

## Concentration of Hematopoietic Progenitor Cells from Human Bone Marrow by a New Type of Blood Component Separator

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**Abstract.** A new type of blood component separator (BCS) was used for the isolation of hematopoietic progenitor cells from human bone marrow aspirates. The BCS was filled with 100–150 ml bone marrow and centrifuged to prepare a buffy coat. This buffy coat was isolated in 10–15% of the original bone marrow volume and contained  $64 \pm 8\%$  of the nucleated cells (NC). Morphological examination revealed that the buffy coat was highly enriched for myeloblasts, promyelocytes, lymphocytes and monocytes, whereas the contamination with granulocytes was reduced to  $46 \pm 8\%$  of the granulocytes initially present in the bone marrow suspension. In addition the contamination with red blood cells (RBC) was very low; the buffy coat contained only  $6 \pm 2\%$  of the RBC. Furthermore it was demonstrated by means of colony assays that the buffy coat was highly enriched for hematopoietic progenitor cells. It contained  $91 \pm 6\%$  of the granulocyte/monocyte progenitor cells (CFU-GM) and  $87 \pm 9\%$  of the erythroid progenitor cells (BFU-E). These results are comparable to those obtained with continuous or semicontinuous blood cell processors. The advantages of the BCS is that it is a simple and inexpensive apparatus which fits in a normal blood bank centrifuge. It permits efficient preparation and isolation of a buffy coat from human bone marrow without substantial loss of hematopoietic progenitor cells.

### Introduction

Bone marrow failure, as a result of anti-cancer therapy, is often treated by reinfusing cryopreserved autologous bone marrow. To facilitate storage, reconstitution and in vitro

manipulation, various methods have been developed to concentrate the marrow cells.

Most investigators use semicontinuous [Weiner et al., 1977; Ma and Biggs, 1982; Van de Ouweland et al., 1982] or continuous

[*Gilmore et al.*, 1983] centrifugation procedures to prepare a buffy coat from the bone marrow suspension to remove red blood cells (RBC) and granulocytes. However, since these techniques are rather expensive and require skillful personnel they are generally applied in the larger medical centers only.

Density gradient centrifugation has also been used to isolate the mononuclear cell fraction (including the hematopoietic progenitor cells) from bone marrow suspensions [*Dicke et al.*, 1968; *Wells et al.*, 1979]. However, the number of cells that can be applied to a density gradient is limited. *Gilmore et al.* [1982] solved this problem by first preparing a buffy coat in an IBM 2991 blood cell processor to reduce the cell number and subsequently isolating the mononuclear cells using Ficoll/metrizoate in the same apparatus.

We recently developed a prototype of an inexpensive blood component separator (BCS) [*Figdor et al.*, 1982a, b]. This sandglass resembling apparatus fitted in a normal preparative bloodbank centrifuge and was used to prepare buffy coats from peripheral blood. After centrifugation, the buffy coat was fractionated in a fraction containing pure platelets and a mononuclear cell fraction containing only 3% granulocytes.

In the present communication it is shown that the BCS is also perfectly suited for the isolation of hematopoietic progenitor cells from bone marrow cells.

## Materials and Methods

### *Bone Marrow Aspiration*

Bone marrow was harvested for cryopreservation under general or epidural anesthesia from the posterior iliac crests of 9 patients (acute leukaemia in first rem-

ission) in disposable syringes (10 ml) containing 1 ml acidic citrate dextrose (ACD) and 50 IU preservative free heparin. The marrow was filtered (70  $\mu$ m pore size) and collected in transfer bags. 100–150 ml (10%) of the bone marrow suspension was separated as described below. The remainder was processed routinely by means of a haemonetics model 30 cell separator and cryopreserved, as described previously [*Van de Ouweland et al.*, 1982].

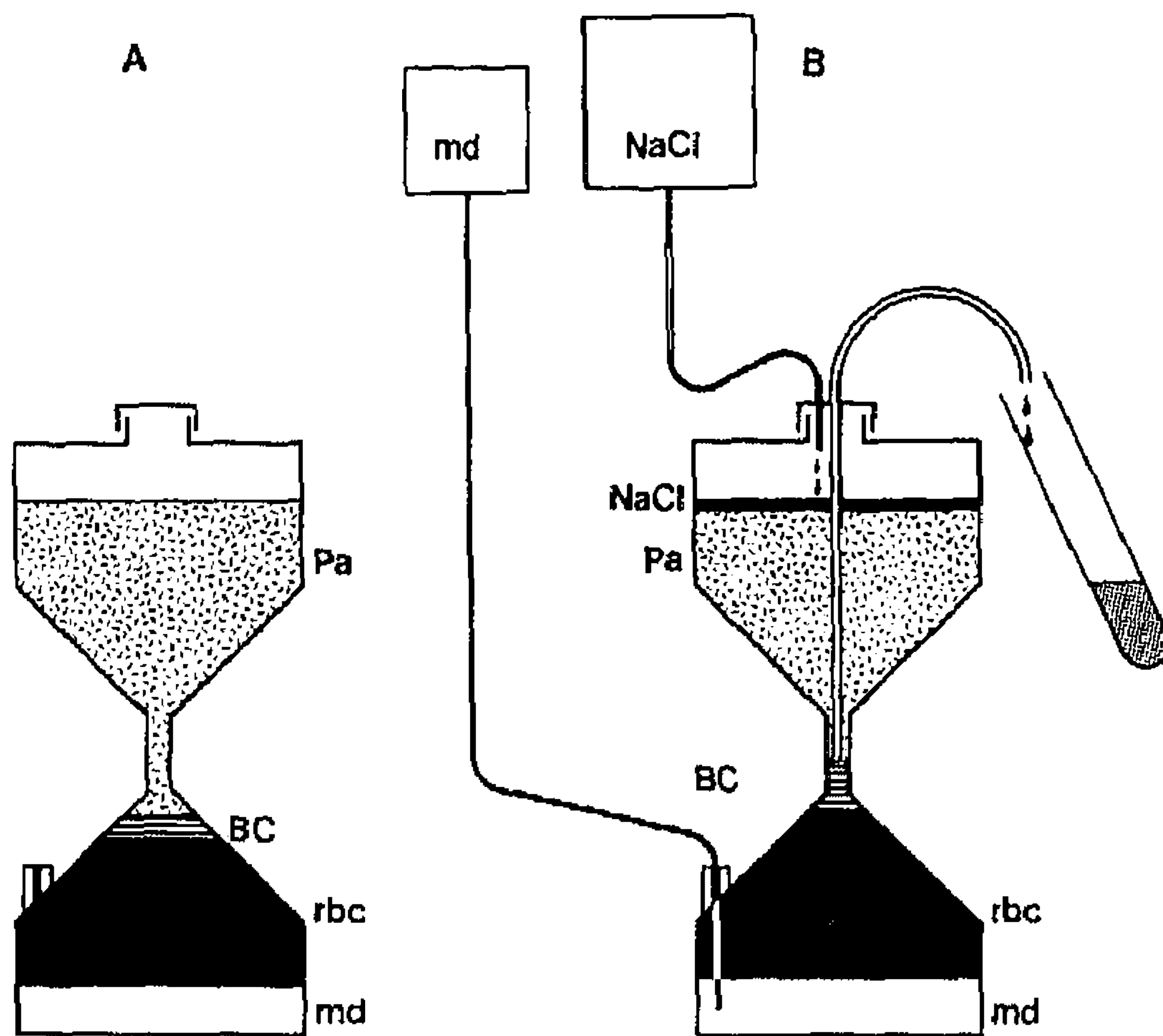
### *Cell Separation*

A buffy coat was prepared from bone marrow aspirates by means of a recently developed BCS [*Figdor et al.*, 1982a]. This sandglass resembling apparatus was made of glass and consists of an upper (180 ml) and lower (150 ml) compartment connected by a narrow tube. The device was filled with 100–150 ml of a bone marrow suspension that was diluted with an equal volume of phosphate-buffered saline containing 15% ACD. In some experiments we added to this suspension 10% Plasmasteril (Fresenius, Bad Homburg, FRG), which contained 6% hydroxyethyl starch (HES) as a rouleau-forming agent.

The volume of the lower compartment of the BCS was adjusted to the hematocrit in such a way that after centrifugation (30 min, 500 g at room temperature) the buffy coat was formed just below the narrow tube (fig. 1a). To this end we injected Maxidens (MD; Nye-gaard and Co., Oslo, Norway) into the lower compartment. MD is a nontoxic autoclavable fluid that is immiscible with aqueous solutions and has a density of 1.9 g/ml. The volume of MD required could be calculated after determination of the hematocrit of the bone marrow suspension. After centrifugation, a fractionation device is screwed on top of the apparatus (fig. 1b). Fractionation is accomplished by the simultaneous introduction of NaCl in the upper compartment and md in the lower compartment. This procedure has the advantage that the plasma acts as a sheath flow surrounding the buffy coat cells, and thus prevents sticking of these cells to the wall of the outlet tube. In addition the buffy coat cells were collected in autologous plasma. Five fractions were collected at the outlet. Fractions 1–4 had a volume of 5 ml each, fraction 5 which contained the remaining RBC layer and the plasma had a volume of 180–280 ml.

The whole procedure including centrifugation and collection of the various fractions took less than 90 min.





**Fig1.** The blood component separator (in cross-section) is filled with bone marrow and centrifuged **A**. After centrifugation the buffy coat (BC) cells are isolated (**B**) by the simultaneous introduction of NaCl and MD. The flow of NaCl and MD is generated by means of hydrostatic pressure. Pa=Plasma.

#### *Cell Counting and Morphology*

The number of RBC and nucleated cells (NC) was determined by means of a Coulter Counter Model ZF (Coulter Electronics Ltd, Dunstable, Beds., UK).

The morphology was examined on cytocentrifuge preparations after staining with May-Grünwald Giemsa.

#### *Committed Stem Cell Assays*

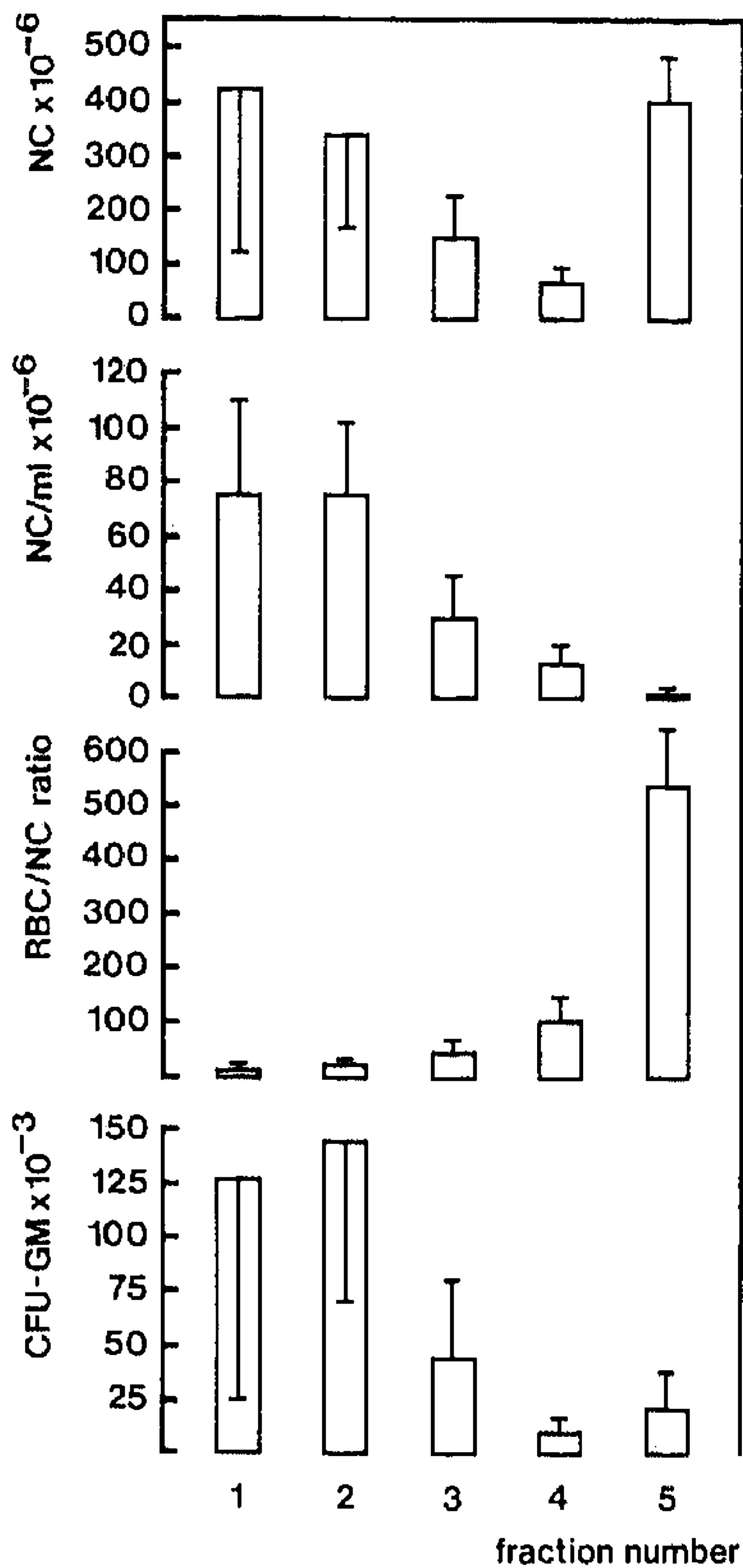
Granulocyte-macrophage colony forming cells (GFU-GM) and erythroid colony forming cells (BFU-E) were determined as described previously [De Witte et al., 1984]. Briefly, the number of GFU-GM was determined after incubation of  $1-2 \times 10^5$  cells in 2 ml semi-solid agar (Difco) containing 4% placenta conditioned medium and 20% fetal calf serum (FCS) for a period of 10–12 days. Aggregates of 40 and more cells were counted as colonies.

The number of BFU-E was determined by incubating  $1 \times 10^5$  cells/ml in Iscove's modified Dulbecco's medium (IMDM, Gibco 78-5220) containing 0.8% methylcellulose (Fluka, Schweiz),  $10^{-4}$  M thyoglycerol, 10% BSA (Sigma), 20% FCS (Rehatain), 20% leukocyte conditioned medium and 2 units sheep erythropoietin (Connaught Medical Research Laboratories). Orange to red colonies of at least three subclusters or one single colony of more than 300 cells were scored as BFU-E on day 14 of the culture. Before culture, a sam-

ple of the original bone marrow suspension and fraction five were centrifuged over Ficoll/Isopaque with a density of 1.085 to remove most of the RBC.

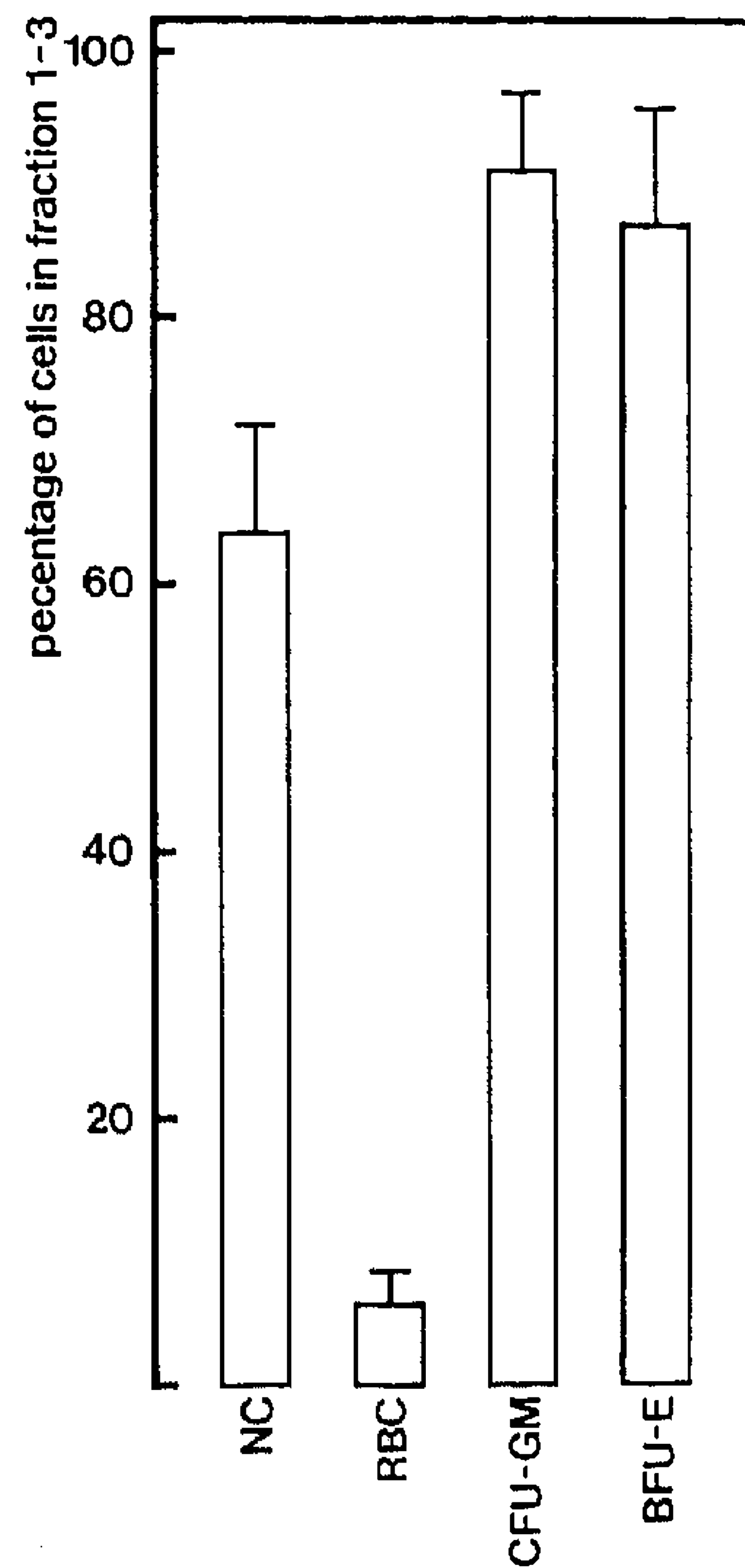
## **Results**

Bone marrow was aspirated from acute leukemia patients in first remission. 100–150 ml of the bone marrow suspension which contained  $980-1,870 \times 10^6$  NC was centrifuged in the BCS and fractionated into five fractions. The buffy coat was isolated in fractions 1–3, fraction 4 was an intermediate fraction, and fraction 5 contained both the remaining plasma and the RBC layer (including the remaining granulocytes). These 5 fractions contained  $95 \pm 7\%$  of the NC. The distribution of the NC in the various fractions is shown in Figure 2a. The number of NC/ml (fig. 2b) gradually decreased from  $751 \pm 34 \times 10^6$ /ml in fraction 1 to only  $2 \pm 0.5 \times 10^6$ /ml in fraction 5. The RBC/NC ratio which is shown in figure 2c indicates



**Fig 2.** Absolute cell counts and RBC/NC ratios of the various fractions. **a** Distribution of the NC in the various fractions. **b** Number of NC/ml. **c** RBC/NC ratio was calculated to determine the RBC contamination. **d** Distribution of the CFU-GM in the various fractions. Unfractionated bone marrow contained  $1,450 \pm 450 \times 10^6$  NC ( $11.7 \pm 3.1 \times 10^6$ /ml). The RBC/NC ratio was  $162 \pm 68$  and the unfractionated marrow contained  $421 \pm 168 \times 10^3$  CFU/GM.

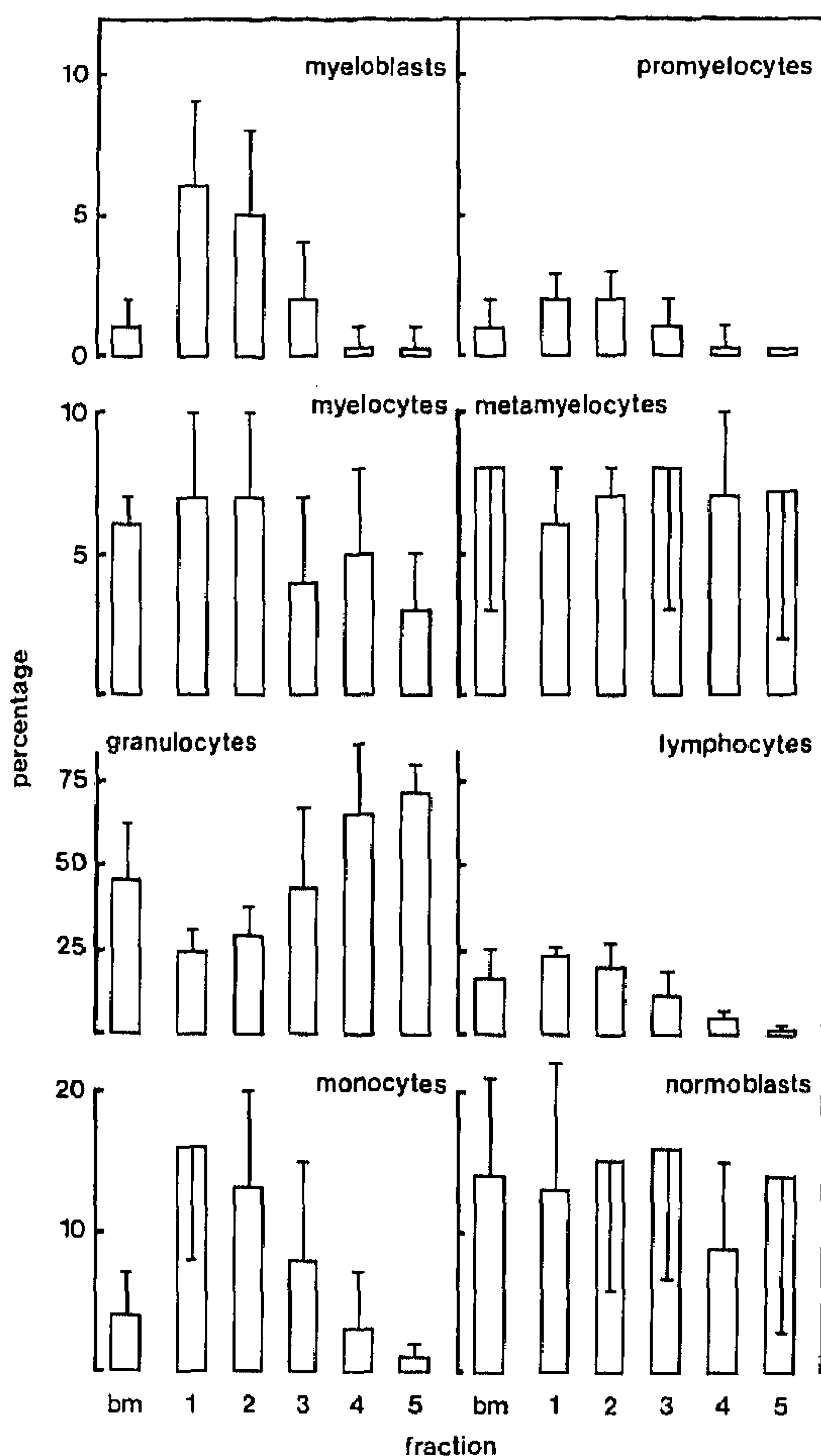
that the RBC contamination was very low in the first three fractions. The RBC/NC ratio increased from  $10 \pm 4$  in fraction 1 to  $540 \pm 105$  in fraction 5. The distribution of the hematopoietic progenitor cells (CFU-GM), which is shown in figure 2d, demonstrated that most of the CFU-GM were recovered in fractions 1, 2 and 3.



**Fig. 3.** Recoveries of the NC, RBC, CFU-GM and BFU-E in the buffy coat (fraction 1-3) of the bone marrow aspirates. Mean  $\pm$  SD of 9 experiments.

The buffy coat which was collected in the first three fractions contained  $64 \pm 8\%$  of the NC (fig. 3). Furthermore, it is shown in figure 3 that the buffy coat contained  $91 \pm 6\%$  of the CFU-GM and  $87 \pm 9\%$  of the BFU-E initially present in the bone marrow suspension. In addition the buffy coat was contaminated with only  $6 \pm 2\%$  of the RBC and  $46 \pm 8\%$  of the granulocytes initially present in the original bone marrow aspirate (fig. 3).

In some experiments we added HES to the bone marrow suspension prior to centrifugation. The results obtained with these bone marrow suspensions did not differ sig-



**Fig 4.** Morphological characterization of the various bone marrow fractions after staining with May-Grünwald Giemsa. Mean  $\pm$  SD of 5 experiments.

nificantly from experiments carried out without the addition of HES (results not shown).

The morphological characterization of the cells in the various fractions is shown in figure 4. Both myeloblasts and promyelocytes were highly enriched in fractions 1-3 ( $95 \pm 4\%$  were recovered in fractions 1-3, respectively) while myelocytes and meta-

myelocytes were distributed equally over fractions 1-5. The number of granulocytes gradually increased with the fraction number, while in contrast the majority of the lymphocytes ( $91 \pm 5\%$ ) and monocytes ( $89 \pm 8\%$ ) was recovered in fractions 1-3. The normoblasts were distributed equally over all five fractions.

## Discussion

The aim of the present study was to investigate whether the BCS could be used for the isolation of hematopoietic progenitor cells by preparing a buffy coat from human bone marrow. The results demonstrated that approximately 90% of the CFU-GM and BFU-E could be isolated in a buffy coat (fraction 1-3) in a volume of only 10-15% of the original bone marrow aspirate. In addition the buffy coat contained more than 90% of the mononuclear cells and only  $6 \pm 2\%$  and  $46 \pm 8\%$  of the RBC and granulocytes, respectively.

The addition of a rouleau-forming agent to the bone marrow suspension did not result in a better separation. Furthermore we found that the optimal temperature to separate bone marrow with the BCS was 25-30 °C (not shown).

The results obtained with the BCS are comparable to those obtained with semicontinuous or continuous blood cell separators [Weiner et al., 1977; Van de Ouweland et al., 1982; Gilmore et al., 1983]. The BCS is a simple and inexpensive apparatus and has the advantage that it can be used in normal blood bank centrifuges. It does not need specially trained personnel and could therefore also be applied in the smaller medical centers.



Bone marrow may be manipulated in vitro prior to infusion into the patient: either to remove T cells from allogeneic bone marrow in order to prevent graft-versus-host disease [Filipovich et al., 1982; Prentice et al., 1982], or to remove tumor cells that contaminate autologous bone marrow from cancer patients [Buckman et al., 1982; Treleaven et al., 1984]. To obtain optimal results it is essential to concentrate the bone marrow progenitor cells as much as possible prior to in vitro manipulation. For this purpose the BCS seems to be suited extremely well.

Until now the prototype of the BCS used in this study can process a volume of up to 150 ml of bone marrow aspirate. However, volumes that have to be processed for clinical use (bone marrow transplantations) are much larger, 800–1,200 ml in general. Therefore, we recently started the development of a larger and disposable version of the BCS by means of which volumes of up to 600 ml can be separated and that also fits in a bucket of a normal blood bank centrifuge.

In our opinion a clinically applicable version of the BCS might further complete the scale of techniques currently available to separate bone marrow, since it allows the efficient isolation of bone marrow progenitor cells under sterile conditions by simple means.

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