa, M. (1984). Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood 64: 393-399.

Suda, J., Suda, T., Kubota, K., Ihle, J.N., Saito, M. and Miura, Y. (1986). Purified interleukin 3 and erythropoietin support the terminal differentiation of hemopoietic progenitors in serum-free culture. Blood 67: 1002-1006.

Suda, T., Suda, J. and Ogawa, M. (1983). Proliferative kinetics and differentiation of murine blast cell colonies in culture: Evidence for variable G0 periods and constant doubling rates of early pluripotent hemopoietic progenitors. J. Cell. Physiol. 117, 308-318.

Suda, T., Suda, J., Ogawa, M. and Ihle, J.N. (1985). Permissive role of interleukin 3 (IL-3) in proliferation and differentiation of multipotential hemopoietic progenitors in culture. J. Cell. Physiol. 124: 182-190.

Sutherland, D.J.A., Till, J.E. and McCulloch, E.A. (1970). A kinetic study of the genetic control of hemopoietic progenitor cells assayed in culture and in vivo. J. Cell. Physiol. 75: 267-274.

Svalander, P.C., Anderson, J. and Nilsson, B.O. (1987). Intrasplenic immunization for production of monoclonal antibodies against mouse blastocysts. J. Immunol. Meth. 105: 221-227.

Swirsky, D.M., Watt, S.M., Gilmore, D.J., Hayhoe, F.G.J. and Waldmann, H. (1983). The characterization of monoclonal antibodies against haemopoietic cells: Comparison of an immunoperoxidase method with fluorescence activated cell sorting. J. Immunol. Meth. 61: 171-182.

Szybalski, W., Szybalski, E.H. and Ragni, B. (1962). Natl. Cancer Inst. Monograph 7: 75.

Takei, F. (1984). A novel differentiation antigen on proliferating murine thymocytes identified by a rat monoclonal antibody. J. Immunol. 132: 766-771.

Terhorst, C., Van Agthoven, A., Leclair, K., Snow, P., Reinherz, E.L. and Schlossman, S.F. (1980). Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. Cell 23: 771-780.

Thalhamer, J. and Freund, J. (1985). Passive immunization: A method of enhancing the immune response against antigen mixtures. J. Immunol. Meth. 80: 7-13.

Tidmarch, C.F., Dailey, M.O., Whitlock, C.A., Pillemer, E., Weissman, I.L. (1985). Transformed lymphocytes from Abelson-diseased mice express levels of a B lineage transformation-associated antigen elevated from that found on normal lymphocytes. J. Exp. Med. 162: 1421-1434.

Till, J.E. (1976). Regulation of hemopoietic stem cells. In: Stem Cells of Renewing Cell Populations (eds.: Cairnie, A.B., Lala, P.K. and Osmond, D.G.), Academic Press, New York, p. 143-155.

Till, J.E. and McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213-222.

Till, J.E. and McCulloch, E.A. (1972). The 'F-factor' of the spleen colony assay for hemopoietic stem cells. Ser. Haemat. 2: 15-21.

Till, J.E. and McCulloch, E.A. (1980). Hemopoietic stem cell differentiation. Biochim. Bioph. Acta 605: 431-459.

Till, J.E., McCulloch, E.A. and Siminovitch, R. (1964). A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. Proc. Natl. Acad. Sci. U.S.A. 51: 29-36.

Titus, J.A., Haughland, R., Sharrow, S.O. and Segal, D.M. (1982). Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. J. Immunol. Meth. 50, 193-204.

Trentin, J.J. and Fahlberg, W.J. (1963). An experimental model for studies of immunological competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptual Advances in Immunology and Oncology. 16th Ann. Symp. Texas, Harper Row, New York, p. 66.

Trentin, J.J., Curry, J.C., Wolf, N. and Cheng, V. (1967). Factors controlling stem cell differentiation and proliferation: The haemopoietic inductive microenvironment (HIM). In: The proliferation and spread of neoplastic cells. M.D. Anderson Hospital, 21st Am. Symp. on Fund. Canc. Res., Baltimore, Williams and Wilkins, p. 713.

Trowbridge, J.S. (1978). Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. J. Exp. Med. 148: 313-323.

Turner, R.W.A., Siminovitch, L., McCulloch, E.A. and Till, J.E. (1967). Density gradient centrifugation of hemopoietic colony forming cells. J. Cell. Physiol. 69, 873.

Umiel, T., Nadler, L.M., Cohen, I.J., Revine, H., Stark, B., Mannon, Z., Dzaldeti, M., Rechavi, G., Simoni, F., Katzir, N., Ramot, B. and Zaizov, R. (1987). Undifferentiated leukemia of infancy with t(11:17) chromosomal rearrangement. Cancer 59: 1143-1149.

Van Agthoven, A., Goridis, C., Naquet, P., Pierres, A. and Pierres, M. (1984). Structural characteristics of the mouse transferrin receptor. Eur. J. Biochem. 140: 433-440.

Van Bekkum, D.W. (1977). The appearance of the multipotential hemopoietic stem cell. In: Experimental Hematology Today (eds.: Baum, S.J. and Ledney, G.D.), Chapter 1, Springer, New York.

Van Bekkum, D.W., Van Noord, M.J., Maat, B. and Dicke, K.A. (1971). Attempts at identification of hemopoietic stem cells in the mouse. Blood 38: 547-558.

Van Bekkum, D.W., Van den Engh, G.J., Wagemaker, G., Bol, S.J.L. and Visser, J.W.M. (1979). Structural identity of the pluripotent hemopoietic stem cell. Blood Cells 5: 143-159.
Van Bekkum, D.W., Visser, J.W.M., Castagnola, C., Boersma, W.J.A., Bol, S.J.L., Van den Engh, G.J. and Wagemaker, G. (1981). Properties of purified stem cells. In: Haemopoietic stem cells (eds.: Killman, S.A., Cronkite, E.P., Muller-Berat, C.N.), Alfred Benzon Symposium 18, Munksgaard, Copenhagen, p. 17-28.
Van den Engh, G.J. (1975). Quantitative in vitro studies on stimulations of murine hemopoietic cells by colony stimulating factor. Cell Tissue Kinet. 8: 537-548.
Van den Engh, G.J. (1976). Early events in haemopoietic cell differentiation. Thesis, State University, Leiden.
Van den Engh, G.J. and Golub, E.A. (1974). Antigenic differences between hemopoietic stem cells and myeloid progenitors. J. exp. Med. 139: 1621-1627.
Van den Engh, G.J. and Visser, J.W.M. (1979). Light scattering properties of pluripotent and committed haemopoietic stem cells. Acta Heamat. 62: 289-298.
Van den Engh, G. and Visser, J. (1984). B. Flow cytometry in experimental hematology. Biblthca. Haemat. 48, Karger, Basel, pp. 42-62.
Van den Engh, G.J., Visser, J.W.M. and Trask, B.J. (1979). Identification of CFU-S by scatter measurements on a light activated cell sorter. In: Experimental Hematology Today (eds.: Baum, S.J. and Ledney, G.D.), Springer Verlag, New York, Heidelberg, Berlin, p. 19.
Van den Engh, G.J., Trask, B.J. and Visser, J.W.M. (1981). Surface antigens of pluripotent and committed haemopoietic stem cells. In: Haematology and Blood Transfusion 26. Modern Trends in Human Leukemia. IV. (eds. R. Neth, R.C. Gallo, Graf, Mannweiler and Winkler). Springer Verlag, Berling, Heidelberg. pp. 305-308.
Van den Engh, G.J., Visser, J.W.M., Bol, S.J.L. and Trask, B.J. (1980). Concentration of hemopoietic stem cells using a light-activated cell sorter. Blood Cells 6: 609-623.
Van den Engh, G.J., Bauman, J., Mulder, D. and Visser, J. (1983). Measurements of antigen expression of hemopoietic stem cells and progenitor cells by fluorescence activated cell sorting. In: Haemopoietic Stem Cells. (eds.: Killman, S.-A., Cronkite, E.P. and Muller-Berat, C.N.), Munksgaard, Copenhagen, pp. 59-74.
Van Ness, J., Laemmli, U.K. and Pettijohn, D.E. (1984). Immunization in vitro and production of monoclonal antibodies specific to insoluble and weakly immunogenic proteins. Proc. Natl. Acad. Sci. U.S.A. 81: 7897-7901.
Van Snick, J., De Plaen, E. and Boon, T. (1985). A neomycin-resistant cell line for improved production of monoclonal antibodies to cell surface antigens. Eur. J. Immunol. 15: 1148-1151.
Van Zant, G. (1984). Studies of hematopoietic stem cells spared by 5-fluorouracil. J. Exp. Med. 159: 679-690.
Visser, J.W.M. and Bol, S.J.L. (1981). A two-step procedure for obtaining 80-fold enriched suspensions of murine pluripotent hemopoietic stem cells. Stem Cells 1: 240-249.
Visser, J.W.M. and Eliason, J.F. (1983). In vivo studies on the regulation kinetics of enriched populations of haemopoietic spleen colony-forming cells from normal bone marrow. Cell Tissue Kinet. 16: 385-392.
Visser, J.W.M. and De Vries, P. (1988). Isolation of spleen-colony forming cells (CFU-S) using wheat germ agglutinin and rhodamine 123 labelling. Blood Cells, in press.

Visser, J.W.M., Haaijman, J. and Trask, B. (1978). Quantitative immunofluorescence in flow cytometry. In: Immunofluorescence and related staining techniques. (eds.: Knapp, W. et al.). Elsevier/North Holland, Amsterdam, pp. 147-160.

Visser, J.W.M., Bol, S.J.L. and Van den Engh, G.J. (1981). Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting. Exp. Hematol. 9: 644-655.

Visser, J.W.M., Bauman, J.G.J., Mulder, A.H., Eliason, J.F. and De Leeuw, A.M. (1984). Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med. 59: 1576-1590.

Vos, O., Davids, J.A.G., Weijzen, W.W.H. and Van Bekkum, D.W. (1956). Evidence for the cellular hypothesis in radiation protection by bone marrow cells. Acta Physiol. Pharmacol. Neerl. 4: 482-486.

Wagemaker, G. (1980). Early erythropoietin-independent stage of in vitro erythropoiesis: relevance to stem cell differentiation. In: Experimental Hematology Today (eds. Baum, S.J., Ledney, G.D. and Van Bekkum, D.W.). Springer Verlag, New York, pp. 47-60.

Wagemaker, G. and Visser, T.P. (1980). Erythropoietin-independent regeneration of erythroid progenitor cells following multiple injections of hydroxyurea. Cell Tissue Kinet. 13: 505-517.

Walker, F. and Burgess, A.W. (1985). Specific binding of radionated granulocyte-macrophage colony-stimulating factor to hemopoietic cells. EMBO Journal 4: 933-939.

Walker, F., Nicola, N.A., Metcalf, D. and Burgess, A.W. (1985). Hieradrical downmodulation of hemopoietic growth factor receptors. Cell 43: 269-276.

Wang, E. and Krüger, J.G. (1985). Application of a unique monoclonal antibody as a marker for nonproliferating subpopulations of cells of some tissue. J. Histochem. Cytochem. 33: 587-594.

Ware, C.F., Reade, J.L. and Der, L.C. (1984). A rat anti-mouse kappa chain specific monoclonal antibody, 187.1.10: Purification, immunochemical properties and its utility as a general second-antibody reagent. J. Immunol. Meth. 74: 93-104.

Warren, D.J. and Moore, M.A.S. (1988). Synergism among interleukin 1, interleukin 3, and interleukin 5 in the production of eosinophils from primitive hemopoietic stem cells. J. Immunol. 140: 94-99.

Warren, M.K. and Ralph, P. (1986). Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor and colony stimulating activity. J. Immunol. 137: 2281-2285.

Watt, S.M., Gilmore, D.J., Metcalf, D., Cobbold, S.P., Hoang, T.K. and Waldmann, H. (1983). Segregation of mouse hemopoietic progenitor cells using the monoclonal antibody YBM/42. J. Cell. Physiol. 115: 37-45.

Watt, S.M., Gilmore, D.J., Clark, M.R., Davis, J.M., Swirsky, D.M. and Waldman, H. (1984). Haemopoietic progenitor cell heterogeneity revealed by a single monoclonal antibody, YW 13-1.1. Mol. Biol. Med. 2: 351-368.

Watt, S.M., Gilmore, D.J., Davis, J.M., Clark, M.R. and Waldmann, H. (1987). Cell surface markers on murine haemopoietic precursors. Reagents for the isolation and analysis of progenitor cell subpopulations. In Press.

Weiss, M.C. and Green, H. (1967). Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. Proc. Natl. Acad. Sci. U.S.A. 58: 1104-1111.

Westerwoudt, R.J. (1985). Review article: Improved fusion methods. IV. Technical aspects. J. Immunol. Meth. 77: 181-196.

Whetton, A.D. and Dexter, T.M. (1986). Haemopoietic growth factors. TIBS 11: 207-211.
Whetton, A.D., Bazill, G.W. and Dexter, T.M. (1984). Haemopoietic cell growth factor mediates cell survival via its action on glucose transport. EMBO J. 3: 409-413.
Wickmann, H.E. and Loeffler, M. (1983). Probability of self-renewal: Assumptions and limitations. Blood Cells 9: 475-483.
Williams, A.F., Galfre, G. and Milstein, C. (1979). Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. Cell 12: 663-673.
Williams, D.E., Lu, L. and Broxmeijer, H.E. (1987b). Characterization of hematopoietic stem and progenitor cells. Immunol. Res. 6: 294-304.
Williams, D.E., Boswell, H.S., Floyd, A.D. and Broxmeyer, H.E. (1985). Pluripotential hematopoietic stem cells in post-5-fluorouracil murine bone marrow express the Thy-1 antigen. J. Immunol. 135, 1004-1011.
Williams, D.A., Lemischka, I.R., Nathan, D.G. and Milligan, R.C. (1984). Introduction of new genetic material into pluripotent haemopoietic stem cells of the mouse. Nature 310: 476-480.
Williams, D.E., Straneva, J.E., Cooper, S., Shadduck, R.K., Wakeed, A., Gilles, S., Urdal, D., Broxmeijer, H.E. (1987a). Interactions between purified murine colony-stimulating factors (natural CSF-1, recombinant GM-CSF, and recombinant IL-3) on the in vitro proliferation of purified murine granulocyte-macrophage progenitor cells. Exp. Hematol. 15: 1007-1012.
Wognum, A.W., Thomas, T.E. and Lansdorp, P.M. (1987). Use of tetrameric antibody complexes to stain cells for flow cytometry. Cytometry 8: 366-371.
Wojchowski, D.M. and Sytkowski, A.J. (1986). Hybridoma production by simplified avidin mediated electrofusion. J. Immunol. Methods 90: 173-177.
Wolf, N.S. and Trentin, J.J. (1968). Hemopoietic colony studies. V. Effect of hemopoietic organ stroma on differentiation of pluripotent stem cells. J. exp. Med. 127: 205-214.
Wolf, N.S. and Priestly, G.V. (1984). The source and potential of late appearing spleen colonies. Abstract. Exp. Hematol. 12: 361.
Wolf, N.S. and Priestley, G.V. (1986). Kinetics of early and late spleen colony development. Exp. Hematol. 14: 676-682.
Worton, R.G., McCulloch, E.A. and Till, J.E. (1969a). Physical separation of hemopoietic stem cells from cells forming colonies in culture. J. Cell Physiol. 74: 171-182.
Worton, R.G., McCulloch, E.A. and Till, J.E. (1969b). Physical separation of hemopoietic stem cells differing in their capacity for self renewal. J. Exp. Med. 130: 91-103.
Wu, A.M., Till, J.E. and McCulloch, E.A. (1967). A cytological study on the capacity for differentiation of normal haemopoietic colony forming cells. J. Cell. Physiol. 69: 177-184.
Wu, C.T. and Liu, M.P. (1984). Characteristics of proliferation and differentiation of spleen colony-forming cells from bone marrow. Int. J. Cell Cloning 2: 69-80.
Wu, A.M., Siminovitch, L., Till, J.E. and McCulloch, E.A. (1968). Evidence for a relationship between mouse hemopoietic stem cells and cells forming colonies in culture. Proc. Natl. Acad. Sci. U.S.A. 59: 1208-1215.
Yelton, D.E., Diamond, B.A., Kwan, S-P., Scharff, M.D. (1978). Fusion of mouse myeloma and spleen cells. Curr. Topics Microb. Immunol. 81: 1-7.
Yoffey, J.M. (1973). In: Haemopoietic Stem Cells. Elsevier/North Holland, Excerpta Medica, Amsterdam, pp. 5-39.

CURRICULUM VITAE

Peter de Vries werd op 4 november 1957 in Vlaardingen geboren. Na het behalen van het middelbare school-diploma (Atheneum-B) in 1976 studeerde hij van 1976 tot 1977 scheikundige technologie aan de Technische Hogeschool Delft. Vanaf 1977 tot 1983 studeerde hij biologie aan de Rijksuniversiteit van Leiden. Het kandidaatsexamen werd in 1980 afgelegd met als 2e hoofdvak scheikunde (B4). In 1983 werd het doctoraal examen behaald met als bijvakken bacteriële genetica en immunoparasitologie en als hoofdvak immunologie.

Van september 1983 tot januari 1984 was hij als tijdelijk wetenschappelijk medewerker werkzaam in de Dr Daniel den Hoed Kliniek in Rotterdam (Directeur: Dr. B. Löwenberg) in het kader van een door het Koningin Wilhelmina Fonds gesubsidieerd onderzoeksproject. In mei 1984 werd hij aangesteld als onderzoeksassistent op de afdeling Experimentele Hematologie en Flowcytometrie van het Radiobiologisch Instituut TNO te Rijswijk (Directeur: Prof.dr. D.W. van Bekkum) op een door de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek gefinancieerd projekt. Tot maart 1988 werkte hij hier onder supervisie van Dr. J.G.J. Bauman, Dr. G.J. van den Engh en Dr. J.W.M. Visser aan het in dit proefschrift beschreven onderzoek.

Sinds 1 april 1988 is hij werkzaam als postdoctoral fellow in het Terry Fox Laboratory in Vancouver, Canada onder leiding van Dr. C.J. Eaves.