British Journal of Haematology, 1990, 76, 174–179

Differential effects of recombinant human colony stimulating factor (rh G-CSF) on stem cells in marrow, spleen and peripheral blood in mice

B. BUNGART,[1,2] M. LOEFFLER, [1] H. GORIS,[2] B. DONTJE,[2] V. DIEHL[1] AND W. NIJHOF[2]
[1]*Medizinische Klinik I, University of Cologne, and* [2]*Laboratory of Physiological Chemistry, University of Groningen*

Received 8 December 1989; accepted for publication 4 June 1990

Summary. Previously it has been hypothesized that the granulopoietic and erythropoietic lineages may compete for differentiating stem cells. According to this hypothesis one would expect that a stimulation of granulopoiesis by G-CSF administration would lead to a reduction of the stem cell pool and be followed by a decline of erythropoietic progenitor numbers. In addition one would expect an enhanced response of granulopoiesis if G-CSF administration were combined with suppression of erythropoiesis by red cell transfusion.

To evaluate whether this hypothesis holds true C57bl mice were injected subcutaneously for 6 d with 3·75 μg rh G-CSF/mouse/d (150 μg G-CSF/kg body weight/d). Marrow CFU-S numbers showed an increase to 160% on day 2, followed by a decrease to 50% of control on day 6. Splenic and peripheral blood CFU-S increased 20-fold and 10-fold, respectively. Marrow CFU-E declined to 40% of the control value. Splenic CFU-E increased 10-fold. The increase in marrow CFU-GM numbers ranged between 140% and 180%. CFU-GM obtained from the spleen and the peripheral blood increased 60-fold and 15-fold, respectively. Regarding the CFU-S and CFU-GM a similar pattern of response was found in an experiment where rh G-CSF administration was combined with an additional red cell transfusion. These data do not provide convincing evidence for an exhaustion of haemopoietic stem cells during treatment with G-CSF. They rather suggest that an important side effect of G-CSF treatment is a release of CFU-S and progenitors from the marrow to the peripheral blood and a reseeding in the spleen.

From previous reports it is evident that recombinant human granulocyte-colony stimulating factor (rh G-CSF) stimulates the proliferation and differentiation of granulopoietic progenitors *in vitro* and *in vivo* (Moore *et al.* 1987; Welte *et al.* 1987). If administered to humans or animals with drug-induced granulopenia rh G-CSF significantly shortens the granulocytopenic period (Morstyn *et al.* 1988; Moore & Warren, 1987). Previously it has been hypothesized that the erythropoietic and granulopoietic lineages compete for the differentiation of haemopoietic stem cells (Dexter, 1987). According to this hypothesis a stimulation of granulopoiesis could induce a decrease in stem cell numbers, leading in consequence to a decline of erythropoietic progenitor numbers. Former investigations by other groups revealed that rh G-CSF *in vivo* has substantial effects not only on granulopoietic progenitors but also on the CFU-S and the erythropoietic lineage (Tamura *et al.* 1987; Moore *et al.* 1987). These effects concern mainly increases of all progenitors within the splenic environment.

It was the objective of the present study to extend these investigations and to examine whether the above hypothesis holds true during treatment with rh G-CSF. Mice were treated with high doses of rh G-CSF. During the treatment the spleen colony forming units (CFU-S), erythroid colony forming units (CFU-E) and granulocyte-macrophage colony forming units (CFU-GM) of the peripheral blood, spleen and bone marrow were determined. Previous studies indicated that suppression of erythropoiesis by red cell transfusion in normal untreated animals leads to an increase in the CFU-S and granulopoietic progenitor numbers (Monette, 1985; Wichmann & Loeffler, 1985). According to the above hypothesis this may be interpreted as a consequence of a failing differentiation pressure by the erythropoietic progenitors on the CFU-S. Assuming this, one would expect that an additional red cell transfusion could enhance the response of granulopoiesis to G-CSF. To evaluate whether such a process can modify the

Correspondence: Dr B. Bungart or Dr M. Loeffler, Dept. Biometry, LFI Ebene 5, Medizinische Klinik I, University of Cologne, Joseph-Stelzmann Str. 9, D-5000 Köln 41, F.R.G.

response of the CFU-S and granulopoiesis to G-CSF an additional experiment was carried out, where rh G-CSF treatment was combined with suppression of erythropoiesis by red cell transfusion.

MATERIALS AND METHODS

Experimental animals. Specific pathogen-free female C57bl/6 mice (Charles River, F.R.G.) between 8 and 12 weeks of age (20–25 g of body weight) were used.

rh G-CSF. Recombinant human G-CSF was generously provided by AMGEN, Thousand Oaks, Calif., U.S.A. It was delivered in an aqueous buffer at a concentration of 530 μg protein/ml. The specific activity of the rh G-CSF was $1{\cdot}2 \times 10^8$ Units/mg protein as determined in a granulocyte-macrophage colony assay. Endotoxin contamination was less than 0·5 μg/mg protein. Rh G-CSF was injected subcutaneously as a freshly prepared dilution of the original material in 0·1 ml pyrogen free physiological NaCl.

Cell suspensions. Bone marrow cell suspensions were obtained by flushing one femur with 1 ml Alpha medium (GIBCO, U.K.) ± 10 mM Hepes, pH 7·2. Single cell suspensions were made by repeated flushing through a 25 gauge needle. Spleens were pressed through a stainless steel sieve and suspended in Alpha medium. Femoral and splenic nucleated cells were counted in a Coulter counter.

Peripheral blood cells. Mice were bled from the orbital plexus. Blood was heparinized (50 U heparin per ml blood). Haematocrit (PCV) and white blood cells (WBC) were determined according to standard procedures. The leucocytes were isolated after centrifugation on a discontinuous gradient of Ficoll-Isopaque (Sigma and Nyegaard, Norway) (Perper *et al*, 1968). The interphase was removed and washed three times with Alpha medium + 10 mM Hepes, pH 7·2, supplemented with 5% fetal calf serum.

Progenitor cell assay. CFU-GM and CFU-E were estimated with the methylcellulose method of Iscove & Sieber (1975). Briefly, 1 ml suspensions of $2{-}5 \times 10^5$ nucleated marrow cells or $2{-}5 \times 10^5$ spleen cells were plated in duplicate. Cultures for CFU-E were supplemented with 0·25 U erythropoietin (Terry Fox Laboratory, Vancouver). 10 μg pokeweed mitogen-stimulated spleen cell conditioned medium (Burgess *et al*, 1980) was added to 1 ml of the CFU-GM cultures. This gave a maximal response. Enumeration of colonies was performed on day 2 and day 8 for CFU-E and CFU-GM respectively. Each data point in the results refers to the median and range of four separate measurements of materials pooled from two groups each of three mice.

Spleen colony assay. The number of CFU-S was determined according to Till & McCulloch (1961). Ten recipient mice per data point were irradiated with 9 Gy. 24 h after the irradiation donor cells were injected via the orbital sinusoids. After 8 d the spleens were removed and the colonies were counted. Each data point refers to the median and range of 10 measurements of material pooled from three mice.

RESULTS

Dose response of granulopoiesis to rh G-CSF

For a dose response of CFU-GM and granulocytes to rh G-CSF, doses ranging between 0 (saline control) and 450 μg rh G-CSF/kg body weight/d were injected subcutaneously for 6 d (day 0 to day 6) divided in two daily injections. Determinations of CFU-GM and blood granulocytes were carried out on day 6.

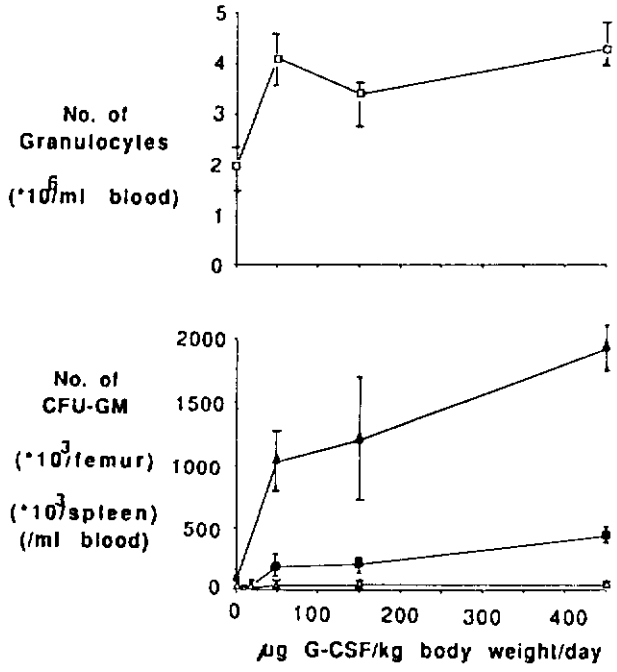

Fig 1. Dose response of the peripheral blood granulocytes and of the splenic (■), peripheral blood (▲) and femoral (△) CFU-GM to 6 d treatment with rh G-CSF. Data expressed as median and range (see Methods).

The numbers of femoral, splenic and circulating CFU-GM showed drastic increases in response to 50 μg rh G-CSF/kg body weight/d and continued to increase slowly with doses above 50 μg rh G-CSF/kg body weight/d (Fig 1). The increase of marrow CFU-GM numbers was 1·3–1·8-fold in response to doses ranging between 50 and 450 μg rh G-CSF/kg body weight/d (Fig 1). Splenic and peripheral blood CFU-GM increased 53-fold and 13-fold respectively for 50 μg rh G-CSF/kg body weight/d. The increases were 125-fold and 24-fold in response to 450 μg rh G-CSF/kg body weight/d (Fig 1). The blood granulocytes increased 1·8–2·1-fold in response to doses ranging between 50 and 450 μg/rh G-CSF/kg body weight/d (Fig 1).

Time course of the haemopoietic response to rh G-CSF

For observation of the behaviour of haemopoiesis under rh G-CSF treatment mice were injected subcutaneously for 6 d (day 0 to day 6) with 150 μg G-CSF/kg body weight/d (corresponding to 3·75 μg rh G-CSF/mouse/d) divided in two daily injections.

During the treatment the blood granulocytes increased 2·2-fold and 1·7-fold on day 4 and day 6 respectively (Fig 2). White blood cell counts did not change significantly ($9{\cdot}8 \times 10^6$/ml blood on day 0, $9{\cdot}0 \times 10^6$/ml blood on day 6). The blood reticulocytes showed a slight but not significant

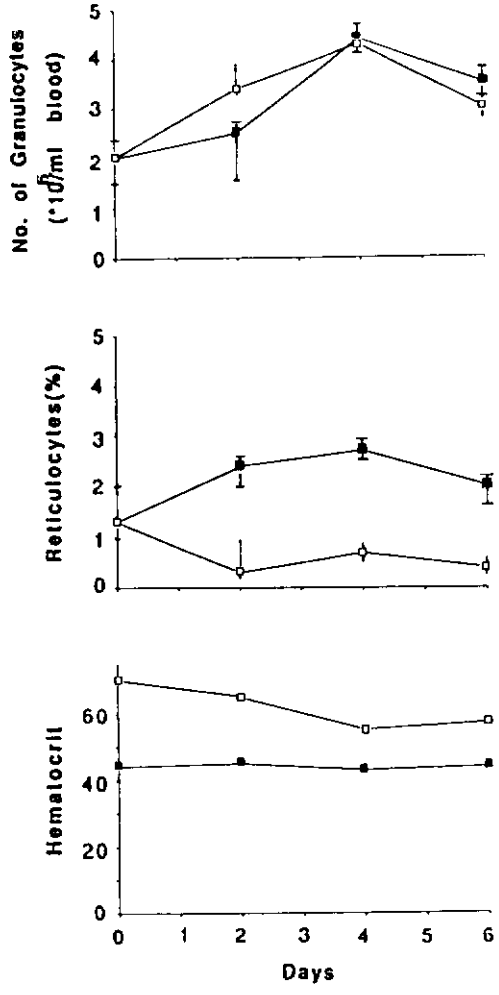

Fig 2. Time course of the peripheral blood granulocytes, reticulocytes and haematocrit during 6 d treatment with rh G-CSF (solid symbols) and during 6 d treatment with a combination of G-CSF and red cell transfusion on day 0 (open symbols). Data expressed as median and range (see Methods).

increase (Fig 2). No change in the haematocrit could be detected (Fig 2).

The splenic nucleated cells increased 1·6-fold from $14·0 \times 10^7$ (range 13·0–18·6) on day 0 to $22·6 \times 10^7$/spleen (range 22·2–23·0) on day 6, while the femoral nucleated cells decreased slightly from $23·5 \times 10^6$ (range 22·8–24·8) on day 0 to $20·0 \times 10^6$ (range 18·0–22·4) on day 6 (no curve shown).

G-CSF induced a dramatic expansion of splenic and peripheral blood haemopoiesis. On day 6 the data for the splenic CFU-S revealed a 17-fold increase and the frequency of CFU-S in the circulation was found to be augmented about 10-fold (Fig 3). Splenic and circulating CFU-GM, increasing 60-fold and 15-fold respectively, paralleled the behaviour of CFU-S (Fig 3). CFU-E obtained from the spleen increased about 10-fold (Table I).

Marrow CFU-S first increased 1·6-fold on day 2 and then declined to 50% of the control value on day 6 (Fig 3). Marrow

Table I. Time course (median and, in parentheses, range) of the femoral and splenic CFU-E during administration of rh G-CSF at a dose of 150 μg/kg body weight/d and, in brackets, during a combination of G-CSF treatment with red cell transfusion

	CFU-E femur ($\times 10^3$/femur)		CFU-E spleen ($\times 10^3$/spleen)	
0	52·0	(48·0–53·0)	25·0	(24·0–26·0)
2	40·0	(21·0–50·0)	35·0	(30·0–50·0)
	[17·8]	[15·3–20·0]	[16·2]	[12·0–21·0]
4	10·5	(8·0–13·0)	110·0	(67·0–135·0)
	[3·7]	[2·5–3·8]	[8·0]	[6·0–8·5]
6	18·3	(16·0–37·1)	240·0	(194·0–285·0)
	[3·9]	[2·8–5·0]	[9·0]	[8·0–10·0]

CFU-GM increased to about 1·6 (1·4–1·8) times the normal value between day 2 and day 6 (Fig 3). Marrow CFU-E declined to about 40% of control (Table I).

Behaviour of haemopoiesis during a combination of rh G-CSF treatment and red cell transfusion

An additional experiment was performed combining rh G-CSF treatment and red cell transfusion by a single injection of 0·5 ml isologous packed red blood cells into the orbital vein on day 0. The experimental design was the same as for the exclusive treatment with rh G-CSF.

Red cell transfusion led to a severe increase of the haematocrit relative to the values for rh G-CSF administration alone and to a decline of the reticulocyte numbers (Fig 2). Both marrow and splenic CFU-E decreased distinctly to 8% of the normal value in the marrow and to 40% of the normal value in the spleen (Table I). With the exception of the data for erythropoiesis all other observations nearly matched those during exclusive treatment with rh G-CSF. Blood granulocytes increased 2-fold and 1·5-fold on day 4 and day 6 respectively (Fig 2). No change of data for the white blood cells could be detected. Splenic nucleated cells increased 1·6-fold and femoral nucleated cells declined slightly to 0·8 times the normal on day 6 (no curve shown). Splenic and peripheral blood CFU-S increased 14-fold and 40-fold respectively. This was paralleled by the CFU-GM that were found to be 50-fold increased for the spleen and 20-fold increased for the circulation (Fig 3). Observations for the marrow CFU-S and CFU-GM matched those under exclusive G-CSF treatment (Fig 3).

DISCUSSION

It was the objective of the present study to gain an indication as to whether the granulopoietic and erythropoietic lineages may compete for the differentiation of CFU-S during treatment with rh G-CSF. According to this hypothesis we expected rh G-CSF to induce a decrease in the CFU-S numbers. This should lead to a diminished inflow from the CFU-S into erythropoietic differentiation inducing a decline in the erythropoietic progenitors. In addition we expected an

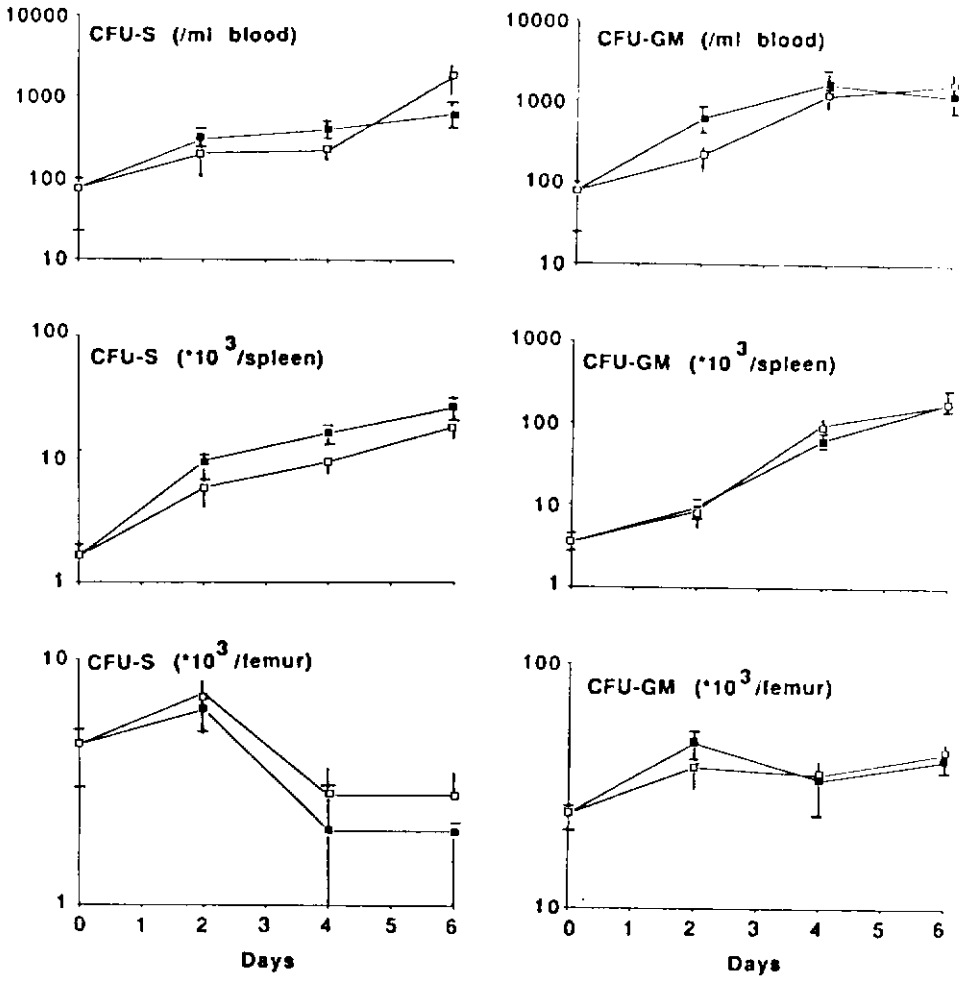

Fig 3. Course of the splenic, peripheral blood and femoral CFU-S and CFU-GM numbers during 6 d treatment with rh G-CSF (solid symbols) and during 6 d treatment with a combination of G-CSF and red cell transfusion on day 0 (open symbols). Data expressed as median and range (see Methods).

enhanced response of granulopoiesis if rh G-CSF administration was combined with a suppression of erythropoiesis by red cell transfusion.

The present results show that rh G-CSF induced a slight increase in the CFU-GM and a decrease in the CFU-S and CFU-E numbers in the bone marrow. On the other hand, the data also show a drastic increase of both peripheral blood and splenic CFU-S and progenitor cell numbers. This strongly suggests a release of CFU-S, granulopoietic and erythropoietic progenitors from the marrow to the peripheral blood and a seeding to the spleen during the administration of rh G-CSF. This would explain the decline in marrow CFU-S and CFU-E numbers as well as the increases in the circulating and splenic CFU-S and progenitors. Similar findings for the CFU-S (increases of peripheral blood and splenic CFU-S, decrease or no change of the marrow CFU-S numbers) have been reported previously after treatment with phenylhydrazine (Hodgson *et al*, 1972), thiamphenicol (Goris *et al*, 1990) and high doses (500 μg/injection) of endotoxin (Vos *et al*, 1972). Since endotoxin is known to lead to a release of G-CSF *in vivo* (Vogel *et al*, 1988) it may be asked whether G-CSF is the active inducer of stem cell release after endotoxin treatment. However, an increased level of stem and progenitor cells in the peripheral blood can be found for about 4 d after a single administration of high doses of endotoxin (Vos *et al*, 1972) although G-CSF can be detected only for hours post endotoxin (Vogel *et al*, 1988). The timing of the events remains obscure. At present it is therefore not clear whether a release of stem cells to the peripheral blood is a process induced by one single factor, namely G-CSF, or whether it is a non specific consequence of a stimulation of haemopoiesis.

Regarding the marrow it has been reported formerly that treatment with high doses of G-CSF leads to an increase of the day-12 CFU-S (Moore *et al*, 1987). This is in contrast to our results with respect to the more committed femoral day-8 CFU-S. During administration of G-CSF the ratio of femoral day-12/day-8 CFU-S seems to change in favour of the more primitive day-12 CFU-S.

The combination of G-CSF treatment with red cell transfusion did not lead to an enhanced or modified response of the

Table II. Time course of the total (marrow*+spleen) CFU-S during administration of rh G-CSF at a dose of 150 μg/kg body weight/d and, in brackets, during a combination of G-CSF treatment with red cell transfusion

	Total CFU-S	
Day	Median ($\times 10^3$)	(Range)
0	69·6	(52·2–87·0)
2	116·4	(92·7–135·1)
	[129·7]	[106·8–151·3]
4	50·2	(31·3–53·2)
	[56·9]	[42·0–71·8]
6	60·5	(30·0–64·0)
	[65·9]	[49·0–86·5]

*Marrow cell content calculated assuming one femur to represent 6% of the whole bone marrow.

CFU-S and granulopoiesis to rh G-CSF. This may be looked at as a contradiction to the concept of competition for stem cell differentiation. In addition, in both series of experiments (with and without red cell transfusion) the total number of CFU-S remained about normal (Table II). Although a decline in the bone marrow was evident this was outweighed by the considerable number found in the spleen. We therefore cannot conclude on the basis of these data that a 6-day application of rh G-CSF has led to any reduction of the stem cell population nor did we find an indication of significant competition for differentiating stem cells under these circumstances.

We as well as others (Moore *et al*, 1987) observed a change in the distribution of erythropoietic progenitors in favour of the spleen by rh G-CSF. If administered to humans rh G-CSF has been shown previously to induce increases in peripheral blood erythropoietic progenitor numbers (Dührsen *et al*, 1988). In the present study despite the decline of marrow CFU-E numbers the peripheral blood reticulocytes did not decrease. This indicates that during G-CSF administration the spleen compensates the decline of marrow erythropoiesis. According to this finding we make the conjecture that the reticulocytes should decline if rh G-CSF is administered to splenectomized animals.

The maximum response of the peripheral blood granulocytes to rh G-CSF treatment was an increase by about a factor of 2. These data match those of other reports for C57bl mice (Tamura *et al*, 1987) but not for C3H mice (Moore *et al*, 1987), where a 6–9-fold increase in granulocytes was observed. In Balb/c mice an increase even to 10–20-fold was reported (Metcalf, 1987). In some subsequent control experiments with C3H mice in our laboratory we found a much more pronounced increase in granulocytes during rh G-CSF treatment than in C57bl mice (unpublished observation). The reason for this variation of the granulocytes' response to rh G-CSF remains obscure and appears to show a strain dependency.

Taken together, the present results do not provide convincing evidence for competition of the granulopoietic and erythropoietic lineages for the differentiation of CFU-S during rh G-CSF treatment. During both experiments, exclusive administration of rh G-CSF and in combination with red cell transfusion, the femoral CFU-S showed a decline. This seems to be a consequence of a release of CFU-S from the marrow rather than being a result of a reduction of the stem cell pool by differentiation into the granulopoietic lineage.

ACKNOWLEDGMENTS

The authors wish to thank Professor Dr K. Welte, Medizinische Hochschule Hannover, F.R.G., for many valuable discussions and Dr L. Souza, AMGEN, for providing the rh G-CSF. Supported in part by the Boehringer Ingelheim Fonds, by the Moritz Stiftung, Cologne, and by the Deutsche Forschungsgemeinschaft (grant LO 342/1–2 given to M.L. as P.I.).

REFERENCES

Burgess, A.W., Metcalf, D., Russell, S.H.M. & Nicola, N.A. (1980) Granulocyte-macrophage, megacaryote, eosinophil and erythroid colony stimulating factors produced by mouse spleen cells. *Biochemical Journal*, **185**, 301–307.

Dexter, T.M. (1987) Growth factors involved in haemopoiesis. *Journal of Cell Science*, **88**, 1–6.

Dührsen, U., Villeval, J.L., Boyd, J., Kannourakis, G., Morstyn, G. & Metcalf, D. (1988) Effects of recombinant human granulocyte colony stimulating factor on hematopoietic progenitor cells in cancer patients. *Blood*, **72**, 2074–2081.

Goris, H., Bungart, B., Loeffler, M. & Nijhof, W. (1990) Antagonist behaviour of stem and progenitor cells in marrow and spleen following a Thiamphenicol treatment of mice. *Experimental Hematology*, in press.

Hodgson, G.S., Bradley, T.R. & Telfer, P.A. (1972) Haemopoietic stem cells in experimental haemolytic anaemia. *Cell and Tissue Kinetics*, **5**, 283–288.

Iscove, N.N. & Sieber, F. (1975) Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. *Experimental Hematology*, **3**, 32–42.

Metcalf, D. (1987) The molecular control of normal and leukaemic granulocytes and macrophages. *Proceedings of the Royal Society* B. **230**, 389–423.

Monette, F.C. (1985) Hypertransfusion—experimental results: effect on erythropoietic progenitors. *Mathematical Modeling of Cell Proliferation* (ed. by H.E. Wichmann and M. Loeffler), Vol. 2, p. 3. CRC Press, Boca Raton.

Moore, M.A.S. & Warren, D.J. (1987) Synergy of interleukin 1 and granulocyte colony-stimulating factor: *in vivo* stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 7134–7138.

Moore, M.A.S., Welte, K., Gabrilove, J. & Souza, L.M. (1987) Biological activities of recombinant human granulocyte colony stimulating factor (rhG-CSF) and tumor necrosis factor: *in vivo* and *in vitro* analysis. *Haematology and Blood Transfusion*, **31**, 210–220.

Morstyn G., Campbell, L., Souza, L.M., Alton, N.K., Keech, J., Green, M., Sheridan, W., Metcalf, D. & Fox, R. (1988) Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. *Lancet*, i, 667–672.

Perper, R.J., Zee, T.W. & Mickelson, M.M. (1968) Purification of lymphocytes and platelets by gradient centrifugation. *Journal of Laboratory and Clinical Medicine*, **72**, 842–848.

Tamura, M., Hattori, K., Nomura, N., Oheda, M., Kubota, N., Imazeki, I., Ono, M., Ueyama, Y., Nagata, S., Shirafuji, N. & Asano, S. (1987) Induction of neutrophilic granulocytosis in mice by administration of purified human native granulocyte colony-stimulating factor (G-CSF). *Biochemical and Biophysical Research Communications*, **142**, 454–460.

Till, J.E. & McCulloch, E.A. (1961) A direct measurement of radiation sensitivity of normal mouse bone marrow cells. *Radiation Research*, **14**, 213–219.

Vogel, S.N., Kaufman, E.N., Tate, M.D. & Neta, R. (1988) Recombinant Interleukin-1a and recombinant tumor necrosis factor synergize *in vivo* to induce early endotoxin tolerance and associated hematopoietic changes. *Infection and Immunity*, **56**, 2650–2657.

Vos, O., Buurman, W.A. & Ploemacher, R.E. (1972) Mobilization of hemopoietic stem cells (CFU) into the peripheral blood of the mouse: effects of endotoxin and other compounds. *Cell and Tissue Kinetics*, **5**, 467–479.

Welte, K., Bonilla, M.A., Gillio, A.P., Boone, T.C., Potter, G.K., Gabrilove, J.L., Moore, M.A.S., O'Reilly, R.J. & Souza, L.M. (1987) Recombinant human granulocyte colony stimulating factor. Effects on hematopoiesis in normal and cyclophosphamide-treated primates. *Journal of Experimental Medicine*, **165**, 941–948.

Wichmann, H.E. & Loeffler, M. (1985) Hypertransfusion—a model analysis. *Mathematical Modeling of Cell Proliferation* (ed. by H. E. Wichmann and M. Loeffler), Vol. 2, p. 3. CRC Press, Boca Raton.