THE RECOGNITION OF EARLY DEVELOPMENTAL STAGES IN HAEMOPOIESIS

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GEBOREN TE 's-GRAVENHAGE

W.D. MEINEMA B.V. - DELFT

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PROF.DR. R. VAN FURTH

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Thoughts are as many-colored and swift-changing as the sunset. Words, by contrast, are pedestrian and limited in number.

(Webster Encyclopedic Dictionary)

Aan Jessica en Mark
voor hun eindeloos geduld.

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE STUDY OF CELLULAR DIFFERENTIATION PROCESSES WHICH OCCUR DURING ADULT LIFE

Almost all tissues of multicellular organisms contain cells which have the capacity to change their proliferative activity according to the demand. Some tissues show little or no cellular turnover under normal steady state conditions, but they can switch to a regeneration process in response to perturbation (e.g., mechanical injury). In other tissues, there is continuous cell production to compensate for cell loss due to the continuous utilization of functional cells even under normal conditions. Variation in demand is met by variation in the rate of cell production. The cells which generate offspring throughout life in the continuously renewing tissues are usually designated as stem cells. Stem cells are capable of extensive proliferation which results in new stem cells as well as differentiating cells. The most extensively studied stem cell systems in vertebrates are those of the epidermis, the intestinal epithelium, the testis and the haemopoietic tissues. These systems are commonly used for investigations on the mechanisms of cellular differentiation. In comparison to differentiation processes during embryogenesis, the organization of the stem cell systems in the adult is relatively simple. In adulthood, differentiation is restricted to one or to a limited number of cell types, while, in embryogenesis, differentiation into a large variety of tissues takes place.

Investigations on cell differentiation can be divided into three phases. Firstly, it is necessary to recognize cells in different stages of development. The second phase is the characterization of factors which regulate proliferation and differentiation. Thirdly, the mechanism of action of these factors can be investigated. At first glance, the recognition of various developmental stages would seem to be rather simple. Cells in the final stages of the differentiation process are present in large numbers and sequential cell types can often be recognized on the basis of morphological and/or functional criteria. However, the more important early differentiation events take

place at the stem cell level. The cells in early stages of development are few in number and mostly lack striking morphological characteristics, which prevents simple recognition. This explains why, in studies of early differentiation events, much time is spent on the detection of sequential cell types. The distinction between cell types in sequential stages of development will allow the study of growth regulating factors. If specific developmental stages of cells can be purified, the possibilities of studying the action of the regulating factors will be greatly increased.

1.2 HAEMOPOIETIC CELL DIFFERENTIATION

Most data on haemopoiesis in mammals are derived from experiments with mice. In the adult mouse, the bone marrow is the main site of haemopoiesis. Differentiating and maturing cells leave the bone marrow to settle in various tissues or to exert their function in the peripheral blood circulation. The presence of primitive stem cells in the bone marrow can be deduced from the observation that mice which have been exposed to a lethal dose of whole body ionising radiation can be rescued by injection of nonirradiated bone marrow cells. All haematological functions can be restored by such a graft. By the use of antigenic, biochemical and chromosome markers, it has been shown that, in many cases, all blood cell types found in the reconstituted mice are of donor origin (Ford et al., 1956; Vos et al., 1956). These findings could only be explained by the existence of a common ancestor cell for all of the haemopoietic differentiation pathways. This ancestor cell has been designated as the pluripotent haemopoietic stem cell (HSC). The HSC are capable of self-renewal and the HSC population is maintained by this means (Figure 1). HSC also give rise to cells which are restricted in their development to a specific cell lineage. These cells have been termed committed progenitor cells. The transition of pluripotent stem cell to committed progenitor cell is regarded as the major differentiation step in haemopoiesis. The committed progenitor cells gradually lose their proliferative capacity as differentiation proceeds and eventually give rise to mature, functional end cells.

The regulation of the first differentiation steps from pluripotent HSC to committed progenitor cells has been the subject of many studies. A model in which differentiation is a stochastic event has been described (Till et al., 1964; Vogel et al., 1968). Each time that a HSC divides, there are fixed probabilities for self-renewal of the HSC and for differentiation. These probabilities are independent of

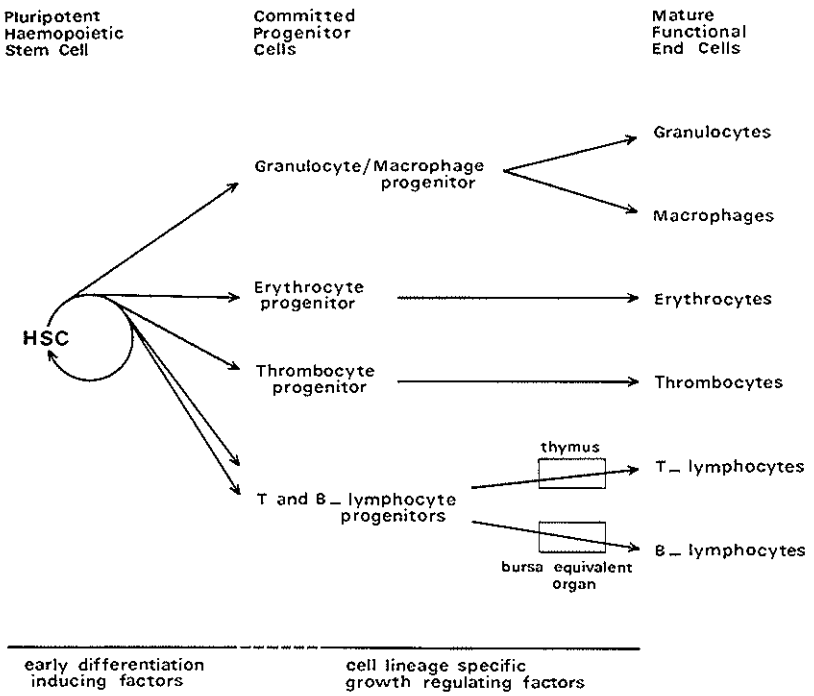


Figure 1:

Haemopoietic cell differentiation

the previous history of the HSC. The eventual direction of differentiation has been suggested to be determined by microenvironmental mechanisms (Curry and Trentin, 1967). Different "haemopoietic inductive microenvironments" are thought to govern the "decision" for differentiation into the various blood cell lineages. In addition to the regulation of differentiation, there is regulation of cell production. The need for specific end cells can vary enormously under different conditions and the cell production has to vary accordingly to meet the demands. A number of factors which regulate the growth of cells belonging to the various differentiation lineages have been identified (see below). Other factors influencing the proliferative activity of HSC and the earliest committed cells have also been discovered. A change in the proliferation rate of HSC will cause a change in the rate of differentiation events (either by stochastic processes or microenvironmental influences). Regulation of the proliferative activity of HSC may therefore represent an important link in the control of the production of mature end cells (Becker et al., 1965).

From the earliest committed progenitor cell onwards, there is a development of response to cell lineage specific growth regulating factors. The hormone erythropoietin induces certain erythrocyte progenitors to proliferate and to differentiate into mature, fully haemoglobinized erythrocytes. An analogue of erythropoietin for the granulocyte/macrophage line is the "colony stimulating factor". This term originates from the capacity of the factor to stimulate the *in vitro* formation of colonies containing granulocytes and/or macrophages. The term thrombopoietin is generally used for humoral factors stimulating the development of megakaryocytes and thrombocytes. The development of "T" and "B" lymphocytes depends largely on the hormonal and local action of thymus and "bursa equivalent" organs, respectively. The final formation of immune reactive cells (activated T cells and plasma cells) takes place after antigenic stimulation.

1.3 BASIC TOOLS FOR STUDYING HAEMOPOIESIS

The study of early haemopoietic differentiation is based on the detection of primitive cells with different biological properties. Although the primitive haemopoietic cells are few in number and cannot be recognized by conventional light microscopic methods, they can be detected indirectly by their high proliferative capacity. This capacity made possible the development of *in vivo* and *in vitro* cell cloning assays.

When lethally irradiated mice are injected intravenously with syngeneic bone marrow cells, the primitive haemopoietic cells will settle in the various haemopoietic organs and start to proliferate, thus giving rise to clones of cells. Eight to ten days after irradiation and bone marrow grafting, such clones can be observed macroscopically on the spleen, where they appear as distinct nodules (Figure 2). These nodules are generally designated as spleen colonies and the cells from which they are derived are termed *in vivo* colony forming cells (CFC). The colonies function as the centers from which the haemopoietic system is restored. Transplantation experiments in which unique chromosome markers were used showed that these markers were restricted to single colonies (Becker et al., 1963). Morphological analysis of the cells in the spleen colonies revealed that many colonies contain cells belonging to the various differentiation lines, although one cell type is predominant initially (Curry and Trentin, 1967; Wu et al., 1967). Generally, lymphoid cells could not be detected in the

Figure 2:

Assay for in vivo colony forming cells
Bone marrow cells are injected into lethally irradiated mice. After 8 to 9 days, colonies initiated by haemopoietic stem cells can be observed in the spleens of these mice. After fixation in Tellyesniczky's solution, the colonies appear as white nodules.
[The colonies on the spleens were formed after injection of 5×10^4 nontreated bone marrow cells (left) or 2×10^5 neuraminidase treated cells (right) (see Chapter 4)].

colonies, but chromosome marker studies indicated a common origin for lymphoid cells and cells in spleen colonies (Wu et al., 1968b). Furthermore, the presence of new "colony forming cells" within the spleen colonies could be demonstrated by injection of colony cells into secondary irradiated recipients (Lewis and Trobaugh, 1964; Gregory et al., 1973). These observations were taken as evidence that each colony was derived from one pluripotent haemopoietic stem cell.

The number of spleen colonies observed can be used as a measure for the number of stem cells present in the inoculum (Till and McCulloch, 1961). However, the number of colony forming cells (CFC) that lodge in the spleen represents only a small proportion of the total CFC population. This proportion is designated as the "spleen seeding efficiency" of CFC and can be determined by transplantation of spleen cells from the primary into secondary irradiated recipient mice. The CFC lodging in the spleen are operationally designated as colony forming units-spleen (CFU-s) and are thought to be representative for the total CFC population.

Certain early haemopoietic cells can proliferate and differentiate in vitro if appropriate culture conditions and stimuli are applied. When the culture medium is supplemented with a solidifying sub-

stance (e.g., agar or methylcellulose), the cells will become fixed in a matrix. This allows the formation of discrete cell clones (Figure 3). These in vitro colonies contain cells of one or two differentiation lines. Which differentiation line is observed depends on the type of stimulus added to the cultures. The addition of colony stimulating factor (CSF) leads to the formation of colonies consisting of neutrophilic granulocytes, macrophages or a mixture of these two cell types (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). The formation of mixed colonies indicates the existence of bipotential cells. The pure granulocyte and pure macrophage colonies can be derived from cells which are already committed to either granulocyte or macrophage development at the time of stimulation (Ichikawa et al., 1966; Van Furth and Diesselhoff-den Dulk, 1970; Goud, 1975). However, factors which control the differentiation of bipotential cells into granulocytes or macrophages have also been described (Horiuchi and Ichikawa, 1977; Williams et al., 1978; Johnson and Metcalf, 1978a; Bol and Williams. 1980). Colonies containing haemoglobinized reticulocytes are formed upon stimulation of bone marrow cell cultures with erythropoietin (Stephenson et al., 1971). Thrombopoietin is responsible for the formation of megakaryocyte colonies (Nakeff and Daniels-McQueen, 1976). On the basis of these data, the in vitro colonies are thought to be derived from committed progenitor cells. The in vitro growth of T- and B-cell colonies has also been reported (Metcalf, 1977). However, the

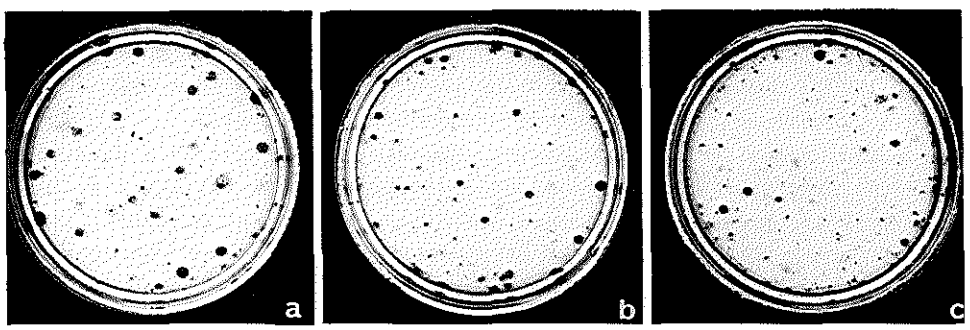


Figure 3:

Assay for in vitro colony forming cells
Bone marrow cells are suspended in medium supplemented with a solidifying substance and seeded into petri dishes. If appropriate stimuli are added, committed progenitor cells start to proliferate and differentiate and colonies can be observed after 7 days of incubation. The cells in the colonies were stained with a tetrazolium salt (see Section 2.8).
[The colonies are derived from GM CFU-c 1 (a), GM CFU-c 2 (b) and GM CFU-c 3 (c), which were separated on the basis of differences in buoyant density (see Section 1.4)].

cells giving rise to these colonies already show morphological and/or functional properties of relatively mature cells.

Because it is unknown in many cases whether all colony forming cells of a specific population are induced to proliferate and differentiate ("plating efficiency"), the cells giving rise to in vitro colonies are designated as colony forming units-culture (CFU-c). The commitment of the colony forming cells to develop into granulocytes/macrophages, erythrocytes and megakaryocytes is indicated by the letters GM, E and Meg, respectively.

In vivo and in vitro assay systems allow the further characterization of the primitive haemopoietic cells. Differentiation is expected to be reflected in cellular properties such as specific metabolic processes, intracellular structures and cell membrane composition. Therefore, a number of cell separation techniques by which cells can be characterized on the basis of their physical, biochemical and antigenic properties have been developed (for a review, see Shortman, 1972a). The cell separation procedures must be devised in such a way that the cells remain viable. Furthermore, the properties to be determined should not be changed by the separation procedure or they should at least be constant during the separation. Equilibrium density centrifugation and sedimentation at unit gravity are frequently employed cell separation techniques. By the first technique, cells are separated on the basis of differences in buoyant density (specific gravity). In the latter procedure, cells are separated on the basis of differences in sedimentation velocity, which is a function of both size and density. The biochemical processes which are reflected in cell buoyant density and sedimentation velocity can be formulated in only rather general terms. Changes in protein synthesis and the development of cell organelles during differentiation can change the cell buoyant density.Cell size changes when a cell traverses the cell cycle, while buoyant density remains constant (MacDonald and Miller, 1970), but changes in cell size may also occur during differentiation.

Other cell separation techniques allow a more specific approach to cell characterization. Especially techniques for characterization of the cell membrane have been shown to be useful for distinguishing different cell types. Cell electrophoresis by which cells are separated on the basis of differences in surface charge is applied to investigate the chemical composition of the cell membrane. Specific membrane molecules can be detected by use of appropriate reagents, e.g., specific enzymes or lectins (Mehrishi, 1972). The technique of fluorescence activated electronic cell sorting provides possibilities for

separating cells on the basis of differences in membrane, cytoplasmic and nuclear properties. Fluorescent labelled antibodies, lectins, RNA and DNA stains can be successfully applied with this technique (Herzenberg et al., 1976).

The clonogenic assays for primitive cells and the cell separation methods are the basic tools for recognizing various differentiation stages in haemopoiesis. Closely associated with the characterization of cells are the studies on the growth regulating factors. A great variety of preparations derived from tissues, sera and cell conditioned media is used to induce the proliferation and differentiation of different cell types. The use of the term "factor" for these growth stimulating preparations might cause confusion since only few of such substances are chemically characterized or available in pure form. Operationally, the term "factor" is used to indicate preparations which exert defined biological effects, e.g., colony formation by a specific cell type.

1.4 DISSECTION OF THE EARLY HAEMOPOIETIC CELL COMPARTMENT

The application of various humoral factors and cell separation techniques revealed the existence of distinct subpopulations within the CFU-s and CFU-c populations. In the bone marrow of normal mice, CFU-s are predominantly noncycling. When cells are exposed for a short time *in vitro* to S phase* specific cytotoxic drugs, those which are synthesizing DNA at that time will be killed. By this procedure, the number of CFU-s is reduced by 5% to 20%. Assuming that the phase of DNA synthesis (S phase) takes half of the cycle time (Vassort et al., 1973), these figures indicate that only 10% to 40% of all CFU-s are in active cell cycle and that 60% to 90% are noncycling (G_0 phase). Noncycling and cycling CFU-s have been physically characterized (Monette et al., 1974; Visser et al., 1977). This characterization was based on comparison between the physical properties of CFU-s from normal bone marrow in which noncycling CFU-s predominate and of CFU-s from regenerating bone marrow of mice recovering from radiation damage in which

*On the basis of DNA synthesis, the cell cycle can be divided into four phases. Mitosis (M) is followed by the G_1 phase (2n DNA). The period in which DNA synthesis takes place is known as S phase (2n to 4n DNA). The time interval preceeding M is designated as the G_2 phase (4n DNA).

all CFU-s can be expected to be in cycle (Becker et al., 1965). The CFU-s from these two bone marrow sources differ in buoyant density and sedimentation velocity (Table 1; Figure 4). Noncycling and cycling CFU-s are characterized by buoyant densities of 1.070 and 1.075 $g.cm^{-3}$ (Visser et al., 1977). Differences in sedimentation velocity between these two types of CFU-s could be largely explained by the differences in buoyant density. These data suggested that the CFU-s population contains a distinct G_0 phase which is characterized by specific physical properties.

When bone marrow cells are injected into lethally irradiated mice, the CFU-s from the graft are rapidly triggered into cycle (Lahiri and Van Putten, 1972). In vitro, the transition from quiescent CFU-s to cycling CFU-s can be induced by factors which are collectively termed "stem cell activating factors" or SAF. SAF can be found in media conditioned by human leukocytes, embryonic mouse fibroblasts, PHA or Con A stimulated mouse spleen cells and in serum from mice injected with bacterial endotoxin 18 hours earlier (Cerny, 1974; Löwenberg and Dicke, 1977; Wagemaker and Peters, 1978; Van den Engh and Bol, unpublished observations).

Primitive haemopoietic cells which are capable of giving rise to CFU-s have been recently described (Hodgson and Bradley, 1979). These cells are resistant to 5-fluorouracil treatment and preferentially home in the bone marrow of irradiated recipients. Furthermore, they seem to play a role in the production of megakaryocytes (Jones et al., 1980). Although the initial data suggest that this cell type represents a pre-CFU-s cell, its relationship to the other haemopoietic cells is not yet completely understood.

The in vitro granulocyte/macrophage colony forming cells (GM CFU-c) represent a heterogeneous population with respect to biological and physical properties (Worton et al., 1969b; Haskill et al., 1970; Sutherland et al., 1971; Messner et al., 1972; Metcalf and MacDonald, 1975a; Byrne et al., 1977; Williams and Eger, 1978). This heterogenous population can be divided into subpopulations of GM CFU-c which respond to different factors and which are distinguishable on the basis of physical properties.

Colony formation by GM CFU-c is induced by colony stimulating factor (CSF). CSF prepared from pregnant mouse uterus extract (CSF-pmue) shows a typical sigmoid dose-response relationship between the logarithm of the CSF concentration and the number of colonies formed and can be used as a reference CSF preparation (Van den Engh, 1974). The dose-response curve shows a clear plateau at about 100 colonies per

TABLE 1

PHYSICAL AND BIOLOGICAL PROPERTIES OF IN VIVO AND IN VITRO COLONY FORMING CELLS

cell type	modal buoyant density at pH 5.1, 4°C ($g.cm^{-3}$)	modal sedimentation velocity at pH 6.0 or 7,2; 4°C ($mm.h^{-1}$)	calculated[a] diameter (μm)	growth regulating factor	estimated incidence (cells per 10^5 nucleated bone marrow cells)
CFU-s resting (normal bone marrow)	1.070[b] 1.071[c] 1.067[d]	4.0[b] 4.0[c] 4.2[e]	7.2	SAF[q]	30-40[x]
CFU-s cycling (regenerating bone marrow)	1.075[b]	4.8[b] 5.3[e]	7.5		8-10
GM CFU-c 1	1.070[f]	4.3[f]	7.4	CEP[r] (18 h PES type)	30-50
GM CFU-c 2	1.075[f]	4.8[f]	7.5	CSF[s] (pmue type)	80-120
GM CFU-c 3	1.080[f,g]	5.3[f]	7.6	CSF + CEP[s] (LYS type)	100-200
E CFU-c 1 (d.9 BFU-E)	1.070[h]	4.0[i] 3.9[k] 3.8[l]	7.2	BPA[t]	25-80
E CFU-c 2 (d.3 BFU-E)	*	4.4[l]	*	BPA + EP	*
E CFU-c 3 (d.2 CFU-E)	1.075[m] 1.070 and 1.075[m] 1.077[h]	6.0 and 7.2[m] 5.5[i] 7.0; 6.1 and 7.1[l] 6.4[k]	9.1[p] 8.1	Ep[v]	300-500
Meg CFU-c	1.075[n]	4.2[o]	7.1	Tp[w]	20

a calculated by use of Stoke's law
b Visser et al., 1977.
c Worton et al., 1969b.
d Haskill et al., 1970
e Monette et al., 1974
f Bol et al., 1979
g Williams and Van den Engh, 1975
h Wagemaker, 1978
i Wagemaker et al., 1977
k Heath et al., 1976
l Gregory and Eaves, 1978
m Hasthorpe and Bol, 1979
n Nakeff, 1977
o Metcalf et al., 1975b
p assuming a sed. vel. of 7.0 $mm.h^{-1}$
q Löwenberg and Dicke, 1977
r Van den Engh and Bol, 1975
s Van den Engh, 1974
t Iscove, 1978
v Stephenson et al., 1971
w Nakeff and Daniels-McQueen, 1976
x Actual number of colony forming cells can be obtained by multiplication by a factor of 20 (spleen seeding efficiency of 5%).
* Data not available.

Abbreviations:

SAP - stem cell activating factor
CSF - colony stimulating factor
18 h PES - 18 h postendotoxin serum
CEF - colony enhancing factor
LYS - lysates of erythrocytes
BPA - burst promoting activity
Ep - erythropoietin
Tp - thrombopoietin

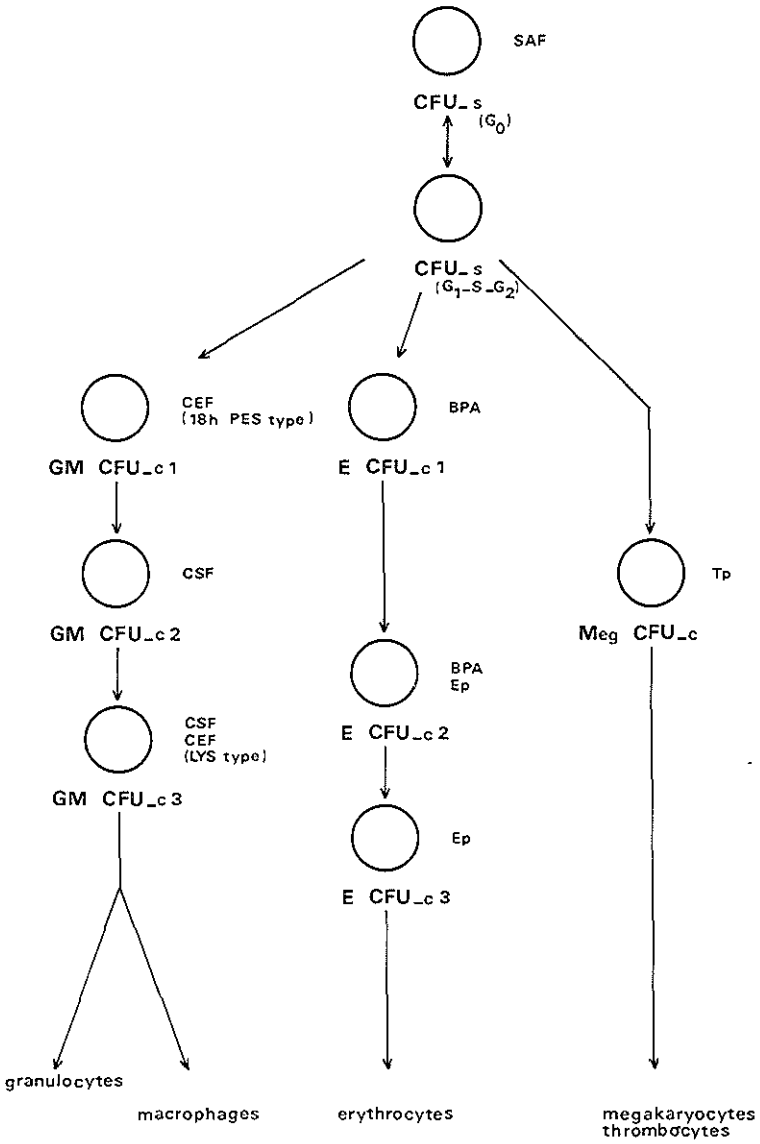


Figure 4:

Various differentiation stages in haemopoiesis detected by in vivo and in vitro cell cloning assays
For abbreviations: see legends of Table 1.

10^5 cells plated and this is reached at a CSF concentration of eight times the threshold concentration for colony formation. The plateau indicates that only a limited number of cells is induced to proliferate by CSF-pmue. However, the number of colonies can be increased above the number induced by CSF by addition of "colony enhancing factors" (CEF). These enhancing factors cannot themselves induce colony formation and their action is observed only when they are used in combination with CSF. Two types of colony enhancing factors can be distinguished. One is present in lysates of rat erythrocytes (LYS). The other is found in serum of mice which have been injected with endotoxin 18 h earlier (18 h postendotoxin serum; 18 h PES) (Van den Engh, 1974; Van den Engh and Bol, 1975).

Cell separation by equilibrium density centrifugation and by sedimentation at unit gravity revealed that the colonies formed in the presence of CSF-pmue alone and the additional colonies formed when the colony enhancing factors of 18 h PES and LYS are added to CSF-pmue stimulated cultures, are derived from three different types of GM CFU-c (Bol et al., 1979). The modal buoyant densities of the GM CFU-c responding to 18 h PES, CSF-pmue and LYS are 1.070, 1.075 and 1.080 $g.cm^{-3}$, respectively. The differences in sedimentation velocity among the three GM CFU-c are largely due to the differences in density, since the cells do not differ significantly in size as calculated by use of Stoke's law (Table 1). The heterogeneity among the GM CFU-c with respect to physical properties as reported by various authors (see references at the beginning of this paragraph) can be explained by the use of heterogeneous growth stimulating preparations which contain CSF-pmue type stimulators as well as colony enhancing factors of the 18 h PES and/or LYS types.

The three GM CFU-c types have been proposed to represent three consecutive stages in differentiation (Bol and Williams, 1980). This was based on the following observations. The GM CFU-c from all three density subpopulations are capable of forming colonies consisting of granulocytes, macrophages or a mixture of these two cell types. The cellular composition of the colonies changes with the culture period. Initially, most colonies are composed of immature blast cells. The percentage of these colonies decreases with time as the cells differentiate. Then, colonies of fully differentiated granulocytes and colonies containing a mixture of granulocytes and macrophages appear. Since granulocytes have a short life time, granulocyte colonies disintegrate and mixed colonies change into macrophage ones. The differentiation of cells and the disintegration of granulocytes is reflected in a steady increase in the percentage of macrophage colonies. This

order of changes in the cellular composition is observed for colonies derived from each of the three GM CFU-c types. The ratios among the various colony types are dependent on the source of growth regulating factors but are not correlated with the type of GM CFU-c. These observations indicate that the three GM CFU-c are not yet committed to granulocyte or macrophage differentiation (see also Metcalf, 1974). Although the GM CFU-c of the three subpopulations are similar in their differentiation potential, the time course of appearance and disappearance of the various colony types depends markedly on the type of GM CFU-c involved. Differentiation into fully mature cells is first observed in colonies derived from high density GM CFU-c (1.080 $g.cm^{-3}$) and last in colonies formed by GM CFU-c of low density (1.070 $g.cm^{-3}$). In addition, differences in colony size are observed; low density GM CFU-c give rise to large colonies, whereas high density GM CFU-c generate small colonies. From these data, it has been concluded that the three GM CFU-c represent three sequential differentiation stages (Bol and Williams, 1980). The low density GM CFU-c (1.070 $g.cm^{-3}$) can be considered as the most primitive cell, followed by the GM CFU-c of intermediate density (1.075 $g.cm^{-3}$), while the high density GM CFU-c (1.080 $g.cm^{-3}$) represents the most differentiated cell in this series. The GM CFU-c are numbered accordingly to this sequence (Figure 4; Table 1). GM CFU-c 1 is induced to proliferate by 18 h PES type factors (Wagemaker and Peters, 1978). Its progeny is sensitive to CSF-pmue type stimuli and colony development is dependent on these kinds of stimulators. GM CFU-c 2 responds directly to CSF-pmue. LYS type factors are suggested to induce extra divisions of GM CFU-c 3. In addition to LYS, GM CFU-c 3 requires CSF-pmue for colony formation.

A new in vitro colony forming cell with a very high proliferation capacity was recently described (Bradley and Hodgson, 1979). These cells give rise to large macrophage colonies under the culture conditions used and are probably slowly cycling cells, since they are not severely depleted by 5-fluorouracil treatment. Thus far, data on the relationship of this primitive cell type to the other haemopoietic cells are limited.

Three distinct populations of in vitro colony forming cells have also been described in the erythroid differentiation line (Gregory, 1976; Gregory and Eaves, 1978). The colonies derived from the three cells are different in size and show different maturation rates. The detection of the three cell types is simplified by the short life span of the mature end cells in culture; colonies with hemoglobinized cells appear and disappear in sequence (Gregory, 1976). The erythrocyte colony forming cells have been designated as day 8 BFU-E, day 3 BFU-E and

day 2 CFU-E. BFU stands for burst-forming-unit, because the colonies which they give rise to are composed of many small clusters. The days indicate the time of culture at which the colonies derived from the various progenitor cells are fully matured. In Figure 4, the three sequential developmental stages of the erythrocyte colony forming cells are indicated by E CFU-c 1, 2 and 3 in analogy to the terminology used for the granulocyte/macrophage progenitor cells. The earliest cell in the erythroid series, the E CFU-c 1, is induced to proliferate by a factor designated as burst promoting activity or BPA. The further development into colonies (involving later differentiation stages) is dependent on erythropoietin (Ep). E CFU-c 2 represents an intermediate stage with respect to the responsiveness to BPA and Ep. E CFU-c 3 responds directly to Ep (Iscove and Guilbert, 1978).

Physical characterization of the three E CFU-c types showed them to be different in buoyant density and sedimentation velocity (Table 1). The E CFU-c 1 is characterized by a modal buoyant density of 1.070 $g.cm^{-3}$ and a sedimentation velocity of about 4.0 $mm.h^{-1}$. The density of E CFU-c 2 is unknown, but the sedimentation velocity seems to be slightly higher than that of E CFU-c 1. E CFU-c 3 have a modal density of about 1.075 $g.cm^{-3}$ but sometimes an additional population with a modal density of 1.070 $g.cm^{-3}$ is observed. For the sedimentation velocity of E CFU-c 3, values of between 5.5 and 7.0 $mm.h^{-1}$ have been reported. In the sedimentation velocity distribution of E CFU-c 3 also two peaks can be present. The size of E CFU-c 3 appears to be significantly larger than that of E CFU-c 1. The differentiation within the GM and E lineages show similar events. In both lineages, it is accompanied by an increase in the buoyant density of cells and by the development of responsiveness to the lineage specific factors CSF and Ep, respectively.

Culture systems which allow the detection of megakaryocyte (thrombocyte) colony forming cells (Meg CFU-c) have also been developed. Up to now, no subpopulations within this differentiation lineage have been reported. The physical characterization shows Meg CFU-c to have a modal buoyant density of 1.075 $g.cm^{-3}$ and a modal sedimentation velocity of 4.2 $mm.h^{-1}$ (Table 1) (Nakeff, 1977; Metcalf et al., 1975b). The density distribution of the Meg CFU-c is rather broad and this suggests heterogeneity within this cell population as well.

During the past ten years, many investigators have reported on physical and biological differences between CFU-s and CFU-c (mainly GM CFU-c). The CFU s and CFU-c populations were found to differ with respect to buoyant density and sedimentation velocity distributions,

capacity to adhere to glass beads, cell surface antigens and cycling status. It was also observed that CFU-s were capable of giving rise to CFU-c *in vivo* as well as *in vitro* (Worton et al., 1969a,b; Haskill et al., 1970; Metcalf et al., 1971b; Van den Engh et al., 1978; Iscove et al., 1970; Rickard et al., 1970; Sutherland et al., 1971; Wu et al., 1968a). However, in view of the types of preparations used to stimulate colony formation by CFU-c and the physical properties of CFU-c, it is quite probable that the reported differences between CFU-s and CFU-c were based upon comparisons between CFU-s and CFU-c 2 or at least a mixture of CFU-c 1/CFU-c 2. Comparison among CFU-s, GM CFU-c 1 and E CFU-c 1 with respect to physical properties and their responsiveness to certain growth inducing factors suggests that these cell types have many similarities (Table 1). CFU-s, GM CFU-c 1 and E CFU-c 1 are characterized by a low buoyant density and are similar in size. The factors which induce these cells to proliferate and differentiate show only small differences in biochemical properties and are distinct from the differentiation lineage specific factors CSF and Ep (Van Bekkum et al., 1979). In addition, CFU-s, GM CFU-c 1 and E CFU-c 1 show only a small reduction in number after *in vitro* incubation with ^{3}H-thymidine, which suggests that they are similar with respect to proliferative activity (Gregory and Eaves, 1978; Visser, personal communication). On the basis of these data, it is suggested that CFU-s and CFU-c 1 represent closely linked differentiation stages and that some of the cells which form spleen colonies (CFU-s) are able to form colonies in culture (CFU-c 1) and vice versa.

1.5 OUTLINE OF THIS THESIS

The studies described in this thesis are aimed at a more complete description of CFU-s and CFU-c. With respect to CFU-c, the studies are restricted to the granulocyte/macrophage colony forming cells: GM CFU-c 1, 2 and 3. Further analysis of the properties of CFU in sequential stages of development will contribute to the understanding of the changes which occur during early differentiation. Since the three GM CFU-c types were shown to represent a differentiation series, they can be expected to be different in many properties. Useful criteria for further characterization of these cells may be found in the presence of specific membrane components. The study of the membrane properties may also elucidate the relationship between CFU-s and GM CFU-c 1. In the lymphoid cell lineage many subpopulations can be distinguished on the basis of specific cell surface antigens. In the

myeloid series, CFU-s and GM CFU-c 2 have already been shown to differ with respect to the H2-KD antigen density on their surface (Van den Engh et al., 1978). The investigations described in this study deal with the chemical composition of the cell membrane. Free-flow cell electrophoresis by which cells are separated on the basis of differences in surface charge is used as the cell separation technique. Neuraminic acids (sialic acids) are generally occurring terminal groups of membrane glycoproteins and glycolipids which are often involved in specific cell functions. The enzymes termed neuraminidases with different substrate specificities (Gottschalk, 1972) can be used to remove neuraminic acids without affecting the underlying structures. Since neuraminic acids are negatively charged, they contribute to the cell surface charge and removal of these molecules will be reflected in a change in the cell electrophoretic mobility. In this way, the presence of neuraminic acids on the cell membrane can be detected.

Part of this study is directed to the enrichment for CFU-s. Highly purified CFU-s preparations can be used not only to identify the morphology of these cells (van Bekkum et al., 1971) but also for in vitro differentiation studies starting with single cells. The availability of purified CFU-s preparations will also extend the possibilities for analysing the chemical and antigenic properties of this cell stage, since it will not be necessary to retain cell viability. It was investigated whether CFU-s could be enriched in concentration by combining cell electrophoresis, buoyant density separation, neuraminidase treatment, supravital DNA staining and light activated cell sorting.

In summary, the studies described in this thesis concern cell surface properties of CFU-s and GM CFU-c 1, 2 and 3 in order to a) investigate whether CFU-s and GM CFU-c 1 can be distinguished; b) determine changes occurring during differentiation from CFU-s through GM CFU-c 3; and c) make use of these properties in a procedure for the purification of CFU-s.

The experimental approach to the cell characterization studies is described in Chapter 2. After a description of the handling of bone marrow cells and the assay systems for CFU-s and GM CFU-c 1,2 and 3, cell separation by free-flow electrophoresis is discussed. This technique was the major one used in this study. Introductory experiments on the resolution and reproducibility of the cell electrophoresis technique and on the effect of neuraminidase treatment on the electrophoretic mobility of cells are reported. Separation of cells by equilibrium density centrifugation, sedimentation at unit gravity and by use of a fluorescence and scattered light-activated cell sorter are briefly considered.

Chapter 3 presents the electrophoretic mobility properties of CFU-s and GM CFU-c 1,2 and 3. It appears that CFU-s can be distinguished from GM CFU-c 1 and this suggests that GM CFU-c 1 is an intermediate stage between CFU-s and GM CFU-c 2. The presence of neuraminic acid groups on the cell surface was investigated by combination of cell electrophoresis and treatment of cells with the enzyme neuraminidase from Vibrio cholerae. Comparison of the electrophoretic mobility of CFU-s and GM CFU-c before and after treatment with neuraminidase indicates that the density of neuraminidase susceptible neuraminic acid groups on the cell surface decreases as differentiation proceeds. The electrophoretic behaviour of resting and proliferating CFU-s was also investigated. It is shown that, despite differences in electrophoretic mobility between CFU-s from normal and regenerating bone marrow, there are no indications for differences in mobility between resting and proliferating CFU-s under normal steady state conditions.

The detection of differences between CFU-s and GM CFU-c 1 with respect to their electrophoretic mobility strongly supports the view that these cell types represent different entities. However, neuraminidase treatment, which is applied in combination with cell electrophoresis for the detection of neuraminic acid groups, appears to reduce the spleen seeding efficiency of in vivo colony forming cells. As a result, the CFU-s present after neuraminidase treatment which were compared with GM CFU-c 1 represent only a proportion of the total CFU-s population and the interpretation of the results depends on whether CFU-s represents a homogeneous population. Therefore, Chapter 4 deals with a search for heterogeneity among CFU-s. It was investigated whether the colony forming cells which are still detectable after neuraminidase treatment are different from the nontreated colony forming cells with respect to self-renewal, capacity to prevent mortality in lethally irradiated mice, electrophoretic mobility, buoyant density and sedimentation velocity properties. No essential heterogeneity can be detected among CFU-s in terms of these parameters.

Cell separation procedures can be used not only for cell characterization but also to enrich for specific cell types. Cell separation on the basis of a single parameter does not result in high concentrations of CFU-s. The enrichment for CFU-s by combining several cell separation procedures is described in Chapter 5. It is demonstrated that a portion of the CFU-s population can be obtained in a high concentration by isolation of cells on the basis of a number of partly independent parameters.

CHAPTER 2

MATERIALS, TECHNIQUES, METHODS AND INTRODUCTORY EXPERIMENTS

2.1 MICE

Specific pathogen free (C57BL/Rij x C3H)Pl mice were used throughout this study. The microflora of the mice consisted of a colonization resistant population of anaerobic, mouse-derived Clostridium species in combination with the bacteria Staphylococcus albus, Streptococcus faecalis and Enterobacter cloacae. There are indications that the composition of the microflora might be of importance for the progression of the haemopoietic processes: bacterial toxins have been shown to influence the production of factors (CSF, CEF) which stimulate granulopoiesis and the proliferative activity of CFU-s and CFU-c may be changed by infections (Metcalf, 1971).

Both male and female mice were used, but, within single experiments, there was a restriction to one sex. Mice varying in age from seven to nine weeks were used as bone marrow donors. Those which were to be irradiated varied in age from 12 to 15 weeks.

2.2 IRRADIATION OF MICE

Many experiments required the use of lethally irradiated mice (e.g., for the CFU-s assay and the provision of regenerating bone marrow). These mice were subjected to total body irradiation by gamma rays from a ^{137}Cs source at a dose rate of about 1 $Gy.min^{-1}$. Male and female mice were irradiated with 9.50 Gy and 10.25 Gy, respectively. Without bone marrow cell transplantation, the mice died between 15 to 25 days after irradiation. The radiation dose employed made possible rescue of the mice by injection of about 1×10^5 syngeneic nucleated bone marrow cells.

2.3 CELL SUSPENSIONS

Cell suspensions were prepared in Hanks' balanced salt solution buffered at pH 7.2 with 10 mM HEPES buffer (Merck) or in phosphate buffered saline of pH 7.2. The salt solutions had an osmolarity of 308 mOsm, which is equivalent to 0.168 M NaCl (Williams et al., 1972). Bone marrow cells were collected from the femurs by flushing the bone cavity with salt solution. A suspension of single cells was obtained by pipetting and filtration through a six-layer nylon sieve. Spleen cell suspensions were prepared by cutting spleens into pieces and pressing the tissue through a nylon sieve with a spatula while adding some salt solution. To prevent cellular changes as a result of metabolic processes, all cell suspensions were kept on melting ice until used. To minimize variation due to individual differences, at least three mice were used as donors of bone marrow or spleen cells.

2.4 VIABLE NUCLEATED CELL COUNTS

Viable nucleated cell counts were performed by pulse cytophotometry (BIO/Physics, cytofluorograph) using fluorescein diacetate (Calbiochem) and propidium iodide (Sigma Chem Comp) as fluorescent stains (Celada and Rotman, 1967; Jongeling and Visser, 1976). The staining solution contained 1.2 mg fluorescein diacetate and 10 mg propidium iodide per 100 ml Hanks' balanced salt solution. One-half millilitre of cell suspension with a maximum concentration of 1×10^6 cells.ml^{-1} was added to one-half millilitre of staining solution and the cells were allowed to stain for 30 min at room temperature. After this staining period, the cell suspensions were placed in melting ice. When fluorescein diacetate is taken up by the cells, it is hydrolysed and this results in accumulation of free fluorescein in viable cells. Cells with a damaged membrane will rapidly lose the fluorescein. Propidium iodide can enter only damaged cells and it stains the DNA of these cells. On excitation by blue laser light (488 nm), viable fluorescein containing cells are green fluorescent (530 nm) and dead cells in which the DNA is stained by propidium iodide are red fluorescent (590 nm), so that viable and dead cells can be counted simultaneously.

2.5 QUANTIFICATION OF CELLS IN THE S PHASE OF THE CELL CYCLE

The proportion of cells in the DNA synthesis phase of the cell cycle (S phase) can be used as a rough measure of the proliferative state of a specific cell population. To determine this proportion, cells are incubated with highly radioactive labelled thymidine (^{3}H-thymidine). After phosphorylation, cells in S phase will incorporate the radioactive thymidine into their DNA and will be subsequently killed. The decrease in cell survival after treatment with ^{3}H-thymidine in comparison to the survival of control-treated cells is a measure for the number of cells in S phase. Experimentally, 1 ml samples of cell suspensions in Hanks' balanced salt solution were incubated for 30 min at 37 ^{o}C with 15 μCi ^{3}H-thymidine of high specific activity (22 Ci.mM^{-1}). To avoid complications due to dilution, the maximum cell concentration used in this set-up was 5 x 10^6 cells.ml^{-1} (see also Iscove, 1970). After the incubation, the cells were washed once with a solution of "cold" thymidine (0.2 mg.ml^{-1}) and twice with Hanks' balanced salt solution. The additional washes with salt solution are essential for certain cell types, since high concentrations of thymidine can inhibit DNA synthesis (Hasthorpe and Harris, 1979). Control cell suspensions were treated identically, except for omission of the addition of ^{3}H-thymidine during the incubation.

Careful analysis of CFU survival curves after *in vitro* incubation with increasing concentrations of ^{3}H-thymidine revealed that no obvious plateau could be reached. Although S phase cells have a higher uptake rate of ^{3}H-thymidine and will be killed first, the reduction in number of cells after exposure to ^{3}H-thymidine may be partly due to the kill of cells in other phases of the cell cycle. Therefore, the absolute numbers of S phase cells obtained in ^{3}H-thymidine kill experiments should be considered with caution. Cytotoxic drugs such as arabinoside cytosine and hydroxyurea may be more suitable to specifically kill S phase cells, but were not used in this study.

2.6 REGENERATING BONE MARROW

In several experiments, bone marrow from mice recovering from radiation damage was used. Mice were lethally irradiated and reconstituted with 2.5 x 10^6 nucleated bone marrow cells from nontreated syngeneic donors. After seven days, the bone marrow of these mice contains a high percentage of rapidly proliferating CFU as determined by

the ^{3}H-thymidine kill technique. Table 2 shows the survival of CFU-s and GM CFU-c 2 from normal and regenerating bone marrow after incubation with ^{3}H-thymidine. CFU-s from normal and regenerating bone marrow show a mean surviving fraction of 83% and 43%, respectively. This indicates an increase in cell kill by a factor of about 3.5 when changing from steady state conditions to a regeneration process. For GM CFU-c 2 derived from normal and regenerating bone marrow, ^{3}H-thymidine surviving fractions of 43% and 23%, respectively, were observed, indicating an increase in cell kill by a factor of about 1.5.

2.7 ASSAY FOR SPLEEN COLONY FORMING CELLS: CFU-s

The number of spleen colony forming cells in various cell suspensions was determined by use of the assay system described by Till and McCulloch (1961). Appropriate numbers of cells suspended in 0.5 ml of salt solution were injected intravenously into lethally irradiated mice. Ten mice were injected per experimental group. After 8 to 9 days, the mice were killed and their spleens were removed. After fixation of the spleens in Tellyesniczky's solution, colonies appeared as white nodules and could be easily counted. The colony number did not change between days 8 and 9. The number of cells injected was chosen such that 15 to 20 colonies were formed per spleen. Using unfractionated bone marrow cells, 0.5×10^{5} cells from normal bone marrow or 2×10^{5} cells from regenerating bone marrow were injected. When the cells had been treated with neuraminidase, the cell doses were increased by a factor of 4 (see Section 2.12). After cell separation procedures, the cells from the collected fractions were injected in numbers corresponding to the expected incidence of CFU-s. In pilot experiments, two doses of each fraction were injected to determine the dose needed to obtain reliable colony counts.

The mean incidence of CFU-s in unfractionated normal bone marrow was found to be between 30 and 40 per 10^{5} viable nucleated cells. If a spleen seeding efficiency of 5% is applied (Lahiri et al., 1970), the incidence of colony forming cells (CFC; all cells capable of forming spleen colonies) is estimated to vary between 6 and 8 per 1000 cells (0.6% to 0.8%).

TABLE 2

SURVIVAL OF CFU-s AND GM CFU-c 2 FROM NORMAL AND REGENERATING BONE MARROW AFTER INCUBATION WITH ^{3}H-THYMIDINE
(DATA FROM VISSER, 1979)

cell type	number of experiments	treatment of cells	normal bone marrow		regenerating bone marrow	
			CFU/10^5 cells	percentage of control	CFU/10^5 cells	percentage of control
CFU-s	16	control	30	100	8	100
		^{3}H-thymidine	25	83 $\pm$ 8	4	43 $\pm$ 8
GM CFU-c 2	8	control	125	100	74	100
		^{3}H-thymidine	55	43 $\pm$ 10	17	23 $\pm$ 6

Figures represent mean $\pm$ standard deviation

2.8 ASSAY FOR GRANULOCYTE/MACROPHAGE COLONY FORMING CELLS: GM CFU-c 1,2 AND 3

The culture system for granulocyte/macrophage colony forming cells (GM CFU-c) has been described in essence by Metcalf and Moore (1971a). Cells were suspended in Dulbecco's medium containing 0.3% w/v agar (Bacto agar, Difco Lab) and 20% v/v of a mixture of 2 volumes of horse serum (noncommercial batch) and 1 volume of foetal bovine serum (Flow Lab). The Dulbecco's Modified Eagle Medium (Gibco Bio-Cult M 07-2501) was supplemented with 2 mg L-asparagine, 56 mg L-glutamine, 370 mg $NaHCO_3$, 7.5 mg DEAE-dextran, 10^4 IU Penicillin and 10 mg Streptomycin per 100 ml (single strength). One millilitre aliquots of this nutrient agar containing 0.5×10^5 nucleated cells were pipetted into 35 mm Petri dishes (Corning). Prior to plating, solutions of one or more types of growth regulating factor preparations were pipetted into the dishes. The dilution of the different preparations was chosen in such a way that the total volume added was 0.15 ml. Each experimental group was tested in triplicate. The cultures were incubated for 7 days at 37° C in an atmosphere of 10% CO_2 in air and 95% relative humidity (National incubator).

After 7 days 0.5 ml of a tetrazolium salt solution (1 mg INT.ml^{-1} Aldrich Chem Co) was added to the cultures and the incubation was continued for another 20 hours. Viable cells reduce the colourless tetrazolium salt to a dark red water-insoluble formozan which precipitates inside the cells. Consequently, cell colonies (cell aggregates of more than 50 cells) appear as red spots against a light background and can be easily counted without magnification (Figure 5) (Bol et al., 1977a). With this technique, the time required for enumeration of colonies is reduced to one-third of that needed for counting microscopically. Moreover, the possibility of comparing various cultures simultaneously contributes to maintaining constant counting criteria when many cultures have to be evaluated. If necessary, the cultures can be fixed with 0.5 ml of a 10% formaldehyde solution in saline, after which they can be stored in closed containers at 4° C for several months without the formozan colour fading or spreading.

The three sequential differentiation stages of GM CFU-c were detected by use of three types of growth regulating factors (Bol et al., 1979; Bol and Williams, 1980). Colony stimulating factor (CSF), which is essential for the final differentiation steps, must always be added to the cultures to promote colony formation by all three GM CFU-c. For this purpose, colony stimulating factor prepared from pregnant mouse uterus extract (CSF-pmue) was used throughout. GM CFU-c 1 do not re-

Figure 5:

Appearance of unstained (left) and INT stained (right) in vitro colonies

spond to CSF-pmue but can be induced to proliferate and differentiate by the use of colony enhancing factor in serum from mice injected with endotoxin 18 h earlier (18 h PES). GM CFU-c 2 directly respond to CSF-pmue and give rise to colonies without further additions. GM CFU-c 3 is assumed to respond to CSF-pmue, but the number of cell divisions is too low to result in colony formation. Addition of lysate of erythrocytes (LYS) to CSF-pmue stimulated cultures leads to expression of this late stage GM CFU-c. In summary, the number of GM CFU-c 2 is represented by the number of colonies in cultures stimulated by CSF-pmue alone. The numbers of GM CFU-c 1 and GM CFU-c 3 are obtained by subtracting the number of colonies present in CSF-pmue stimulated cultures from the colony number detected in cultures containing CSF-pmue + 18 h PES and CSF-pmue + LYS, respectively (Figure 6).

In unfractionated bone marrow, the mean incidences of GM CFU-c 1, GM CFU-c 2 and GM CFU-c 3 were found to be between 30-50, 100-120 and 80-150 per 10^5 nucleated cells, respectively. Since a plateau stimulation could not be reached with 18 h PES and LYS, the incidences of GM CFU-c 1 and GM CFU-c 3 are approximate values. The incidence of GM CFU-c 3 is probably underestimated since it would be expected that the more differentiated cells exceed the more primitive ones in number.

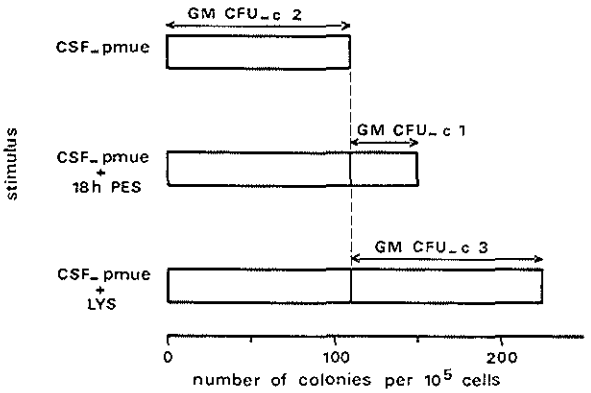


Figure 6:

Schematic representation of the method used to determine the numbers of the three GM CFU-c types

Colony stimulating factor from pregnant mouse uterus extract (CSF-pmue) was prepared as described by Bradley et al. (1971a) and Stanley et al. (1972). Homogenates of pregnant mouse uteri, including embryos, were treated with ammonium sulphate. The 50% to 100% saturation fraction was taken and dialysed against glass distilled water. The remaining soluble material was subjected to calcium phosphate gel adsorption and the adsorbed fraction was recovered by elution with 0.09 M phosphate buffer, pH 7.4. This fraction was dialysed against glass distilled water and incubated at 60° C for 1 hour. After centrifugation, the preparation was sterilized by membrane filtration (Millipore, 0.22 µm pore size) and 10 ml aliquots were stored at -20° C. The activity of the preparation is expressed in an arbitrary unit system proportional to the CSF concentration. One unit CSF.ml^{-1} represents the lowest concentration at which cells are stimulated to some proliferation, 5 units.ml^{-1} represent the threshold for colony formation (some cells form clones of more than 50 cells) and a plateau level is reached at 40 units.ml^{-1} (Van den Engh, 1974). All cultures were stimulated with a saturating CSF-pmue concentration of 160 units.ml^{-1}.

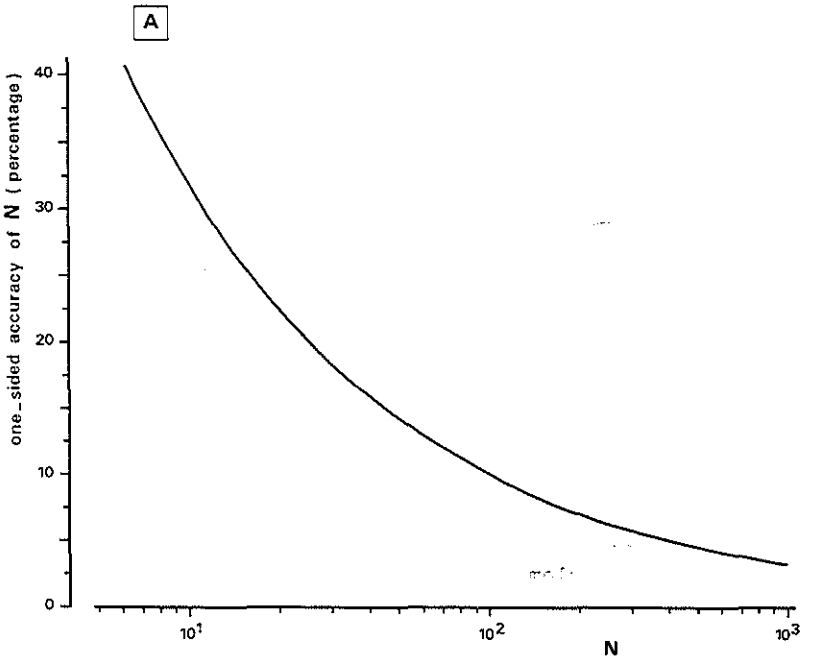
Eighteen-hour postendotoxin serum (18 h PES) was obtained from mice which had been injected intravenously with 25 µg Salmonella typhosa lipopolysaccharide (Difco Lab). The mice were bled from the orbital plexus and the blood was allowed to clot for 1 to 2 h at room

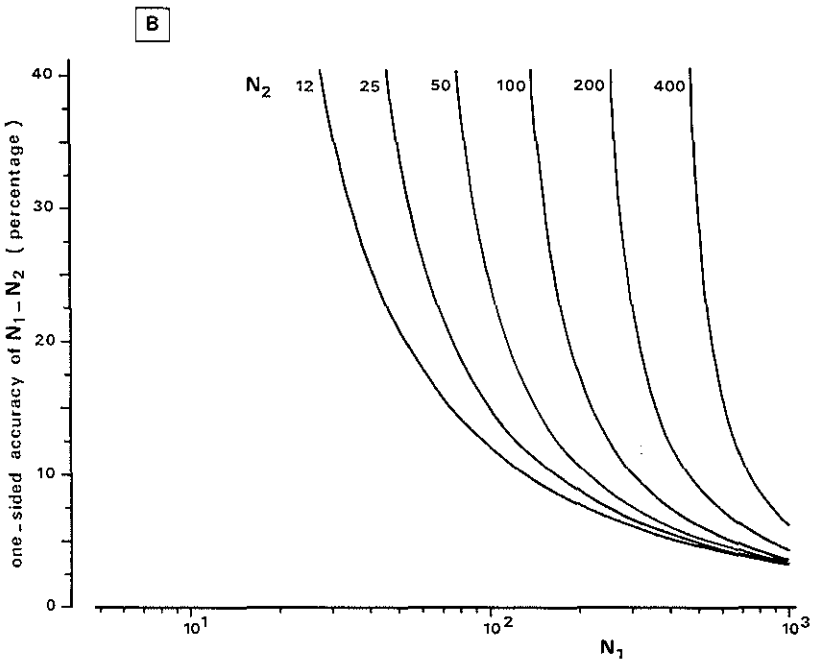
temperature. After centrifugation (800 g, 15 min), the serum was collected, sterilised by filtration through a 0.22 µm pore size membrane (Millipore) and stored at -20° C in aliquots of 1 ml. The cultures received 0.05 ml of a 1/2 to 1/6 serum dilution, depending on the ratio of inhibitors to colony enhancing factors, which varied with the batch. Corresponding dilutions of sera from untreated mice were used as controls (Van den Engh and Bol, 1975).

Erythrocyte lysate (LYS) was prepared from rat erythrocytes. Blood from rats was collected aseptically from the descending aorta into tubes containing 2 ml of a 3.1% sodium citrate solution sufficient for anticoagulation of 8 ml blood. The plasma and buffy coat were removed after centrifugation and the red cells were washed three times with a large volume of phosphate buffered saline (pH 7.2; free of Ca^{2+} and Mg^{2+} ions). The washed, packed erythrocytes were lysed by adding an equal volume of sterile glass distilled water (1 h, 4° C). After centrifugation (800 g, 15 min), the supernatant was collected. The cultures were supplemented with 0.1 ml of this erythrocyte lysate. It was used on the day of preparation (Bradley et al., 1971b; Bradley et al., 1973; Bertoncello and Bradley, 1977; Van den Engh, 1974).

2.9 THE ACCURACY OF CELL AND COLONY COUNTS

Within one experiment, the number of cells in equal volumes taken randomly from one cell suspension and transferred to a haemocytometer, culture dishes or animals is expected to follow a Poisson distribution. If technical errors are neglected, the accuracy of the cell and colony counts can therefore be expected to depend solely on the total number of cells or colonies counted (N). If N exceeds 10, the Poisson distribution approximates a normal distribution and the estimated standard deviation is given by $\sqrt{N}$. The accurary can be described by $N \pm \sqrt{N}$ (Blackett, 1974). If the accuracy is expressed in percent of the total count N, it can be applied to any value which is derived from N by a correction factor. Figure 7 A gives the accuracy of N in percentages. The number of cells counted in a haemocytometer or in the pulse cytofluorograph is usually around 200 and 5000, respectively, giving accuracies of $\pm$ 7% and $\pm$ 1.5%. In cultures of 5×10^4 unfractionated bone marrow cells stimulated by CSF-pmue, the number of colonies (representing GM CFU-c 2) varies around 50, which results in a total colony count of 150 in 3 cultures. The total number of spleen colonies (CFU-s) also varies around 150 (10 spleens with an average of 15 colo-


A
one-sided accuracy of N (percentage)
40
30
20
10
0
10^1
10^2
10^3
N


B
one-sided accuracy of N_1-N_2 (percentage)
40
30
20
10
0
N_2
12
25
50
100
200
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10^1
10^2
10^3
N_1

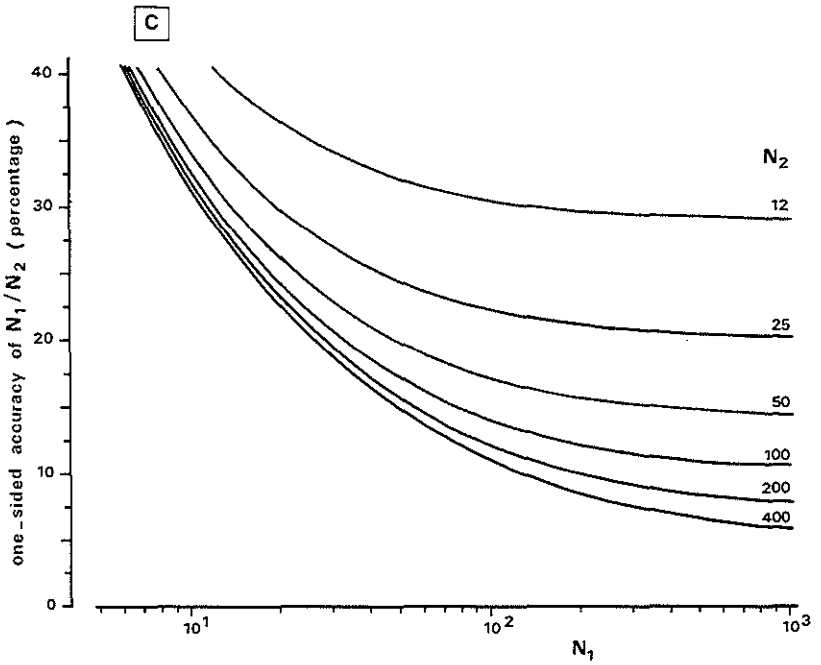

Figure 7:

Accuracy of cell and colony counts on the basis of Poisson statistics

A. Accuracy of a single count
The accuracy is given by $\sqrt{N}/N$

B. Accuracy of the subtraction of two counts
The accuracy is given by $\sqrt{(N_1 + N_2)}/(N_1 - N_2)$. The nomogram can be read for $N_1 > N_2$

C. Accuracy of the quotient of two counts
The accuracy is given by $\sqrt{(1/N_1) + (1/N_2)}$.

nies after injection of 5×10^4 cells). According to the nomogram of Figure 7 A these colony counts have an accuracy of ± 8%.

The enumeration of some cell types (e.g., GM CFU-c 1 and GM CFU-c 3) requires the subtraction of two colony counts (total counts of N_1 and N_2). The accuracy of the difference between two counts is determined by the absolute accuracy of each count. With the restriction that N_1 and N_2 are derived from equally sized samples (same number of cells tested per experimental group), the accuracy of (N_1-N_2) is given by $(N_1-N_2) \pm \sqrt{(\sqrt{N_1})^2 + (\sqrt{N_2})^2} = (N_1-N_2) \pm \sqrt{N_1 + N_2}$. Figure 7 B gives the accuracy of (N_1-N_2) for several values of N_1 and N_2. For example, let the total number of colonies detected in cultures of unfractionated bone marrow cells stimulated by CSF-pmue, CSF-pmue + 18 h PES and CSF-pmue + LYS be 150, 220 and 300, respectively. Then, the number of GM CFU-c 1 (net 18 h PES effect) = 70 ± 27% and the number of GM CFU-c 3 (net LYS effect) = 150 ± 14%.

If results are to be expressed as the ratio of two counts (e.g., the proportion of CFU-s which survives after ^{3}H-thymidine treatment), the accuracy of this ratio is dependent on the proportional accuracy of each count. Taking total counts of N_1 and N_2 from two experimental groups to be compared, the accuracy of (N_1/N_2) is given by

$$(N_1/N_2) \pm \sqrt{\left(\frac{\sqrt{N_1}}{N_1}\right)^2 + \left(\frac{\sqrt{N_2}}{N_2}\right)^2} = (N_1/N_2) \pm \sqrt{(1/N_1) + (1/N_2)}$$

The proportional accuracy of (N_1/N_2) for various values of N_1 and N_2 is shown in Figure 7 C. For example, let the total number of spleen colonies (CFU-s) after injection of nontreated and ^{3}H-thymidine-treated cells be 200 and 160, respectively. Then the proportion of CFU-s which survive the treatment is 0.8 ± 11%.

The examples of the accuracies of the various cell types described above are representative for experiments with unfractionated bone marrow. Cell separation will result in fractions which contain varying cell numbers and varying concentrations of particular cell types. The number of cells to be counted and the number of cells to be cultured or to be injected were chosen such that reliable cell and colony counts were obtained.

2.10 CELL SEPARATION BY FREE-FLOW ELECTROPHORESIS

Cells can be characterized on the basis of their surface charge by electrophoresis. At neutral pH values, most cells carry a net negative charge on their surface and consequently when they are placed in

an electric field they will move towards the anode. Cells with a different net surface charge show different velocities in the electric field and as a result these cells can be separated from each other. Generally, the electrophoretic mobility (EPM), defined as the velocity of a cell per unit field strength, is taken as a measure for the cell surface charge. The EPM of a cell depends not only on its net surface charge but also on the properties of the surrounding medium. Firstly, the viscosity of the medium will result in a retarding force according to Stoke's law. Secondly, more complex physical phenomena occur when the medium contains electrolytes (which are required to maintain cell viability) (Sherbet, 1978; Hannig et al., 1975). Cells in aqueous solutions are surrounded by a hydrate layer (Stern layer). Ionic groups on the cell surface attract ions of opposite charge from the surroundings. The attracted ions enter the hydrate shell and form a layer close to the cell surface. Outside this adsorbed ion layer, a diffuse ion cloud is formed (Debye-Hückel layer). This electric double layer reduces the charge of the cell. The cells are separated according to the resultant "zeta potential", which is the potential at the transition of the Stern layer to the diffuse Debye-Hückel layer (Figure 8). Both the thickness of the electric double layer and the zeta potential are inversely related to the ionic strength of the

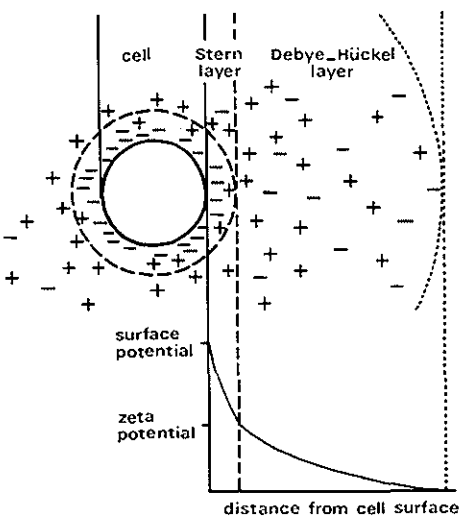

Figure 8:

Schematic representation of the electric double layer (adapted from Sherbet, 1978 and Hannig and Heidrig, 1977)
The dimension of the ion cloud is highly exaggerated in comparison to the cell diameter. In reality, the thickness of the ion cloud is 10 to 20 Å.

medium. The EPM of a cell in an electric field is decreased further by the formation of a dipole moment in the electric double layer ("relaxation effect"). Furthermore, the applied electric field can be modified in the ion cloud by the altered conductance. However, the latter two effects become relevant only when the ratio of cell radius to thickness of the ion cloud is small (i.e., at relatively large extension of the ion cloud). Quantitative studies on the above-mentioned phenomena have led to the conclusion that the EPM of cells in media of relatively high ionic strength (above 0.01 M) is directly related to the surface charge density (Sherbet, 1978; Hannig et al., 1975; Hannig and Heidrich, 1977). If electrophoresis is carried out under constant conditions and with defined buffers of constant composition, the observed EPM can be used as a measure of the cell surface charge density in qualitative and comparative studies.

Cell electrophoresis offers possibilities for investigations on the physicochemical composition of the cell surface. The presence of specific charged molecules can be detected by removing or covering these molecules with specific reagents. This will change the exposure of free charges and as a result the electrophoretic mobility of the cells. This possibility to measure a variety of parameters makes cell electrophoresis a powerful tool for cell identification.

The continuous free-flow cell electrophoresis developed by Hannig (1969; 1971; 1972) is a preparative method by which cells with different EPM can be collected in different fractions and functionally tested after the separation. In this study, cell electrophoresis was carried out using an ELPHOR VaP5 apparatus (Bender and Hobein, GmbH, München, W. Germany) (Figure 9). The separation chamber is formed by two parallel vertical glass plates placed at a distance of 0.75 mm from each other. A continuous flow of separation buffer from top to bottom is controlled by a peristaltic pump. At each side of the separation chamber, there is an elongated platinum electrode. This allows an electric field to be installed perpendicular to the separation buffer flow. To remove electrolysis products, the electrodes are placed in chambers through which a salt solution is pumped. The electrode chambers are separated from the separation chamber by selective ion exchange membranes. A cation and an anion exchanger are placed at the anode and cathode, respectively. This prevents deflected ions and particles from moving from the separation buffer into the electrode buffer. The temperature in the separation chamber should be constant to prevent thermal convection. This is achieved by a homogeneous cooling at the back of the chamber in combination with a good insulation at

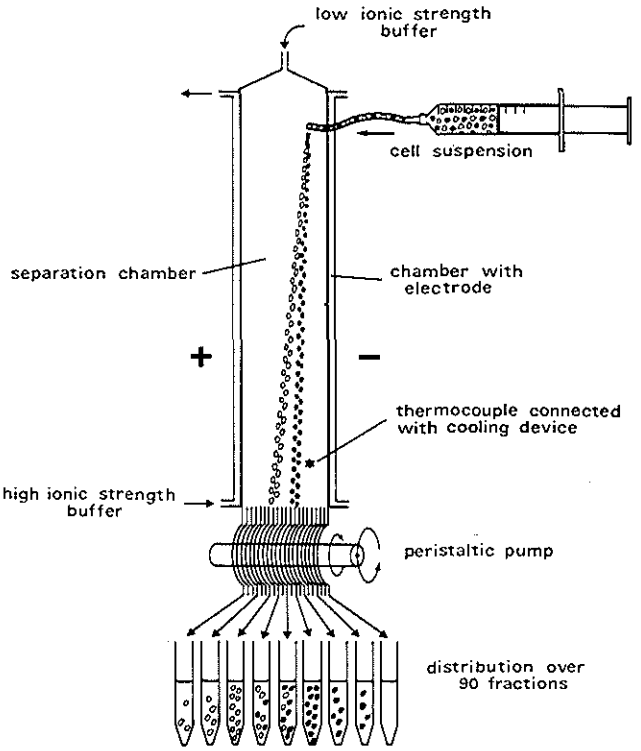

Figure 9:

Apparatus for carrying out free-flow cell electrophoresis (Elphor VaP5, Bender and Hobein, GmbH).

the front. The cell suspension can be continuously injected into the separation buffer stream. During their movement downwards, the cells are deflected according to their surface charge density. At the bottom of the separation chamber, the buffer flow is divided into 90 channels. The deflection of cells can be manipulated by varying the buffer flow velocity and/or the electric field strength.

The separation and electrode buffers were prepared as described by Von Boehmer et al. (1975). The separation buffer should contain buffer components of low electrophoretic mobility to maintain constant pH, only a low concentration of ions to limit heat production but high enough to maintain cell viability and nondissociated components (e.g., sugars) to establish the proper osmolarity. The separation buffer consisted of 5 volumes of 0.336 M HEPES buffer solution

(adjusted to a pH value of 7.2 with 0.336 M NaOH), 2.5 volumes of HEPES-buffered balanced salt solution (pH 7.2), 12.5 volumes of 0.308 M glucose solution and 80 volumes of 0.308 M sorbitol solution. This buffer had a specific conductance of 595 x 10^{-6} $\Omega^{-1}.cm^{-1}$ at at 20°C (395 x 10^{-6} $\Omega^{-1}.cm^{-1}$ at the separation temperature of 6°C). The ionic strength of the separation buffer is equivalent to approximately that of 0.013 M NaCl, which is about 1/13 times the physiological strength. The electrode buffer (pH 7.2) was composed of 80.4 volumes of 0.112 M Na_2HPO_4 solution and 19.6 volumes of 0.168 M NaH_2PO_4 solution. It should be noted that each of the stock solutions has an osmolarity of 308 mOsm. Thus, the solutions can be mixed in any ratio to obtain buffers with various pH values and ionic strengths without affecting the osmolarity.

Cell suspensions for electrophoresis were prepared as follows. Shortly before the electrophoretic separation, an appropriate number of cells was centrifuged (300 g, 10 min) and the cells in the pellet were resuspended in the separation buffer of low ionic strength. This causes immediate aggregation of dead cells. The cell aggregates were removed by filtration through a thin plug of cotton wool in a siliconised Pasteur pipette (Von Boehmer and Shortman, 1973; Von Boehmer et al., 1975). With this procedure, a cell suspension containing 95% viable cells was obtained (Table 3). The recovery of nontreated viable cells was about 70%, but neuraminidase treated cells (see Section 2.12) showed increased aggregation in the low ionic strength buffer. This caused a significant loss of viable cells (recovery of 30%). In addition to the values for cell viability and recoveries, Table 3 shows the number of colony forming cells per 10^5 viable nucleated cells after the various treatments. No striking differences were observed in the incidence of these cells; this indicates that the cell loss due to aggregation in the separation buffer is most probably non-selective and is in agreement with data reported by Von Boehmer and Shortman (1973) and Zeiller et al. (1975). For separation, the cells were adjusted to a maximum concentration of 2 x 10^7 $cells.ml^{-1}$. With this or lower cell concentrations, further aggregation was limited within the 1 to 1 1/2 h necessary for electrophoresis.

Sterilisation of the electrophoresis apparatus was achieved by rinsing all tubing of the peristaltic pump and that for sample and buffer supply with a 0.5% Hibitane (Imp Chem Ind) solution in 70% ethanol. The separation chamber was sterilised for 1 to 2 h with a 1% Hibitane solution in sterile distilled water. After sterilisation,

TABLE 3

VIABILITY AND RECOVERY OF NUCLEATED CELLS AND INCIDENCE OF COLONY FORMING CELLS AFTER VARIOUS TREATMENTS

treatment of bone marrow cells	number of experiments	percentage viable nucleated cells	recovery of viable nucleated cells in percentage of control	number of colony forming cells per 10^5 viable nucleated cells			
				CFU-s/10^5	GM CFU-c 1/10^5	GM CFU-c 2/10^5	GM CFU-c 3/10^5
Hanks' balanced salt solution (control)	9	79 (66-87)	100	35 (10-59)	54 (26-91)	124 (59-168)	64 (47-85)
centrifugation; resuspension in separation buffer; filtration	5	94 (91-97)	70 (49-93)	33 (10-49)	59 (17-98)	125 (99-166)	109 (38-204)
37°C, 1 h, HBSS; wash	4	82 (78-87)	90 (76-97)	35 (29-43)	54 (37-75)	124 (114-135)	83 (65-119)
37°C, 1 h, HBSS + neuraminidase; wash	4	72 (68-76)	85 (79-92)	12 (7-15) 46[a]	72 (40-100)	130 (82-177)	90 (51-130)
37°C, 1 h, HBSS + neuraminidase; wash; resuspension in separation buffer; filtration	4	91 (90-93)	30 (11-40)	8 (5-13) 31[a]	69 (36-90)	110 (78-136)	87 (40-136)

The figures represent means and limits of range observed.

a Corrected values. Neuraminidase treatment reduces the number of CFU-s to 26% of the number of untreated CFU-s (mean of 21 experiments; Chapter 4). The corrected values are obtained by multiplication of the observed number by a factor 100/26. The reduction in CFU-s number by neuraminidase treatment is not due to cell kill but to a decrease in spleen seeding efficiency of colony forming cells.

the apparatus was rinsed with two litres sterile distilled water. To reduce electroosmosis during the separation, the chamber walls were coated with bovine serum albumin by leaving the chamber overnight filled with a 2% w/v albumin solution in separation buffer (Hannig et al., 1975). The chamber was then rinsed once more with sterile distilled water and was filled from bottom to top with separation buffer.

Electrophoresis of cells was carried out in an electric field of 115 $V.cm^{-1}$. The electric current was stabilised at 210 mA and the temperature maintained at 6 °C. The mean effective separation time for individual cells was 170 s (buffer flow rate of 790 $ml.h^{-1}$). The change in electrophoretic mobility per fraction under these conditions was $0.057670 \pm 0.002214 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ (mean ± standard deviation calculated from 24 experiments). The cells were collected in plastic tubes (Falcon, 2057) of 15 ml capacity which contained 1 ml Hanks' balanced salt solution supplemented with 20% foetal bovine serum at the start. After the separation procedure, 0.5 ml of each fraction was used for viable nucleated cell counts. The cells in the fractions were centrifuged (300 g, 10 min), resuspended in salt solution and adjusted to a concentration of 1×10^6 $cells.ml^{-1}$.

The mean recovery of cells after the actual separation procedure was between 80% and 90% for all viable nucleated cells as well as for colony forming cells.

The results were expressed in electrophoretic mobilities and/or fraction numbers. Without application of the electric field, over 90% of the cells was collected in fraction no. 70. Under the conditions used, about 50% of mouse erythrocytes were collected (on the average) in fractions 31 and 32.

2.11 RESOLUTION AND REPRODUCIBILITY OF CELL SEPARATION BY FREE-FLOW ELECTROPHORESIS

The detection of cell types with different surface charge densities depends on the resolution of the electrophoresis techniques. Since erythrocytes are considered to be homogeneous in many properties, these cells were used to test the resolution. When cells migrate in the electric field, the shape of the distribution curve of a homogeneous cell type and the degree of separation between two different cell types is related to the length of the migration path. Figure 10 shows the separation of rat erythrocytes. The migration path was varied by changing the electric field strength. The width of the dis-

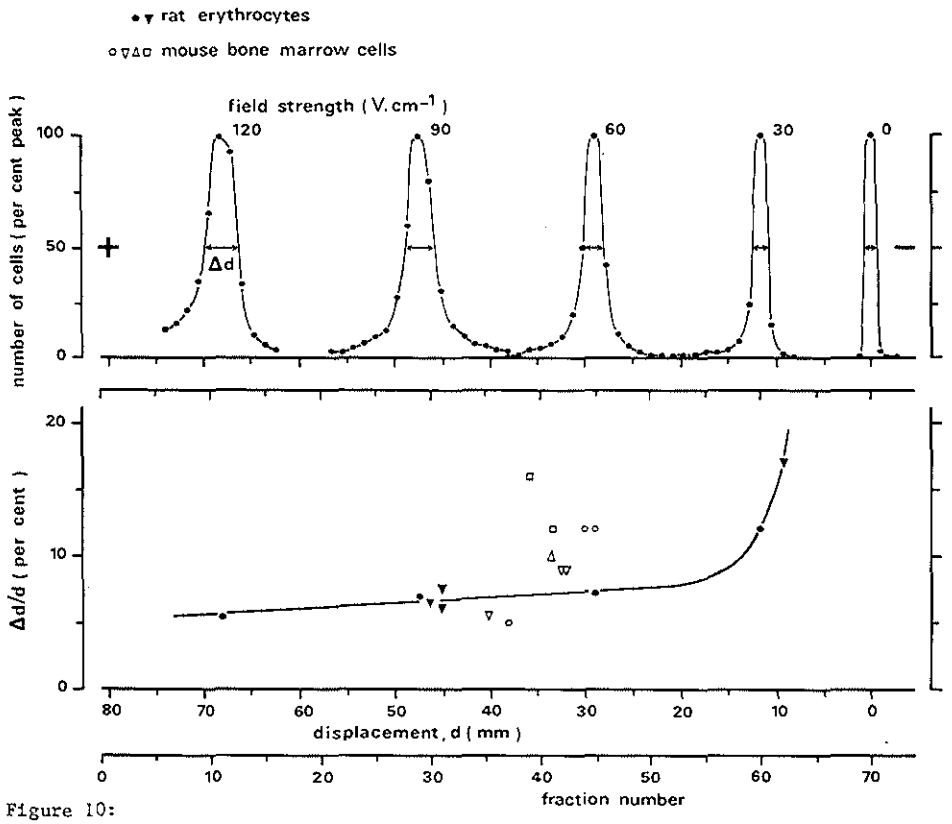

Figure 10:

Resolution of the free-flow cell electrophoresis technique
Rat erythrocytes were separated at various electric field strengths. The band width at 50% of the peak height (Δ d) increases with increasing displacement. The bottom panel shows the band width as a fraction of the displacement (Δ d/d). Different symbols represent results of different experiments. Closed symbols represent results of experiments with rat erythrocytes. Open symbols represent results of rebanding experiments with mouse bone marrow cells.

tribution at 50% of the peak height (Δ d) in percentage of the length of the migration path (displacement, d) was taken as a measure of the resolution. It can be seen that the band width increases continuously with increasing displacement. The resolution improves considerably with increasing d but reclines to about 8% when d exceeds 15 fractions. These values are in good agreement with those reported by Hannig et al. (1975).

The resolution was also tested by repeated separation of mouse bone marrow cells. After an initial electrophoresis, cells in a single fraction can be assumed to have similar EPM. The width of the distri-

bution observed after re-electrophoresis of cells from such a single fraction can be used as a measure of the resolution. In addition, if the cells are not affected by the separation procedure, they should separate in fractions around the original fraction from which they are derived. Figure 11 shows the results of an experiment in which mouse bone marrow cells were separated by electrophoresis. After an initial electrophoresis, an equal number of cells from a high and a low EPM fraction were mixed and reseparated under identical conditions. The cells from the two fractions are again clearly separated from each other. The band width at 50% of the peak height is 1.5 and 2.5 fractions for the high and low EPM cells, respectively. This results in a resolution of about 5 to 10%. The resolution calculated from several reseparation experiments (indicated by the open symbols in Figure 10) appears to be somewhat lower than that calculated from the separation of erythrocytes. The cell profiles of Figure 11 also show that, after the reseparation, the distributions are shifted 1 to 3 fractions to higher EPM values in comparison with the original fractions of the first separation. This shift in EPM is not specific for the chosen fractions. If cells are separated by electrophoresis and the cells from all fractions are mixed and reseparated, the entire cell profile

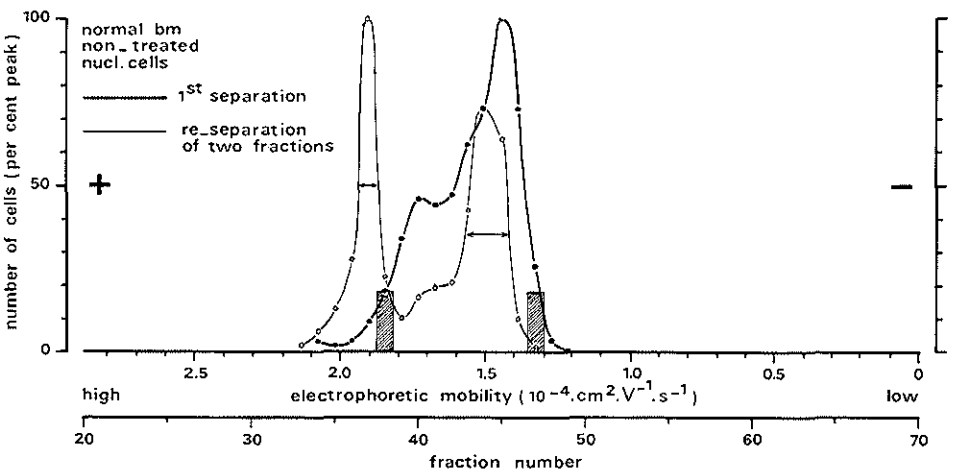

Figure 11:

Rebanding of nucleated bone marrow cells of low and high electrophoretic mobility
The solid line represents the electrophoretic mobility distribution of nucleated bone marrow cells. The hatched columns indicate the cell fractions which were subjected to a second electrophoresis. The thin line represents the electrophoretic mobility distribution of the cells from the two selected fractions. The profiles are calculated as nucleated cells per fraction and are expressed as a percentage of the maximum value.

is shifted to higher EPM values (Figure 12). These data indicate that the cells are slightly affected by exposure to the electric field.

On the basis of these measurements, the resolution of the free flow electrophoresis was taken to be ± 5%. Differences of 5% or more between the EPM of different cell types measured within one experiment were considered to be significant.

In the first biological test, it was investigated whether morphologically recognizable cells could be separated by electrophoresis. Viable nucleated mouse bone marrow cells show an electrophoretic mobility distribution as illustrated in the top panel of Figure 13. The profile shows two peaks which represent cells with a relatively high and a relatively low EPM. Morphological characterization of the cells from the different fractions using Giemsa stain shows that myeloblasts, mature neutrophilic granulocytes and lymphocytes have a relatively low but clearly different modal EPM. The difference in modal EPM among these cell types is about 6.5%. Surprisingly, these cell types also have a small peak in the high EPM region. Normoblasts are found exclusively in the high EPM cell fractions. Erythrocytes, which are not included in the total cell profile, have a very high EPM and

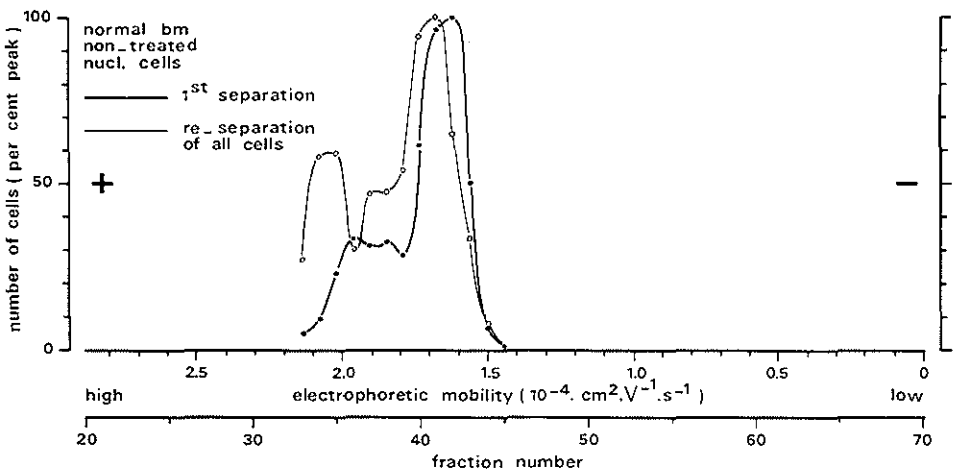

Figure 12:

Repeated electrophoresis of all bone marrow cells
The solid line represents the EPM distribution of nucleated bone marrow cells. After electrophoresis, the cells from all fractions were mixed and subjected to a second electrophoresis. EPM distribution of the cells after the second run is represented by the thin line. The profiles are calculated as nucleated cells per fraction and are expressed as a percentage of the maximum value.

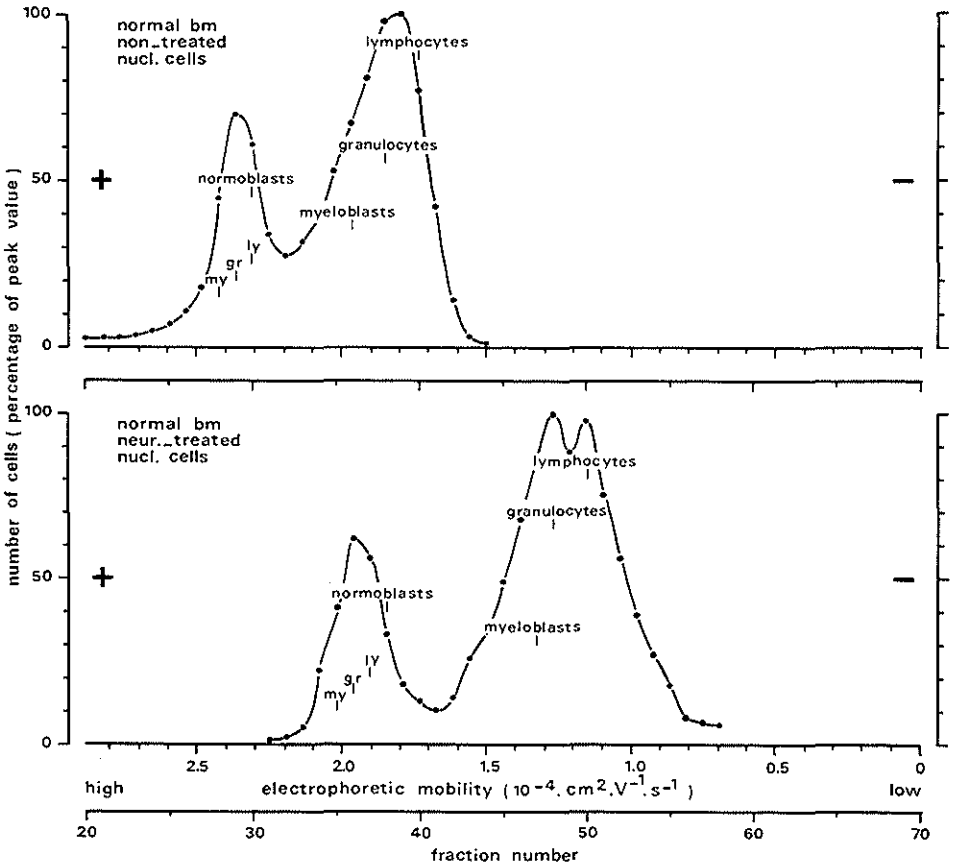

Figure 13:

Electrophoretic separation of morphologically recognizable bone marrow cells
Top panel: EPM distribution of nontreated nucleated bone marrow cells.
Bottom panel: EPM distribution of neuraminidase treated nucleated bone marrow cells.
The profiles are calculated as nucleated cells per fraction and are expressed as a percentage of the maximum value. After electrophoresis, the cells from the various fractions were sedimented onto microscope slides after which they were fixed and stained with May Grünwald/Giemsa. The positions of the various morphologically recognizable cell types are indicated.

separate at the anodic side of the high EPM peak. The bottom panel of Figure 13 shows the effect of neuraminidase treatment (see Section 2.12) on the EPM of nucleated bone marrow cells. Removal of negatively charged neuraminic acid from the cell surface glycoproteins drastically reduces the EPM of most cells. The EPM distribution of neuraminidase treated cells also shows two peaks. With respect to the morphologically recognizable cells, this EPM distribution seems to be comparable to the EPM distribution of nontreated cells. Myeloblasts, granulocytes and lymphocytes are equally affected and shift about 0.58×10^{-4} $cm^2.V^{-1}.s^{-1}$ in EPM, which still allows a clear distinction among the modal EPM of these cells (differences of about 6.0%). Again, additional small peaks are observed in the high EPM fractions. Normoblasts appear to be somewhat less affected by the neuraminidase (shift of about 0.46×10^{-4} $cm^2.V^{-1}.s^{-1}$ in EPM), which results in a clear separation from the bulk of the other three cell types. Erythrocytes (not included in the profile) are again found at the anodic side of the high EPM peak*. These experiments show that free-flow electrophoresis allows distinction among certain mouse bone marrow cell types.

EPM distributions of mouse bone marrow cells obtained in independent experiments carried out under apparently identical conditions showed considerable variation in position and shape. The EPM distributions of nucleated cells from a series of 32 experiments could be classified into 4 groups. Figure 14 shows representative distributions of these groups. Firstly, the position of the distributions varies from experiment to experiment. The mean modal EPM of nucleated cells (main peak of relatively low EPM cells) and standard deviation from 32 experiments is $1.70 \pm 0.13 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ ($1.70 \pm 7.5\%$). The mean EPM of cells in the minor additional peak (in most experiments) was calculated to be $2.07 \pm 0.20 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ ($2.07 \pm 9.7\%$). After neuraminidase treatment, the mean modal EPM of nucleated cells calculated from 9 experiments is $1.18 \pm 0.06 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ ($1.18 \pm 5.1\%$). The mean EPM of cells in the additional peak is $1.94 \pm 0.09 \times 10^{-4}$ $cm^2.V^{-1}.s^{-1}$ ($1.94 \pm 4.6\%$). On the basis of these measurements, the variation in modal EPM of a certain cell type over various experiments was expected to be $\pm$ 8% (standard deviation). If the EPM of different cell types determined in independent experiments were compared, a difference in EPM of 8% or higher was considered to be significant.

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*This is in contrast to human erythrocytes, which show a great reduction in EPM after neuraminidase treatment (Section 2.12).

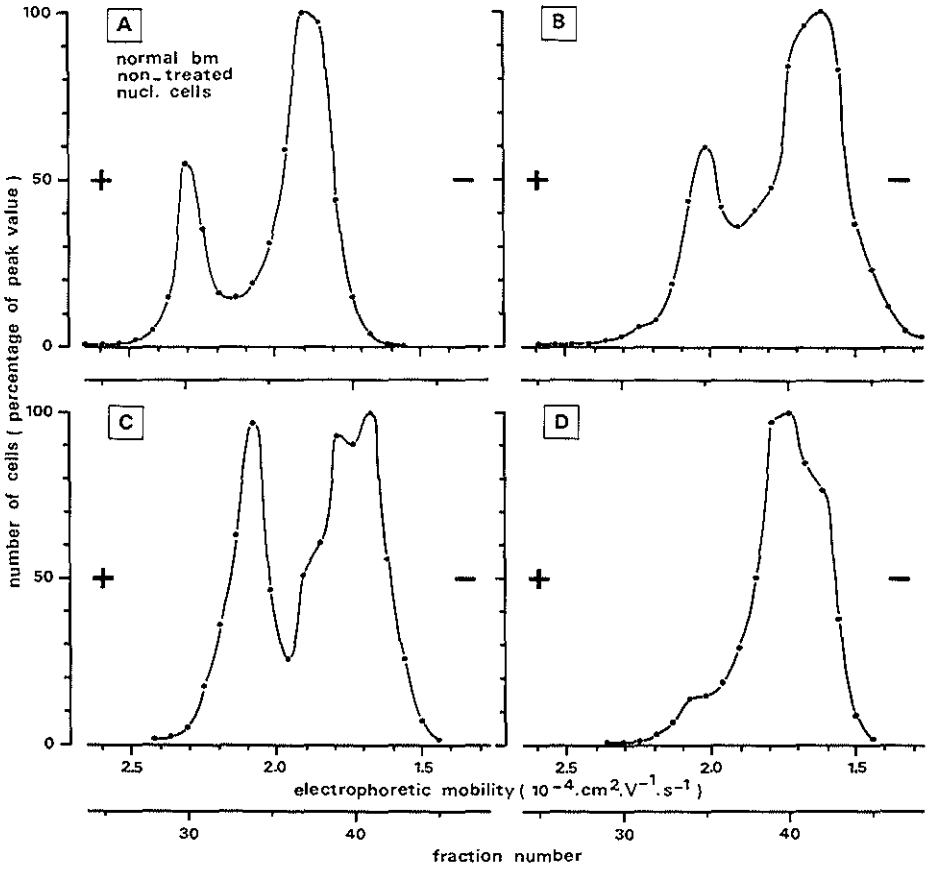


Figure 14:

Four examples of the electrophoretic mobility distribution of nucleated bone marrow cells

The EPM distributions of nucleated cells from a series of 32 experiments could be classified into 4 groups. Variation is observed in the position of the profiles, the ratio between the height of the minor and major peak and the depth of the "valley" between the peaks. Profiles as shown in panels A and B are observed in 70% of the experiments. Profiles shown in panels C and D are observed in 20% and 10% of the experiments, respectively. The profiles are calculated as nucleated cells per fraction and are expressed as a percentage of the maximum value.

Secondly, variation was observed in the ratio between the height of the two peaks and in the depth of the "valley" in between the peaks. In 22 out of 32 experiments (about 70%), cell profiles as shown in Figure 14 A and B were observed. The high EPM peak was reduced to a pronounced shoulder in some cases. In 7 experiments (about 20%), the height of the high EPM peak was equal to or exceeded the height of the "main" low EPM peak. An example of such a distribution is shown in Figure 14 C. In only 3 experiments (about 10%) was the high EPM peak almost absent as shown in Figure 14 D. The EPM distributions of neuraminidase treated cells showed two peaks in each of 9 experiments. In 3 experiments of this series (about 35%), the height of the high EPM peak exceeded that of the low EPM peak.

The variability in the ratio between the height of the two peaks and the observation that many cell types (several morphologically recognizable cells but also, as will be shown later, in vivo and in vitro colony forming cells) show a small high EPM peak in addition to a main low EPM peak cast doubt on the importance of the high EPM peak. As mentioned earlier, cell aggregation was limited by use of relatively low cell concentrations, but could not be totally prevented. If cell aggregation occurred, visible cell aggregates separated rather uniformly at the cathodic side of the high EPM peak. Thus, a portion of the high EPM peak might be due to cell aggregates which are subsequently disaggregated in the normal ionic strength medium used after the separation procedure. There are, however, several observations which indicate that the high EPM peak is not just composed of unfractionated cells. It was suggestive that some of the cell aggregation occurred only on exposure of the cells to the electric field. This may indicate that the aggregation during the actual separation is cell type dependent. Also the ratio among different cell types in the high EPM peak differs markedly from that found in unfractionated bone marrow. Furthermore, several cell types show a single EPM peak: erythrocytes and normoblasts separate in a single, relatively high EPM peak and mouse thymocytes, cells from regenerating mouse bone marrow and rat and human bone marrow cells show a single relatively low EPM peak. Finally, the results from the reseparation experiments (Figure 11) show that high and low EPM cells selected after an initial electrophoresis separate according to these properties in a second electrophoresis procedure. This indicates that these cells are indeed of different EPM.

2.12 TREATMENT OF CELLS WITH NEURAMINIDASE

The occurrence of neuraminic acids or sialic acids (mostly N-acetylneuraminic acid: NANA) on the surface of haemopoietic cells was investigated by use of the enzyme neuraminidase from Vibrio cholerae in combination with cell electrophoresis. Vibrio cholerae neuraminidase has a broad substrate spectrum. It can split all linkages of NANA to the carbohydrate moieties of glycoproteins and some linkages to glycolipids (Gottschalk and Drzeniek, 1972). Incubation of bone marrow cells with neuraminidase has two obvious effects: a) it differentially reduces the electrophoretic mobility of CFU-s and GM CFU-c 1, 2 and 3 (Chapter 3); and b) it causes a decrease in the number of CFU-s (Chapter 4).

Neuraminidase from Vibrio cholerae was obtained from Behringwerke AG in a concentration of 2×10^{-1} IU.ml^{-1} (unit definition according to Warren, 1963). This preparation contains no measurable activities of proteases, aldolase and phospholipase C. Bone marrow cells were routinely incubated with neuraminidase at 37°C for 1 h. The presence of Ca^{2+} ions during the incubation is necessary to activate the enzyme reaction. The cell concentration during the incubation was between 1×10^{7} and 5×10^{7} cells.ml^{-1}. This variation in cell concentration had no detectable influence on the action of neuraminidase with respect to the reduction in electrophoretic mobility of cells and the decrease in CFU-s numbers. Control cell suspensions were incubated without neuraminidase. After the incubation, the cells were washed with a large volume of Hanks' balanced salt solution.

The influence of neuraminidase on cell viability and cell recovery is given in Table 4. The data show that incubation of bone marrow cells at 37°C for 1 h without neuraminidase does not affect cell viability in comparison with the viability of cells kept at 0°C, but 10% of the cells is lost by the additional wash. The cell viability is decreased by about 10% after the neuraminidase treatment. The recovery of viable cells coincides with the decrease in viability and this indicates that there is no selective loss of dead or viable cells by other effects, e.g., possible cell aggregation.

The relationship between the neuraminidase concentration during the incubation and the reduction in electrophoretic mobility (EPM) was tested with human erythrocytes. The erythrocytes shift as a homogenous population to lower EPM values when the neuraminidase concentration is increased. The modal values of the EPM distributions observed after incubation with varying concentrations of neuraminidase are plotted against the neuraminidase concentration in Figure 15 A (solid circles).

TABLE 4

CELL VIABILITY AND CELL RECOVERY AFTER TREATMENT WITH NEURAMINIDASE

treatment of cells	number of experiments	percentage viable cells	percentage recovery viable cells
0°C	15	79.5 ± 5.3	100
37°C, 1h; wash	6	80.8 ± 4.3	90.3 ± 8.6
neuraminidase, 37°C, 1h; wash	8	72.9 ± 3.1	80.7 ± 9.0

Figures represent mean ± standard deviation.
Viable nucleated cell counts were performed by pulse cytophotometry using fluorescein diacetate and propidium iodide as fluorescent dyes (Section 2.4).

The modal EPM of the erythrocytes decreases continuously with increasing neuraminidase concentrations and no plateau is reached up to 1×10^{-1} IU neuraminidase.ml^{-1}. The modal value of the EPM distribution of total mouse bone marrow cells also decreases continuously after incubation with increasing concentrations of neuraminidase. On the basis of this observation, the neuraminidase concentration for the treatment of bone marrow cells in the EPM studies was arbitrarily chosen at 2×10^{-2} IU.ml^{-1}.

The reduction in CFU-s numbers after incubation with neuraminidase is also dependent on the neuraminidase concentration. The number of CFU-s decreases with increasing concentrations of neuraminidase and reaches a plateau of 25% of the control number at a concentration of about 1×10^{-3} IU neuraminidase.ml^{-1} (Chapter 4, Figure 23, p. 78). In experiments in which only the phenomenon of the CFU-s reduction was investigated, the neuraminidase concentration during the incubation was 4×10^{-3} IU.ml^{-1}.

The neuraminidase induced decrease in EPM and the reduction in CFU-s numbers are generally ascribed to the removal of negatively charged NANA molecules. Studies of the EPM behaviour of erythrocytes by Eylar et al. (1962) showed a positive correlation between the relative change in EPM and the relative amount of NANA removed. However, this relationship was not 1 to 1; the change in EPM exceeded the loss of NANA groups. This greater change in EPM than expected has been explained by assuming that not all NANA at the cell surface contribute equally to the zeta potential and that the more externally exposed NANA groups are more easily removed than those located on more internal structures. Another phenomenon that might influence the EPM of

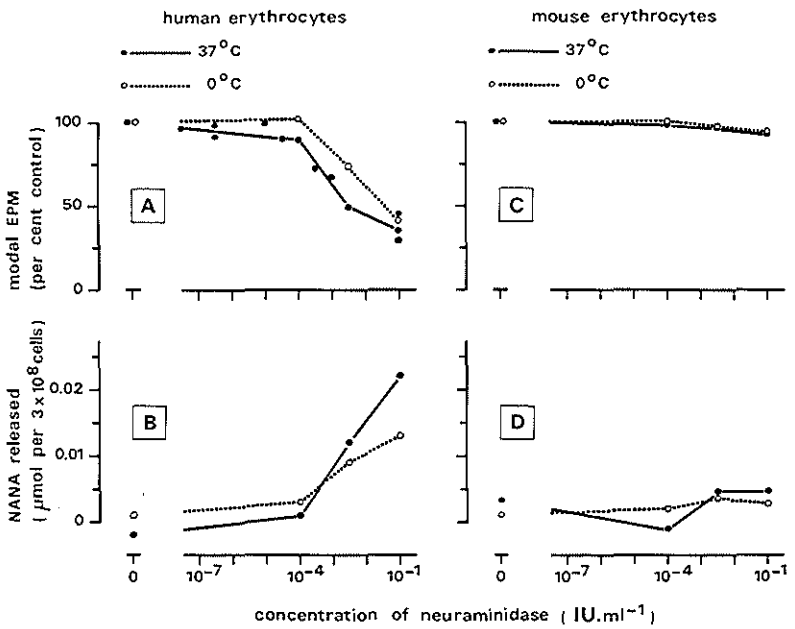


Figure 15:

The effect of neuraminidase treatment on human and mouse erythrocytes with respect to change in EPM and NANA release

Erythrocytes were incubated with increasing concentrations of neuraminidase for 1 h. After the incubation the cells were centrifuged and the supernatant was tested for the presence of neuraminic acid according to the method described by Warren (1963). The supernatant and a control solution of NANA were treated with sodium periodate, sodium arsenite and 2-thiobarbituric acid followed by extraction of the aqueous solution with cyclohexanon. Extinction was measured at 549 nm in a Beckman model 25 spectrophotometer. Correction was made for interfering material (mainly 2-deoxysugars) with an extinction at 532 nm. Extinction of medium without cells and cuvette was subtracted. The cells in the pellet were washed, resuspended in separation buffer and their EPM was determined by electrophoresis. Nonincubated erythrocytes were used as control cells. The solid circles in panel A represent the modal EPM of human erythrocytes measured in 3 experiments. The solid line connects the values obtained in 1 experiment.

cells is the adsorption of neuraminidase to the cell surface (Sedlacek and Seiler, 1974; Seiler and Sedlacek, 1974). By using fluorescent labelled antineuraminidase antibodies, these authors showed that attached neuraminidase cannot be removed by extensive washing and that it retains its activity for at least several hours. Neuraminidase remains attached even after the cleavage of NANA from the surface glycoproteins has been completed. This indicates that the attachment of the enzyme is probably not dependent on the presence of NANA groups. It has been suggested that the decrease in the EPM of cells after neuraminidase treatment is due not only to the enzymatic removal of NANA groups but also to covering of negatively charged groups on the cell surface by the adsorbed enzyme (Sachtleben et al., 1973).

The possible influence of surface attached neuraminidase on the EPM of cells was investigated. Human erythrocytes were treated with various concentrations of neuraminidase for 1 h at 37°C and at 0°C. After the incubation, the modal EPM of the cells and the amount of NANA released by the enzyme were determined. At 0°C, the enzymatic activity should be minimized but nonspecific attachment of the enzyme to the cell surface should not. Consequently, the effect of attached enzyme on the EPM of the cells should become evident. The results are shown in Figure 15 A and B. Incubation of human erythrocytes with neuraminidase at 37°C decreases the EPM of these cells and causes the release of NANA molecules. After incubation at 0°C, a decrease in EPM is also observed, but to a somewhat lesser extent than that observed after incubation at 37°C. Surprisingly, the enzymatic removal of NANA appears to take place even at 0°C. In comparison to the amount of NANA removed at 37°C about 70% of the NANA is removed at 0°C. Therefore, this experimental approach does not allow the determination of the contribution of enzyme attachment to the change in EPM.

In the next experiments, the enzyme reaction was inhibited by addition of 20 mM EDTA (ethylenediaminetetraacetate), which depletes the incubation medium of Ca^{2+} ions. The results are shown in Figure 16. When human erythrocytes are incubated with neuraminidase in the presence of EDTA, the EPM of the cells is not decreased and no NANA is removed from the cells. Although it is possible that EDTA also prevents the adsorption of the enzyme, these observations strongly suggest that the change in EPM after neuraminidase treatment is due to the release of NANA molecules.

Supporting evidence that adsorption of neuraminidase does not affect the EPM of cells is obtained from observations on the electrophoretic behaviour of mouse and human erythrocytes (Figure 15). Mouse

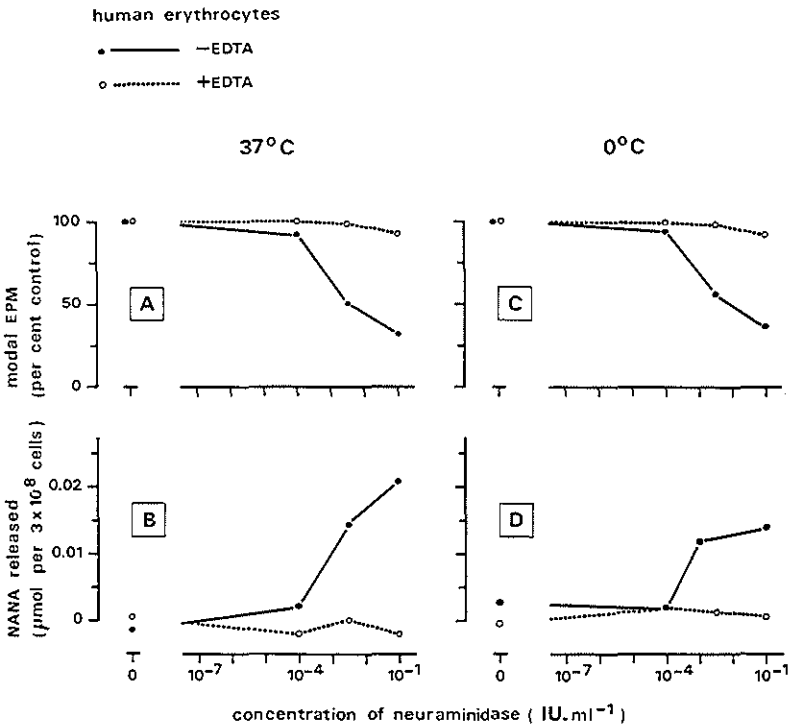


Figure 16:

The effect of EDTA on the action of neuraminidase
Human erythrocytes were incubated with increasing concentrations of neuraminidase without or with addition of 20 mM EDTA. After the incubation the cells were centrifuged. The supernatant was tested for the presence of neuraminic acid. The cells were washed and subjected to electrophoresis to determine their EPM.

and human erythrocytes were incubated with increasing concentrations of neuraminidase. After the incubation, the modal EPM of the erythrocytes and the amount of NANA released were determined. Mouse and human erythrocytes differ markedly with respect to the change in EPM after neuraminidase treatment. In contrast to the EPM of human erythrocytes, the EPM of mouse erythrocytes is hardly affected by neuraminidase. This difference between the two types of erythrocytes in EPM change coincides with a difference in the amount of NANA released. These observations again suggest that the neuraminidase induced decrease in EPM is due to the removal of NANA groups and not to nonspecific adsorption of the enzyme to the cell surface.

Finally, the isoelectric point of neuraminidase is 4.8. This indicates that the enzyme is negatively charged at the pH value of 7.2 of the separation buffer. It is therefore unlikely that attachment of the enzyme to the cell surface will reduce the negative surface charge.

The cause of the reduction in CFU-s numbers after incubation with neuraminidase has also been a subject of discussion. This reduction may be due to the loss of NANA residues or the adsorption of neuraminidase to the cell surface. Removal of NANA residues can result in the exposure of terminal galactose groups which are recognized by, e.g., liver cell receptors (Morell et al., 1971; Ploemacher and Van Soest, 1979a, b). Therefore, trapping of neuraminidase affected colony forming cells in the liver may explain the decrease in CFU-s numbers. Neuraminidase treated cells may also be recognized and eliminated by lymphocytes and macrophages, either because of the changes in the surface composition after the NANA release or because of the presence of

TABLE 5

EFFECT OF EDTA ON THE NEURAMINIDASE INDUCED REDUCTION IN CFU-s

	treatment of bone marrow cells	CFU-s per 10^5 cells	percentage
A.	1 h incubation at 37°C		
	control	35.7 (42.6; 28.8)	100
	EDTA	0.5 (0.8; 0.2)	1.4 (1.9; 0.7)
	neuraminidase	8.8 (9.5; 8.0)	24.6 (22.3; 27.8)
	neuraminidase + EDTA	0.5 (0.8; 0.1)	1.4 (1.9; 0.3)
B.	1 h incubation at 0°C		
	control	35.0 (39.2; 30.8)	100
	EDTA	36.5 (41.0; 32.0)	104.3 (104.6;103.9)
	neuraminidase	9.3 (11.0; 7.5)	26.6 (28.1; 24.4)
	neuraminidase + EDTA	30.4 (33.6; 27.2)	86.7 (85.7; 88.3)

Figures represent mean and individual values of 2 experiments.

the foreign enzyme molecules at the cell surface (Knop et al., 1978). It was investigated whether NANA release or adsorption of neuraminidase is responsible for the decrease in CFU-s numbers.

Mouse bone marrow cells were incubated with neuraminidase (4×10^{-3} IU.ml^{-1}) and with or without EDTA (10 mM). By inhibition of the enzyme reaction with EDTA, the effect of surface adsorbed neuraminidase should be made manifest. The results in Table 5 show that incubation of bone marrow cells with neuraminidase reduces the CFU-s numbers to 25% of control numbers. Incubation of cells with EDTA at 37°C appears to have a toxic effect, since hardly any CFU-s were observed after such an incubation. Therefore, the experiment was repeated at 0°C, at which temperature neuraminidase is still active (see above) and EDTA does not exert cytotoxic effects (Table 5 B). As observed after incubation with neuraminidase at 37°C, the number of CFU-s is reduced to about 25% of control numbers. Inhibition of the neuraminidase activity by EDTA results in a much smaller reduction in the number of CFU-s to 87% of control numbers. Although it has not been excluded that EDTA prevents the attachment of neuraminidase to the cell surface, these data suggest that the neuramidase induced reduction in CFU-s numbers is caused by the release of NANA groups.

The decrease in the EPM of cells and the reduction in CFU-s numbers after incubation of cells with neuraminidase is considered to be due mainly to the cleavage of NANA from the cell surface. Conclusive data on the effects of surface attached neuraminidase may be obtained by using immobilized enzyme (Bazarian and Wingard, 1979).

2.13 CELL SEPARATION BY EQUILIBRIUM DENSITY CENTRIFUGATION

Mouse bone marrow cells were separated on the basis of differences in buoyant density (specific gravity) according to the technique described by Shortman (1968). Full details have been described elsewhere (Bol et al., 1977b). Bovine serum albumin (Fraction V, Sigma Chem Co) solutions were used as separation media (Shortman et al., 1972b). These solutions were isotonic to mouse serum (310 mOsm) (Williams et al., 1972) and had a pH value of 5.1. All procedures were carried out at 4°C.

Linear gradients (14 ml; density varying between 1.060 and 1.090 g.cm^{-3}) can be created by continuously mixing low and high density albumin solutions. Cells were suspended directly into the high density medium so that they were distributed over the gradient. The

maximum load applied was 3 x 10^8 cells per 14 ml gradient. The gradients were spun at 4000 g for 30 min. About 20 fractions of equal volume were collected by upward displacement with bromobenzene (BDH Chem Ltd.). The density of each fraction was determined by use of a linear bromobenzene/petroleum ether gradient (in a graduated burette) which was calibrated with standard density albumin solutions. The average density increment of the fractions was 0.0016 $g.cm^{-3}$ (ranging from 0.0013 to 0.0019 $g.cm^{-3}$). Cells were recovered from the fractions by dilution with balanced salt solution, centrifugation (300 g, 10 min) and resuspension in balanced salt solution. Nucleated cell counts were made on each fraction by use of a haemocytometer. All fractions were diluted to the same concentration of cells and tested for the presence of colony forming cells. To correct for possible nonlinearity of the gradient, the number of cells and detected colony forming cells in each fraction was divided by the density increment of that fraction.

A single-step density separation procedure was applied to obtain separation of cells into two fractions. Cells were suspended in an albumin solution of the desired density and loaded in a centrifuge tube on top of a small volume of albumin solution of the same density. The suspension was centrifuged at 4000 g for 10 min. This resulted in separation into two fractions containing cells with a density lower (supernatant) and higher (pellet) than that of the albumin solution. The cells were washed, counted and diluted to the desired concentrations. The maximum load was 1 x 10^8 cells.ml^{-1}. This separation procedure allows a rapid selection of a large number of cells of the desired buoyant density.

The cell recovery after the continuous gradient procedure and after the single-step density procedure was 60% to 80% for both viable nucleated cells and colony forming cells.

2.14 CELL SEPARATION BY SEDIMENTATION AT UNIT GRAVITY

Cell separation on the basis of differences in sedimentation velocity (which depends on cell density and cell volume) has been described in detail by Miller and Phillips (Miller and Phillips, 1969; Miller, 1973). All procedures were carried out at 4°C. Cells were suspended in 0.2% w/v bovine serum albumin in phosphate buffered saline and loaded by application of a "buffered step" gradient from 0.2% to 2% w/v albumin (Miller, 1973). The separation medium had an osmolarity of 310 mOsm, a pH value of 6.0 and an average density of 1.0075 $g.cm^{-3}$. A cylindrical sedimentation chamber with a diameter

of 19.5 cm was used. The maximum cell load was 5×10^8 cells. Cells were allowed to sediment for 3 to 4 h; then, 25 fractions of 50 ml each were collected. After centrifugation, the cells of the various fractions were suspended in a small volume of balanced salt solution, counted and adjusted to the desired concentration. The recovery of cells varied between 80% and 90% for both viable nucleated cells and colony forming cells.

2.15 CELL SEPARATION BY FLUORESCENCE AND SCATTERED LIGHT ACTIVATED ELECTRONIC CELL SORTING

Cells were analyzed and sorted on the basis their light scattering properties by use of a FACS II cell sorter (Becton and Dickinson, Mountain View, Calif.). In this instrument, cells are centered in a buffer stream which is ejected from a vibrating nozzle. The stream passes a laser beam before breaking into droplets. From the cells which pass the laser beam, the scattered light intensities and (if they are labelled with fluorescent probes) fluorescent light intensities can be measured by various detectors. Cells which meet preselected properties can be sorted out. The droplets which contain the desired cells are charged and are electrostatically deflected. In the FACS II, the detector placed along the axis of the laser beam was used to measure scattered light signals between -1° to -10° and 1° to 10°. The signals are refered to as forward light scattering or FLS. One of the detectors placed perpendicular to the laser beam generally used for fluorescence measurements was used to measure light scattered between 70° and 110°: perpendicular light scattering or PLS. Forward and perpendicular light scattering intensities have been shown to be measures for cell size (cross-sectional area) and cell structure (or deviation from a sphere), respectively. Detailed data on this technique and its application to mouse bone marrow cells have been published elsewhere (Visser et al., 1978; Van den Engh et al., 1979).

CHAPTER 3

CHARACTERIZATION OF CFU-s, GM CFU-c 1, GM CFU-c 2 AND GM CFU-c 3 BY THEIR SURFACE CHARGE PROPERTIES

3.1 INTRODUCTION

The cells forming spleen colonies in lethally irradiated mice (CFU-s) and those forming colonies of granulocytes and/or macrophages in culture (GM CFU-c 1,2 and 3) have been characterized mainly by their physical properties such as buoyant density and sedimentation velocity (Chapter 1). It has been shown that the development from CFU-s through GM CFU-c 3 is accompanied by an increase in buoyant density with minor changes in cell size. Studies of the light scattering properties of the various CFU showing small differences in cell size between CFU-s and GM CFU-c 1,2,3 (which confirms the size calculations on the basis of buoyant density and sedimentation velocity) and an increase in cell "structuredness" in the sequence of CFU-s through GM CFU-c 3 have been recently reported (Visser et al., 1980b).

This Chapter concerns studies on CFU-s and GM CFU-c 1,2 and 3 with respect to their surface charge density by use of the free-flow electrophoresis technique. It will be shown that CFU-s are different from GM CFU-c 1,2 and 3 in their surface charge density; this indicates differences in the cell surface composition of these cells. The contribution of N-acetylneuraminic acid (NANA) to the cell surface charge was investigated by use of the enzyme neuraminidase from Vibrio cholerae. When negatively charged NANA groups are removed by neuraminidase, the electrophoretic mobility of cells decreases. The relative decrease in mobility may be used as a measure for the density of neuraminidase susceptible NANA groups on the cell surface. The electrophoretic mobility of the various CFU will be shown to be differentially affected by treatment with neuraminidase, suggesting changes in the surface NANA density during early haemopoietic development.

The electrophoretic mobility of cells can in part reflect their proliferative activity. Differences in mobility between cells from normal and regenerating rat liver have been reported (Ben-Or et al., 1960). A relationship between the mobility of cells and the cell cycle phase has also been demonstrated (Mayhew and O'Grady, 1965; Brent and

Forrester, 1967). Since CFU-s and GM CFU-c show differences in their proliferative activity and since CFU-s has been subdivided into a resting and cycling population, the influence of the proliferative activity on the electrophoretic mobility was investigated. It will be shown that part of the difference in mobility between CFU-s and GM CFU-c might be ascribed to differences in proliferative activity. However, resting and cycling CFU-s cannot be distinguished by their electrophoretic mobility under normal steady state conditions.

3.2 CFU-s HAVE A HIGHER MODAL ELECTROPHORETIC MOBILITY THAN GM CFU-c 1,2 AND 3

Mouse bone marrow cells were separated on the basis of differences in electrophoretic mobility (EPM) by free-flow electrophoresis. The collected fractions were tested for the presence of *in vivo* spleen colony forming cells (CFU-s) and *in vitro* granulocyte/macrophage colony forming cells (GM CFU-c 1,2 and 3). Figure 17 shows the EPM distributions of all viable nucleated cells and CFU-s in six independent experiments. This figure shows that the EPM distribution of CFU-s is different from that of all nucleated cells in all experiments. The EPM distributions of GM CFU-c 1, 2 and 3 (not shown in this figure) completely coincide with the EPM distribution of all nucleated cells.

The mean EPM distributions of the various cell types calculated from 5 experiments in which CFU-s and GM CFU-c 1,2 and 3 were assayed simultaneously are shown in Figure 18. The viable nucleated cell profile shows a minor peak at 2.12×10^{-4} $cm^2 . V^{-1} . s^{-1}$ and a major peak (modus) at 1.74×10^{-4} $cm^2 . V^{-1} . s^{-1}$. The EPM distribution of CFU-s also reveals two peaks. The minor peak coincides with the high EPM peak of the total nucleated cell profile. The modus of the CFU-s profile is found at 1.87×10^{-4} $cm^2 . V^{-1} . s^{-1}$. The bottom panel of Figure 18 shows the mean EPM distributions of the three differentiation stages of GM CFU-c. No differences in modal EPM among these three cell types can be detected and a complete concurrence of their EPM distributions is observed. Unlike CFU-s, GM CFU-c do not segregate from the other nucleated cells. These results indicate that CFU-s and GM CFU-c have different EPM. In particular, it should be noted that CFU-s and GM CFU-c 1 can be distinguished on the basis of their modal EPM.

For both CFU-s and GM CFU-c, an additional high EPM peak is observed. This suggests heterogeneity in surface charge within the CFU populations. However, as described in Section 2.11 (p. 51), part of

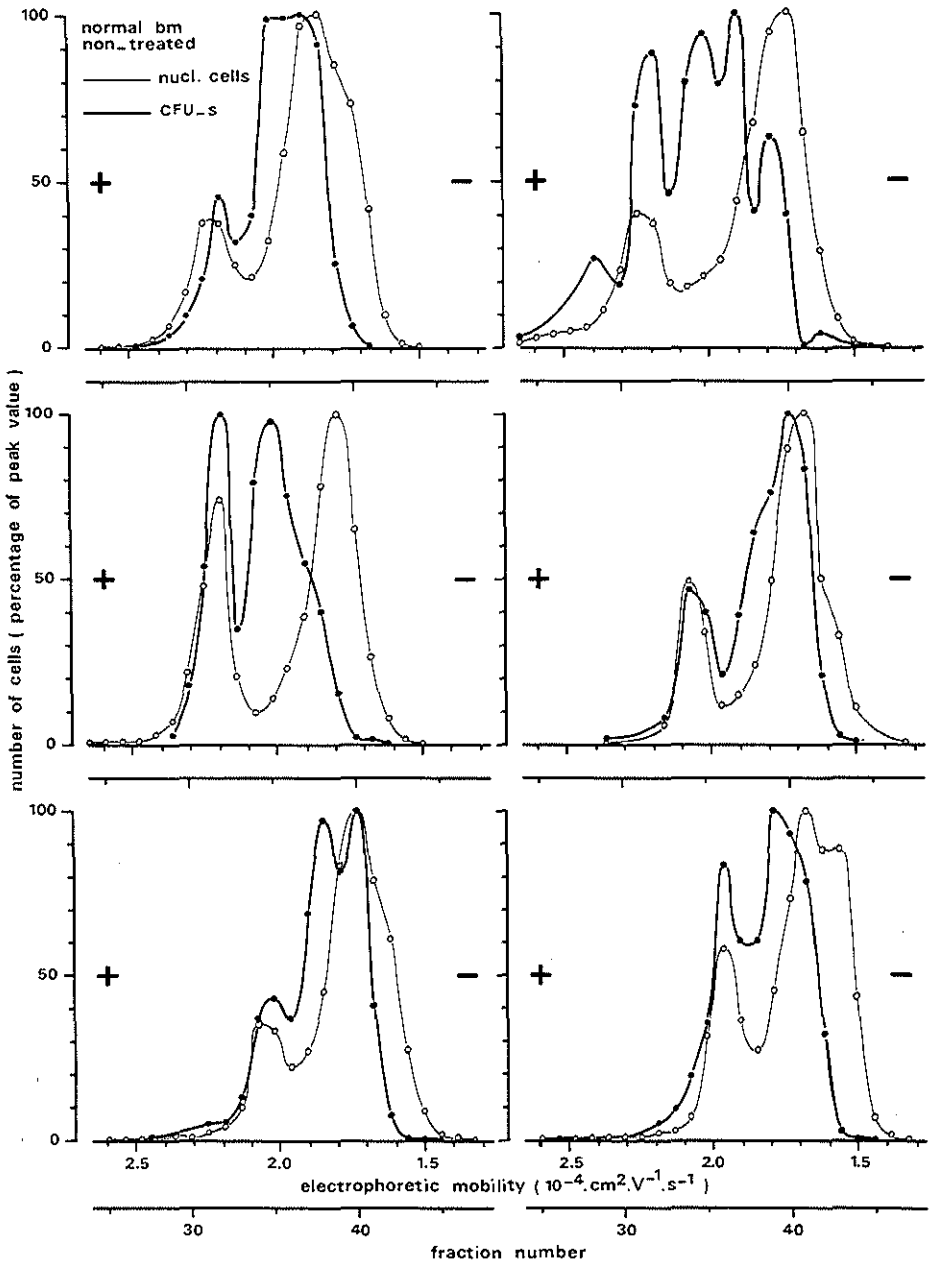


Figure 17:

Electrophoretic mobility distributions of viable nucleated bone marrow cells and CFU-s obtained in 6 experiments
The profiles are calculated as nucleated cells or CFU-s per fraction and each profile is expressed as a percentage of its maximum value.

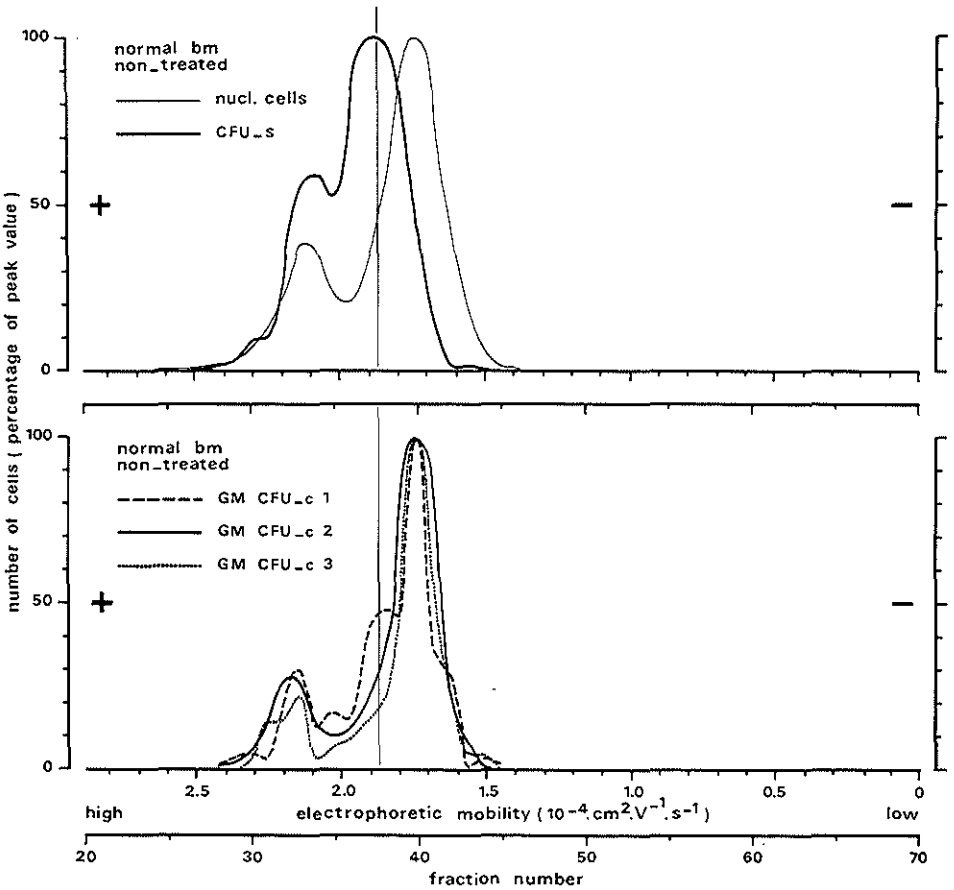


Figure 18:

Electrophoretic mobility distributions of nontreated nucleated cells, CFU-s and GM CFU-c 1,2 and 3 from normal bone marrow
Top panel: EPM distributions of nucleated cells and CFU-s (mean of 5 experiments).
Bottom panel: EPM distributions of GM CFU-c 1,2 and 3 (mean of 5 experiments).
The profiles are calculated as cells per fraction and each profile is expressed as a percentage of its maximum value.

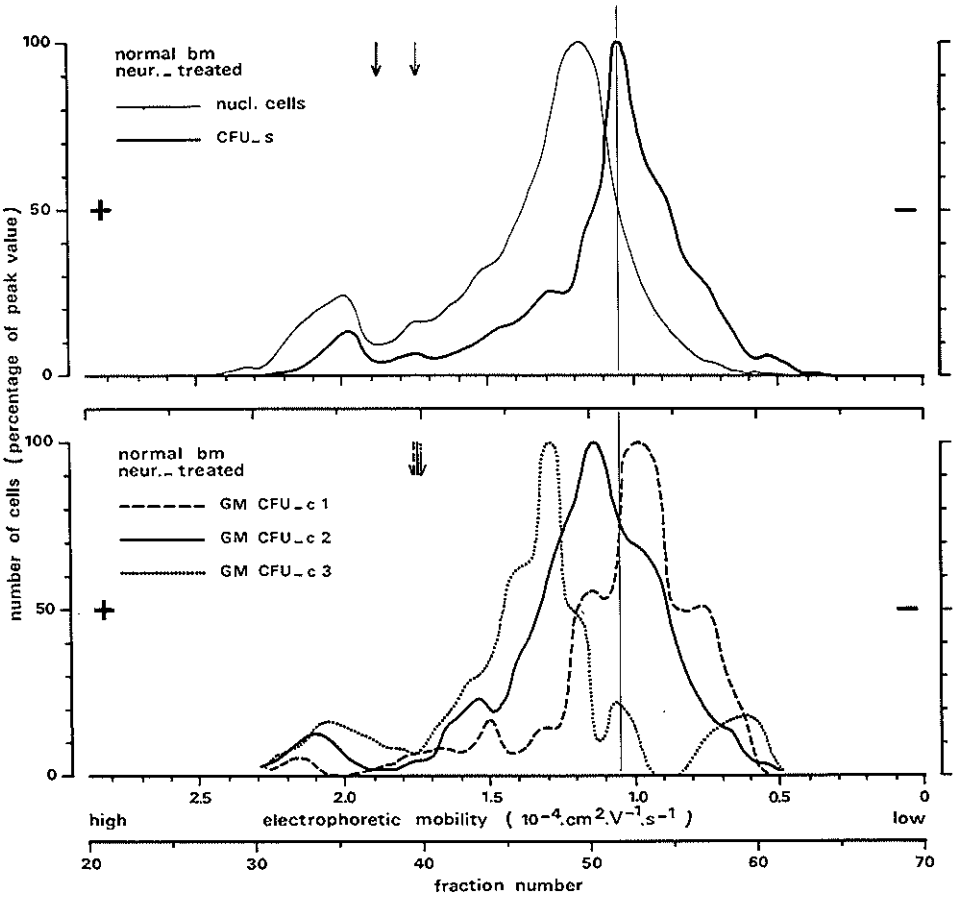


Figure 19:

Electrophoretic mobility distributions of neuraminidase treated nucleated cells, CFU-s and GM CFU-c 1,2 and 3 from normal bone marrow
Top panel: EPM distributions of nucleated cells and CFU-s (mean of 4 experiments)
Bottom panel: EPM distributions of GM CFU-c 1,2 and 3 (mean of 4 experiments)
The profiles are calculated as cells per fraction and each profile is expressed as a percentage of its maximum value. The arrows indicate the modal EPM of nontreated cells.

the high EPM peak can be explained by cell aggregation, so that not too much value can be placed on this observation.

3.3 THE CONTRIBUTION OF NEURAMINIDASE SUSCEPTIBLE N-ACETYLNEURAMINIC ACID TO THE CELL SURFACE CHARGE DECREASES WITH DIFFERENTIATION

When bone marrow cells are incubated with neuraminidase, their EPM is drastically decreased. The mean EPM distributions of all viable nucleated cells, CFU-s and GM CFU-c 1,2,3 after neuraminidase treatment as calculated from four experiments are shown in Figure 19. For comparison, the modal EPM of nontreated cells (derived from Figure 18) is indicated by arrows. The viable nucleated cell profile is broadened and the modal EPM is reduced to 1.18×10^{-4} $cm^2.V^{-1}.s^{-1}$, which is about 60% of the modal EPM of nontreated bone marrow cells. A small population of nucleated cells is characterized by a higher EPM. The EPM of CFU-s is reduced to 1.05×10^{-4} $cm^2.V^{-1}.s^{-1}$, which is 56% of the modal EPM of nontreated CFU-s. Thus, in contrast to CFU-s from nontreated bone marrow, CFU-s from neuraminidase treated bone marrow have a lower modal EPM than most other nucleated cells.

The EPM of the three GM CFU-c is differentially affected by the neuraminidase treatment (Figure 19, bottom). As observed for CFU-s, the modal EPM of GM CFU-c 1 is strongly reduced to 56% of that of nontreated cells (0.98×10^{-4} $cm^2.V^{-1}.s^{-1}$). The EPM of GM CFU-c 2 and GM CFU-c 3 is less affected by the enzyme treatment. Their modal EPM is reduced to 1.13 and 1.29×10^{-4} $cm^2.V^{-1}.s^{-1}$, respectively, which is 65% and 74% of the EPM of nontreated cells.

A summary of the data is given in Table 6 A. When the EPM of nontreated CFU-s and GM CFU-c are compared, it is noted that CFU-s have a higher EPM than GM CFU-c 1, 2 and 3. CFU-s and GM CFU-c 1 appear to be equally affected by the neuraminidase treatment, since both show the same relative reduction in EPM. This indicates that, also after neuraminidase treatment, CFU-s have a somewhat higher EPM than GM CFU-c 1. However, the relative order of the modal EPM of CFU-s and GM CFU-c 2 and 3 is reversed after neuraminidase treatment, the EPM of GM CFU-c 2 and 3 being higher than that of CFU-s.

These data indicate that the effect of neuraminidase on the EPM of CFU decreases as differentiation proceeds. This is interpreted as a decrease in the density of neuraminidase susceptible NANA groups on the cell surface during early haemopoiesis. The similar relative decrease in the EPM of CFU-s and GM CFU-c 1 after neuraminidase treatment indicates that these CFU types are very similar with respect to

TABLE 6

MODAL ELECTROPHORETIC MOBILITY OF CFU-s AND GM CFU-c 1,2 AND 3 BEFORE AND AFTER TREATMENT WITH NEURAMINIDASE

	modal EPM * of nontreated cells (control)	modal EPM of neuraminidase treated cells	modal EPM of neuraminidase treated cells in percentage of control
A. Normal bone marrow			
All nucleated cells	** 1.74 (1.67 - 1.84)[a]	1.18 (1.15 - 1.21)[b]	68
CFU-s	1.87 (1.72 - 2.01)[a]	1.05 (1.03 - 1.09)[b]	56
GM CFU-c 1	1.74 (1.67 - 1.84)[a]	0.98 (0.98 - 0.98)[b]	56
GM CFU-c 2	1.74 (1.67 - 1.84)[a]	1.13 (1.09 - 1.15)[b]	65
GM CFU-c 3	1.74 (1.67 - 1.84)[a]	1.29 (1.21 - 1.38)[b]	74
B. Regenerating bone marrow			
All nucleated cells	1.64 (1.61 - 1.67)[c]	1.24 (1.21 - 1.27)[d]	76
CFU-s	1.63 (1.61 - 1.67)[c]	0.92 (0.87 - 0.98)[d]	56
GM CFU-c 1	1.59[e]	0.87[e]	55
GM CFU-c 2	1.59[e]	0.98[e]	62
GM CFU-c 3	[1.59?]	1.18[e]	[74?]

* The EPM is expressed in $10^{-4}.cm^2.V^{-1}.s^{-1}$.

** The figures represent mean and limits of range observed.

a, 5 experiments; b, 4 experiments; c, 3 experiments; d, 2 experiments; e, 1 experiment.

the expression of neuraminidase susceptible NANA groups on the cell surface. Furthermore, comparison among the EPM of nontreated and neuraminidase treated CFU shows that there must be other important differences in the surface composition among the various CFU types. Nontreated GM CFU-c 1, 2 and 3 cannot be distinguished by their EPM, but appear to have different densities of NANA on their surface. CFU-s have a higher EPM than have GM CFU-c 1, but these cells are likely to have a similar surface NANA density. This indicates that significant quantities of negatively charged groups other than neuraminidase susceptible NANA ones contribute to the surface charge of the CFU.

The EPM distributions of neuraminidase treated CFU are rather broad. The band width at half the peak value is greater than can be expected for a homogeneous cell type on the basis of the resolution. This suggests that there is a large variation in the surface NANA density within each of the CFU populations.

3.4 RAPIDLY PROLIFERATING CFU HAVE A RELATIVELY LOW ELECTROPHORETIC MOBILITY

The EPM distributions of CFU as described in the preceeding paragraphs were further analyzed by investigating the relationship between EPM and the proliferative status of the cells. It has been reported that the CFU-s population contains a large proportion of resting (G_0) cells which can be distinguished from cycling (G_1-S-G_2) cells on the basis of differences in buoyant density and sedimentation velocity properties (Van den Engh, 1976; Visser et al., 1977). The GM CFU-c populations (with GM CFU-c 1 as a possible exception) are generally considered to mainly contain cycling cells. Therefore, some of the differences in EPM between CFU-s and GM CFU-c might be due to differences in the cell cycle properties of these cells. To investigate this hypothesis, the EPM properties of CFU from normal bone marrow described above were compared with those of CFU from regenerating bone marrow. During bone marrow regeneration, all CFU can be expected to be rapidly proliferating and, with respect to CFU-s, no resting cells will be present. The proliferative status of CFU was monitored by measuring the proportion of CFU killed after treatment with ^{3}H-thymidine (Section 2.5, p. 29).

The rapidly proliferating CFU were tested for their EPM properties. The mean EPM distributions of all nucleated cells and CFU-s from three experiments are shown in the top panel of Figure 20. The bottom panel shows the EPM distributions of GM CFU-c 1 and 2 from one experi-

ment. CFU-s and GM CFU-c are found exclusively in the relatively low EPM region. In none of the three experiments were high EPM cells detected. The modal EPM of CFU-s in each of the experiments was lower than that of normal bone marrow CFU-s. From the three experiments, a mean modal EPM of 1.63×10^{-4} $cm^2 . V^{-1} . s^{-1}$ was calculated for CFU-s. This is about 87% of the modal EPM of CFU-s from normal bone marrow. The GM CFU-c 1 and 2 profiles in the single experiment were also shifted to lower EPM values in comparison with the EPM of GM CFU-c from normal bone marrow, but the overlap in EPM properties of CFU-s and GM CFU-c seems to be greater. In the experiment shown in Figure 20, GM CFU-c 1 and GM CFU-c 2 had modal EPM values of about 1.59×10^{-4} $cm^2 . V^{-1} . s^{-1}$, which is about 91% of the modal EPM of GM CFU-c from normal bone marrow. The difference in EPM between CFU-s from normal and regenerating bone marrow is greater than that between GM CFU-c from the two bone marrow sources. This observation might be correlated with the magnitude of the ^{3}H-thymidine kill in the CFU-s and GM CFU-c populations when changing from normal to regenerating conditions (Section 2.6, p. 29). The observations indicate that rapidly proliferating CFU from regenerating bone marrow have a lower EPM than have CFU from normal bone marrow. This may indicate that the difference in EPM between CFU-s and CFU-c under normal conditions partly reflects the proliferative status of these cells.

On the basis of the differences in EPM between CFU-s from normal and regenerating bone marrow, it can be hypothesized that resting and cycling CFU-s will show different EPM properties under normal conditions. This possibility was investigated by determination of the EPM of CFU-s in S phase. Bone marrow cells were separated by electrophoresis and the cells from each fraction were divided into two groups. One group was incubated with ^{3}H-thymidine, while the other was handled as a control. Figure 22 shows the EPM distribution of CFU-s after incubation at 37°C with ^{3}H-thymidine (dotted line) and without (solid line). It can be determined from the cell profiles that an equal proportion of CFU-s in all EPM fractions is impaired by the ^{3}H-thymidine treatment. The CFU-s surviving ^{3}H-thymidine show an EPM distribution which is identical to that of the total CFU-s population. This is clearly shown when the EPM distribution of the surviving CFU-s is normalized to its own peak value. In contrast to what would be expected on the basis of the differences in EPM between CFU from normal and regenerating bone marrow, this observation on the EPM properties of CFU-s in S phase suggests that resting and cycling CFU-s cannot be distinguished on the basis of their EPM properties. In general, the results

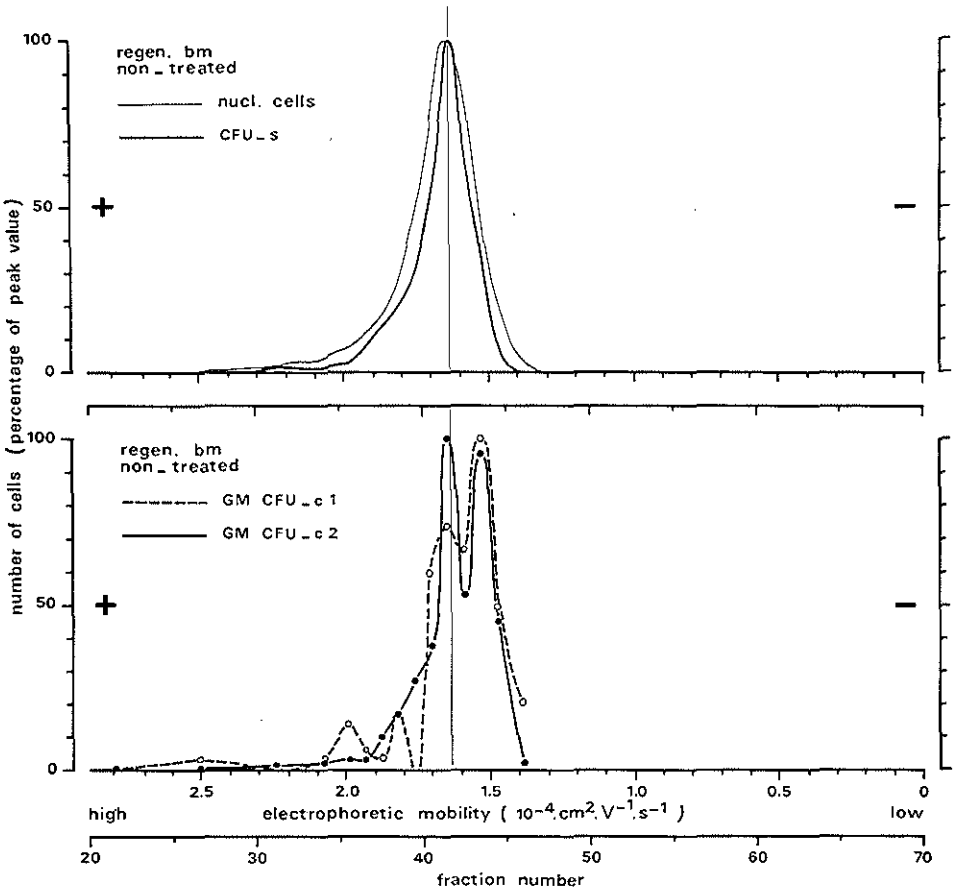


Figure 20:

Electrophoretic mobility distributions of nontreated nucleated cells, CFU-s and GM CFU-c 1 and 2 from regenerating bone marrow
Top panel: EPM distributions of nucleated cells and CFU-s (mean of 3 experiments)
Bottom panel: EPM distributions of GM CFU-c 1 and 2 (1 experiment)
The profiles are calculated as cells per fraction and each profile is expressed as a percentage of its maximum value.

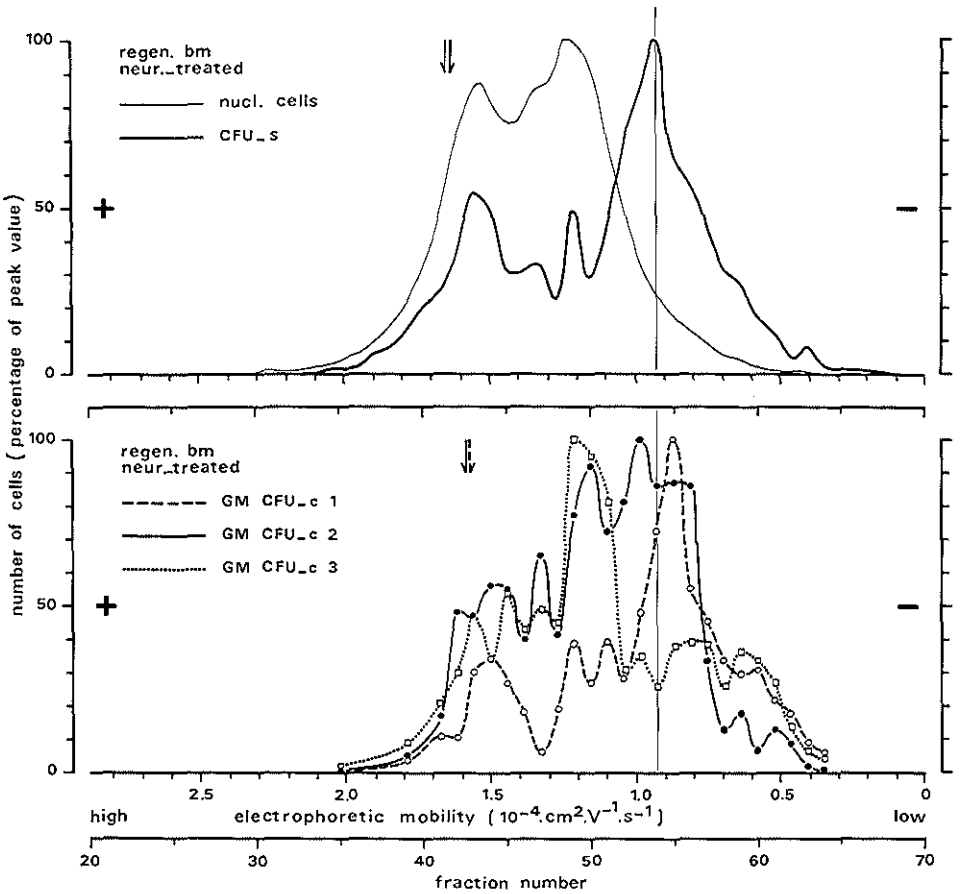


Figure 21:

Electrophoretic mobility distributions of neuraminidase treated nucleated cells, CFU-s and GM CFU-c 1,2 and 3 from regenerating bone marrow
Top panel: EPM distributions of nucleated cells and CFU-s (mean of 2 experiments)
Bottom panel: EPM distributions of GM CFU-c 1,2 and 3 (1 experiment)
The profiles are calculated as cells per fraction and each profile is expressed as a percentage of its maximum value. The arrows indicate the modal EPM of nontreated cells.

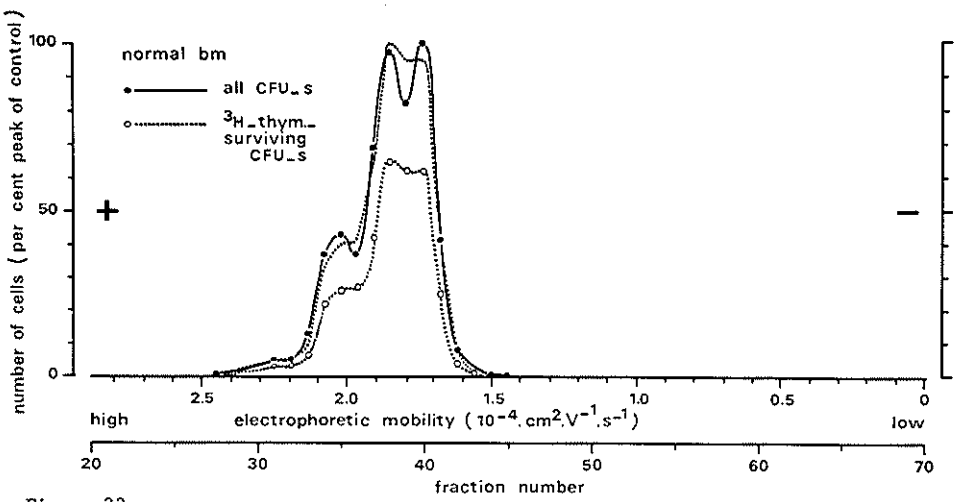


Figure 22:

Electrophoretic mobility distributions of all CFU-s and of CFU-s surviving ^{3}H-thymidine treatment

Bone marrow cells were separated by electrophoresis and each cell fraction was divided into 2 groups which were incubated for 30 min at 37°C without or in the presence of ^{3}H-thymidine. The solid line (closed circles) represents the EPM distribution of CFU-s which received the control treatment. The dotted line (open circles) represents the EPM distribution of CFU-s which survived the ^{3}H-thymidine treatment. The profiles are calculated as CFU-s per fraction and are expressed as a percentage of the maximum value of the control CFU-s profile.
The dotted line without points represents the EPM distribution of the ^{3}H-thymidine surviving CFU-s expressed as a percentage of its own maximum value.

described in this paragraph suggest that the rapidly proliferating CFU have characteristic cell surface properties which are different from those of CFU proliferating under normal conditions.

3.5 RAPIDLY PROLIFERATING CFU HAVE A SURFACE DENSITY OF NEURAMINIDASE SUSCEPTIBLE N-ACETYLNEURAMINIC ACID WHICH IS SIMILAR TO THAT OF NORMAL CFU

To investigate whether the relatively low modal EPM of rapidly proliferating CFU and the complete absence of high EPM cells could be related to an altered exposure of NANA groups on the cell surface, the

effect of neuraminidase treatment on the EPM of CFU was determined. As shown in Figure 21, the mean modal EPM of CFU-s is reduced to 0.92×10^{-4} $cm^2.V^{-1}.s^{-1}$, which is 56% of the EPM of nontreated CFU-s. The peak in the high EPM region can be mainly ascribed to cell aggregation. The modal EPM of GM CFU-c 1 and GM CFU-c 2 is reduced to 0.87 and 0.98×10^{-4} $cm^2.V^{-1}.s^{-1}$; these values represent 55% and 62% of the EPM of nontreated cells. The EPM distribution of GM CFU-c 2 is wide and the position of the absolute peak may be doubtful. However, the EPM distributions of GM CFU-c 1 and GM CFU-c 3 are located at the low and the high EPM side, respectively, of the GM CFU-c 2 profile. This indicates that the modal EPM of GM CFU-c 2 is intermediate between that of GM CFU-c 1 and 3 as is also observed for GM CFU-c 2 from normal bone marrow.

The data suggest that, after neuraminidase treatment, the rapidly proliferating CFU still have a lower EPM than do normal CFU. Furthermore, on the basis of the similar relative reduction in EPM after incubation with neuraminidase, it can be concluded that the CFU from regenerating and normal bone marrow are likely to be similar with respect to the density of neuraminidase susceptible NANA groups on the cell surface. A summary of the modal EPM values of the various cell types from regenerating bone marrow incubated with and without neuraminidase is given in Table 6 B.(p.67)

3.6 DISCUSSION

This chapter describes experiments on the electrophoretic mobility (EPM) properties of *in vivo* colony forming cells (CFU-s) and three developmental stages of *in vitro* granulocyte/macrophage colony forming cells (GM CFU-c 1,2,3). CFU-s and GM CFU-c differ in their EPM. CFU-s have a relatively high modal EPM, while that of the GM CFU-c is relatively low. No differences in the modal EPM can be detected among GM CFU-c 1, GM CFU-c 2 and GM CFU-c 3. The most interesting observation is that CFU-s and GM CFU-c 1 can be distinguished on the basis of their modal EPM, suggesting them to be different in membrane composition. There is still, however, a considerable overlap in EPM properties between these two cell types, especially in the high EPM region.

Rapidly proliferating CFU from regenerating bone marrow have a lower EPM than normal CFU. The modal EPM of CFU-s is decreased to 87% and that of GM CFU-c to 91% of the EPM of normal CFU. High EPM CFU as observed in normal bone marrow are completely absent in regenerating bone marrow. The overlap in EPM between CFU-s and GM CFU-c is some-

what greater during the regenerating process than under normal conditions. This may indicate that the surface charge density of CFU is in part determined by the proliferative state of these cells.

Under normal conditions, the CFU-s population is suggested to consist of a resting and a proliferating cell compartment. On the basis of the above described results, it would be expected that the CFU-s in these two compartments would be characterized by a high and a low EPM. respectively. However, such a difference in EPM could not be detected. S phase cells in the CFU-s population cannot be distinguished on the basis of their EPM and it is therefore unlikely that cells in other phases of the cell cycle have a specific EPM. This leads to the conclusion that the EPM properties of rapidly proliferating cells cannot be related to those of cells proliferating under normal conditions and that the existence of electrophoretically distinguishable resting and cycling cells under normal conditions is doubtful.

The nature of the high EPM CFU in normal bone marrow is unclear and only speculative remarks can be made. Part of the high EPM cell peak can be explained by cell aggregation (Section 2.11, p. 51). If these aggregates are composed of the same cells which are found in the major low EPM peak, the surface charge density of the aggregates should not be changed. Although cells are separated by their surface charge density and not by their absolute surface charge (size), it is possible that great differences in size (one cell - cell aggregate) can be detected by electrophoresis. However, the high EPM of cell aggregates may also be due to the presence of a high number of normoblasts which are exclusively found in the high EPM peak (Section 2.11, p. 47). If the size of the cell aggregates is not reflected in the EPM, two possibilities remain to explain the presence of high EPM CFU: either CFU are nonselectively involved in aggregation with high EPM cells (e.g. normoblasts) or CFU of high EPM selectively aggregate and represent a CFU subpopulation. As shown for CFU-s, the heterogeneity in EPM does not represent CFU-s in different phases of the cell cycle.

Characterization of CFU-s by their EPM was first reported by Zeiller et al. (1972). Comparison between the results reported here and the observations described by those authors reveals the following differences and similarities. Zeiller et al. observed an EPM distribution of nucleated bone marrow cells with a single peak. If the EPM of erythrocytes is taken as a reference, the total EPM range of nucleated cells was about 2/3 times the range observed in this study and the modal EPM was higher. This may indicate differences in resolution. The

modal EPM of CFU-s was reported to be the same as that of all nucleated cells, although the total EPM distribution of CFU-s showed a tendency towards higher EPM values. This tendency may be in agreement with the relatively high modal EPM of CFU-s observed here. CFU-s which survive after in vivo treatment with a single dose of vinblastine were reported to have a relatively low EPM. Since resting cells are less affected by this treatment, it was concluded that the low EPM is characteristic for resting CFU-s. However, it seems more likely that perturbation of the haemopoietic system will result in an immediate recruitment of resting CFU-s into the cell cycle. CFU-s found in the bone marrow 24 h after vinblastine treatment can therefore be assumed to be rapidly proliferating. In that case, the reported low EPM of those CFU-s may be in agreement with the low EPM of CFU-s from regenerating bone marrow found in this study, but the differences detected in EPM between normal and proliferating CFU-s are much greater than those reported by Zeiller et al. However, the relatively high EPM of CFU-s present in spleen colonies in primary recipients as reported by Zeiller et al. is in contradiction with these considerations. Those CFU-s can also be assumed to be in active cell cycle and they would be expected to have a relatively low EPM. There is no explanation for this discrepancy. The only general remark that can be made is that cell physical properties studied after perturbation of the haemopoietic system must be interpreted with caution, since these properties are not necessarily related to the properties of cells under normal conditions.

The net negative surface charge can be decreased by treatment of the cells with neuraminidase. This decrease in negative charge is the result of the removal of N-acetylneuraminic acid groups (NANA) by the enzyme. Neuraminidase treatment appears to have a differential effect on the EPM of the various CFU types. It strongly affects the modal EPM of CFU-s and GM CFU-c 1 (reduction to 56% of the EPM of nontreated CFU), has a moderate effect on the modal EPM of GM CFU-c 2 (reduction to 65%) and only a small effect on the modal EPM of GM CFU-c 3 (reduction to 74%). CFU with a low proliferative activity in normal bone marrow and rapidly proliferating CFU in regenerating bone marrow show similar relative reductions in modal EPM after neuraminidase treatment. On the basis of these results, it is hypothesized that the density of neuraminidase susceptible NANA groups on the cell surface decreases during the development from CFU-s through GM CFU-c 3.

Although the EPM of CFU from regenerating bone marrow is lower than that of CFU from normal bone marrow, the results of the electro-

phoresis of neuraminidase treated cells show that the contribution of NANA to the cell surface charge does not change with proliferative activity of the cells. This indicates that the low EPM of rapidly proliferating CFU cannot be explained by a decrease in the surface NANA density.

It should be noted that the differences and similarities in EPM among nontreated CFU (normal as well as rapidly proliferating) cannot be related to the surface density of neuraminidase susceptible NANA groups as measured by the reduction in EPM. This indicates that NANA groups which are not split off by neuraminidase and other charged molecules contribute to a significant extent to the CFU surface charge.

The results discussed in this chapter can be summarized as follows:

a) CFU-s have a higher modal EPM than GM CFU-c 1,2 and 3. This difference in modal EPM is smaller when the CFU are rapidly proliferating.
b) Rapidly proliferating CFU have a somewhat lower modal EPM than have normal CFU.
c) Although rapidly proliferating CFU-s are characterized by a relatively low modal EPM, the resting and cycling CFU-s which are thought to be present under normal conditions could not be distinguished on the basis of their EPM.
d) It is postulated that the density of neuraminidase susceptible NANA groups on the cell surface decreases during the development from CFU-s through GM CFU-c 3. These differences are not dependent on the proliferative state of the CFU.
e) Although CFU-s and GM CFU-c 1 can be distinguished on the basis of their modal EPM, these cell types probably have a similar neuraminidase susceptible NANA density on their surfaces.

CHAPTER 4

SENSITIVITY OF CFU-s TO NEURAMINIDASE

4.1 INTRODUCTION

In the previous chapter, it was shown by means of cell electrophoresis that incubation of cells with neuraminidase changes the cell surface properties. A differential effect of this enzyme on the electrophoretic mobility of CFU-s and GM CFU-c 1, 2 and 3 was demonstrated. An additional effect of neuraminidase treatment is a reduction in the number of CFU-s to about 25% of control numbers. This raises the question as to whether CFU-s which are present after neuraminidase treatment are representative for the total CFU-s population or whether the CFU-s population contains subpopulations which are differentially affected by neuraminidase. This chapter describes experiments in which the colony forming cells still detectable after neuraminidase treatment were investigated in terms of certain functional and physical properties.

4.2 NEURAMINIDASE TREATMENT IS NOT CYTOTOXIC FOR CFU

The reduction in CFU-s numbers after incubation with neuraminidase is illustrated in the upper graph of Figure 23. The number of CFU-s decreases with increasing concentrations of neuraminidase. The curve reaches a plateau at a neuraminidase concentration of about 1×10^{-3} IU.ml^{-1}, but the plateau value shows variation. Bone marrow cells were routinely incubated with a neuraminidase concentration of 4×10^{-3} IU.ml^{-1} and this resulted in a mean "survival" of CFU-s of 26%. This value can be considered as the mean plateau level obtained after neuraminidase treatment. The neuraminidase induced reduction in CFU-s numbers might be due to either cytotoxicity or altered organ distribution (decrease in spleen seeding efficiency) of colony forming cells. These two possible effects of neuraminidase may be random; alternatively, they may be selective, i.e., there may be two subpopulations of colony forming cells with respect to enzyme sensitivity and/or ability to lodge in the spleen: one neuraminidase sensitive, the other neuraminidase resistant.

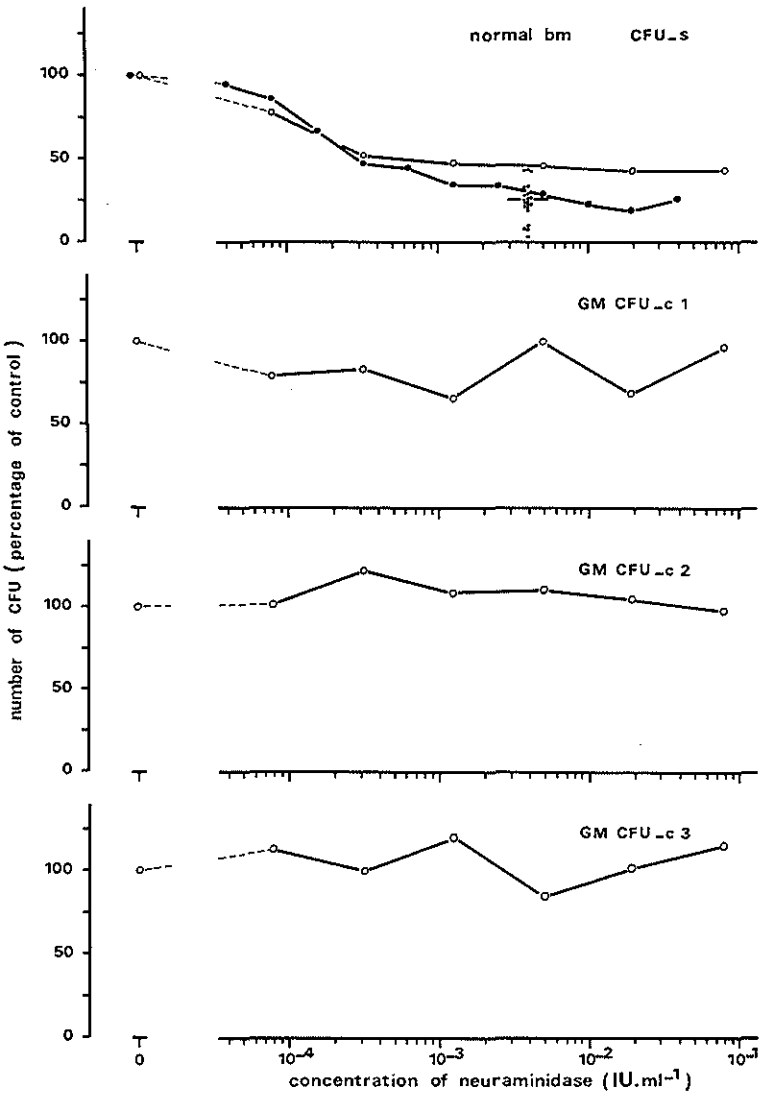


Figure 23:

Recovery of CFU-s and GM CFU-c 1,2 and 3 after in vitro treatment with increasing concentrations of neuraminidase

Unfractionated bone marrow cells were incubated for 1 h at 37°C in the presence of neuraminidase. The points represent the number of CFU per 10^5 nucleated cells injected or plated and are expressed as a percentage of the number of CFU detected after incubation without neuraminidase. Different symbols represent results of different experiments. The small dots in the top panel represent the number of CFU-s observed in 21 experiments in which cells were incubated with a fixed concentration of neuraminidase of 4×10^{-3} IU.ml^{-1}.

The reduction in CFU-s after incubation with neuraminidase might be simply explained by immediate cell kill. There are, however, several observations indicating that this explanation is unlikely. Incubation of cells with neuraminidase hardly affects cell viability as determined by fluorescein diacetate/propidium iodide fluorescence (Section 2.12, p. 52 , Table 4). Furthermore, neuraminidase treatment does not affect colony formation by GM CFU-c 1, 2 and 3. This is illustrated in the three lower graphs of Figure 23, which represent the number of the various GM CFU-c after incubation with increasing concentrations of neuraminidase. Up to a concentration of 8×10^{-2} IU.ml^{-1}, neuraminidase does not cause a significant change in colony formation. These results suggest that neuraminidase is not cytotoxic to CFU.

The other explanation for the reduction in CFU-s numbers might be found in an altered organ distribution of cells after modification of their surface composition by neuraminidase. Such a change in the behaviour of cells in vivo has been reported for lymphocytes, erythrocytes and bone marrow cells. After in vitro incubation with neuraminidase, lymphocytes and bone marrow cells accumulate in the liver and erythrocytes are rapidly cleared from the circulation by the liver as well as the spleen (Woodruff and Gesner, 1969; Freitas and de Sousa, 1976; Landaw, Tenforde and Schooley, 1977; Ploemacher and Van Soest, 1979 a; b). To determine whether such a mechanism might apply to the in vivo colony forming cells, the following experiment was carried out. Lethally irradiated recipient mice were injected intravenously with varying doses (1.6×10^{-2} to 2.5×10^{-1} IU) of neuraminidase one-half hour prior to the injection of neuraminidase treated cells. Cells and plasma glycoproteins affected by neuraminidase in the recipient mice would be expected to compete for receptors with the injected neuraminidase treated colony forming cells (e.g., in the liver). As a consequence, the colony forming cells should show a normal distribution in the various organs. Indeed, a much smaller reduction in CFU-s was observed in these recipients (Figure 24). Pretreatment of recipient mice with high doses of neuraminidase changes the "survival" of neuraminidase treated CFU-s from 25% to about 80% of control numbers. Injection of nontreated bone marrow cells into neuraminidase treated mice results in normal colony numbers. These observations are in agreement with data on CFU-s recovery after neuraminidase treatment by simultaneous injection of neuraminidase treated bone marrow cells and mouse erythrocytes (Ploemacher and Van Soest, 1979 a). The possibility of recovering a major proportion of the enzyme treated CFU-s demonstrates that neuraminidase does not have an important cytotoxic effect and that the neuraminidase induced reduction in CFU-s can be explained by an altered organ distribution of colony forming cells.

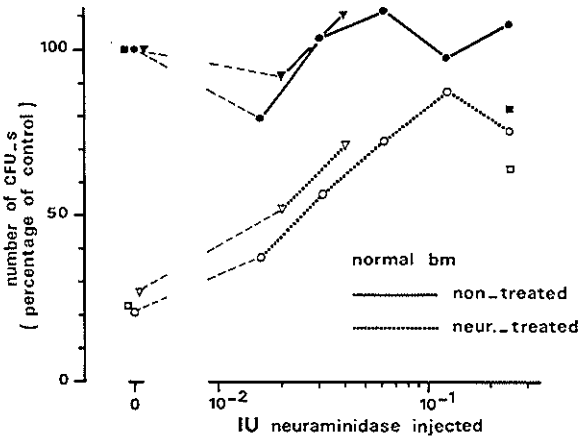


Figure 24:

In vivo recovery of neuraminidase treated colony forming cells after pretreatment of recipient mice with increasing amounts of neuraminidase
Lethally irradiated recipient mice were injected intravenously with neuraminidase 30 min prior to the injection of nontreated or neuraminidase treated bone marrow cells. The points represent the number of CFU-s per 10^5 nucleated cells injected and are expressed as a percentage of the number of CFU-s observed after injection of nontreated cells into nontreated irradiated mice. Differently shaped symbols represent the results of different experiments.

4.3 SOME FUNCTIONAL PROPERTIES OF NEURAMINIDASE "SURVIVING" CFU-s

A mechanism of trapping a fixed proportion of neuraminidase treated colony forming cells in organs other than spleen may explain the limited decrease in CFU-s number, even if all colony forming cells are identically affected by the enzyme. However, since it is technically difficult to characterize the "missing" neuraminidase treated colony forming cells, one has to consider the possibility that the enzyme treatment discriminates between functionally and physically different CFU-s. Therefore, the properties of colony forming cells which "survive" the neuraminidase treatment were investigated and compared with those of nontreated colony forming cells.

Nontreated and neuraminidase treated CFU-s were compared with respect to their self-renewal capacity and spleen seeding efficiency according to the method described by Lahiri et al. (1970). Bone marrow cells were incubated with and without neuraminidase and injected into lethally irradiated mice. After 4 hours, 1 day, 4 days and 7 days, the number of CFU-s in the spleens of these mice was determined by injection of spleen cells into secondary irradiated recipients (Table 7).

TABLE 7

PROLIFERATION OF NONTREATED AND NEURAMINIDASE TREATED CFU-s IN THE SPLEENS OF IRRADIATED RECIPIENTS

A. Incidence of CFU-s in the initial bone marrow cell suspension

treatment of cells	CFU-s per 10^5 cells	CFU-s per $5x10^6$ cells	percentage
control	26.3 (27.2; 25.4)	1315 (1360; 1270)	100
neuraminidase	5.8 (5.6; 5.9)	288 (280; 295)	21.9 (20.6; 23.2)

B. CFU-s per spleen at various time intervals after injection of 5 x 10^6 bone marrow cells

	4 hours		1 day		4 days		7 days	
treatment of cells	CFU-s per spleen	percen-tage	CFU-s per spleen	percen-tage	CFU-s per spleen	percen-tage	CFU-s per spleen	percen-tage
control	199 (167; 230)	100	72.9 (89.7;56.1)	100	391 (-; 391)	100	2988 (2936;3040)	100
neuraminidase	43.5 (18.0; 69.0)	21.9 (10.8;30.0)	21.0 (24.3;17.6)	28.8 (27.1;31.4)	83.8 (-; 83.8)	21.4 (-; 21.4)	1174 (776;1572)	39.3 (26.4;51.7)

Figures represent mean and individual values of 2 experiments

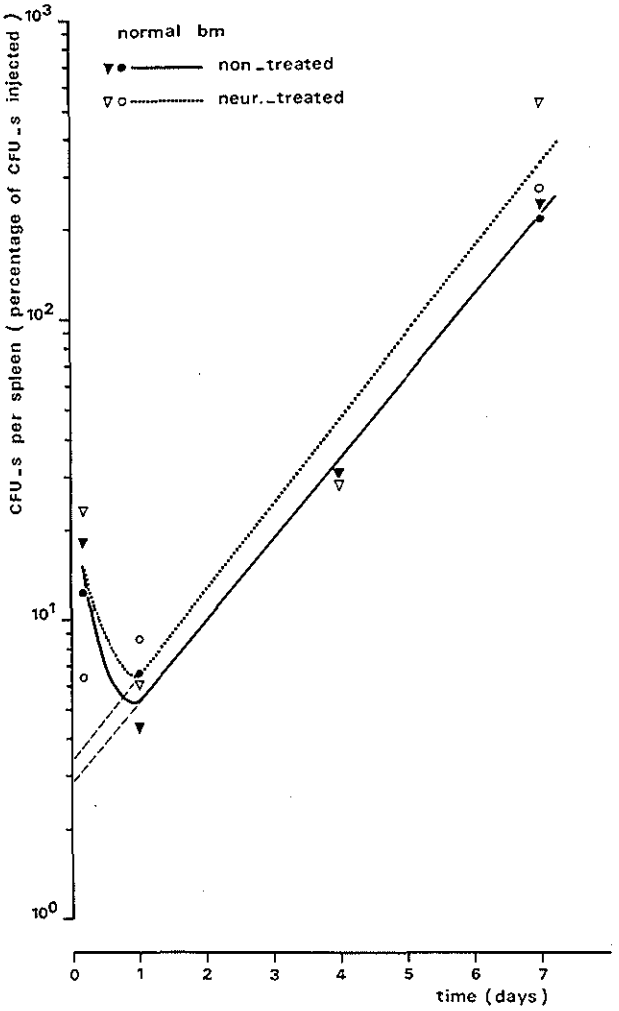


Figure 25:

<u>The proliferation of injected nontreated and neuraminidase treated CFU-s in the spleens of lethally irradiated recipient mice</u>
The curves represent the number of CFU-s recovered from spleens of primary recipients at different time intervals after injection of 5 x 10^6 nontreated (solid line; closed symbols) or neuraminidase treated (dotted line; open symbols) bone marrow cells. Each curve is expressed as a percentage of the number of CFU-s injected. The number of CFU-s injected is calculated from the colony number in primary recipients 9 days after injection of 0.5 x 10^5 nontreated and 2 x 10^5 neuraminidase treated cells, respectively. Cells were injected within 3 h after irradiation. Differently shaped symbols represent the results of different experiments.

This retransplantation procedure is based on the assumption that the spleen seeding efficiency of the colony forming cells in the secondary recipient is identical to that in the primary recipient. Comparison of the numbers of CFU-s per spleen after injection of nontreated and neuraminidase treated cells shows that the ratio between these numbers is the same at any time point. This indicates that the difference in the initial number of CFU-s in the two experimental groups is maintained and that nontreated and neuraminidase treated CFU-s have an identical self-renewal capacity. The numbers of CFU-s per spleen from Table 7 are plotted in Figure 25 as a percentage of the number of CFU-s injected. The dip in the CFU-s growth curves at about 1 day has been explained by cell loss from the spleen (Lahiri et al., 1970), probably due to a reduction in the spleen volume after irradiation (Lord and Hendry, 1973). Extrapolation of the exponential part of the growth curves to time point zero gives the percentage of colony forming cells which permanently lodge in the spleen (spleen seeding efficiency). For both enzyme treated and nontreated colony forming cells, a seeding efficiency of about 3 % can be determined; this is close to the reported value of 5% (Lahiri et al., 1970). It can be concluded that neuraminidase "surviving" and nontreated colony forming cells cannot be distinguished on the basis of their spleen seeding efficiency.

Because it would be expected that the neuraminidase induced altered organ distribution of colony forming cells also occurred in the secondary recipients, it was striking that, even at 4 or 24 h after injection of both neuraminidase treated and nontreated cells, the percentages of CFU-s in the spleens (as tested in secondary recipients) were similar. This may indicate that neuraminidase treatment selects for a subpopulation of colony forming cells which are not affected in their spleen seeding efficiency. However, to accept this explanation it must be assumed that the neuraminidase induced change in surface properties of CFU-s (as measured by electrophoresis; Section 3.3., p. 66) is a phenomenon completely independent of the reduction in CFU-s numbers after neuraminidase treatment. Another explanation might be that colony forming cells overcome the neuraminidase induced changes in cell surface properties within a few hours (e.g., by rapid cell membrane repair) and thus recover normal seeding properties. This capacity might be a property of either all colony forming cells or of cells of a selective population.

In another series of experiments, the in vitro growth rate of nontreated and neuraminidase treated CFU-s was investigated. In the same experiments, data were obtained on the recovery of normal in vivo

seeding properties (cell membrane repair) of the neuraminidase treated colony forming cells. For this purpose, liquid cultures stimulated by feeder layers of bone-marrow-derived adherent fibroblastoid cells were used (Friedenstein et al., 1974; Dexter et al., 1977; Zipori and Bol, 1979). These cultures have been shown to be optimal for CFU-s maintenance. Identical numbers of nontreated and neuraminidase treated bone marrow cells were cultured in parallel and the presence of CFU-s in the cultures was tested for at various time intervals over a period of 7 days (Figure 26). In the cultures initiated with nontreated bone marrow cells, the number of CFU-s decreases to about 35% of the original number in four days. In the cultures of neuraminidase treated bone marrow cells, the initial number of CFU-s is low due to the altered organ distribution of colony forming cells in the irradiated recipient mice used for the CFU-s assay. The number of CFU-s increases to the same plateau value as observed in the control cultures of nontreated cells in 3 to 4 days. The increase in CFU-s numbers in the cultures of enzyme treated cells cannot be explained by the proliferation of a surviving CFU-s population, since it was shown that all CFU-s can be recovered in neuraminidase injected recipients (Section 4.2). This indicated that all neuraminidase treated CFU-s are able to proliferate and differentiate. Therefore, the identical plateau value of CFU-s in

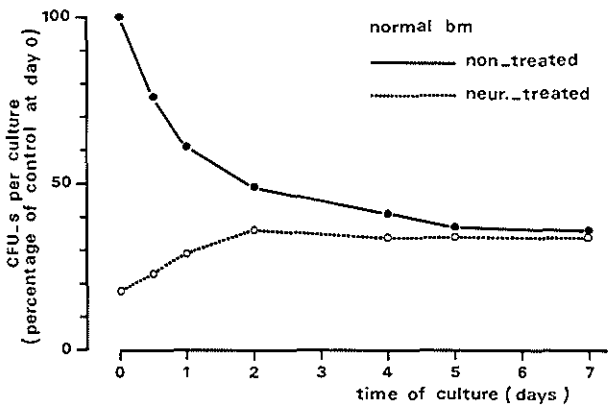


Figure 26:

Recovery of nontreated and neuraminidase treated CFU-s from liquid cultures
Feeder layers of adherent fibroblastoid cells are obtained by plating bone marrow cells from 2-week-old mice into liquid cultures. After a culture period of 3 weeks in which medium is refreshed weekly, greatly extended colonies of adherent fibroblastoid cells are formed. These adherent cells exert activities which allow the maintenance of CFU-s. Nontreated and neuraminidase treated bone marrow cells were seeded on top of the feeder cell layers. The curves represent the number of CFU-s per culture at different time intervals after plating and are expressed as a percentage of the number of nontreated CFU-s at day 0. The results represent the mean of 4 experiments.

cultures of nontreated and neuraminidase treated cells indicates that neuraminidase treatment does not influence the in vitro growth pattern of colony forming cells. The recovery of normal in vivo seeding properties after neuraminidase treatment (by cell membrane repair or by formation of normal progeny cells) is however much slower than observed in vivo. This difference might be explained by assuming that the in vitro environment is not optimal for cell membrane repair. Furthermore, since neuraminidase cannot be washed from the cell surface (Sedlacek and Seiler, 1974), it may be transferred to the cultures and exert its activity there for a longer period of time than in vivo.

Finally, neuraminidase treated bone marrow cells were tested for their capacity to prevent mortality in lethally irradiated mice. If neuraminidase "surviving" haemopoietic cells are not functionally different from the total population, they should be able to restore the haemopoietic system. However, the number of CFU-s in the spleen (see Table 7) and also in the bone marrow (Ploemacher, personal communication) is lower after injection of neuraminidase treated cells than after injection of nontreated cells. This suggests that, when bone marrow cells used for transplantation are treated with neuraminidase, fewer cells will arrive in haematological tissues and that more cells must be injected to reconstitute the haemopoietic system. Figure 27 shows the percentage of surviving animals at day 30 after irradiation

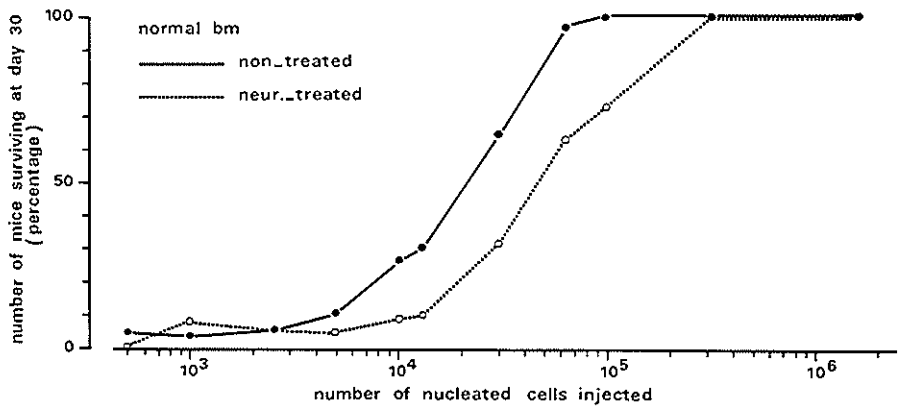


Figure 27:

Radiation protection capacity of nontreated and neuraminidase treated bone marrow cells

Mice were lethally irradiated and injected with varying numbers of nontreated or neuraminidase treated bone marrow cells. The curves represent the number of mice surviving at day 30 after irradiation and transplantation and are expressed as a percentage of the initial number of mice per group. The mean result of 3 experiments is shown. Ten mice per group were used in each experiment.

and transplantation of varying numbers of nontreated and neuraminidase treated bone marrow cells. When the donor cells had been treated with neuraminidase, about 2.5 times more cells were needed for 50% survival at day 30. This indicates that the neuraminidase "surviving" cells do not differ from nontreated cells in their potential to restore the haemopoietic system, but that, in comparison to nontreated cells, a larger proportion of neuraminidase treated cells is probably trapped in nonhaematological organs.

The data described in this paragraph show that neuraminidase "surviving" colony forming cells cannot be distinguished from the total nontreated population of colony forming cells on the basis of self-renewal, spleen seeding efficiency, in vitro maintenance and potential to prevent mortality in mice after lethal irradiation. The only unique characteristic of neuraminidase "surviving" colony forming cells might be the capacity for rapidly restoring normal seeding properties.

4.4 THE EFFECTS OF NEURAMINIDASE ARE NOT RELATED TO THE ELECTROPHORETIC MOBILITY OF CFU-s.

In a continuation of the search for heterogeneity of CFU-s in relation to the effects of neuraminidase, it was investigated whether neuraminidase treatment allows physical distinction between subpopulations of CFU-s. The heterogeneity in the EPM properties of nontreated CFU-s (Chapter 3, Section 3.2) may reflect differences in surface NANA content. This was a reason to test whether the EPM of CFU-s was related to the negative effect of neuraminidase on the spleen colony formation. Bone marrow cells were separated by electrophoresis and the resulting cell fractions were incubated with and without neuraminidase. The EPM distributions of CFU-s in the two experimental groups are shown in Figure 28. In each EPM fraction, the number of CFU-s was identically reduced by neuraminidase. When the EPM distribution of the neuraminidase "surviving" CFU-s is normalized to its peak value, it is clearly shown that it does not differ significantly from the EPM distribution of total CFU-s. The bottom panel of Figure 28 shows the results of a similar experiment carried out with cells from regenerating bone marrow. Also in the rapidly proliferating CFU-s population, no differences were detected between the EPM distributions of total CFU-s and of the neuraminidase "surviving" CFU-s. It is concluded therefore that the reduction in CFU-s number by neuraminidase is not related to the heterogeneity in EPM within the CFU-s population.

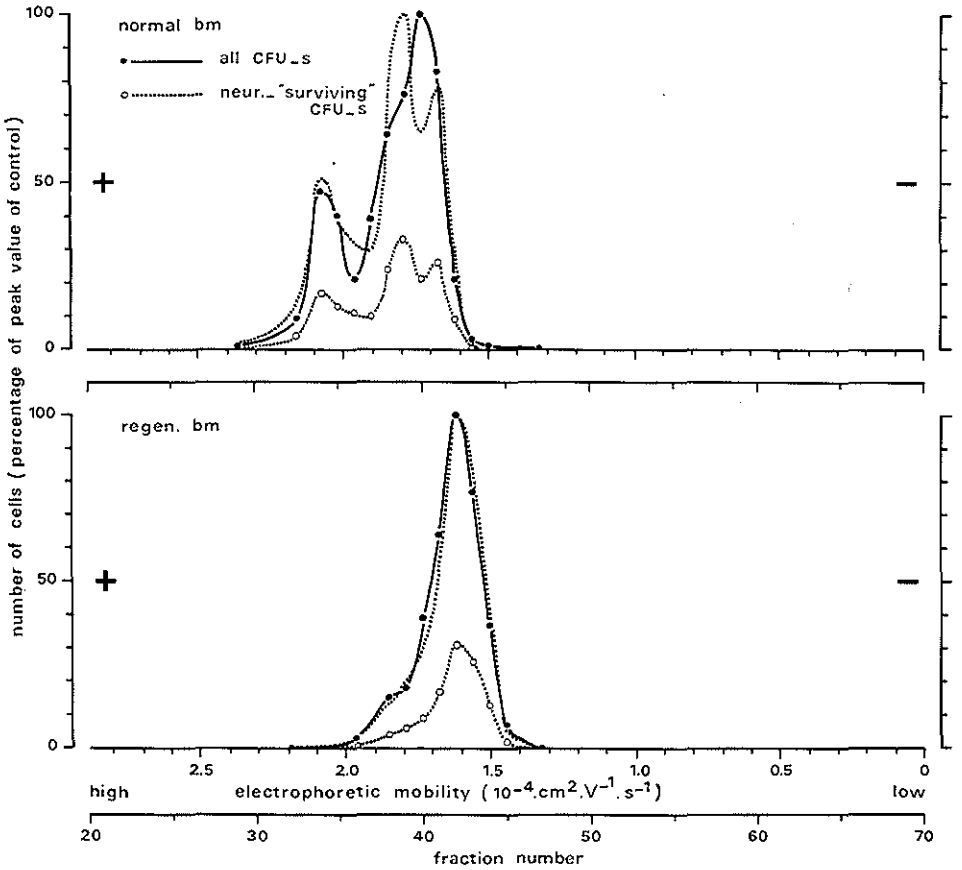


Figure 28:

Electrophoretic mobility distributions of all CFU-s and of CFU-s "surviving" neuraminidase treatment

Bone marrow cells were separated by electrophoresis and each cell fraction was divided into 2 groups which were incubated for 1 h at 37°C without or in the presence of neuraminidase. The solid line (closed circles) represents the EPM distribution of CFU-s which received the control treatment. The dotted line (open circles) represents the EPM distribution of CFU-s which "survived" the neuraminidase treatment. The profiles are calculated as CFU-s per fraction and are expressed as a percentage of the maximum value of the control CFU-s profile.

The dotted line without points represent the EPM distribution of the neuraminidase "surviving" CFU-s expressed as a percentage of its own maximum value.

Top panel: EPM distributions of CFU-s from normal bone marrow.

Bottom panel: EPM distributions of CFU-s from regenerating bone marrow.

4.5 THE EFFECTS OF NEURAMINIDASE MAY BE RELATED TO THE CYCLING STATE OF CFU-s

In experiments with normal and regenerating bone marrow, a difference between these bone marrow cell sources was observed with respect to the neuraminidase induced reduction in CFU-s. When regenerating bone marrow is incubated with increasing concentrations of neuraminidase, the number of CFU-s decreases. As observed for CFU-s from normal bone marrow, CFU-s from regenerating bone marrow reach a plateau at a neuraminidase concentration of about 1×10^{-3} IU.ml^{-1}. However, the CFU-s plateau value obtained with regenerating bone marrow is higher than that obtained with normal bone marrow. Figure 29 shows the percentage survival of CFU-s from the two bone marrow sources after incubation with a neuraminidase concentration of 4×10^{-3} IU.ml^{-1}.

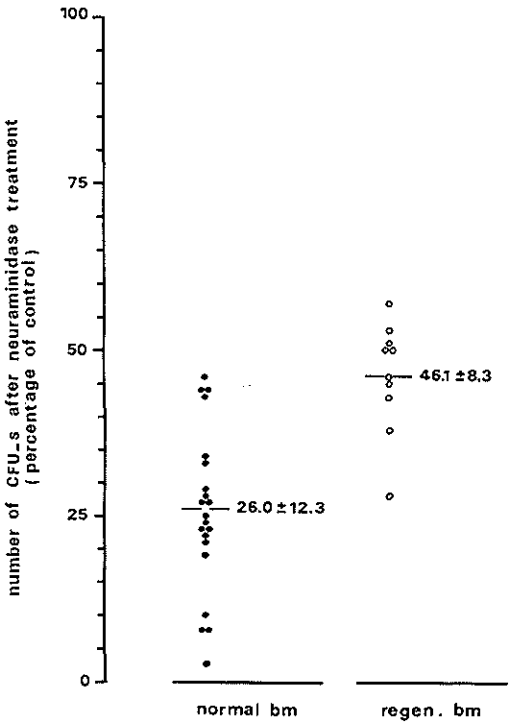


Figure 29:

Effect of neuraminidase treatment on CFU-s from normal and regenerating bone marrow
Cell suspensions from normal and regenerating bone marrow were incubated with neuraminidase and tested for the presence of CFU-s. The number of CFU-s was expressed in percentage of the number present in the control treated cell suspension. The points represent the results of individual experiments. The mean and standard deviation of the "survival" of CFU-s from normal and regnerating bone marrow is 26.0 ± 12.3 (21 experiments) and 46.1 ± 8.3 (10 experiments), respectively.

CFU-s from normal bone marrow are reduced to 26%, whereas those from regenerating bone marrow are reduced to 46% of control numbers. This may indicate a difference between normal and rapidly proliferating in vivo colony forming cells with respect to spleen seeding properties after neuraminidase treatment.

A technical aspect of the CFU-s assay was first investigated. Cell suspensions prepared from regenerating bone marrow contain a higher concentration of erythrocytes and a lower concentration of CFU-s than those from normal bone marrow. To obtain a suitable number of spleen colonies, the number of regenerating bone marrow cells injected is usually chosen to be 4 times as large as the number of normal bone marrow cells. As a result, the number of accompanying cells injected for the CFU-s assay is relatively large. Large numbers of neuraminidase treated accompanying cells may have a similar positive effect on the spleen seeding efficiency of colony forming cells as that observed after pretreatment of recipient mice with neuraminidase (Section 4.2) or with neuraminidase treated erythrocytes (Ploemacher and Van Soest, 1979a). To investigate this possibility, neuraminidase treated cells from regenerating bone marrow were irradiated (to inactivate CFU-s) and injected along with neuraminidase treated normal bone marrow cells. The results are shown in Table 8. The upper part of this table shows the effect of neuraminidase on CFU-s from regenerating bone marrow. After incubation with the enzyme, the number of CFU-s is reduced to 52% of the control numbers. When neuraminidase treated cells are irradiated with 4.00 Gy, no colony formation is observed in the spleens of the recipient mice. Injection of nontreated cells along with neuraminidase treated and subsequently irradiated cells has a slight negative effect on CFU-s. The lower part of Table 8 shows the effect of neuraminidase treated and irradiated regenerating bone marrow cells on the CFU-s from normal bone marrow. After incubation with neuraminidase, normal bone marrow CFU-s show a reduction to 25% of control numbers. When nontreated or neuraminidase treated normal cells are injected along with neuraminidase treated and subsequently irradiated regenerating bone marrow cells, CFU-s numbers which are identical to the number found in the absence of extra accompanying cells are observed. The results of these experiments demonstrate that the difference in reduction in CFU-s from normal and regenerating bone marrow is not likely to be due to technical variations but seems to reflect a difference in cellular properties.

TABLE 8

EFFECT OF NEURAMINIDASE TREATMENT ON CFU-s FROM NORMAL AND REGENERATING BONE MARROW
The influence of accompanying neuraminidase treated cells

R = Regenerating bone marrow
N = Normal bone marrow

code	treatment of cells	nucleated cells per mouse	CFU-s/10^5 cells	percentage of control
R1	control	2×10^5	4.7 ± 0.5[a]	100 ± 14.7
R2	neuraminidase	4×10^5	2.4 ± 0.2	52.2 ± 7.6
R3	neuraminidase and radiation[b]	4×10^5	0	0
R1 +R3		2×10^5 + 4×10^5	4.1 ± 0.5	87.1 ± 13.2
N1	control	0.5×10^5	39.2 ± 2.8	100 ± 10.0
N2	neuraminidase	1×10^5	9.8 ± 1.0	25.0 ± 3.1
N1 +R3		0.5×10^5 + 4×10^5	37.4 ± 2.7	95.4 ± 9.7
N2 +R3		1×10^5 + 4×10^5	11.2 ± 1.1	28.6 ± 3.4

a Accuracy, calculated by assuming a Poisson distribution of colony counts.
b In vitro irradiation of cells with 4.00 Gy.

It has been reported that, in normal bone marrow, a relatively high proportion of CFU-s is in a resting state (G_0), while CFU-s in regenerating bone marrow are mainly in active cell cycle (G_1-S-G_2). It is suggestive therefore that the differential effect of neuraminidase on the CFU-s from the two bone marrow sources is related to the proliferative state of the in vivo colony forming cells. This possible relationship was initially investigated by comparing the number of S phase cells in the total nontreated and in the neuraminidase "surviving" CFU-s populations. As shown in Table 9, when normal bone marrow is used, the survival of CFU-s after incubation with ^{3}H-thymidine is 70% in the neuraminidase "surviving" in comparison to 90% in the total nontreated CFU-s population. Although this is a moderate difference, it can be hypothesized that neuraminidase treatment selects in part for cycling cells and that the spleen seeding efficiency of cycling cells is less reduced after neuraminidase treatment than that of resting cells. This hypothesis was further tested.

TABLE 9

EFFECT OF ^{3}H-THYMIDINE TREATMENT ON TOTAL CFU-s AND NEURAMINIDASE "SURVIVING" CFU-s FROM NORMAL BONE MARROW

		Normal bone marrow	
	treatment of cells	CFU-s per 10^5 cells	percentage
A. total CFU-s	control	32.7 ± 1.9[a]	100 ± 8.1
	^{3}H-thymidine	29.3 ± 2.6	89.6± 9.3
B. neuraminidase "surviving" CFU-s	control	7.1 ± 0.5	100 ± 9.7
	^{3}H-thymidine[b]	4.9 ± 0.4	69.0± 7.7

a Accuracy, calculated by assuming a Poisson distribution of colony counts.

b Cells were incubated with ^{3}H-thymidine before, simultaneously or after incubation with neuraminidase. These three experimental groups gave identical results.

Since CFU-s in various phases of the cell cycle (G_0-G_1-S-G_2) have been reported to be different in sedimentation velocity, the neuraminidase "surviving" CFU-s were characterized with respect to this parameter. Figure 30 shows the sedimentation velocity distributions of total CFU-s, CFU-s surviving ^{3}H-thymidine treatment and CFU-s "surviving" neuraminidase treatment. These distributions were determined simultaneously. It is shown that ^{3}H-thymidine treatment selectively affects CFU-s with a relatively high sedimentation velocity, which represent S phase cells of the cycling CFU-s compartment (van den Engh, 1976; Visser et al., 1977). A decrease in CFU-s after neuraminidase treatment is found over the entire sedimentation velocity range. However, a relatively smaller neuraminidase induced decrease in CFU-s is observed in the velocity range where ^{3}H-thymidine sensitive CFU-s are found. This is illustrated in the bottom panel of Figure 30, in which the three distributions are normalized to their peak value. In the high sedimentation velocity range, the relative number of ^{3}H-thymidine surviving CFU-s is smaller and the relative number of neuraminidase "surviving" CFU-s is larger than that of nontreated CFU-s. These data confirm the hypothesis that a relatively large proportion of the neuraminidase "surviving" CFU-s is in active cell cycle.

Cell separation on the basis of differences in buoyant density properties have been reported to allow distinction between resting (G_0) and cycling (G_1-S-G_2) CFU-s (Visser et al., 1977). On the basis of the observations described above, the neuraminidase induced reduction in CFU-s would therefore be expected to be related to the buoyant density of CFU-s. Since the modal densities of resting and cycling CFU-s have been reported to be 1.070 and 1.075 g.cm^{-3}, respectively, bone marrow cells were separated into two fractions by a "density cut" at 1.072 g.cm^{-3}. The effects of ^{3}H-thymidine and neuraminidase on CFU-s from unfractionated material and from fractions of cells with a density lower or higher than 1.072 g.cm^{-3} are shown in Table 10. Surprisingly, no differences were detected between low and high density CFU-s, neither with respect to the effect of ^{3}H-thymidine nor to that of neuraminidase. Similar results were obtained after cell separation in a continuous density gradient. The complete buoyant density distributions of total CFU-s, CFU-s surviving ^{3}H-thymidine treatment and CFU-s "surviving" neuraminidase treatment are shown in Figure 31. Reduction in CFU-s by ^{3}H-thymidine or neuraminidase is observed over the entire density range. The similarity of the various cell profiles is illustrated in the bottom panel of Figure 31, in which each profile is expressed as a percentage of its own peak value. The density distributions of ^{3}H-thymidine surviving and neuraminidase "surviving"

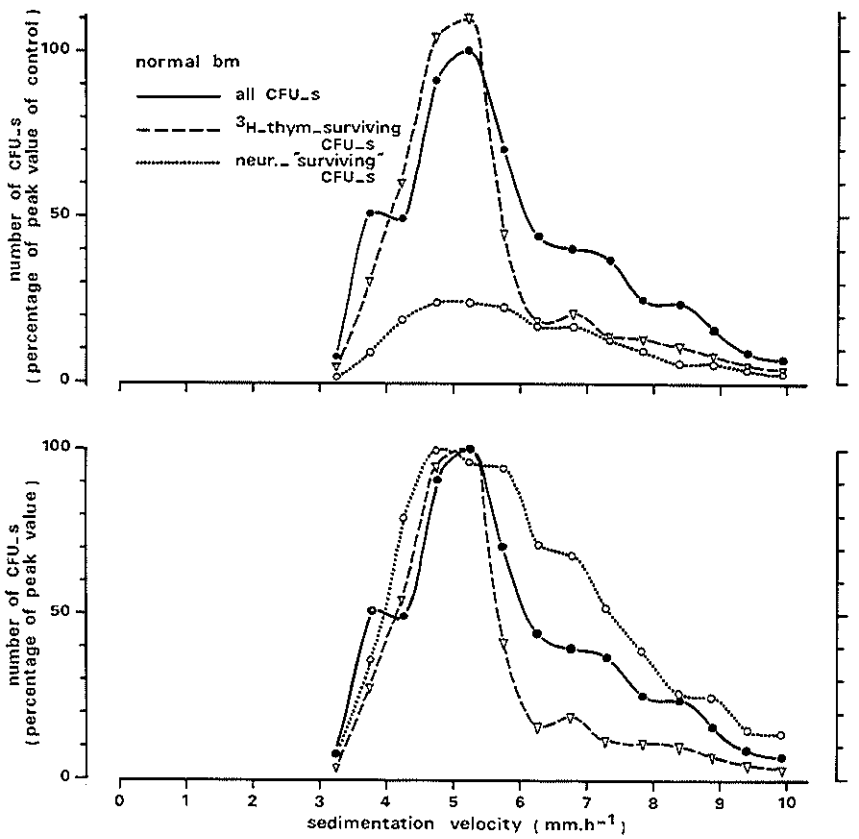


Figure 30:

Sedimentation velocity distributions of all CFU-s, CFU-s surviving ^{3}H-thymidine treatment and CFU-s "surviving" neuraminidase treatment

Bone marrow cells were separated by sedimentation at unit gravity and each cell fraction was divided into 3 groups which were incubated at 37° C in the presence of ^{3}H-thymidine (30 min) or in the presence of neuraminidase (1 h) or without additions (handled as a control for the ^{3}H-thymidine treatment). The solid line represents the sedimentation velocity distribution of CFU-s after control treatment, the dashed line that of CFU-s surviving ^{3}H-thymidine treatment and the dotted line that of CFU-s "surviving" neuraminidase treatment. The profiles are calculated as CFU-s per fraction.

Top panel: Profiles expressed as a percentage of the maximum value of the control CFU-s profile.

Bottom panel: Profiles expressed as a percentage of their own maximum value.

TABLE 10

EFFECT OF NEURAMINIDASE AND ^{3}H-THYMIDINE TREATMENT ON CFU-s OF DIFFERENT BUOYANT DENSITY

		Unfractionated cells		Low density cells (density < 1.072 g.cm^{-3})		High density cells (density > 1.072 g.cm^{-3})	
treatment of cells	number of experiments	CFU-s/10^5 cells	percentage	CFU-s/10^5 cells	percentage	CFU-s/10^5 cells	percentage
control	4	29.3 (26.0 - 34.0)	100	144.6 (94.4 -196.0)	100	12.2 (9.0 - 15.8)	100
neuraminidase	4	6.5 (5.0 - 7.3)	22.2 (19.1-26.5)	38.0 (24.5 - 48.0)	26.3 (23.4-33.6)	3.3 (2.1 - 4.5)	27.1 (21.7-34.1)
control	2	31.2 (28.4; 34.0)	100	83.0 (48.0; 118.0)	100	13.5 (11.2; 15.8)	100
^{3}H-thymidine	2	28.3 (26.0; 30.6)	90.7 (91.6;90.0)	92.0 (50.0; 134.0)	110.8 (104.2;113.6)	14.9 (14.2; 15.5)	110.0 (126.8;98.1)

Figures represent mean and limits of range observed.

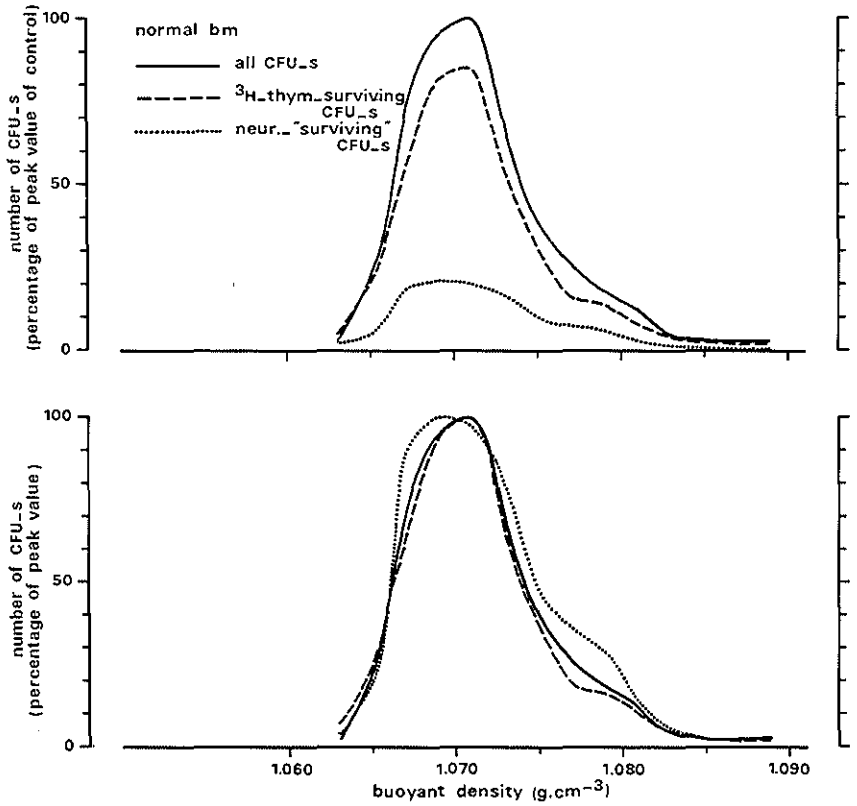


Figure 31:

Buoyant density distributions of all CFU-s, CFU-s surviving ^{3}H-thymidine treatment and CFU-s "surviving" neuraminidase treatment

Bone marrow cells were separated by equilibrium density centrifugation and each cell fraction was divided into 3 groups which were incubated at 37°C in the presence of ^{3}H-thymidine (30 min) or in the presence of neuraminidase (1 h) or without additions (handled as a control for the ^{3}H-thymidine treatment). The solid line represents the buoyant density distribution of CFU-s after control treatment, the dashed line that of CFU-s surviving ^{3}H-thymidine treatment and the dotted line that of CFU-s "surviving" neuraminidase treatment. The profiles are calculated as CFU-s per fraction per density increment. The profiles represent the means of 2 experiments.

Top panel: Profiles expressed as a percentage of the maximum value of the control CFU-s profile.

Bottom panel: Profiles expressed as a percentage of their own maximum value.

CFU-s do not deviate systematically from that of all CFU-s. Although there is a tendency that high density CFU-s are somewhat less reduced in number than low density CFU-s, this could not be correlated with the kill of CFU-s by ^{3}H-thymidine. This again indicates that low and high density CFU-s cannot be distinguished with respect to their sensitivity to ^{3}H-thymidine or neuraminidase.

The results from the experiments with CFU-s from normal and regenerating bone marrow and cell separation by sedimentation velocity indicate that the neuraminidase induced reduction in CFU-s may be related to the proliferative activity of CFU-s. Cycling CFU-s appear to be relatively less reduced than resting CFU-s. However, this could not be confirmed by separation of cells on the basis of differences in buoyant density because resting and cycling CFU-s could not obviously be distinguished.

4.6 DISCUSSION

The use of neuraminidase in the electrophoresis studies of the different CFU types has the disadvantage that the number of CFU-s is severely reduced by the enzyme treatment. As a result, only a proportion of the total CFU-s population can be investigated. Therefore the question arises whether neuraminidase treatment selects for a subpopulation of CFU-s. This is of special importance for the interpretation of the differences observed between CFU-s and GM CFU-c 1 with respect to their surface charge properties.

Incubation of bone marrow cells with increasing concentrations of neuraminidase reduces the number of CFU-s until a plateau value is reached. The reduction in CFU-s numbers is not due to cytotoxic effects of the enzyme, but to an altered organ distribution of the colony forming cells. Therefore, the plateau in CFU-s reduction might indicate the existence of two subpopulations of *in vivo* colony forming cells which differ with respect to the capacity to form spleen colonies after neuraminidase treatment. The results of the experiments described in this chapter show that neuraminidase "surviving" CFU-s cannot be distinguished from the total nontreated CFU-s population on the basis of self-renewal capacity, potential to lodge in the spleen, spleen seeding efficiency, potential to prevent mortality in lethally irradiated mice, electrophoretic mobility and buoyant density. Some differences in the neuraminidase induced reduction in CFU-s could be detected between CFU-s from normal and regenerating bone marrow and among CFU-s with different sedimentation velocities. CFU-s from re-

generating bone marrow are less reduced in number after neuraminidase treatment than are CFU-s from normal bone marrow. Separation of cells by differences in sedimentation velocity and treating the cells in the various fractions with neuraminidase or ^{3}H-thymidine shows that CFU-s numbers over the entire velocity range are decreased by neuraminidase treatment but that there is a relatively smaller CFU-s reduction in the region where CFU-s in S phase are found. These data suggest that the extent of the neuraminidase induced decrease in CFU-s numbers is dependent on the proliferative activity of CFU-s. The relatively small decrease in CFU-s with a high sedimentation rate from normal bone marrow can be thought to be due to a high production rate of cellular components in cells which are preparing for cell division. These cells can be assumed to exhibit a rapid formation of cell surface proteins by which the changes induced by neuraminidase are eliminated. This view may be supported by reports on variations in the synthesis rate of membrane proteins over the cell cycle. Using radioactive labelled precursors of cell membrane macromolecules and synchronized cultures of different cell lines, a greatly increased synthesis rate of membrane components during S phase and G_2 phase (Baserga, 1962; Bosmann and Winston, 1970) and a reduced synthesis rate during mitosis (Prescott and Bender, 1962) has been demonstrated. A high synthesis rate of membrane components in the neuraminidase "surviving" cells may also explain the rapid in vivo recovery of the normal seeding properties of colony forming cells observed in the retransplantation experiments (Section 4.3).

The data show that neuraminidase treatment selects only moderately for cycling cells. Since differences related to cell cycle phenomena cannot be regarded as differences related to the differentiation stage of cells, it can be concluded that neuraminidase treated colony forming cells which are still detectable by spleen colony formation are not different from the nondetectable colony forming cells. Furthermore, it was shown that in normal bone marrow, differences in EPM among CFU-s in different cell cycle phases could not be detected and that CFU-s from normal and regenerating bone marrow are similar in their surface NANA density (Chapter 3). Therefore, the CFU-s present after neuraminidase treatment can be regarded as representative for the total CFU-s population.

It has been suggested that NANA groups are essential for the specific homing of colony forming cells in the spleen (Tonelli and Meints, 1977). This is not supported by the present data. Firstly, the normal CFU-s number is recovered by pretreatment of the irradiated

recipient mice with neuraminidase. Secondly, no heterogeneity among colony forming cells can be detected and all cells can be regarded as being equally affected by neuraminidase. This indicates that the alteration in surface properties of colony forming cells by neuraminidase does not change the potential of these cells to lodge in the spleen and to start normal proliferation and differentiation.

The data described in this chapter can be summarized as follows:

a) The number of CFU-s is reduced to 25% of control numbers by treatment with neuraminidase. This reduction is due to an altered organ distribution of the in vivo colony forming cells.

b) The colony forming potential of neither CFU-s nor GM CFU-c 1, 2 and 3 is impaired by treatment with neuraminidase.

c) The extent of the neuraminidase induced reduction in CFU-s can be related to the cycling state of CFU-s. It is hypothesized that CFU-s in S and G_2 phases are relatively less reduced in numbers by the enzyme treatment than are CFU-s in G_0 or G_1 phase, due to differences in the rate of cell membrane repair.

d) CFU-s which "survive" neuraminidase treatment and total nontreated CFU-s are identical in many functional and physical properties, while the differences in cycle state between them are not reflected in their EPM. It is therefore concluded that CFU-s present after incubation with neuraminidase are representative for the total CFU-s population.

CHAPTER 5

MULTIPARAMETER SEPARATION OF CFU-s FROM OTHER BONE MARROW CELLS

5.1 INTRODUCTION

The cell separation techniques which are used to characterize cells with respect to physical, chemical and biological properties can also be applied to enrich for particular cell types. Since CFU-s are generally considered to represent pluripotent stem cells, many attempts have been made to isolate them for the purpose of characterizing them morphologically (Niewisch et al., 1967; Van Bekkum et al., 1971; Murphy et al., 1971; Rubinstein and Trobaugh, 1973). In addition to the morphological recognition of candidate pluripotent stem cells, the availability of a pure or highly enriched population of CFU-s would offer many other advantages. For example, *in vitro* studies on early events in differentiation, starting with single CFU-s, would be possible. Purified CFU-s could be extensively characterized with respect to physical and chemical properties, since it would no longer be necessary to keep the cells viable. In addition, antigenic properties of CFU-s could be more easily investigated in pure preparations. In turn, the newly analysed properties of CFU-s might be used to improve or to simplify the CFU-s isolation procedure.

The incidence of CFU-s in murine bone marrow is between 30 and 40 per 10^5 nucleated bone marrow cells. If a spleen seeding efficiency of 5% is accepted (Lahiri et al., 1970), these CFU-s numbers represent 600 to 800 colony forming cells per 10^5 nucleated bone marrow cells (0.6% to 0.8%). Consequently, to obtain a pure population of *in vivo* colony forming cells, the concentration has to be increased 125 to 165 times. These values represent the theoretical maximum enrichment factors.

Enrichment for CFU-s has been reported by several authors (Turner et al., 1967; Niewisch et al., 1967; Van Bekkum et al., 1971). The cells were separated on the basis of differences in buoyant density by means of continuous or discontinuous Ficoll or albumin gradients. Normal bone marrow cells, cells from spleen colonies or bone marrow cells from vinblastine/nitrogen mustard treated mice have been used as the starting material. The different procedures have been reported to re-

sult in CFU-s enrichment factors of 10 to 20. Only Van Bekkum and co-workers were able to combine relatively high enrichment factors with a sufficient yield. They have used the purified CFU-s fractions to investigate the morphology of the cells in those fractions and found a quantitative relationship between the number of CFU-s and the number of a cell type of particular morphology (Van Bekkum et al., 1971; Van Bekkum and Knaan, 1978).

This chapter describes the application of the free-flow cell electrophoresis technique in CFU-s purification procedures. Cell separation on the basis of differences in cell surface charge will be shown to be useful for enriching for CFU-s. In combination with specific modification of the cell membrane composition (in this case, the removal of NANA by neuraminidase treatment), electrophoresis can be carried out repeatedly to select for CFU-s on the basis of more than one surface property. Furthermore, the surface charge properties of CFU-s appear to be partly independent of other properties such as a relatively low buoyant density and a relatively high forward and low perpendicular light scattering intensity. As a result, high CFU-s concentrates can be obtained by combination of different cell separation techniques.

5.2 ENRICHMENT FOR CFU-s BY REPEATED ELECTROPHORESIS

For practical reasons, the determination of CFU-s enrichment was made on a "per fraction" basis using the peak position of nucleated cells as a reference. The CFU-s enrichment was determined by calculation of the ratio between the percentage of all CFU-s and the percentage of all nucleated cells collected in the various fractions. This calculation can be applied if the recoveries of CFU-s and nucleated cells are in the same order of magnitude after the separation procedures (i.e., nonspecific cell loss for both populations). For each separation step, the proportion of the total cell and CFU-s populations retained is determined. In this determination, only the selection by the separation procedure is included and cell loss is assumed to be nonselective (Section 2.10, p. 42 ; Table 3). Also the reduction in CFU-s after neuraminidase treatment is considered to be nonselective (Chapter 4) and, if separation procedures include incubation of cells with neuraminidase, the number of CFU-s is corrected for the reduction. In the calculation of the cell recovery, only cell loss during the separation procedures and not the selection of cells is taken into account.

As was described in Chapter 3, CFU-s can be partly separated from other nucleated bone marrow cells. The data indicate that electrophoresis of nontreated cells results in enrichment for CFU-s in relatively high EPM fractions and that, on separation of neuraminidase treated cells, the relatively low EPM fractions are enriched for CFU-s. The mean EPM distributions of nontreated and neuraminidase treated CFU-s and all nucleated cells expressed as a percentage of the total cell number are shown in the top and middle panels of Figure 32 A. The areas under the curves are normalized to 100% for both CFU-s and all nucleated cells. The CFU-s enrichment factors in various EPM fractions as calculated from the distributions shown in Figure 32 A are given in Table 11 A and B (first enrichment columns). Electrophoresis of nontreated bone marrow cells (Table 11 A) results in mean enrichment factors of about 2.0 in the midpart of the EPM distribution (fractions 34 to 38). On electrophoretic separation of neuraminidase treated cells (Table 11 B), the CFU-s enrichment factors are higher. Mean CFU-s concentrations increasing from 4 to 23 times normal can be found in the relatively low EPM fractions (fractions 55 to 70).

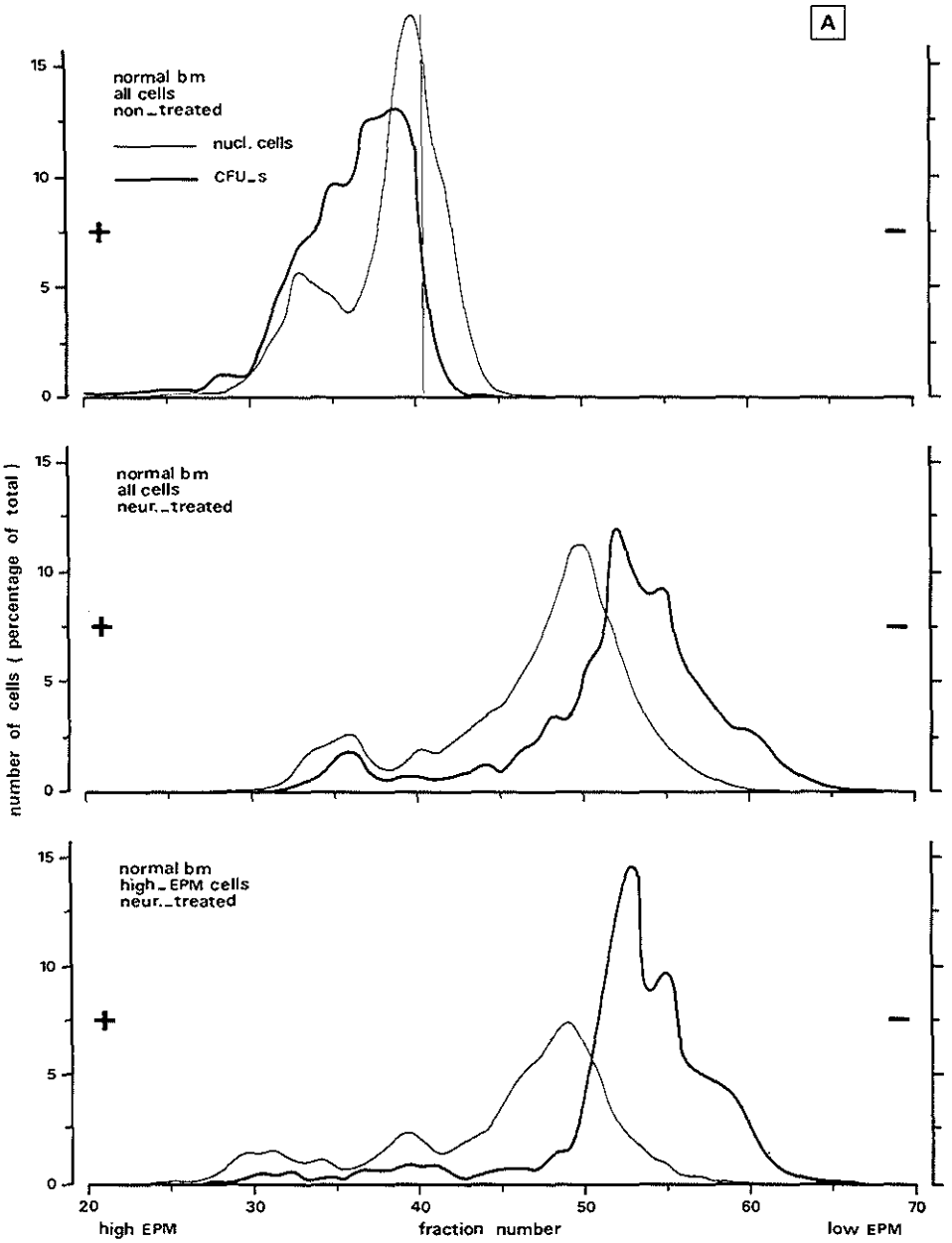
Electrophoresis of nontreated bone marrow cells can be used to eliminate about 25% of nucleated cells without a significant loss of CFU-s. This can be easily seen from the cumulative percentage curves of CFU-s and all nucleated cells shown in the top panel of Figure 32 B. The pool of high EPM fractions, including the peak fraction of nucleated cells, contains 96% of all CFU-s (varying between 91% and 99% in 6 experiments) and 73% of all nucleated cells (varying between 63%

Figure 32:

Enrichment for CFU-s by repeated electrophoresis in combination with neuraminidase treatment

A. The EPM distributions of nucleated cells and CFU-s after different electrophoresis procedures are shown. The profiles are calculated as cells per fraction and each profile is expressed as a percentage of the total cell number. The position of the CFU-s profiles is determined relative to the mean peak position of the nucleated cell profile. If neuraminidase treatment is involved, the CFU-s numbers are corrected for the neuraminidase induced reduction.
Top panel: EPM distributions of nontreated nucleated cells and CFU-s. The total cell number is set at 100% for both nucleated cells and CFU-s. The vertical line indicates the selection for "high EPM" cells.
Middle panel: EPM distributions of neuraminidase treated nucleated cells and CFU-s. The total cell number is set at 100% for both nucleated cells and CFU-s.
Bottom panel: EPM distributions of neuraminidase treated high EPM nucleated cells and CFU-s. High EPM cells were obtained by pooling fractions 11 to 40 after electrophoresis of nontreated cells (see top panel). The total cell number is set at 73% for nucleated cells and 96% for CFU-s.

B. The cumulative percentage curves for the EPM distributions of nucleated cells and CFU-s shown in Fig. 32A. The CFU-s enrichment factors in various pools of low EPM fractions containing 5%, 10%, 25% or 50% of the total CFU-s population are indicated along the curves.



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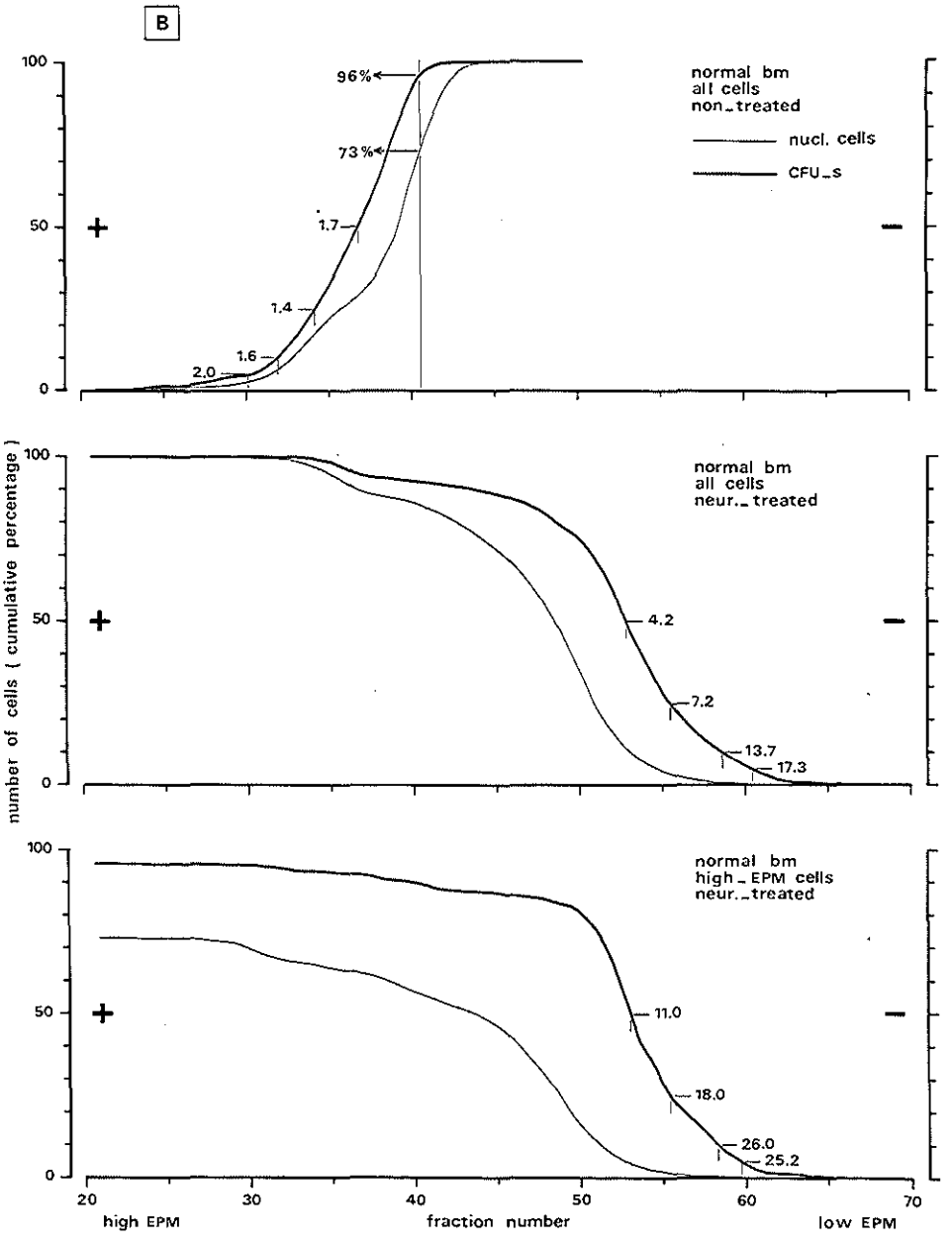


For legends, see p. 101

TABLE 11 A

ENRICHMENT FOR IN VIVO COLONY FORMING CELLS IN VARIOUS EPM FRACTIONS AFTER ELECTROPHORESIS OF NONTREATED BONE MARROW CELLS

	All cells (6 experiments)		Low density cells[a] density < 1.072 g.cm^{-3} (1 experiment)	
fraction number	percentage of total CFU-s	enrichment factor	percentage of total CFU-s	enrichment factor
21-30	5.5	1.8 (0.2 -4.9)[b]	11.9	5.2 ± 0.3[c]
31	3.2	1.5 (0.3 -4.1)	2.5	5.3 ± 0.5
32	4.9	1.4 (0.3 -3.2)	2.6	4.6 ± 0.5
33	6.7	1.2 (0.4 -3.0)	3.0	3.9 ± 0.4
34	7.4	1.5 (1.1 -1.9)	4.2	3.0 ± 0.3
35	9.6	2.1 (0.9 -3.0)	5.7	4.7 ± 0.5
36	9.6	2.5 (1.7 -4.3)	8.4	5.2 ± 0.6
37	12.3	2.4 (1.9 -3.4)	10.6	4.1 ± 0.4
38	12.6	1.5 (0.6 -2.0)	8.8	3.9 ± 0.4
39	13.0	0.9 (0.5 -1.3)	6.0	2.4 ± 0.3
40	11.0	0.6 (0.2 -1.1)	3.1	1.7 ± 0.2
41	3.3	0.3 (0.03-0.6)	1.0	1.1 ± 0.2
42-50	0.9	0.1 (0.02-0.1)	0.4	0.6 ± 0.1
21-50 (all)	100	1.0	68	3.6
Recovery	60%		40%	

[a] Low density cells are obtained by single-step density separation of bone marrow cells.

[b] Figures represent mean and limits of range observed

[c] Accuracy, calculated by assuming a Poisson distribution of cell counts and colony counts.

TABLE 11 B

ENRICHMENT FOR IN VIVO COLONY FORMING CELLS IN VARIOUS EPM FRACTIONS AFTER ELECTROPHORESIS OF NEURAMINIDASE TREATED BONE MARROW CELLS

fraction number	All cells (4 experiments)		High EPM cells[a] EPM > 1.74×10^{-4} $cm^2.V^{-1}.s^{-1}$ (3 experiments)		Low density cells[a] density < 1.072 $g.cm^{-3}$ (1 experiment)	
	percentage of total CFU-s	enrichment[d] factor	percentage of total CFU-s	enrichment[d] factor	percentage of total CFU-s	enrichment[d] factor
21-50	28.0	0.4 (0.2- 0.5)[b]	17.6	0.3 (0.2- 0.4)[b]	17.0	1.5 ± 0.1[c]
51	6.6	0.7 (0.4- 1.0)	8.2	1.8 (0.5- 3.0)	3.4	1.5 ± 0.2
52	12.0	1.8 (0.5- 2.9)	12.6	4.4 (1.6- 5.7)	5.5	2.5 ± 0.3
53	10.2	2.1 (0.9- 3.4)	14.6	7.2 (3.7-10.7)	11.0	9.9 ± 1.0
54	9.0	2.9 (1.2- 3.6)	8.9	6.9 (4.2- 8.4)	10.6	13.6 ± 1.2
55	9.1	4.3 (3.2- 4.7)	9.5	10.6 (4.0-16.2)	6.6	19.4 ± 1.2
56	6.0	4.3 (1.2- 6.5)	5.8	12.5 (7.3-16.3)	3.8	29.1 ± 3.7
57	4.8	5.9 (1.2-10.8)	5.0	14.2 (8.4-20.5)	2.2	20.4 ± 3.0
58	3.8	7.5 (2.9-19.8)	4.6	24.6 (18.4-28.0)	2.6	25.5 ± 3.8
59	3.0	9.2 (4.7-18.8)	4.0	27.2 (19.5-36.5)	2.0	30.4 ± 5.3
60	2.7	14.5 (5.4-31.9)	2.6	30.0 (20.9-40.2)	0.9	32.1 ± 8.4
61	2.1	23.0 (6.1-37.5)	1.2	33.5 (17.8-49.2)	1.0	39.6 ± 10.8
62-70	2.8	14.7 (9.5-19.6)	1.5	17.6 (3.8-33.0)	1.3	81.9 ± 26.0
21-70 (all)	100	1.0	96	1.3	68	3.6
Recovery	25%		15%		20%	

[a] High EPM cells and low density cells are obtained by electrophoresis and single-step density separation of nontreated bone marrow cells, respectively.

[b] Figures represent mean and limits of range observed

[c] Accuracy, calculated by assuming a Poisson distribution of cell counts and colony counts.

[d] CFU-s enrichment factors are calculated on the basis of CFU-s numbers corrected for the neuraminidase induced reduction.

and 83%). In 3 experiments, these "high EPM" cells were selected and incubated with neuraminidase. After the incubation, the cells were subjected to a second electrophoresis. The EPM distributions of neuraminidase treated high EPM nucleated cells and CFU-s are shown in the bottom panel of Figure 32 A. The areas under the curves are adjusted to 73% for nucleated cells and 96% for CFU-s. These percentages are based on the selection made after the initial electrophoresis. As would be expected, the EPM distribution of CFU-s is hardly affected by the selection for high EPM cells. However, comparison of the EPM distribution of all nucleated cells (middle panel) with that of high EPM nucleated cells (bottom panel) shows that the preseparation selectively decreases the number of cells in the low EPM fractions. The CFU-s enrichment factors obtained by this procedure are listed in Table 11 B (second enrichment column). The CFU-s enrichment is 1.5 to 3 times higher than that obtained by electrophoresis of neuraminidase treated cells without preseparation. In fractions 55 to 70, the CFU-s concentration increases from 10 to 34 times (mean values).

The number of CFU-s in the individual fractions represents only a small proportion of the total CFU-s population. From the cumulative percentage curves of nucleated cells and CFU-s shown in Figure 32 B, it can be determined which fractions can be pooled to obtain a specific percentage of the CFU-s population while retaining an acceptable enrichment factor. The CFU-s enrichment factors when selecting for 5%, 10%, 25% or 50% of the total CFU-s population are indicated in the figures. The data show that, after the repeated electrophoresis procedure, CFU-s enrichment factors increasing from 11 to 26 are achieved for proportions of 50% to 10% of all CFU-s, respectively.

The cell recoveries after electrophoresis in combination with neuraminidase treatment are low: 25% after the single electrophoresis and 15% after the repeated separation (Table 11 B). The cell loss is mainly due to nonselective aggregation of neuraminidase treated cells in the electrophoresis buffer of low ionic strength (Section 2.10, p.42). Although high CFU-s concentrations can be obtained by the repeated electrophoresis procedure, the final yield, if selecting, for example, for 10% of the total CFU-s population, is only 1.5% (corrected for a neuraminidase induced reduction).

CFU-s have been shown to have a higher EPM than GM CFU-c (Chapter 3). Furthermore, the EPM of the various CFU is differentially affected by neuraminidase treatment. It was therefore investigated whether the repeated electrophoresis procedure could be used to enrich for CFU-s with respect to GM CFU-c. Table 12 shows the enrichment fac-

TABLE 12

COMPARISON OF ENRICHMENT FOR CFU-s, GM CFU-c 1 AND GM CFU-c 2 FOLLOWING VARIOUS ELECTROPHORESIS PROCEDURES

bone marrow cells subjected to electrophoresis	number of experiments	fraction numbers	CFU-s percentage of total CFU-s[c]	CFU-s enrichment factor	GM CFU-c 1 percentage of total GM CFU-c 1[c]	GM CFU-c 1 enrichment factor	GM CFU-c 2 percentage of total GM CFU-c 2[c]	GM CFU-c 2 enrichment factor
nontreated cells	6	11-40	96	1.3(1.2 - 1.4)[b]	81	1.1(0.8- 1.3)[b]	75	1.0(0.9- 1.0)[b]
		41-50	4	0.2(0.02- 0.4)	19	0.8(0.3- 2.4)	25	0.9(0.7- 1.3)
neuraminidase treated cells[d]	4	21-58	89	0.9(0.6- 1.0)	83	0.8(0.5- 1.0)	94	1.0(0.9- 1.0)
		59-70	11	13.4(4.7-24.3)	17	21.8(9.3-33.5)	6	7.5(1.7-18.3)
neuraminidase treated high EPM cells[a,d]	3	21-58	86	1.2(1.2- 1.2)	74	1.0(0.9- 1.0)	73	1.0(1.0- 1.0)
		59-70	10	26.2(14.9-43.3)	7	16.3(15.9-16.8)	2	5.1(3.6- 6.0)

[a] Obtained by electrophoresis of nontreated bone marrow cells.

[b] Figures represent mean and limits of range observed.

[c] Approximate percentages

[d] CFU-s enrichment factors are calculated on the basis of CFU-s numbers corrected for the neuraminidase induced reduction.

tors of CFU-s, GM CFU-c 1 and GM CFU-c 2 in two pools of fractions following each of the three electrophoresis procedures. Electrophoresis of nontreated cells results in enrichment factors of 1.3, 1.1 and 1.0 for CFU-s, GM CFU-c 1 and GM CFU-c 2, respectively, in the pool of high EPM fractions (21 to 40). In the low EPM fractions (41 to 50), the CFU-s concentration is reduced with respect to the concentration of CFU-c.

The pool of low EPM fractions (59 to 70) after electrophoresis of neuraminidase treated cells contains relatively high numbers of GM CFU-c 1 (enrichment factor of about 22), while CFU-s and GM CFU-c 2 are less enriched (enrichment factors of about 13 and 8, respectively). This indicates a reduction in CFU-s with respect to GM CFU-c 1.

The final separation procedure in which preselected high EPM cells are treated with neuraminidase and then subjected to a second electrophoresis results in a decrease in the GM CFU-c 1 and GM CFU-c 2 numbers, specifically in the low EPM fractions (59 to 70). Since the CFU-s concentration is increased in the low EPM fractions by this procedure, CFU-s are enriched with respect to GM CFU-c 1 and GM CFU-c 2 by factors of 1.5 and 5, respectively. The various fractions were not tested for the presence of GM CFU-c 3, but, on the basis of the great difference in EPM between CFU-s and GM CFU-c 3 after neuraminidase treatment, it can be predicted that CFU-s are more than 5-fold enriched with respect to this CFU type after the repeated electrophoresis procedure.

5.3 ENRICHMENT FOR CFU-s BY COMBINATION OF A SINGLE-STEP DENSITY SEPARATION AND ELECTROPHORESIS

CFU-s have been shown to possess a relatively low buoyant density (Visser et al., 1977). A single step density separation can therefore be used to obtain a cell suspension which is enriched for CFU-s. Table 13 shows the results of cell separation in an albumin solution with a density of 1.072 $g.cm^{-3}$. The fraction of cells with a density lower than 1.072 $g.cm^{-3}$ contains 68% of CFU-s (varying between 52% and 82% in 7 experiments) and 19% of all nucleated cells (varying between 9% and 29%). The mean CFU-s enrichment factor in the low density fraction is 3.6.

When low density cells are subjected to electrophoresis, EPM distributions of nucleated cells and CFU-s as shown in the bottom panel of Figure 33 A are observed. The areas under the curves are adjusted to 19% for nucleated cells and to 68% for CFU-s. For comparison, the

TABLE 13

ENRICHMENT FOR IN VIVO COLONY FORMING CELLS
BY SINGLE-STEP DENSITY SEPARATION

fraction	all cells (7 experiments) percentage of total CFU-s	enrichment factor
density < 1.072 g.cm^{-3}	68	3.6 (2.4 - 7.4)
density > 1.072 g.cm^{-3}	32	0.4 (0.3 - 0.6)
both fractions	100	1.0
Recovery	70%	

Figures represent mean and limits of range observed.

EPM distributions of all nucleated cells and CFU-s are shown in the top panel. The density separation primarily eliminates high EPM cells (fractions 36 and lower). Although this effect is observed for both CFU-s and other nucleated cells, the number of CFU-s is relatively less reduced. Consequently, selection of low density cells prior to electrophoresis leads to a gain in CFU-s enrichment by a factor of about 3 in the low and high EPM regions (Table 11 A).

The cumulative percentage curves for the EPM distributions of CFU-s and nucleated cells are shown in Figure 33 B. The CFU-s enrichment factors obtained when choosing various proportions of the CFU-s population are indicated along the curves. If the high EPM fractions are pooled, the combination of density separation and electrophoresis results in CFU-s enrichment factors ranging from 4.5 to 5.2 when retaining 50% to 10% of the CFU-s population, respectively.

The results described above seem to indicate that preseparation of cells on the basis of buoyant density properties is not very effective in improving the CFU-s enrichment by electrophoresis. However,

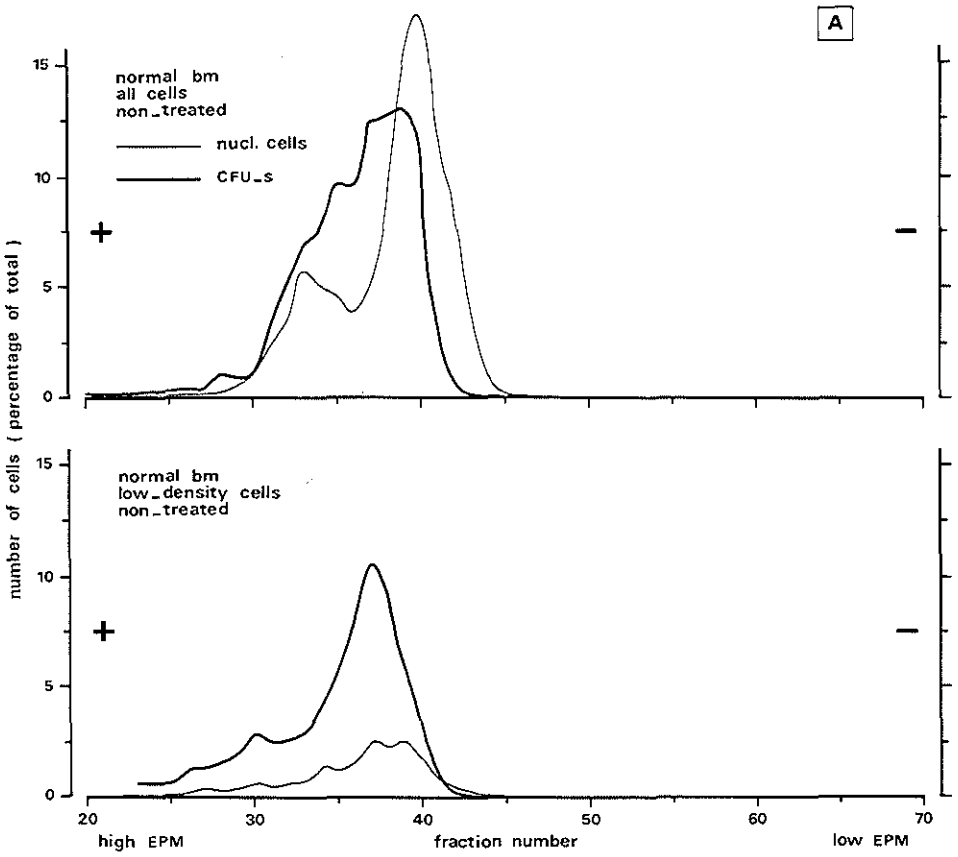
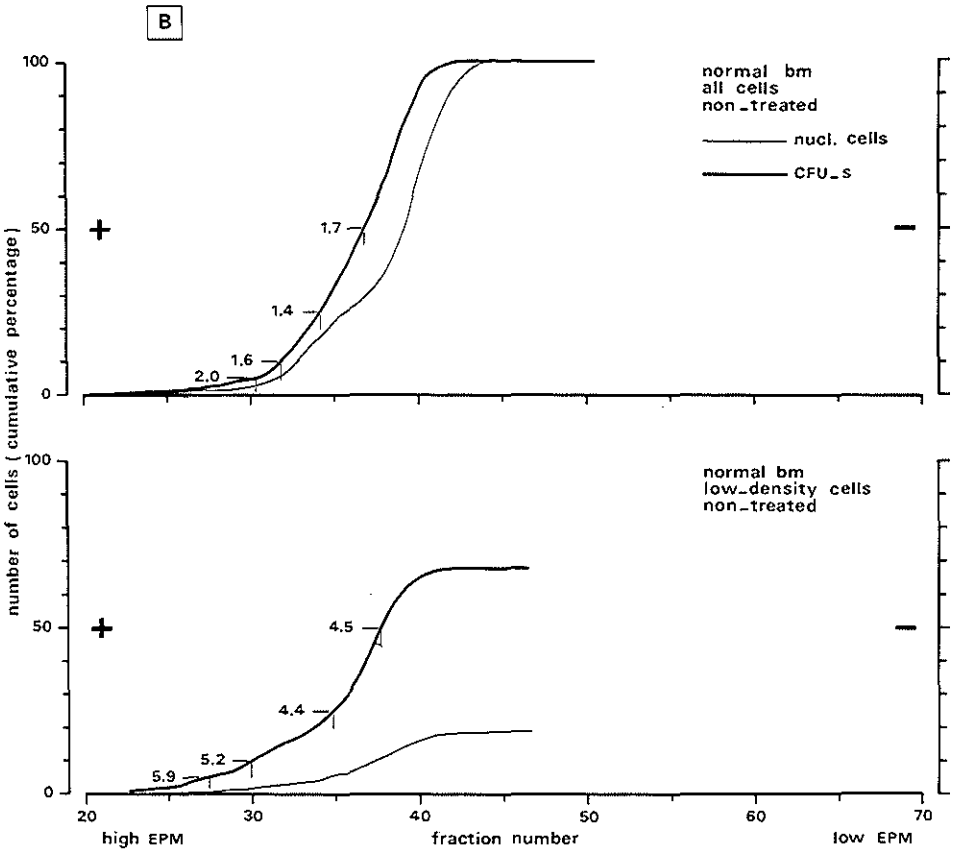


Figure 33:

Enrichment for CFU-s by combining buoyant density separation and electrophoresis

A. The EPM distributions of nucleated cells and CFU-s after different electrophoresis procedures are shown. The profiles are calculated as cells per fraction and each profile is expressed as a percentage of the total cell number. The position of the CFU-s profiles is determined relative to the mean peak position of the nucleated cell profile.
Top panel: EPM distributions of nontreated nucleated cells and CFU-s. The total cell number is set at 100% for both nucleated cells and CFU-s.
Bottom panel: EPM distributions of nontreated low density nucleated cells and CFU-s. Low density cells were obtained by a single step density separation at 1.072 $g.cm^{-3}$ (see Table 13). The total cell number is set at 19% for nucleated cells and 68% for CFU-s.

B. The cumulative percentage curves of the EPM distributions of nucleated cells and CFU-s shown in Fig. 33A. The CFU-s enrichment factors in various pools of high EPM fractions containing 5%, 10%, 25% or 50% of the total CFU-s population are indicated along the curves.



much higher CFU-s enrichment factors are obtained when low density cells are treated with neuraminidase prior to electrophoresis. The EPM distributions of neuraminidase treated low density nucleated cells and CFU-s are shown in the bottom panel of Figure 34 A in comparison to the EPM distributions of all neuraminidase treated cells shown in the top panel. The CFU-s enrichment factors obtained by this procedure are given in Table 11 B (last column). The enrichment for CFU-s is similar to that obtained after electrophoresis of neuraminidase treated high-EPM cells and increases from 10 to 40 in fractions 53 to 61.

The cumulative percentage curves for the EPM distributions of neuraminidase treated cells are shown in Figure 34 B. It can be seen from these curves that the combination of density separation, neuraminidase treatment and electrophoresis results in CFU-s enrichment fac-

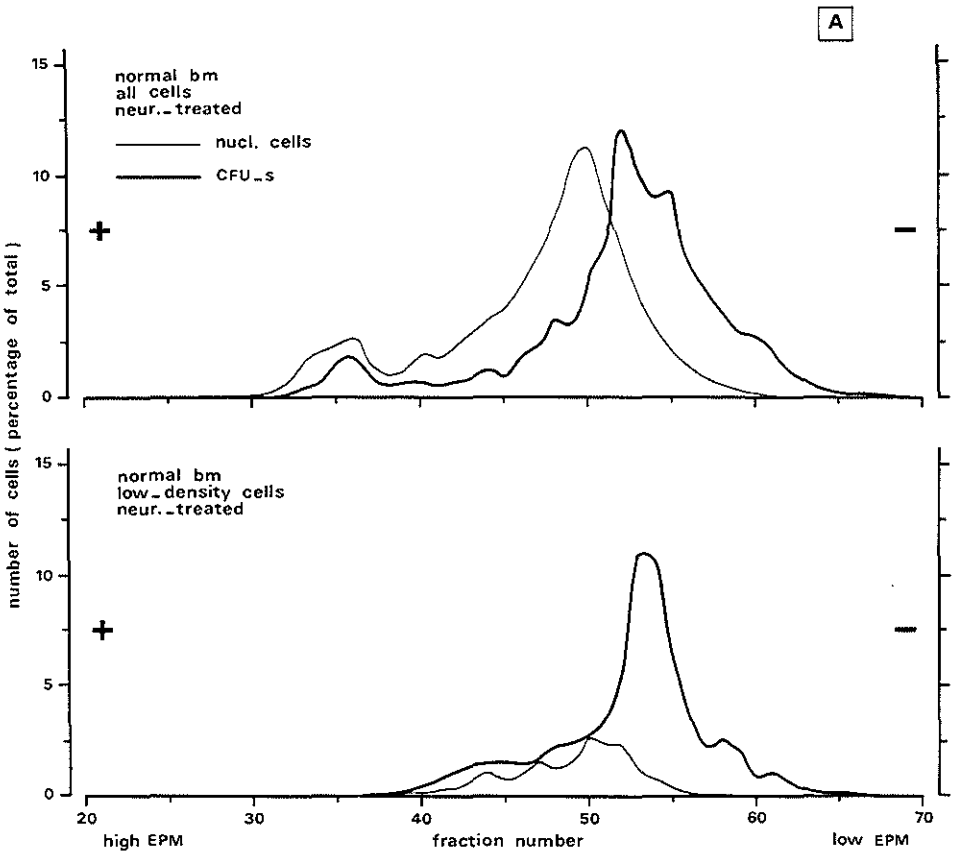
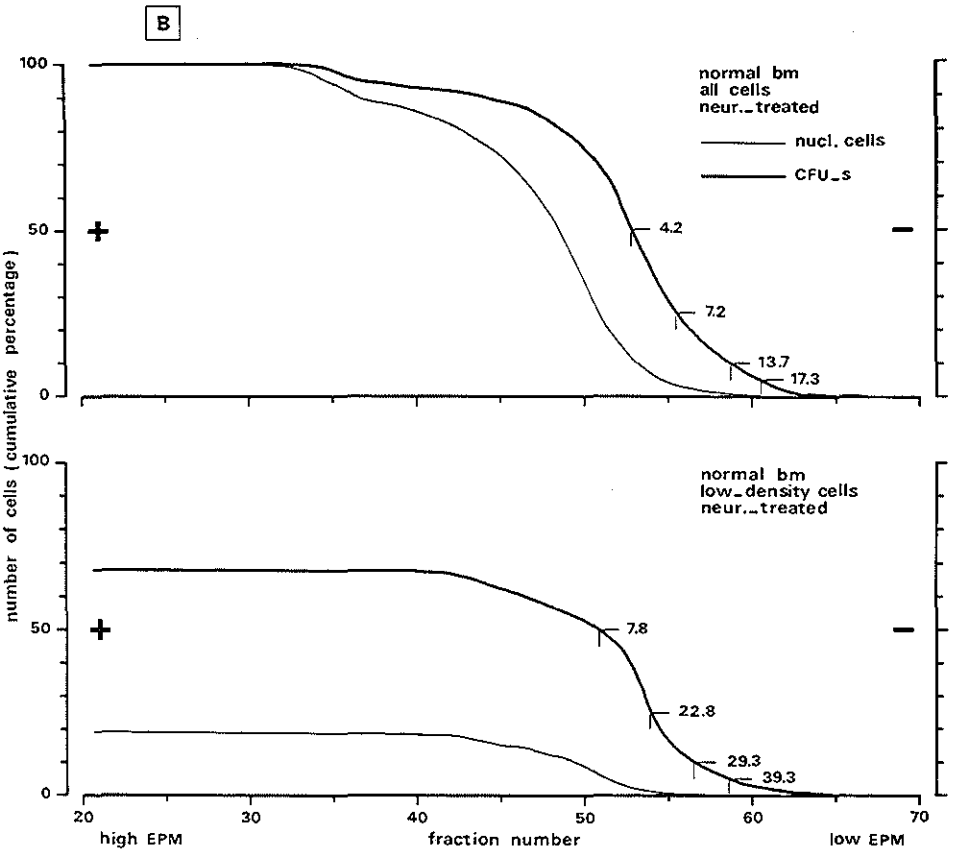


Figure 34:

Enrichment for CFU-s by combining buoyant density separation, neuraminidase treatment and electrophoresis

A. The EPM distributions of nucleated cells and CFU-s after different electrophoresis procedures are shown. The profiles are calculated as cells per fraction and each profile is expressed as a percentage of the total cell number. The position of the CFU-s profiles is determined relative to the mean peak position of the nucleated cell profile.
Top panel: EPM distributions of neuraminidase treated nucleated cells and CFU-s. The total cell number is set at 100% for both nucleated cells and CFU-s.
Bottom panel: EPM distributions of neuraminidase treated low density nucleated cells and CFU-s. Low density cells were obtained by a single step density separation at 1.072 $g.cm^{-3}$ (see Table 13). The total cell number is set at 19% for nucleated cells and 68% for CFU-s.

B. The cumulative percentage curves of the EPM distributions of nucleated cells and CFU-s shown in Fig. 34A. The CFU-s enrichment factors in various pools of low EPM fractions containing 5%, 10%, 25% or 50% of the total CFU-s population are indicated along the curves.



tors increasing from 8 to 29 when selecting for 50% to 10% of all CFU-s, respectively. The yield of CFU-s is also low after this selection procedure. Electrophoresis of neuraminidase treated low density cells results in a cell recovery of about 20%. This means a CFU-s yield of 2%, if selecting, e.g., for 10% of the total CFU-s population.

The enrichment of CFU-s with respect to GM CFU-c was not investigated in this combined separation procedure. However, on the basis of the physical properties of these cells, the following remarks can be made. CFU-s have a lower buoyant density than have GM CFU-c 2 and 3 (Visser et al., 1977; Bol et al., 1979). Furthermore, after neuraminidase treatment, CFU-s have a lower EPM than have GM CFU-c 2 and 3 (Section 3.3, p. 66). The combination of the separation techniques can

therefore be expected to result in enrichment of CFU-s over GM CFU-c 2 and 3. CFU-s and GM CFU-c 1 have been shown to be similar in buoyant density properties and, after neuraminidase treatment, the CFU-s have a higher EPM than have GM CFU-c 1. Therefore, enrichment of CFU-s with respect to GM CFU-c 1 will not be possible by the separation procedure described and the opposite result will even be more likely.

5.4 ENRICHMENT FOR CFU-s BY COMBINATION OF SINGLE-STEP DENSITY SEPARATION, ELECTROPHORESIS AND LIGHT ACTIVATED CELL SORTING

General morphological characteristics of bone marrow cells are reflected in the light scattering signals of these cells as measured by flow cytophotometry (Fluorescence and scattered light activated cell sorter, FACS II, Becton and Dickinson) (Section 2.15, p. 60). The intensity of light scattered between -1° to -10° and 1° to 10° with respect to the direction of the incident light (forward light scattering, FLS) is proportional to cell size. The intensity of light scattered between 70° and 110° with respect to the direction of the incident light (perpendicular light scattering, PLS) has been shown to be a measure for cell "structuredness" (Van den Engh et al., 1979). The top panel of Figure 35 A shows a dot display of PLS versus FLS of unfractionated bone marrow cells. Several clusters of cells can be distinguished. Morphological analysis of the cells in the various clusters after sorting the cells onto microscope slides and cell staining reveals that erythrocytes, lymphocytes and blast cells show increasing FLS intensities, while granulocytes are characterized by a high FLS as well as a high PLS intensity. The outlined area indicates the light scattering properties of CFU-s. CFU-s are characterized by a relatively high FLS and low PLS. By comparison of the light scattering properties of CFU-s with those of morphologically recognizable bone marrow cells and mouse thymocytes, CFU-s can be described as smooth, spherical blast cells with a rounded nucleus and few cytoplasmic structures. Taking the FLS signals of thymocytes as a reference, the modal diameter of CFU-s has been estimated to be 7.2 μm. This description of CFU-s is in agreement with the electron microscopic picture of CFU-s reported by Van Bekkum and co-workers (Van Bekkum et al., 1971; Van den Engh et al., 1979; Van Bekkum et al., 1979). The outlined area indicated in the top panel of Figure 35 A contains about 15% of all nucleated cells and about 80% of all CFU-s. Sorting out the cells from this area will therefore result in a cell suspension which is about 5 times enriched for CFU-s.

These data show that the cellular composition of cell suspensions can be rapidly analysed by measuring FLS and PLS signals. This method was therefore applied to analyse cell suspensions enriched for CFU-s after various cell separation procedures. The data described above also suggest that CFU-s can possibly be further purified by light activated cell sorting.

Cell suspensions obtained after the combinations of electrophoresis/neuraminidase treatment/electrophoresis or density separation/ neuraminidase treatment/electrophoresis were analysed by flow cytophotometry. Figures 35 B and D show the dot diagrams of PLS versus FLS of cells in the pool of low EPM fractions after the two CFU-s enrichment procedures. These cell suspensions containing 10% of the total CFU-s population are 25 to 30 times enriched for CFU-s (see bottom panels of Figures 32 B and 34 B). Two main groups can be easily distinguished by differences in FLS intensity and a slight difference in PLS intensity. The cluster near the origin containing particles with very low light scattering intensities (lower than those of the erythrocytes) can be considered as noise. The light scattering diagrams suggest a high concentration of blast cells and lymphocytes. Erythrocytes and granulocytes are virtually absent, as would be expected on the basis of their EPM and buoyant density properties and the selection procedure applied. The windows were set identically to those in the top panels and indicate the expected light scattering properties of the CFU-s in the cell suspensions after the enrichment procedures. The windows in Figures 35 B and D contain about 30% of the cells detected. Sorting out the cells from these windows would be expected to increase the concentration of CFU-s.

The major contaminating cells in the CFU-s enriched fractions are morphologically recognizable lymphocytes. These lymphocytes can be partly eliminated by separation on the basis of forward light scattering properties. It has been recently reported that a clear-cut distinction between lymphocytes and blast cells can be achieved by combining measurements on FLS and the fluorescence of the supravital DNA dye "Hoechst 33342" (Visser et al., 1980b). At a relatively low pH value of 6.5, lymphocytes and blast cells show a different uptake rate of Hoechst 33342 which, after excitation by laser light, results in different fluorescence intensities of these cells. Figure 36 C shows the dot diagram of fluorescence versus FLS intensities of unfractionated bone marrow cells. Morphological analysis of the cells in the various clusters revealed that lymphocytes could be separated from blast cells and granulocytes. Granulocytes can be partly separated from the

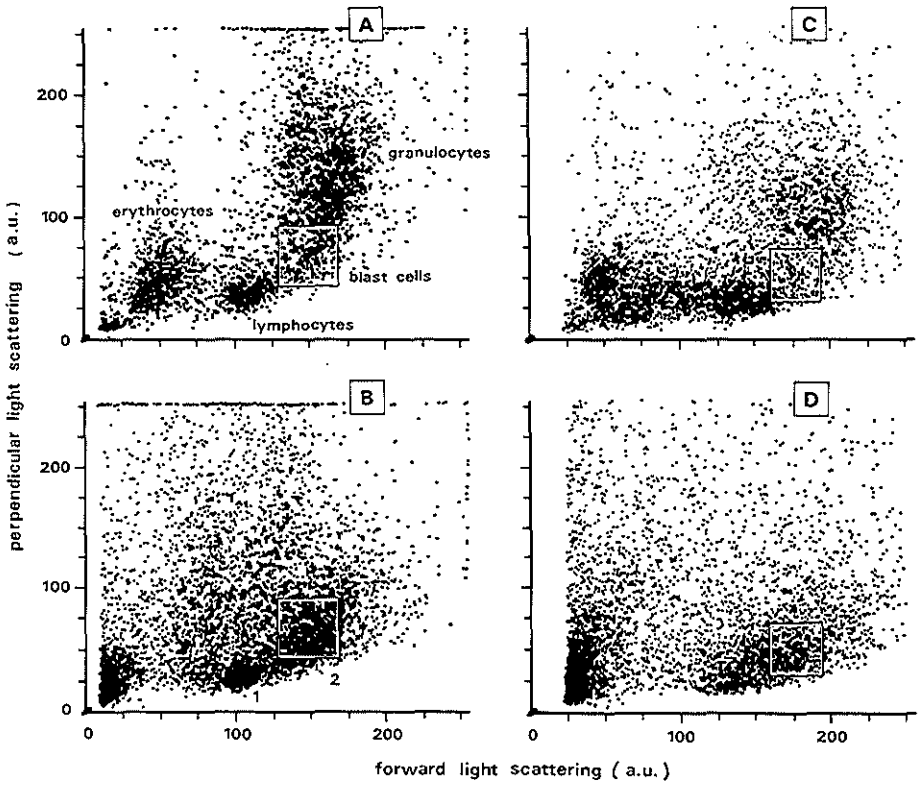


Figure 35:

Light scattering properties of unfractionated bone marrow cells and cells in CFU-s enriched fractions

The figure shows the dot displays of perpendicular versus forward light scattering intensities.

A. Light scattering of unfractionated bone marrow cells. CFU-s are found in the outlined area.

B. Light scattering of cells in the pool of low EPM fractions (58 to 70) after electrophoresis of neuraminidase treated high EPM cells. The outlined area corresponds with that in the top panel.
The dot displays in A and B are from the same experiment.

C. Light scattering of unfractionated bone marrow cells. CFU-s are found in the outlined area.

D. Light scattering of cells in the pool of low EPM fractions (56 to 70) after electrophoresis of neuraminidase treated low density cells. The outlined area corresponds with that in the top panel.
The dot displays in C and D are from the same experiment.

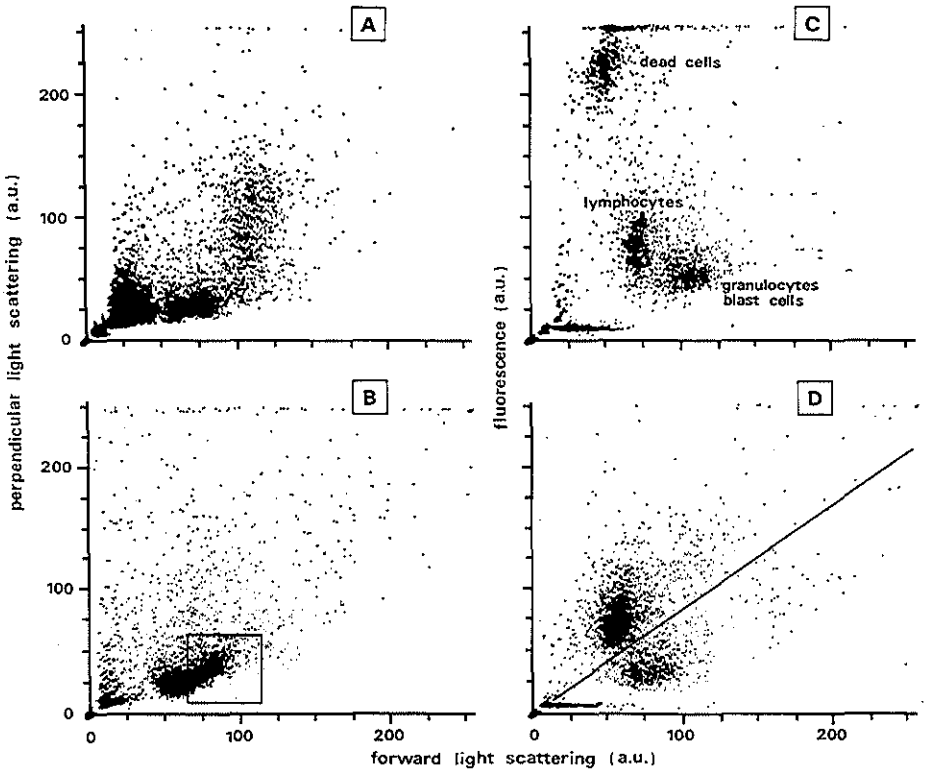


Figure 36:

Light scattering and fluorescence properties of unfractionated bone marrow cells and cells in CFU-s enriched fractions

A and C Perpendicular versus forward light scattering (A) and Hoechst 33342 fluorescence versus forward light scattering (C) of unfractionated bone marrow cells.

B and D Perpendicular versus forward light scattering (B) and Hoechst 33342 fluorescence versus forward light scattering (D) of cells in the pool of low EPM fractions after electrophoresis of neuraminidase and Hoechst 33342 treated low density cells. The outlined area in panel B indicates the sort window for CFU-s on the basis of light scattering signals. The line in panel D indicates the separation between cells with high and low (CFU-s) fluorescence intensities.

All four dot displays are from the same experiment.

blast cells on the basis of PLS signals. Starting with unfractionated bone marrow, CFU-s can be 15-fold enriched in concentration by sorting on the basis of Hoechst 33342 fluorescence, FLS and PLS signals simultaneously, retaining about 80% of the total CFU-s population (Visser et al., 1980b).

CFU-s in the fractions obtained after the electrophoresis procedures might also be more efficiently sorted out by using the combination of Hoechst fluorescence and light scattering measurements. In four experiments, CFU-s were enriched in concentration by carrying out the following steps in sequence:

a) selection of low density cells by a single-step density separation (which is easier and faster than the selection of high EPM cells);
b) simultaneous incubation of cells with neuraminidase and Hoechst 33342;
c) selection of low EPM cells by free-flow electrophoresis;
d) selection of cells with high FLS, low PLS and low fluorescence intensities by scattered light and fluorescence activated cell sorting.

The results are presented in Table 14. Selection of cells with a buoyant density lower than 1.072 $g.cm^{-3}$ resulted in a 6.6 times normal CFU-s concentration. The subsequent incubation of the low density cells with neuraminidase and Hoechst 33342 followed by selection of low EPM cells increased the CFU-s concentration to 15.5 times normal. The cell recovery after neuraminidase treatment and electrophoresis is only about 20%. To obtain enough cells for continuing the enrichment procedure with cell sorting, it was necessary to select a rather high proportion of low EPM cells. Selection was made for about 55% of the total CFU-s population, although this was disadvantageous for the CFU-s enrichment. The low EPM cells were analysed by flow cytophotometry. The dot diagrams of PLS versus FLS and fluorescence versus FLS are shown in the Figures 36 B and D. The sort windows for FLS, PLS and fluorescence are indicated. By sorting out cells with high FLS, low PLS and low fluorescence intensities, the CFU-s concentration in the resulting cell suspension was 39 times that in unfractionated bone marrow.

In three of the four experiments, the cell suspensions obtained after the different selection steps were tested for the presence of GM CFU-c 1 and 2. As can be seen in Table 14, the enrichment for GM CFU-c 1 was similar to that of CFU-s. GM CFU-c 2 were less enriched in concentration than CFU-s and GM CFU-c 1.

TABLE 14

ENRICHMENT FOR CFU-s AND GM CFU-c 1 AND 2 BY COMBINING BUOYANT DENSITY SEPARATION, TREATMENT OF CELLS WITH NEURAMINIDASE AND HOECHST 33342, FREE-FLOW ELECTROPHORESIS AND LIGHT ACTIVATED CELL SORTING

cell separation procedure	percentage of total CFU-s	CFU-s per 10^5 cells	enrichment factor	percentage yield of CFU-s	GM CFU-c 1 enrichment factor	GM CFU-c 2 enrichment factor
unfractionated	100	36.3 (29.9-39.5)	1	100	1	1
a. density separation; select low density cells →	66 (51.4-80.2)	239 (150-409)	6.6 (4.0-10.4)	43.2 (25.8-57.0)	6.8 (1.7-11.2)	4.0 (1.7-7.4)
b. incubation of cells with neuraminidase and Hoechst 33342						
c. free-flow electrophoresis; select low EPM cells →	55.5 (44.7-52.3)	564[a] (423-764)	15.5 (11.4-19.3)	8.5 (5.3-12.1)	11.1 (7.3-15.2)	4.8 (0.3-12.5)
d. light activated cell sorting; select cells with high FLS, low PLS and low fluorescence intensities →	50[b] (40-70)	1414[a] (910-2364)	39.0 (25.5-59.9)	7.5[b] (5-10)	36.6 (30.4-44.3)	16.1 (4.6-34.2)

Figures represent mean and limits of range observed from 4 experiments.

[a]Values corrected for the neuraminidase induced reduction in CFU-s.

[b]Estimated values. Only two fractiohs could be sorted on the basis of 3 parameters. The windows were set such that 90% of CFU-s would be present on the basis of CFU-s properties in unfractionated bone marrow.

The cell recovery after this four-step procedure was about 15%. In combination with the selection for 50% of the total CFU-s population, the final yield of CFU-s was 7.5%.

These results show that light activated cell sorting can be successfully applied as a last step in the CFU-s enrichment procedure. Cell sorting increased the CFU-s concentration by a factor of 2.5. The CFU-s enrichment factor of about 15 obtained by selection for 55% of the total CFU-s population after electrophoresis was increased to about 40 by cell sorting. As reported in Section 5.3, selection for 10% of all CFU-s after electrophoresis resulted in a CFU-s enrichment factor of 30 (bottom panel Figure 34 B). If cell recovery could be increased, selection for 10% of all CFU-s after electrophoresis could be combined with cell sorting. Such a procedure can be predicted to result in a 75 times increased CFU-s concentration.

5.5 DISCUSSION

Many studies on the mechanism of haemopoietic differentiation would be greatly facilitated if cells in a very early stage of development were available in high concentrations and in sufficient quantities. Since *in vivo* colony forming cells (CFU-s) are considered to represent pluripotent stem cells, they have frequently been subjected to purification studies. Described here has been the development of a CFU-s enrichment procedure which employs several physical properties of CFU-s known from analytical cell separation studies. CFU-s are characterized by low buoyant density, high electrophoretic mobility, a great change in electrophoretic mobility after neuraminidase treatment a high forward and a low perpendicular light scattering and low fluorescence after Hoechst 33342 staining. Cell separation on the basis of each individual property results in rather low enrichment for CFU-s. Combination of cell separation techniques each selecting for a different property of CFU-s considerably increases the enrichment.

A summary of the approximate mean values obtained in the various CFU-s enrichment procedures is presented in Table 15. In the procedures described, electrophoresis of neuraminidase treated cells is an important step. After this separation (Table 15 A), the pool of low EPM fractions containing 50% [10%] of the total starting CFU-s population is 4 [14]-fold enriched for CFU-s. This enrichment factor can be increased to 10 [25] by preselection of high EPM cells (Table 15 B). Preselection of low density cells prior to neuraminidase treatment and

electrophoresis increases the CFU-s enrichment factor to 15 [30] (Table 15 C 3). The CFU-s can be further purified by light activated cell sorting. Selection of cells with high FLS, low PLS and low Hoechst 33342 fluorescence intensities results in a CFU-s concentration of 40 [75?] times the concentration found in unfractionated bone marrow (Table 15 C 4). The value of 75 is a hypothetical one. It was assumed that selecting for 10% of all CFU-s after electrophoresis would not decrease the contribution of cell sorting to the purification. This was based on the observation that the heterogeneity in light scattering properties among the low EPM cells is similar after selection for 50% and 10% of all CFU-s.

If the incidence of *in vivo* colony forming cells in unfractionated bone marrow is estimated to be 0.7%, a 40-fold enrichment for CFU-s corresponds to an incidence of about 30% of colony forming cells, while the expected enrichment factor of 75 corresponds to a 50% incidence of colony forming cells. It was assumed that the spleen seeding efficiency of the selected colony forming cells was not changed.

GM CFU-c 1 is enriched to a similar extent as CFU-s. If the incidence of GM CFU-c 1 in unfractionated bone marrow is estimated to be 0.05% (50 per 10^5 bone marrow cells), enrichment factors of 40 and 75 correspond to an incidence of 2% and 4%, respectively.

The morphology of the cells in the CFU-s enriched fractions was not extensively investigated. May Grünwald-Giemsa staining and light microscopic study of the cells in the final cell suspensions after sorting revealed that they contained about 70% myeloblast-like cells and 10% lymphocytes. Examination of cells by electron microscopy showed the presence of cells with morphological properties similar to those of the candidate stem cell described by Van Bekkum and coworkers (1971). However, the number of cells was too low to allow a quantitative comparison of these cells with the number of *in vivo* colony forming cells. The cell recovery after the combined separation procedure has to be increased to conduct such studies. In addition, an increase in cell recovery is necessary for testing the spleen seeding efficiency of the isolated colony forming cells.

The cell separation techniques used in sequence do not separate on completely independent parameters. By use of the light activated cell sorter alone, CFU-s concentrations up to 15 times normal can be obtained when starting with unfractionated bone marrow cells and sorting on the basis of three parameters simultaneously (Visser et al., 1980b). The contribution of cell sorting to the enrichment of CFU-s was decreased to a factor of 2.5 after the various preselection proce-

TABLE 15

ENRICHMENT FOR CFU-s BY COMBINATION OF VARIOUS CELL SEPARATION TECHNIQUES

	cell separation procedure	percentage of total CFU-s	enrichment factor	percentage yield	percentage CFC[a]	no. of experiments
	unfractionated	100	1	100	0.7	
A 1	neuraminidase treatment →					
2	electrophoresis; select low EPM cells →	50	4	12	3	4
		10	14	2.5	10	4
B 1	electrophoresis; select high EPM cells →	95	1.3	55	1	6
2	neuraminidase treatment →					
3	electrophoresis; select low EPM cells →	50	10	7.5	7	3
		10	25	1.5	18	3

C 1 density separation; select low density cells	70	4.5	50	3	11
2 neuraminidase and Hoechst 33342 treatment					
3 electrophoresis; select low EPM cells	55	15	8.5	10	4
	10	30	2	20	1
4 light activated cell sorting; select cells with low fluorescence, high FLS and low PLS	50	40	7.5	30	4
	(10)	(75)	(1.5)	(50)	

Figures represent approximate mean values.
Correction was made for the neuraminidase induced reduction in CFU-s.

[a]The number of *in vivo* colony forming cells was calculated by assuming a spleen seeding efficiency of 5%.

dures. However, the use of cell sorting as the last step has the advantages of: a) rapid cell analysis, because the cell suspensions contain few cells which are of no importance. For example, most granulocytes have been removed by the density separation; b) reliable counts of low cell numbers; and c) possibility for precise deposition of single cells onto microscope slides or in culture wells.

The data show that increases in the incidence of in vivo colony forming cells to 30 to 50% are within the possibilities of the purification techniques described. This would meet to a large extent the requirement of high concentrations of colony forming cells for in vitro differentiation studies and further morphological, cytochemical and antigenic characterization. However, the final yield of colony forming cells after the enrichment procedure is low and this may hinder further analysis. This low yield is largely due to nonselective cell loss resulting from strong aggregation of neuraminidase treated cells in the electrophoresis buffer of low ionic strength. The technical difficulty of cell aggregation can be solved by using electrophoresis buffers of a higher ionic strength. However, the use of such buffers will require free-flow electrophoresis devices with a greatly improved cooling capacity.

A summary of the data presented in this chapter is as follows:

a) CFU-s can be 40-fold enriched by combining density separation, neuraminidase treatment, supravital DNA staining, free-flow electrophoresis and light activated cell sorting.
b) Although high CFU-s concentrations can be obtained, it is necessary that the yield be increased.
c) CFU-s can be enriched with respect to GM CFU-c 2 and GM CFU-c 3, but enrichment of CFU-s with respect to GM CFU-c 1 on the basis of the presently used parameters seems hardly possible. However, the incidence of in vivo colony forming cells exceeds that of GM CFU-c 1 by a factor of 15 in the final CFU-s enriched fraction.

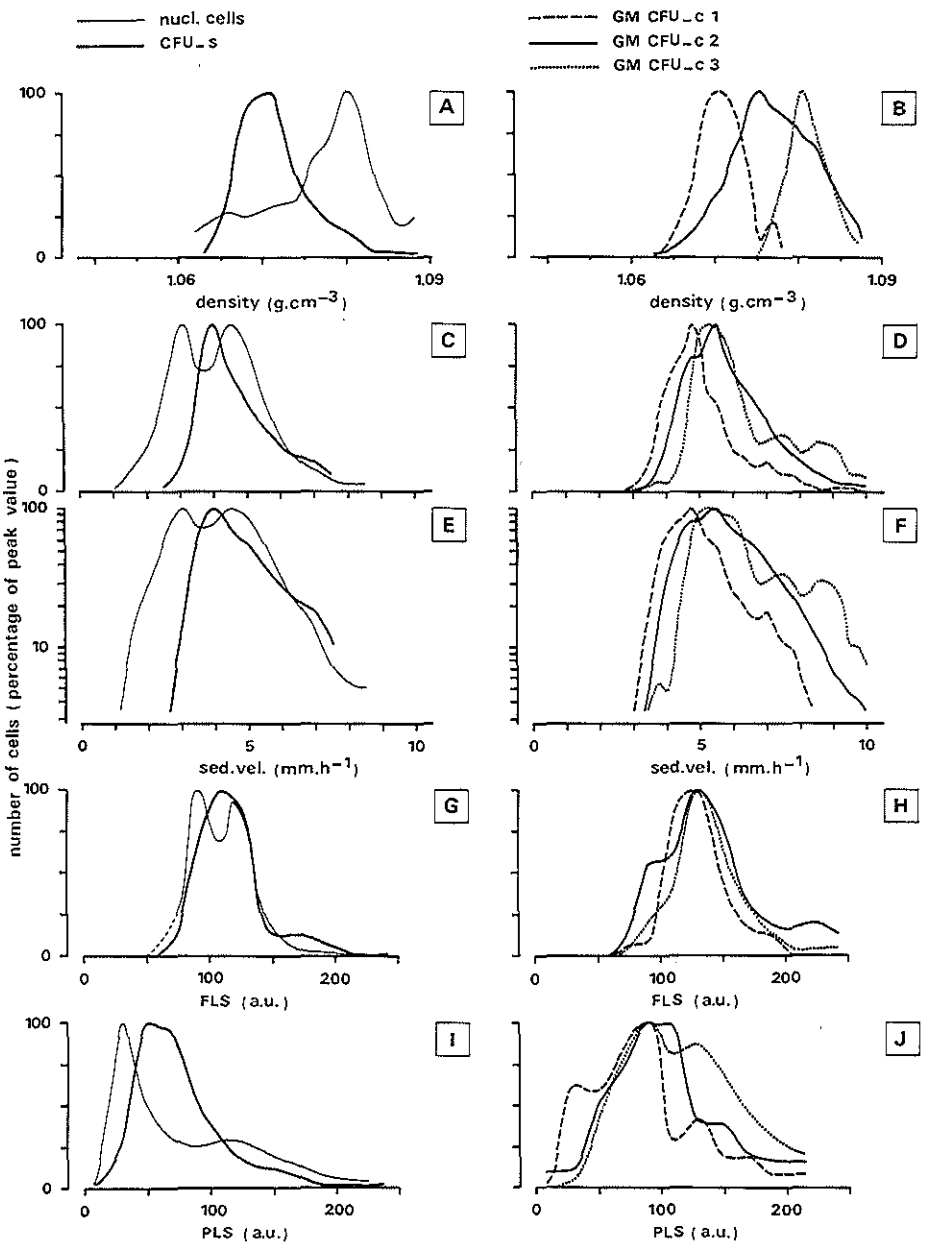
CHAPTER 6

GENERAL DISCUSSION

The electrophoretic behaviour of early haemopoietic cells which is described in this thesis is an addition to the data on the physical and biological properties of these cells obtained in other studies. Figure 37 shows a comparison among the distributions of buoyant density (Visser et al., 1977; Bol et al., 1979), sedimentation velocity (Van den Engh, 1976; Visser et al., 1977; Bol et al., 1979), forward and perpendicular light scattering (Van den Engh et al., 1979; Visser et al., 1980) and electrophoretic mobility before and after neuraminidase treatment (this study) of CFU-s, GM CFU-c 1, GM CFU-c 2 and GM CFU-c 3. The modal values of these distributions are given in Table 16. This table also includes data on CFU with respect to size, antigenic properties (Van den Engh et al., 1978; Russell and Van den Engh, 1979), cycling status (Visser, unpublished observations) and growth regulating factors (Löwenberg and Dicke, 1977; Wagemaker and Peters, 1978; Bol and Williams, 1980). On the basis of the cellular properties, it is proposed that differentiation from CFU-s through GM CFU-c 3 is accompanied by:

a) an increase in buoyant density and in sedimentation velocity with a minor change in cell size, which can be explained by an increase in biosynthesis and the development of cytoplasmic structures;
b) an increase in cell structure (perpendicular light scattering), which can be related to the development of the characteristic morphology of the functional end cells;
c) a decrease in electrophoretic mobility, due to changes in the surface density of charged groups other than neuraminidase susceptible NANA groups;
d) a decrease in the density of neuraminidase susceptible NANA groups on the cell surface;
e) a decrease in the density of H2-K,D antigens;
f) changes in the proliferative activity;
g) changes in the responsiveness to various growth regulating factors.

The data show that different developmental stages can be recognized and that obvious cellular changes occur during early differentiation. Buoyant density, size and structure are cell properties which


nucl. cells
CFU_s
GM CFU_c 1
GM CFU_c 2
GM CFU_c 3
A
B
C
D
E
F
G
H
I
J
100
0
10
1.06
1.09
density (g.cm^{-3})
number of cells (percentage of peak value)
0
5
10
sed.vel. (mm.h^{-1})
100
200
FLS (a.u.)
PLS (a.u.)

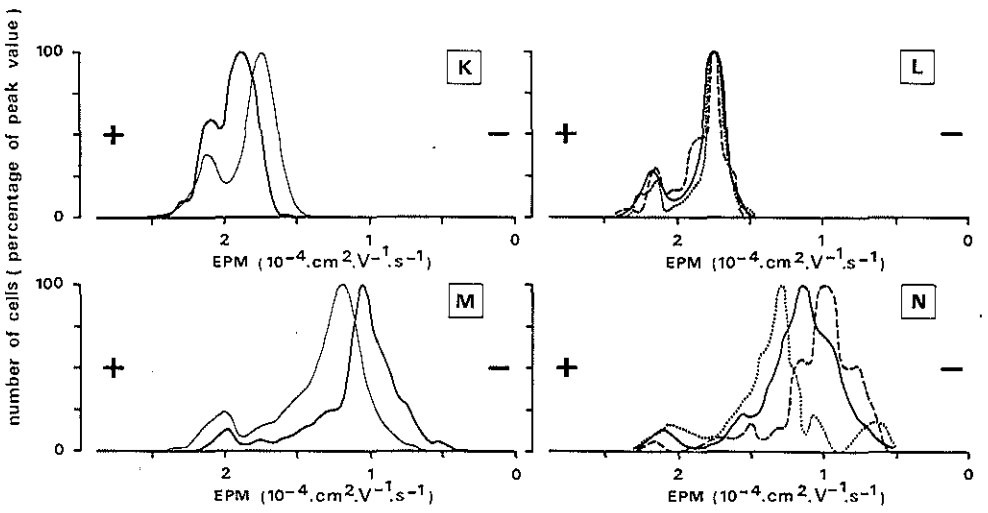


Figure 37:

Comparison among the distributions of various properties of nucleated cells, CFU-s and GM CFU-c 1,2 and 3

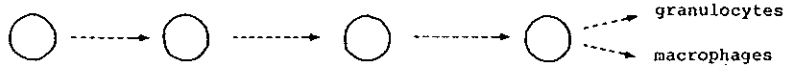
AB Buoyant density distributions.
CD Sedimentation velocity distributions.
EF Sedimentation velocity distributions in a semi-logarithmic plot.
GH Forward light scattering distributions.
IJ Perpendicular light scattering distributions.
KL Electrophoretic mobility distributions.
MN Electrophoretic mobility distributions after neuraminidase treatment.

References

ABCDEF: Williams and Van den Engh, 1975; Van den Engh, 1976; Visser et al., 1977; Bol et al., 1979
GHIJ : Van den Engh and Visser, 1979; Visser et al., 1980 (adapted)
KLMN : This thesis.

TABLE 16

PHYSICAL AND BIOLOGICAL PROPERTIES OF EARLY HAEMOPOIETIC CELLS IN SEQUENTIAL STAGES OF DEVELOPMENT



	(mouse thymocytes)[a]	CFU-s	GM CFU-c 1	GM CFU-c 2	GM CFU-c 3
buoyant density ($g.cm^{-3}$) [b,c,d]		1.070	1.070	1.075	1.080
sedimentation velocity [b,c,d] ($mm.h^{-1}$)		4.0	4.3	4.8	5.3
calculated diameter (μm) [e]	(5.9)	7.2	7.4	7.5	7.6
forward light scattering [f,g] (arb. units)	(78)	117	133	137	137
calculated diameter (μm) [h]		7.2	7.7	7.8	7.8
perpendicular light scattering [f,g] (arb. units)	(29)	69	86	105	120
electrophoretic mobility [i] ($10^{-4}.cm^2.V^{-1}.s^{-1}$)		1.87	1.74	1.74	1.74
electrophoretic mobility after neuraminidase treatment [i]		1.05	0.98	1.13	1.29
relative surface NANA content [i] (% reduction in EPM by neuraminidase)		44	44	35	26
antigens [j,k,l,m] ; BAS		+;-[n]	*	-[o]	*
H2-KD		+	*	-	*
H2-Ia		-	*	-	*
Thy-1		-	*	-	*
relative number in S phase [p] (% kill by ^3H-thymidine)		17	-4(13)[q]	57	23 (25)[q]
growth regulating factors [r,s,t,v]		SAF	CEF (18 h PES type)	CSF (pmue type)	CSF+CEF (LYS type)

a Mouse thymocytes can be used as a reference in light scattering studies
b Visser et al., 1977
c Williams and Van den Engh, 1975
d Bol et al., 1979
e Diameter calculated by use of Stoke's law
f Visser et al., 1980
g Van den Engh and Visser, 1979
h Diameter calculated on the basis of linear relation between FLS and cross sectional area (see ref. f and g)
i this thesis
j Thierfelder, 1977
k Basch et al., 1977
l Van den Engh et al., 1978
m Trask and van den Engh, 1980
n Positive in cytotoxicity assay; negative by immune fluorescence
o Negative by cytotoxicity assay
p Visser, 1979 (unpublished observations)
q Corrected value on the basis of 10% reduction in plating efficiency caused by aspecific inhibitory activity in mouse serum and lysate
r Cerny, 1974
s Löwenberg and Dicke, 1977
t Wagemaker and Peters, 1978
v Bol and Williams, 1980
* not tested

reflect a complex of metabolic processes which cannot be precisely specified. Increase in RNA and protein synthesis and the development of cell organelles necessary for specialized functions are likely to be reflected in cell mass and cell structure. Analysis of the changes in metabolism and the development of, e.g., specialized enzyme systems by use of cytochemical techniques is rather difficult, since the cells must remain viable. The cell membrane is more accessible for investigations concerning the presence of cell type specific components without affecting cell viability. Investigations of the cell membrane properties revealed specific changes during differentiation. Studies of the surface antigens showed that early haemopoietic differentiation is accompanied by a decrease in H2-K,D antigens (Van den Engh et al., 1978). The investigations on the electrophoretic behaviour of the haemopoietic cells described in this thesis revealed that the surface density of NANA groups on the cell surface decreases with differentiation. The observation that the NANA density does not change with the proliferative activity of the cells (Section 3.5) might indicate that the change in NANA density relfects a specific differentiation event.

The presence of NANA groups on the cell surface can be tested for by means other than electrophoresis. Wheat germ agglutinin (WGA) has been reported to bind specifically to cell surface NANA groups (Peters et al., 1979). The proposed decrease in the surface density of NANA groups can be investigated by use of fluorescent labelled WGA and light activated cell sorting. Early results show that CFU-s are among a small population of bone marrow cells with the highest fluorescence intensities (Visser et al., 1980a). Treatment of cells with neuraminidase prior to WGA labelling results in a much smaller fluorescence intensity of all nucleated cells and CFU-s. This supports the view that CFU-s have a high surface NANA density. However, differences among CFU-s and GM CFU-c 1, 2 and 3 with respect to WGA binding could not be detected. This suggests that in addition to WGA binding sites, other differences among the various CFU are detected by electrophoresis. Since WGA binding is correlated with the occurrence of NANA groups, treatment of cells with WGA would be expected to influence their electrophoretic mobility. Measuring WGA binding by electrophoresis would allow a useful comparison with the results obtained by cell sorting. The differences in surface NANA density among CFU-s and GM CFU-c 1, 2 and 3 were detected by use of Vibrio cholerae neuraminidase. This enzyme has a wide substrate spectrum. It may be that WGA binds specifically to particular positioned NANA groups. Viral neuraminidases have been reported to be more specific. They split, e.g.,

only one type of linkage between NANA and the carbohydrates of glycoproteins. Therefore, the use of viral neuraminidases in electrophoretic studies should be considered.

The properties of CFU-s and GM CFU-c 1 may provide information on cellular changes occurring during the transition of pluripotent to committed cells. Comparison of CFU-s and GM CFU-c 1 with respect to physical and biological properties (Table 16) reveals similarities as well as differences. Although CFU-s and GM CFU-c 1 have a similar buoyant density and surface NANA density, they differ slightly with respect to cell size and cell structure and show different electrophoretic mobilities. The differences in surface charge density indicate differences in the chemical composition of the cell membrane. Since the results from ^{3}H-thymidine experiments suggest that CFU-s and GM CFU-c 1 are similar in their proliferative activity, the differences in physical properties are likely to reflect changes occurring during differentiation. Therefore, CFU-s and GM CFU-c 1 are postulated to represent very closely associated but different developmental stages.

Cell suspensions which contain high concentrations of CFU-s may be helpful for further investigations on the differences between CFU-s and GM CFU-c 1 with respect to their differentiation potential and their biochemical and antigenic properties. CFU-s can be 40-fold increased in concentration and up to 75-fold enrichment has been shown to be within the possibilities of the cell separation techniques described (Chapter 5). These enrichment factors indicate an incidence of 30 to 50% colony forming cells. Such a high concentration of colony forming cells makes it feasible to do in vitro differentiation studies starting with single cells. The culture systems which stimulate the proliferation and differentiation of multipotent haemopoietic cells are the best ones to use for this purpose (Johnson and Metcalf, 1978). The colonies which develop in these culture systems contain a mixture of cell types of the various cell lineages and even newly formed CFU-s. This suggests that these cultures may be used as an equivalent of the CFU-s assay (Dicke et al., 1971; Metcalf et al., 1979; Humphries et al., 1979). By the use of liquid medium in the single cell cultures, the progeny of the initial cell can be easily transferred to other cultures and tested for their proliferation and differentiation capacity. In this way, differentiation can be followed step by step and the relationship between CFU-s and GM CFU-c 1 can be analyzed. Of course, the use of specific growth regulating factors must be involved in these studies.

Highly purified CFU-s preparations might also be used to investigate the composition of the CFU-s membrane. Although completely pure CFU-s cannot be obtained, the CFU-s enriched cell suspensions contain only cells in very early stages of development and are devoid of morphologically recognizable mature cells. Since the colony assay is no longer necessary, the cells in the CFU-s enriched fractions can be rapidly analyzed for a variety of properties. By use of flow cytophotometry, a great number of fluorescent labelled lectins and antibodies could be tested. The binding of such substances to the cells in the CFU-s enriched fractions could be easily compared with the binding to unfractionated bone marrow cells. In this way, a rapid selection for useful parameters could be made. If the cell recovery of the CFU-s enrichment procedure could be increased, it might be possible to obtain membrane preparations from the CFU-s concentrates for chemical analysis. CFU-s enriched cell suspensions might be used also in techniques for the production of monoclonal antibodies to stem cell surface antigens (Milstein et al., 1979). All of these methods would allow the development of chemical and antigenic membrane markers which are invaluable tools for studying the changes which occur during the transition of pluripotent stem cells to committed progenitor cells.

SUMMARY

In the adult mouse, all of the different mature blood cells which perform their function in various tissues and in the peripheral blood circulation are derived from one cell type: the pluripotent haemopoietic stem cell or HSC. HSC produce progeny cells throughout the life of the animal to compensate for the continuous utilization of mature end cells. Under normal steady state conditions, only a small proportion of the HSC population is in active cell cycle; the largest proportion is in a nonproliferating or resting state. The ratio between the proportions of resting and proliferating HSC depends on the demand for functional end cells. Proliferating HSC give rise to cells which are copies of themselves (self-renewal) as well as to cells which are committed to differentiate along one of the various blood cell lineages. The proliferative activity of HSC and the proliferation and differentiation of the committed progenitor cells are regulated by a number of different humoral and microenvironmental factors.

The bone marrow and to a lesser extent the spleen are the sites of haemopoiesis. Since the HSC and committed progenitor cells represent only 1 to 2 percent of all nucleated bone marrow cells, they cannot be investigated directly. Only the high proliferation capacity of the early haemopoietic cells can reveal their presence. Under appropriate conditions, HSC and committed progenitor cells can proliferate and differentiate in in vivo and in vitro systems and thus give rise to discrete colonies (clones) of mature cells. The number and the differentiation stage of the various haemopoietic cells present in certain cell suspensions must be extrapolated from the number and the cellular composition of the colonies formed. Pluripotent stem cells can form colonies in the spleen of lethally irradiated mice. After intravenous injection of bone marrow cells, a proportion of the stem cells among them will permanently settle in the spleen. These cells have been designated operationally as colony forming units-spleen or CFU-s. Committed progenitor cells of erythrocytes (E), granulocytes/macrophages (GM) and megakaryocytes (Meg) can be detected by their colony formation in vitro after stimulation by various growth regulating factors and have been termed colony forming units-culture or CFU-c. In this thesis, studies are restricted to CFU-s and GM CFU-c.

The detection systems for pluripotent and committed haemopoietic cells have led to investigations on the physical, biochemical and antigenic properties of these cells in order to recognize them more

directly, to investigate whether they represent single cell types or whether intermediate cell types can be distinguished and to determine which cellular changes occur during differentiation. Several techniques by which viable cells can be fractionated on the basis of differences in the cellular properties mentioned above have been developed. The cell fractions obtained after such cell separation procedures can be tested for the presence of haemopoietic cells in the in vivo and in vitro assay systems and in this way the properties of these cells can be determined. Cell separation by equilibrium density centrifugation and sedimentation at unit gravity revealed differences in buoyant density between resting and proliferating CFU-s. Furthermore. by combination of these techniques with different growth stimulating factors, three consecutive differentiation stages characterized by increasing buoyant density could be detected within the GM CFU-c compartment: GM CFU-c 1, 2 and 3. With respect to physical properties, response to various factors and proliferative activity, CFU-s, GM CFU-c 2 and GM CFU-c 3 can be easily distinguished. CFU-s and GM CFU-c 1 show a number of similarities which suggest that these cell types represent closely associated developmental stages. The studies described in this thesis concern further characterization of the properties of the various CFU types to obtain more information on the cellular changes occurring during the development from CFU-s through GM CFU-c 3.

Buoyant density and sedimentation velocity are rather general physical cell properties. Cell surface properties can be investigated more specifically. The data described here concern the chemical characterization of the surface of haemopoietic cells by use of the free-flow electrophoresis technique. Using this technique, cells are separated on the basis of differences in surface charge density which reflect the chemical composition of the cell surface. The presence of specific charged molecules on the cell surface can be studied by removing or covering these molecules with specific reagents. These manipulations change the exposure of free charges on the cell surface and as a result the electrophoretic mobility of the cells will be changed. At neutral pH values, most cells carry a net negative charge on their surface. This negative charge is partly due to the presence of neuraminic acids which are generally occurring terminal groups of the carbohydrate moieties of glycoproteins and glycolipids. Neuraminic acids can be enzymatically removed by the enzymes known as neuraminidases. In this study, neuraminidase from Vibrio cholerae was used to determine the contribution of neuraminic acids susceptible to this enzyme to the surface charge of haemopoietic cells.

After introductory studies on the resolution and reproducibility of the cell electrophoresis technique and on the effect of neuraminidase treatment on the electrophoretic mobility of cells (Chapter 2), the electrophoretic behaviour of CFU-s and GM CFU-c 1, 2 and 3 was investigated (Chapter 3). CFU-s can be distinguished from GM CFU-c 1, 2 and 3 on the basis of differences in their modal electrophoretic mobility (EPM). CFU-s have a higher modal EPM than GM CFU-c 1, 2 and 3, while no differences in modal EPM can be detected among the three GM CFU-c types. The distinction between CFU-s and GM CFU-c 1 supports the view that these cell types represent different developmental stages. To investigate whether differences in proliferative activity between CFU-s and GM CFU-c would be reflected in the EPM of these cells, the EPM of CFU from normal bone marrow was compared with that of CFU from regenerating bone marrow, in which all CFU are rapidly proliferating. All CFU from regenerating bone marrow have a somewhat lower EPM than those from normal bone marrow, while the difference between the modal EPM of CFU-s and GM CFU-c is somewhat smaller under regenerating conditions. This indicates that the surface charge density of CFU might in part reflect the proliferative state of these cells. The lower EPM of rapidly proliferating CFU-s would suggest that resting and proliferating CFU-s which are present in normal bone marrow have different EPM. However, such a difference could not be detected. Therefore, the properties of cells proliferating under regenerating conditions are not necessarily related to those of cells proliferating under normal steady state conditions and the exact causes of the changes in EPM of the CFU when changing to a regeneration process remain unclear.

The contribution of neuraminic acids to the surface charge of CFU-s and GM CFU-c was determined by comparison of the EPM of these cells before and after treatment with Vibrio cholerae neuraminidase. Neuraminidase treatment strongly reduces the modal EPM of CFU-s and GM CFU-c 1 by a similar percentage, has a moderate effect on the modal EPM of GM CFU-c 2 and causes only a small decrease in the modal EPM of GM CFU-c 3. CFU from normal bone marrow and rapidly proliferating CFU from regenerating bone marrow show similar relative reductions in modal EPM after neuraminidase treatment. On the basis of these data, it is hypothesized that the density of neuraminidase susceptible neuraminic acid groups on the cell surface decreases during the differentiation from CFU-s through GM CFU-c 3.

The observations also suggest other (yet undefined) differences in surface composition among the various CFU. The differences and similarities in modal EPM among nontreated CFU (normal as well as rapidly proliferating) cannot be related to the surface density of neuramini-

dase susceptible neuraminic acid groups. This indicates that neuraminic acid groups which are not removed by Vibrio cholerae neuraminidase and other charged molecules contribute to a significant extent to the CFU surface charge.

Neuraminidase treatment also reduces the number of CFU-s to a minimum of 25% of control numbers (Chapter 4). This might indicate the presence of two subpopulations of in vivo colony forming cells which differ in their sensitivity to neuraminidase with respect to their potential to form colonies in the spleen of lethally irradiated mice. This is of importance for interpreting the similarity in electrophoretic behaviour of neuraminidase treated CFU-s and GM CFU-c 1 and for the study of possible heterogeneity within the CFU-s population. Since the largest proportion of neuraminidase treated CFU-s can be recovered by pretreatment of the irradiated recipient mice with neuraminidase, it was concluded that neuraminidase does not kill CFU-s and that the enzyme treatment does not change the potential of colony forming cells to lodge in the spleen and to start proliferation. The neuraminidase "surviving" CFU-s were further investigated and compared with the total nontreated CFU-s population in terms of functional and physical properties. Neuraminidase "surviving" CFU-s cannot be distinguished from nontreated CFU-s on the basis of self-renewal, spleen seeding efficiency, potential to prevent mortality in lethally irradiated mice, electrophoretic mobility and buoyant density.

Comparison between the effects of neuraminidase treatment on the number of CFU-s from normal and regenerating bone marrow and on the number of CFU-s in different phases of the cell cycle (different sedimentation velocity fractions) revealed that cycling CFU-s are relatively less reduced in number than resting CFU-s. It is hypothesized that this cell cycle related difference in neuraminidase sensitivity is due to differences in the rate of cell membrane repair. In combination with the results described in Chapter 3 showing that differences in EPM among CFU-s in different phases of the cell cycle cannot be detected and that CFU-s from normal and regenerating bone marrow are similar in their surface density of neuraminic acids, it is concluded that the EPM properties of neuraminidase treated CFU-s are representative for the total CFU-s population. Furthermore, it can be concluded that neuraminidase "surviving" CFU-s and total nontreated CFU-s are similar in functional and physical properties except for small differences in proliferative activity.

All cell separation techniques applied to determine the cellular properties of the early haemopoietic cells must be followed by the *in vivo* and *in vitro* assay systems for these cells. This considerably impedes the investigations and also limits the number of methods for physical and chemical cell characterization, since the cells must remain viable. The possibilities for cell characterization would be greatly increased if specific cell types could be isolated from the complex mixture represented by bone marrow cells. For example, *in vitro* differentiation studies starting with single cells and studies of factor - cell interactions would be simplified, while the analysis of physical and chemical properties of the isolated cells would not be limited to vital techniques. Since the *in vivo* colony forming cells (CFU-s) are considered to represent pluripotent stem cells, they have frequently been subjected to purification studies. In this study, attempts were made to separate CFU-s from the other bone marrow cells on the basis of a number of partly independent physical and chemical parameters (Chapter 5). CFU-s are characterized by low buoyant density, high electrophoretic mobility, high surface density of neuraminic acid groups, high forward light scattering (size), low perpendicular light scattering (structure, cell organelles) and low fluorescence after staining with the vital DNA dye Hoechst 33342 (low uptake rate of the dye at low pH values). Separation of cells on the basis of either of these parameters alone does not result in high concentrations of CFU-s. Combination of cell separation techniques considerably increases the CFU-s enrichment. Several combinations are described. The highest enrichment for CFU-s was obtained by carrying out the following steps in sequence: a) selection of cells with low buoyant density by a single-step density separation; b) simultaneous incubation of cells with *Vibrio cholerae* neuraminidase and the vital DNA dye Hoechst 33342 at a pH value of 6.5; c) selection of cells with low electrophoretic mobility by free-flow electrophoresis; and d) selection of cells with high forward and low perpendicular light scattering and low fluorescence intensities by light activated cell sorting. When selecting for 50% of the total CFU-s population present in unfractionated bone marrow, this procedure results in mean CFU-s concentrations of 40 times normal. However, the yield is low (about 8%) due to nonselective cell loss. Selecting for 10% of the total CFU-s can be predicted to result in 75 times normal CFU-s concentrations but would be possible only if the cell recovery could be increased. If the incidence of stem cells in unfractionated bone marrow is estimated to be 0.7%, the 40-fold and the predicted 75-fold enrichment for CFU-s correspond to 30% and 50% stem cells, respectively. GM CFU-c 1 is en-

riched to a similar extent as CFU-s. However, the incidence of GM CFU-c 1 in unfractionated bone marrow is lower than that of CFU-s and the incidence of GM CFU-c 1 after the combination of cell separation techniques is estimated to be between 2% and 4%.

A comparison among all presently known properties of CFU-s and GM CFU-c 1, 2 and 3 is presented in the last chapter (Chapter 6). The changes which occur during differentiation from CFU-s to GM CFU-c 3 are discussed. CFU-s and GM CFU-c 1 are similar in a number of properties but small differences in size, "structuredness" and electrophoretic mobility between these cells can be detected. This supports the view that CFU-s and GM CFU-c 1 represent closely associated but different developmental stages and that GM CFU-c 1 might be among the immediate progeny of CFU-s. The further development from GM CFU-c 1 to GM CFU-c 3 is accompanied by more obvious changes such as increase in buoyant density, development of intracellular structures, decrease in neuraminidase susceptible neuraminic acid groups on the cell surface and changes in their response to different regulating factors.

Further analysis of the neuraminic acid groups on the cell surface of the early haemopoietic cells may be made possible by use of lectins and other types of neuraminidases. The use of fluoresceinated lectins and light activated cell sorting may be useful to test the results obtained by cell electrophoresis.

Finally, studies which could be performed with highly concentrated stem cell suspensions are discussed. Application of media and regulatory factors which stimulate stem cell proliferation and differentiation in cultures of single cells would allow the differentiation process to be followed step by step. Furthermore, since the colony assay would no longer be necessary, the cells in the stem cell enriched fractions could be rapidly and extensively analyzed for physical, chemical and antigenic properties. In this way, a rapid determination of parameters useful for studying differentiation events and for simplifying the isolation of specific cell types could be made.

SAMENVATTING

Alle soorten bloedcellen die hun funktie uitoefenen in de verschillende weefsels en in het perifere bloed van volwassen muizen worden gevormd vanuit één celtype: de pluripotente hemopoëtische stamcel (HSC). Gedurende het gehele leven produceren de HSC nakomelingen om steeds de rijpe eindcellen te vervangen. Onder normale "steady state" omstandigheden (dynamisch evenwicht) voert slechts een klein aantal cellen van de HSC populatie celdelingen uit en verkeren de meeste HSC in een rusttoestand. De verhouding tussen het aantal rustende en delende HSC wordt bepaald door de grootte van de vraag naar funktionele eindcellen. Door deling kunnen de HSC nieuwe HSC vormen of cellen die bestemd zijn om te differentiëren in één van de bloedcellijnen. De delingsaktiviteit van de HSC en van de vroege voorlopercellen van de verschillende rijpe bloedceltypen wordt gereguleerd door een aantal humorale en micromilieu faktoren.

Het beenmerg en in mindere mate de milt zijn de weefsels waarin bloedcelvorming plaats vindt. De HSC en de differentiatierichting gebonden voorlopercellen vormen slechts 1% tot 2% van alle gekernde beenmergcellen en kunnen niet rechtstreeks bestudeerd worden. Hun aanwezigheid kan worden onthuld door hun hoge delingscapaciteit. In geschikte *in vivo* en *in vitro* systemen kunnen HSC en differentiatierichting gebonden voorlopercellen aangezet worden tot deling en differentiatie. Dit resulteert in de vorming van kolonies (klonen) van rijpe cellen. Gegevens over het aantal en het differentiatiestadium van de verschillende vroege hemopoëtische cellen in bepaalde celsuspensies moeten worden afgeleid uit het aantal en de cellulaire samenstelling van de gevormde kolonies. HSC kunnen kolonies vormen in de milt van letaal bestraalde muizen. Wanneer beenmergcellen intraveneus worden ingespoten, zal een bepaald percentage van de HSC in de milt terecht komen en daar kolonies vormen. Deze HSC worden aangeduid met de term "colony forming units-spleen" (CFU-s) en worden als representatief beschouwd voor de totale HSC populatie. De vroege voorlopercellen van erythrocyten (E), granulocyten/macrofagen (GM) en megakaryocyten (Meg) kunnen onder invloed van verscheidene regulerende faktoren kolonies vormen *in vitro* en worden aangeduid als "colony forming units-culture" (CFU-c). Het onderzoek beschreven in dit proefschrift is beperkt tot CFU-s en GM CFU-c.

De detektiesystemen voor pluripotente en differentierichting gebonden hemopoëtische cellen hebben het mogelijk gemaakt om de

fysische, biochemische en antigene eigenschappen van deze cellen te onderzoeken. Deze karakterisering is van belang voor een direkte herkenning van deze cellen, voor de bepaling of deze cellen één celtype vertegenwoordigen of dat er onderscheid gemaakt kan worden tussen meerdere celtypen en voor het onderzoek naar de cellulaire veranderingen die plaats vinden tijdens de differentiatie. Er zijn verschillende technieken ontwikkeld waarmee cellen gescheiden kunnen worden op grond van verschillen in de bovengenoemde eigenschappen zonder dat daarbij de levensvatbaarheid van de cellen wordt geschaad. Informatie over de eigenschappen van hemopoëtische cellen kan verkregen worden door de celfrakties die verzameld zijn na celscheidingsprocedures in vivo en in vitro te testen op de aanwezigheid van kolonievormende cellen. Celscheiding op grond van verschillen in zweefdichtheid (soortelijk gewicht) en in zinksnelheid (soortelijk gewicht en celgrootte) hebben verschillen in dichtheid tussen rustende en delende CFU-s aan het licht gebracht. Door combinatie van deze celscheidingstechnieken met verschillende soorten groeistimulerende faktoren konden in de GM CFU-c populatie drie opeenvolgende differentiatiestadia herkend worden: GM CFU-c 1, 2 en 3. Deze drie GM CFU-c typen zijn gekenmerkt door een toenemende zweefdichtheid. CFU-s, GM CFU-c 2 en GM CFU-c 3 kunnen eenvoudig worden onderscheiden op grond van fysische eigenschappen, respons op verschillende faktoren en delingsaktiviteit. CFU-s en GM CFU-c 1 vertonen een aantal overeenkomsten in eigenschappen wat kan duiden op een zeer nauwe verwantschap tussen deze celtypen. Het huidige onderzoek richt zich op verdere karakterisering van de eigenschappen van de verschillende CFU typen om meer informatie te verkrijgen over de cellulaire veranderingen die plaats vinden tijdens de ontwikkeling van CFU-s tot GM CFU-c 3.

Zweefdichtheid en zinksnelheid kunnen moeilijk vertaald worden in specifieke celeigenschappen. Zij kunnen beschouwd worden als de resultanten van een complex van structuren en metabolische processen (zoals kern/cytoplasma verhouding, aanwezigheid en type van celorganellen, mate van RNA en eiwit synthese, enz.) welke moeilijk specifiek onderzocht kunnen worden zonder de levensvatbaarheid van cellen aan te tasten. Eigenschappen van het celoppervlak zijn meer toegankelijk voor onderzoek. Het hier beschreven onderzoek betreft de chemische karakterisering van het oppervlak van hemopoëtische cellen met behulp van de "free-flow" celelektroforesetechniek. Met deze techniek kunnen cellen gescheiden worden op grond van verschillen in ladingsdichtheid aan het celoppervlak die bepaald wordt door de chemische membraansamenstelling. De aanwezigheid van bepaalde geladen moleculen op het celoppervlak kan bestudeerd worden door deze moleculen te verwijderen

of af te schermen met specifieke reagentia. Dit soort behandelingen zal de lading op het celoppervlak veranderen wat tot uiting komt in verandering van de elektroforetische mobiliteit van cellen. Bij neutrale pH waarden dragen de meeste cellen een negatieve lading op hun oppervlak. Deze negatieve lading wordt gedeeltelijk veroorzaakt door de aanwezigheid van neuraminezuren die algemeen voorkomen als terminale groepen van de suikergedeelten van glycoproteinen en glycolipiden. Neuramininezuren kunnen verwijderd worden door enzymen die bekend staan als neuraminidases. In dit onderzoek werd neuraminidase van Vibrio cholerae gebruikt om de bijdrage van neuraminezuren aan de oppervlaktelading van hemopoëtische cellen te bepalen.

Na inleidende experimenten betreffende de resolutie en reproduceerbaarheid van de celelektroforesetechniek en het effekt van neuraminidase behandeling op de elektroforetische mobiliteit van cellen (Hoofdstuk 2), werd het elektroforetische gedrag van CFU-s en GM CFU-c 1, 2 en 3 onderzocht (Hoofdstuk 3). CFU-s kunnen van GM CFU-c 1, 2 en 3 onderscheiden worden op grond van verschillen in elektroforetische mobiliteit (EPM). CFU-s hebben een hogere modale EPM dan GM CFU-c 1, 2 en 3. Tussen de drie GM CFU-c typen kunnen geen verschillen worden waargenomen. Het onderscheid tussen CFU-s en GM CFU-c 1 ondersteunt de opvatting dat deze celtypen verschillende ontwikkelingsstadia vertegenwoordigen. Teneinde vast te stellen of de verschillen in EPM tussen CFU-s en GM CFU-c veroorzaakt zouden kunnen worden door verschillen in delingsaktiviteit, werd de EPM van CFU van normaal beenmerg vergeleken met die van CFU van regenererend beenmerg waarin alle CFU een hoge delingsaktiviteit vertonen. Alle CFU van regenererend beenmerg hebben een iets lagere EPM dan die van normaal beenmerg, terwijl het verschil in modale EPM tussen CFU-s en GM CFU-c iets kleiner is onder regenererende omstandigheden. Dit betekent dat de oppervlakteladingsdichtheid van CFU gedeeltelijk de delingsaktiviteit van deze cellen zou kunnen weergeven. De lagere EPM van sneldelende CFU-s zou doen vermoeden dat rustende en delende CFU-s die aanwezig zijn in normaal beenmerg een verschillende EPM hebben. Zo'n verschil kon echter niet worden aangetoond. Dit geeft aan dat de eigenschappen van sneldelende cellen die aangetroffen worden gedurende regeneratie processen niet noodzakelijkerwijs de eigenschappen weerspiegelen van cellen die onder normale "steady state" omstandigheden delen. De juiste oorzaak van de veranderingen in EPM van de CFU bij de overgang van normale naar regenererende omstandigheden blijft onduidelijk.

De bijdrage van neuraminezuren aan de oppervlaktelading van CFU-s en GM CFU-c werd bepaald door de EPM van deze cellen voor en na behandeling met Vibrio cholerae neuraminidase te vergelijken. Behandeling van beenmergcellen met neuraminidase brengt de modale EPM van CFU-s en GM CFU-c 1 sterk terug met een gelijk percentage. De modale EPM van GM CFU-c 2 wordt minder gereduceerd door de enzymbehandeling dan de EPM van CFU-s en GM CFU-c 1, terwijl de modale EPM van GM CFU-c 3 het minst wordt beinvloed. CFU van normaal beenmerg en sneldelende CFU van regenererend beenmerg vertonen gelijke percentuele redukties in de modale EPM na neuraminidase behandeling. Op grond van deze gegevens werd aangenomen dat de dichtheid van neuraminidase gevoelige neuraminezuurgroepen op het celoppervlak afneemt tijdens de differentiatie van CFU-s naar GM CFU-c 3.

De waarnemingen duiden ook op andere (nog onbekende) verschillen in de oppervlakte eigenschappen van de diverse CFU typen. De verschillen en overeenkomsten in de modale EPM tussen de onbehandelde CFU typen (zowel normale als sneldelende) kunnen niet gecorreleerd worden met de oppervlaktedichtheid van neuraminidase gevoelige neuraminezuurgroepen. Dit duidt erop dat neuraminezuurgroepen die niet door Vibrio cholerae neuraminidase verwijderd worden en andere geladen moleculen in een belangrijke mate bijdragen tot de CFU oppervlakte lading.

Neuraminidase behandeling heeft niet alleen een effekt op de EPM van cellen, maar brengt ook het aantal CFU-s terug tot een minimum van 25% van de controle aantallen. Dit zou kunnen wijzen op het bestaan van twee subpopulaties van in vivo kolonievormende cellen die verschillen in hun gevoeligheid voor neuraminidase met betrekking tot hun mogelijkheid om kolonies te vormen in de milt van letaal bestraalde muizen. Dit is van belang voor de interpretatie van de overeenkomsten in elektroforetisch gedrag van met neuraminidase behandelde CFU-s en GM CFU-c 1 en voor de bestudering van mogelijke heterogeniteit binnen de CFU-s populatie. Daar het grootste gedeelte van de met neuraminidase behandelde CFU-s teruggewonnen kan worden door de bestraalde ontvanger muizen in te spuiten met neuraminidase, kan aangenomen worden dat neuraminidase de CFU-s niet doodt en dat de enzymbehandeling de potentie van kolonievormende cellen om zich in de milt te vestigen en te prolifereren niet verandert. De neuraminidase "overlevende" CFU-s werden verder onderzocht en vergeleken met de gehele onbehandelde CFU-s populatie wat betreft funktionele en fysische eigenschappen. Neuraminidase "overlevende" CFU-s kunnen niet onderscheiden worden van onbehandelde CFU-s op grond van zelfvernieuwing, de eigenschap om zich in de milt te nestelen, de geschiktheid om de

dood van letaal bestraalde muizen te voorkomen, elektroforetische mobiliteit en zweefdichtheid.

Vergelijking tussen de uitwerking van neuraminidase behandeling op het aantal CFU-s van normaal en regenererend beenmerg en op het aantal CFU-s in verschillende fasen van de celcyclus (CFU-s met verschillende zinksnelheden) onthulde dat delende CFU-s verhoudingsgewijs minder in aantal teruggebracht worden dan rustende CFU-s. Dit verschil zou mogelijk verklaard kunnen worden door een verschil in herstelsnelheid van het celmembraan. Op grond van de gegevens over de funktionele en fysische eigenschappen van neuraminidase "overlevende" CFU-s en onbehandelde CFU-s in combinatie met de resultaten die in hoofdstuk 3 beschreven zijn en die aantonen dat verschillen in EPM tussen CFU-s in verschillende fasen van de celcyclus niet gedetekteerd kunnen worden en dat CFU-s van normaal en regenererend beenmerg gelijk zijn in hun oppervlaktedichtheid van neuraminezuren, kan gekonkludeerd worden dat de EPM eigenschappen van met neuraminidase behandelde CFU-s representatief zijn voor de gehele CFU-s populatie. Verder kan de konklusie getrokken worden dat neuraminidase "overlevende" CFU-s en onbehandelde CFU-s met uitzondering van kleine verschillen in delingsaktiviteit, gelijksoortig zijn in funktionele en fysische eigenschappen.

Alle celscheidingstechnieken die toegepast worden voor de bepaling van de eigenschappen van de vroege hemopoëtische cellen moeten worden gevolgd door de in vivo en in vitro testsystemen voor deze cellen. Dit vertraagt het onderzoek aanzienlijk en beperkt ook het aantal technieken voor de fysische en chemische celkarakterisering omdat de cellen levensvatbaar moeten blijven. De mogelijkheden tot celkarakterisering zouden enorm vergroot kunnen worden als specifieke celtypen geisoleerd konden worden uit het complexe mengsel van beenmergcellen. In vitro differentiatie studies die beginnen met afzonderlijke cellen en onderzoek betreffende de werkingsmechanismen van proliferatie en differentiatie inducerende faktoren zouden eenvoudiger worden, terwijl de analyse van fysische en chemische eigenschappen van het geisoleerde celtype niet beperkt zou zijn tot het gebruik van vitale technieken. Daar algemeen wordt aangenomen dat de in vivo kolonievormende cellen (CFU-s) pluripotente stamcellen vertegenwoordigen, is herhaaldelijk geprobeerd deze cellen te isoleren. In het hier beschreven onderzoek werden pogingen gedaan om de CFU-s van de andere beenmergcellen te scheiden op grond van een aantal gedeeltelijk onafhankelijke fysische en chemische eigenschappen (Hoofdstuk 5). CFU-s worden gekenmerkt door een lage zweefdichtheid, hoge oppervlaktedichtheid van neuraminezuurgroepen, hoge voorwaartse lichtverstrooiing (celgrootte),

lage zijwaartse lichtverstrooiing (celstructuren, celorganellen) en lage fluorescentie na kleuring met de vitale DNA kleurstof "Hoechst 33342" (lage opnamesnelheid van de kleurstof bij lage pH-waarden). Door scheiding van cellen op grond van één van deze eigenschappen alleen kunnen geen hoge CFU-s concentraties verkregen worden. Een aanzienlijke verhoging in de verrijking van CFU-s kan bereikt worden door combinatie van celscheidingstechnieken. Verscheidene combinaties zijn beschreven. De grootste verrijking van CFU-s werd verkregen door de hierna genoemde stappen achtereenvolgens uit te voeren: a) selektie van beenmergcellen met een lage zweefdichtheid door middel van een éénstaps dichtheidsscheiding; b) gelijktijdige incubatie van de cellen met Vibrio cholerae neuraminidase en de vitale DNA kleurstof "Hoechst 33342" bij een pH waarde van 6.5; c) selektie van cellen met een lage elektroforetische mobiliteit door middel van free-flow elektroforese; en tenslotte d) selektie van cellen met hoge voorwaartse en lage zijwaartse lichtverstrooiing en lage fluorescentie intensiteiten door middel van "light activated cell sorting". Deze procedure resulteert in een CFU-s concentratie die gemiddeld 40 maal hoger is dan de normale, als men selekteert voor 50% van de totale ongefractioneerde CFU-s populatie. De opbrengst is echter laag (ongeveer 8%) hetgeen te wijten is aan niet-selektief celverlies. Als men selekteert voor 10% van de totale CFU-s populatie zou voorspeld kunnen worden dat dit resulteert in 75 maal de normale CFU-s concentratie. Dit is echter alleen mogelijk als de opbrengst van cellen vergroot kan worden. Als het aantal pluripotente stamcellen in ongefraktioneerd beenmerg gesteld wordt op 0,7 % dan komen de 40-voudige en de voorspelde 75-voudige verrijkingen van CFU-s overeen met respectievelijk 30% en 50% stamcellen. In de gevolgde celscheidingsprocedure wordt GM CFU-c 1 in gelijke mate verrijkt als CFU-s. In ongefraktioneerd beenmerg komen echter minder GM CFU-c 1 voor dan CFU-s en de concentratie van GM CFU-c 1 in celsuspensies verkregen na de combinatie van celscheidingstechnieken wordt geschat op 2 à 4%.

Een vergelijking tussen alle tot dusver bekende eigenschappen van CFU-s en GM CFU-c 1, 2 en 3 wordt gepresenteerd in het laatste hoofdstuk (Hoofdstuk 6). De veranderingen die plaats vinden tijdens de differentiatie van CFU-s naar GM CFU-c 3 worden besproken. CFU-s en GM CFU-c 1 hebben een aantal overeenkomstige eigenschappen, maar kleine verschillen in grootte, celstrukturen en elektroforetische mobiliteit tussen deze celtypen kunnen worden waargenomen. Dit staaft de opvatting dat CFU-s en GM CFU-c 1 nauw verwante maar verschillende ontwikkelingsstadia vertegenwoordigen en dat GM CFU-c 1 tot de on-

middelijke nakomelingen van CFU-s zouden kunnen behoren. De verdere ontwikkeling van GM CFU-c 1 naar GM CFU-c 3 gaat gepaard met duidelijker veranderingen zoals toename in zweefdichtheid, ontwikkeling van intracellulaire strukturen, afname van neuraminidase gevoelige neuraminezuurgroepen op het celoppervlak en veranderingen in hun respons op verschillende regulatiefaktoren.

Verdere analyse van de neuraminezuurgroepen op het celoppervlak van de vroege hemopoëtische cellen zou mogelijk zijn door gebruik te maken van andere typen neuraminidase en van lectines. Het gebruik van met fluorescerende stoffen gemerkte lectines en "light activated cell sorting" zou van nut kunnen zijn om de resultaten te testen die verkregen zijn uit de experimenten met celelektroforese.

Tot slot worden studies besproken die uitgevoerd kunnen worden met hoog geconcentreerde stamcelpreparaten. Gebruik van media en faktoren die de stamcelproliferatie en differentiatie stimuleren in kweken van afzonderlijke cellen zou het mogelijk maken het differentiatieproces stap voor stap te volgen. Verder zouden, omdat de in vivo kolonie test niet langer nodig zou zijn, de cellen in de stamcel verrijkte frakties snel en uitgebreid geanalyseerd kunnen worden op fysische, chemische en antigene eigenschappen. Op deze manier zou een snelle selektie gemaakt kunnen worden voor parameters die bruikbaar zijn voor het bestuderen van differentiatieprocessen en voor het vereenvoudigen van de isolatie van specifieke celtypen.

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ABBREVIATIONS

BFU	Burst Forming Units. Early erythrocyte progenitor cells which give rise to large colonies composed of many small clusters *in vitro* in the presence of Burst Promoting Activity (BPA) and Erythropoietin (Ep).
BPA	Burst Promoting Activity. Factor(s) required for colony formation by Burst Forming Units (BFU).
CEF	Colony Enhancing Factors. Factors which enhance granulocyte/macrophage colony formation in bone marrow cell cultures stimulated by Colony Stimulating Factor (CSF). Different types of CEF are required for colony formation by different developmental stages of granulocyte/macrophage progenitor cells.
CFC	Colony Forming Cells. Used in this thesis only for *in vivo* colony forming cells, i.e. all cells capable of forming colonies in the spleen of lethally irradiated mice. The CFC which permanently lodge in the spleen are designated as Colony Forming Units-spleen (CFU-s). The CFC are considered to represent Pluripotent Haemopoietic Stem Cells (HSC).
CFU-c	Colony Forming Units-culture. Early progenitor cells of the various cell lineages giving rise to colonies *in vitro* in the presence of appropriate stimuli. In the literature, generally restricted to granulocyte/macrophage progenitor cells.
CFU-s	Colony Forming Units-spleen. Pluripotent cells which give rise to colonies in the spleen of lethally irradiated mice. The colonies contain new CFU-s and cells belonging to the various differentiation lines. CFU-s represent a fixed proportion of injected *in vivo* Colony Forming Cells (CFC).

CSF	Colony Stimulating Factor. Factor which is essential for colony formation by granulocyte/macrophage progenitor cells *in vitro*.
E	Erythrocytes.
Ep	Erythropoietin. Factor which is essential for colony formation by erythrocyte progenitor cells *in vitro*.
EPM	Electrophoretic Mobility. Velocity of a cell per unit field strength. In media of relatively high ionic strength, the EPM is a measure for the charge density on the cell surface.
FLS	Forward Light Scattering. Scattered light signals of a cell over 20^{o} in the direction of the laser beam, measured in a light activated cell sorter. FLS signals are proportional to the cross-sectional area of a cell.
GM	Granulocytes and Macrophages.
H 33342	Hoechst 33342. A supravital DNA dye.
HBSS	Hanks' Balanced Salt Solution.
HSC	Pluripotent Haemopoietic Stem Cells. The common ancestor cell for all haemopoietic differentiation pathways.
LYS	Lysates of erythrocytes. A Colony Enhancing Factor (CEF) which promotes colony formation by a late stage granulocyte/macrophage progenitor cell (GM CFU-c 3) in CSF stimulated bone marrow cultures.
Meg	Megakaryocytes.
modus	The absolute peak of a distribution. Often used to compare the properties of various cell types.
NANA	N-acetylneuraminic acids. Generally occurring terminal groups of the carbohydrate moieties of glycoproteins and glycolipids.

neur.	Neuraminidase from Vibrio cholerae. It can split most linkages of NANA to carbohydrates.
normal bm	Normal bone marrow. Bone marrow from nontreated healthy mice.
nucl. cells	Nucleated cells.
18 h PES	Postendotoxin Serum. Serum from mice injected with bacterial endotoxin 18 hours earlier. A Colony Enhancing Factor (CEF) which promotes colony formation by an early stage granulocyte/macrophage progenitor cell (GM CFU-c 1) in CSF stimulated bone marrow cultures.
PLS	Perpendicular Light Scattering. Scattered light signals of a cell between 70° and 110° with respect to the direction of the laser beam, measured in a light activated cell sorter. PLS can be used as a measure for the "structuredness" of a cell.
PMUE	Pregnant Mouse Uterus Extract. A source of Colony Stimulating Factor (CSF)
regen. bm	Regenerating bone marrow. Bone marrow from mice which were lethally irradiated and then injected with 2.5×10^{6} nucleated bone marrow cells from non-treated syngeneic donors.
SAF	Stem Cell Activating Factors. Factors which stimulate the proliferation of CFU-s.
sed. vel.	sedimentation velocity.
spleen seeding efficiency	The proportion of injected in vivo Colony Forming Cells (CFC) lodging permanently in the spleen of lethally irradiated mice.
Tp	Thrombopoietin. Factor which is essential for colony formation by megakaryocyte progenitor cells in vitro.

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