Kinetics of Neutrophil Production in Normal and Neutropenic Animals during the Response to Filgrastim (r-metHu G-CSF) or Filgrastim SD/01 (PEG-r-metHu G-CSF)[1]

Brian I. Lord,[2] Lorna B. Woolford, and Graham Molineux

CRC Experimental Haematology Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 4BX, United Kingdom [B. I. L., L. B. W.], and Departments of Pharmacology, Pharmaceutics, and Clinical Development, Amgen Inc., Thousand Oaks, California 91320-1789 [G. M.]

ABSTRACT

Filgrastim G-CSF has a short, biologically active half-life, and its effective use depends on repeated inoculations. A major aim, therefore, has been to develop a once-per-chemotherapy cycle formulation. To this end, a polyethylene glycolylated form of Filgrastim, known as SD/01, has been developed. In this study, we compared the cellular kinetics of granulocyte production in mice stimulated with SD/01 and granulocyte colony-stimulating factor (G-CSF). Mice were injected with a single dose of SD/01 (1 mg/kg) or G-CSF (125 μg/kg) twice per day for 4 days. Mice rendered leukopenic with a single injection of cyclophosphamide (200 mg/kg) and temozolomide (90 mg/kg) were similarly treated at their 3-day neutrophil nadir. Tritiated thymidine was injected for autoradiographic labeling studies. Bone marrow labeling indices and the release of labeled neutrophils and monocytes into the peripheral blood were assessed. Granulocytopoiesis was stimulated similarly by both SD/01 and G-CSF in both normal and neutropenic animals, with counts rising to $>20 \times 10^9$ polymorphonuclear neutrophils/l in both cases. Bone marrow thymidine labeling indices were increased, indicating a greater proportion of cells in DNA synthesis and an elevated proliferative activity. Compared with the normally slow release of neutrophils into the peripheral blood, labeled neutrophils (and monocytes) were rapidly released, increasing to peak levels at ~24 h. The peripheral half-life of neutrophils was not significantly different from normal, and the mitotic amplification factors for increase in granulocytopoiesis, accounted for by 3–3.9 extra cell divisions, were comparable for both factors. We conclude that neutrophil kinetics are stimulated in the same way and to the same extent by both SD/01 and G-CSF.

INTRODUCTION

Filgrastim, or r-metHu G-CSF,[3] is rapidly removed from the body by a combination of renal and active neutrophil clearance processes. As a result, for most practical purposes, repeated injection or continuous infusion is necessary to generate usefully elevated neutrophil and mobilized progenitor/stem cell levels in the peripheral blood. Recently, however, a novel PEGylated form, known as Filgrastim (SD/01), that has a sustained duration of action was reported; a single injection of Filgrastim (SD/01) sustained elevated levels of neutrophils for 5 days in mice and for 9 days in humans (1, 2). It is thought that PEGylation of the molecule results in a reduced renal clearance rate, leading to a prolonged circulating half-life. Removal of the drug by neutrophils thus becomes more dominant, and clearance becomes dependent primarily on the number of neutrophils in circulation (1). Production of neutrophils following a single injection of SD/01 thus becomes subject to the equivalent of a classical negative feedback control mechanism.

It has been demonstrated that SD/01 has the same effects as conventional Filgrastim (G-CSF): elevation of peripheral neutrophil counts, mobilization of progenitor cells, and reduction in duration of chemotherapy-induced neutropenia (1). A major aim of this program was to develop a once-per-chemotherapy cycle form of G-CSF. SD/01 would appear, therefore, to be a viable alternative to Filgrastim in clinical applications because its protracted course of action makes it more convenient and acceptable to patients and clinicians alike. The remaining major question, therefore, is whether the kinetics of neutrophil generation are also comparable to those generated by repeated injection of G-CSF. It was previously demonstrated that G-CSF acts by increasing the number of cell amplification divisions, mainly through the maturational phase of granulopoiesis, and by inducing rapid release from the marrow into the peripheral circulation of mature functional neutrophils (3, 4). We have now made a parallel investigation of SD/01 and show comparable changes in granulocytopoietic cell kinetics.

MATERIALS AND METHODS

Male BDF1 mice (C57Bl × DBA2), 9–11 weeks of age, were used throughout. All animal handling and experimental procedures were carried out under Home Office License accord-

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[1] This work was supported by the UK Cancer Research Campaign.
[2] To whom requests for reprints should be addressed, at CRC Experimental Haematology Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester M20 4BX, United Kingdom. Phone: 44 161 446 3236; Fax: 44 161 446 3033; E-mail: blord@picr.man.ac.uk.

[3] The abbreviations used are: r-metHu G-CSF, recombinant human granulocyte colony-stimulating factor; PEG, polyethylene glycol; CY/T, cyclophosphamide plus temozolomide; PMN, polymorphonuclear neutrophil.

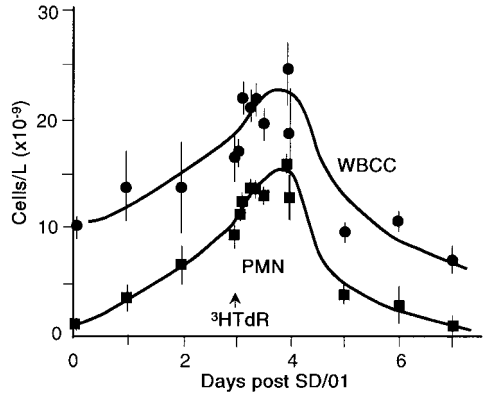

Fig. 1 WBC counts (*WBCC*) and PMN counts in normal mice receiving a single injection of Filgrastim SD/01. For cell kinetic analyses, [^{3}H]thymidine (*[^{3}H]TdR*) was injected on day 3. ●, total WBC count; ■, PMNs. Data points represent the means ± SE (*bars*) of the individual counts in groups of three mice.

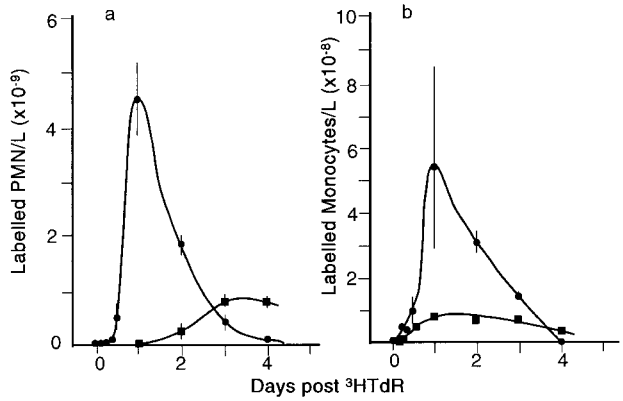

Fig. 2 Release and clearance of [^{3}H]thymidine-labeled neutrophils (*PMN*; *A*) and monocytes (*B*) into the peripheral circulation following injection of [^{3}H]thymidine (*[^{3}H]TdR*) on day 3 after a single injection of SD/01. ■, controls (4); ●, SD/01 injected. Data points represent the means ± SE (*bars*) of the individual counts in groups of three mice.

ing to the provisions of the United Kingdom Animals (Scientific Procedures) Act, 1986.

Granulocytopoiesis was stimulated by s.c. injection of 125 μg/kg G-CSF twice per day for 4 days to give a total dose of 1 mg/kg over 4 days, or by s.c. injection of Filgrastim SD/01 as a single dose of 1 mg/kg.

Neutropenia (leukopenia) was induced by a combined i.p. injection of cyclophosphamide (200 mg/kg) and temozolomide (90 mg/kg) in a volume of 0.2 ml (CY/T).

For cell kinetic studies, mice received i.v. injections of [^{3}H]thymidine (37 kBq/kg at 185 GBq/mmol). Three mice from each group were killed 1 h later, their femora were excised, and bone marrow cell suspensions were prepared for each individual animal. Cells were counted, and cytospin preparations were made on clean glass slides. After fixation in methanol, autoradiographs were then prepared by dipping the slides in Ilford K5 liquid emulsion and exposing them for 2 weeks before processing and staining with May-Grünwald-Giemsa. Differential cell counts and the percentages of labeled cells were scored, with a minimum of 500 cells per slide counted. Peripheral blood samples were also obtained from additional groups of three mice at 3, 6, 9, and 12 h and thereafter daily for 4 days after injection of [^{3}H]thymidine. Blood smears were made and processed for autoradiographic analysis, as for the bone marrow.

SD/01 in Normal Mice. Normal mice received injections of SD/01, and at daily intervals, three were sacrificed. Peripheral blood counts and differentials were obtained for each mouse. In some experiments, [^{3}H]thymidine was injected at 3 days post-SD/01. One h later, three mice were sacrificed for bone marrow autoradiographic analysis. At 3, 6, 9, 12, 24 h and subsequently at daily intervals, further groups were sacrificed for peripheral blood analyses. All slides prepared from mice that had received injections of [^{3}H]thymidine were processed for autoradiography.

Filgrastim in Leukopenic Mice. Peripheral blood samples (50 μl) were obtained from the tail veins of 15 mice, and nucleated cell counts were obtained. The mice then received injections of the cytotoxic mixture CY/T. The mice were divided into three groups of five mice, and serial peripheral tail blood samples were taken at 3-day intervals; the groups were staggered so that daily samples could be obtained out to 18 days. The comparative effects of SD/01 *versus* G-CSF were assessed by injecting SD/01 or the first of the G-CSF treatment course at the neutropenic nadir, 3 days after treatment with CY/T. In some experiments, blood smears were made for differential cell counting. [^{3}H]thymidine was injected 3 days after Filgrastim treatment, and cell kinetic measurements were made as for normal mice.

RESULTS

SD/01 in Normal Mice. Following injection of SD/01, peripheral WBC counts rose steadily from their control level of $\sim 10 \times 10^9$/l to 24×10^9/l by day 4, after which they returned to normal by day 6–7 (Fig. 1). This pattern primarily reflected the change in the circulating PMN count, which similarly rose from 10^9/l to 16×10^9/l. Monocytes increased from $\sim 4 \times 10^8$/l to 14×10^8 over the same time period.

After injection of [^{3}H]thymidine, labeled neutrophils started to appear in the peripheral circulation by 1–3 h, accelerating from ~9 h to a peak of $4.51 \pm 0.68 \times 10^9$/l (28.7% labeled) by 24 h (Fig. 2*A*). This rapid release should be compared with the slow release peaking at 3–4 days in the controls. In the same way, release of labeled monocytes was also accelerated, peaking by 24 h at $5.7 \pm 2.6 \times 10^8$/l (Fig. 2*B*). In the controls, peak numbers were attained between 1 and 3 days and showed very slow clearance compared with the stimulated monocytes.

Differential analysis of the bone marrow autoradiographs 1 h after injection of [^{3}H]thymidine is shown in Fig. 3 and compared with previous results obtained for G-CSF (4). The increase in the neutrophilic cell populations was confined to the late metamyelocyte compartment, resulting in a small net increase in the total granulocytic cells, 79% of the marrow compared with 70%. Overall, however, SD/01 caused less disturbance to the general differential picture than did G-CSF. This was mainly because the proportion of nongranulocytic elements, the

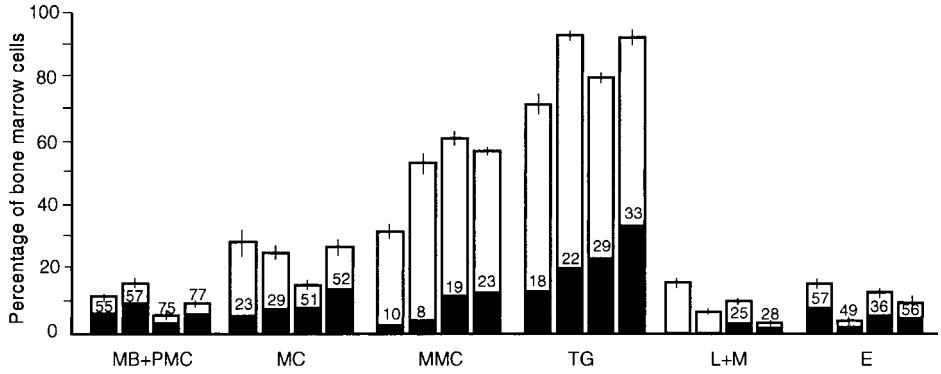

Fig. 3 Differential analysis of cell proliferation in the bone marrow of normal mice treated with G-CSF (*second column* of each set of four), with SD/01 (*third column*), and of leukopenic mice treated with SD/01 (*fourth column*). Control data are shown in *first column* of each group. The total height of each column represents the percentage of total cells. The *filled portions* represent [^{3}H]thymidine-labeled cells (cells in DNA synthesis), the *number* in each column showing the percentage of labeled cells. *MB + PMC*, myeloblasts + promyelocytes; *MC*, myelocytes; *MMC*, metamyelocytes; *TG*, total MB + PMC + MC + MMC; *L + M*, lymphocytes and macrophages; *E*, erythroid cells. Values for each column represent the means ± SE (*bars*) of the individual counts in groups of three mice.

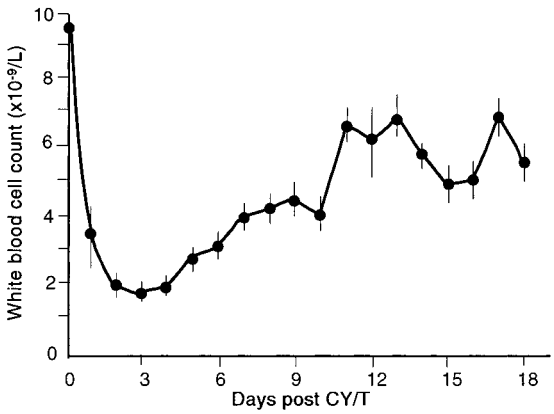

Fig. 4 Recovery of total WBC counts following a dose of CY/T. Cumulative data for all experiments are shown, resulting in a total of 195 mice for the pretreatment value. This baseline was established by staggering results of groups of five (counted individually), sampling each group every 3 days so that a daily assessment could be established. *Bars*, SE for all counts for each day.

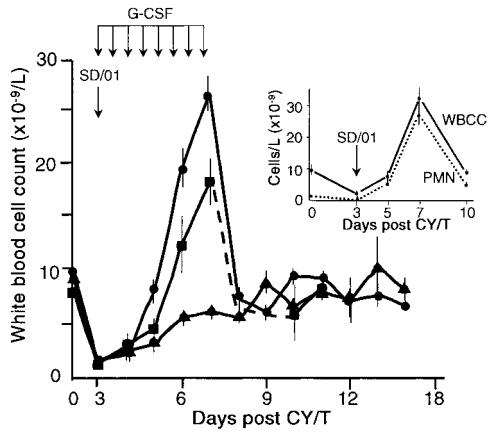

Fig. 5 Recovery of total WBC counts in mice treated with CY/T at day 0. At 3 days, mice received injections of SD/01 (●) or started on a course of G-CSF injections (■). The control curve (no G-CSF; ▲) is for CY/T-treated mice and is as shown in Fig. 4. Shown in the *inset* are the differential counts performed in some experiments. *WBCC*, WBC counts. *Bars*, SE for all counts for each day.

erythroid, lymphocytic, and macrophagic cells, was not so severely depressed: by only 30% compared with 75% with G-CSF. As with G-CSF, however, the proliferative activity of the neutrophil precursors was considerably increased. Seventy-five percent of myeloblasts and promyelocytes and 51% of myelocytes were in DNA synthesis compared with 48% and 18%, respectively, in the controls, and proliferation was extended more into the metamyelocyte compartment, with 19% labeling compared with only 9% in the controls.

Filgrastim in Leukopenic Mice. CY/T was used to induce a deep and protracted leukopenia. The nadir in white cells arose at 3 days when the counts had fallen from 9×10^9/l to $\sim 1.5 \times 10^9$/l (Fig. 4). At this point, PMN counts were $<0.1 \times 10^9$/l. SD/01 (or the first dose of the G-CSF sequence) was injected at this point. In both cases, the white cell counts rapidly recovered and overshot normal levels to reach 26×10^9 (SD/01) and 19×10^9 (G-CSF) white cells/l by 7 days (Fig. 5). At the peak of cell counts, neutrophils represented >80% of the total (Fig. 5, *inset*).

Following [^{3}H]thymidine injection at 3 days after stimulation with SD/01, labeled neutrophils were rapidly released into the peripheral circulation. After 25 h, 34.5% ($2.62 \pm 0.29 \times 10^9$/l) were labeled. After an additional 23 h, only $0.61 \pm 0.23 \times 10^9$/l (16.4%) labeled neutrophils remained into the blood (Fig. 6*A*). Labeled monocytes similarly were released early, increasing to $14 \pm 3 \times 10^8$/l (37.9% labeled) by 10.5 h (Fig. 6*B*). Their number was already falling by 25 h, leaving the peak number undefined, and by 48 h, only $2.7 \pm 0.1 \times 10^8$/l (12% labeled) remained in the circulation.

Granulocytopoiesis was again heavily stimulated, accounting for nearly 90% of the cells in the bone marrow. Overall, 33% of these cells were labeled (in DNA synthesis) with [^{3}H]thymidine (Fig. 3). Proliferation rates in the developmental

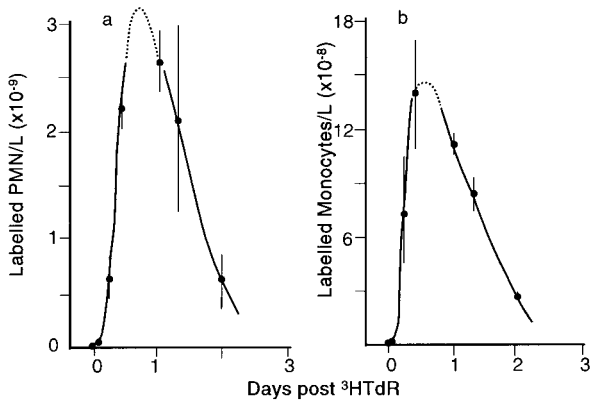

Fig. 6 Release and clearance of labeled neutrophils (*PMN*; *A*) and monocytes (*B*) into the peripheral circulation following SD/01 stimulation of leukopenic mice. SD/01 was injected into mice that had been treated 3 days earlier, with CY/T. [^{3}H]Thymidine (*[^{3}H]TdR*) was then injected after an additional 3 days. Data points represent the means ± SE (*bars*) of the individual counts in groups of three mice.

stages were increased to levels similar to or higher than those in normal animals stimulated with SD/01. Erythropoiesis and the lymphoid elements were depressed, as with G-CSF, but proliferation rates of those cell populations were high, with 56% erythroid cells in DNA synthesis.

DISCUSSION

SD/01 was compared, as a single administration, to a 4-day regimen of twice daily injections of G-CSF. The results obtained confirm the effectiveness of SD/01 in both normal mice (Fig. 1) and in leukopenic mice (Fig. 5). The neutrophilia generated in both cases compares well with other reported data in mice (1, 5, 6), primates (7), and humans (8–10). The primary purpose of this study, however, was to investigate the effect on the kinetics of granulocytopoiesis following stimulation with SD/01.

In the bone marrow of both normal and neutropenic mice, SD/01 stimulated granulopoiesis in a manner comparable to that of G-CSF in mice (Fig. 3; Ref. 4) and humans (3). On the basis of steady-state cell kinetics, the increase in thymidine labeling indices indicated significantly reduced cell cycle times of the myeloblasts, promyelocytes, and myelocytes (Table 1). As with G-CSF, this was not reflected in any major change in the distribution of the phases of granulocyte maturation or increases in the compartment sizes. The faster cell cycle times thus indicated a more rapid throughput because of a reduction in the overall maturation time and an early release of mature cells. It is this rapid maturation and release that prevents the build up of these cells in the marrow and results in their early appearance in the circulation. Measuring the time to attain 50% of peak counts (Figs. 2 and 6*A*) in the blood, it is clear that in normal mice, mature functional neutrophils were available ~40 h earlier than normal whether SD/01 or G-CSF was used, whereas with SD/01 in neutropenic mice, they appeared 10 h earlier still. This suggests that both increased production and decreased transit time contribute to the overall effects of SD/01.

The principles of analyzing peripheral blood cell kinetics using [^{3}H]thymidine were established in the late 1950s (11–15).

Table 1 Bone marrow labeling and calculated cell cycle times

	Labeling index, I_L (%)	Cell cycle time,[a] t_c (h)
Myeloblasts + promyelocytes		
Control	55.5 ± 6.3	9.1 ± 1.0
SD/01 (normal mice)	75.2 ± 3.4	6.6 ± 0.3
G-CSF[b] (normal mice)	56.5 ± 2.3	7.6 ± 0.4
SD/01 (neutropenic mice)	76.8 ± 1.4	6.5 ± 0.2
Myelocytes		
Control	22.7 ± 3.9	22.0 ± 3.8
SD/01 (normal mice)	51.1 ± 1.7	9.8 ± 0.3
G-CSF[b] (normal mice)	29.4 ± 1.5	16.2 ± 1.1
SD/01 (neutropenic mice)	52.3 ± 0.8	9.6 ± 0.2

[a] Assumes duration of DNA synthesis, t_s, is 5 h.
[b] Data from Lord *et al.* (4).

Use of a similar approach allows assessment of the relative neutrophil production rates and peripheral half-lives resulting from these growth factor treatments.

The half-lives ($t_{1/2}$) of the circulating neutrophils are calculated from the rate of loss of labeled cells from the blood, using the formula $n_t = n_o e^{-\lambda t}$, where n_o is the number at a given time, n_t is the number t hours later, and λ is the decay constant. Thus, for SD/01 (Fig. 2*A*), $n_o = 451$ at 24 h and $n_t = 40$ at 72 h. Thus, $\lambda = 0.0505$ and $t_{1/2} = \ln 2/\lambda = 13.7$ h. Taking into account the error observed on the labeled neutrophil counts, $t_{1/2}$ lies in the range 12.4–14.6 h, and this is somewhat longer than the 9.7 h observed for G-CSF, which we originally concluded was not significantly different from a normal value of ~8 h (4).

The current discrepancy, however, is probably not real because the corresponding calculations for neutropenic mice stimulated with SD/01 (Fig. 6*A*) gave a half-life in the range of 8.6–9.8 h, similar to that with G-CSF. The reason for this apparent discrepancy lies in the mechanism by which excess G-CSF is cleared. For G-CSF, it is necessary to maintain the stimulus by giving repeated administrations. The effective half-life of G-CSF is so short that by the next injection, the concentration has always fallen to an insignificant level. Likewise, the stimulus is lost very rapidly after completion of the course of treatment, thus allowing neutrophil levels to fall according to their natural half-life. By contrast, SD/01 has a long effective half-life, and its activity falls only as the number of neutrophils increases. Thus, there will be a period when the CSF is still active, albeit at a decreasing concentration, and continues to stimulate further neutrophil production. This offsets the natural loss of neutrophils, leading to an apparently reduced rate of removal. The effect is minimized in neutropenic mice, in which the large stimulation induced reduces the period of submaximal stimulation and allows a more realistic estimation of the true half-life. We conclude, therefore, that SD/01, like G-CSF, does not significantly affect the peripheral clearance rates of circulating neutrophils, which in these experiments has, therefore, been taken as $t_{1/2} = 9.2$ h in both cases.

Taking the increase in neutrophils over 24 h after labeling and correcting for half-life loss over the same period, one can estimate the true increase in numbers over this period and hence calculate the excess production over normal. In normal animals, a steady state exists, and the rate of production equals the rate of

Table 2 Neutrophil production

		SD/01		G-CSF	
	Control	Normal mice	Neutropenic mice	Normal mice[a]	Neutropenic mice
Maximum neutrophil count[b] $\times 10^{-9}$/l	1.8 ± 0.2	15.5 ± 2.5	24.0 ± 2.8	25.7 ± 3.2	18.0 ± 2.5
Time of peak appearance in peripheral blood (days)	3.4	1	1	1	1
Peripheral half-life, $t_{1/2}$ (h)	8[c]	13.7[d]	9.2	9.7	ND
Amplification factor	1	8.8	14.9	14.5	10.1
Extra maturation divisions	0	3	3.9	3.8	3.3

[a] Data from Lord *et al.* (4).
[b] Mean values for three mice in each group ± SE.
[c] Assumed because the descending arm of the control curve was not sufficiently defined.
[d] As calculated (see text).

loss. Thus, for example, with SD/01 in normal mice, the PMN concentration increases between 3 and 4 days from 10.5×10^9/l to 15.5×10^9/l (Fig. 1), a gain of 5×10^9/l over the 24 h. With $t_{1/2}$ in the order of 9.2 h, $\lambda = 0.0753$, and the loss of cells over the same period would be given by $n = 10.5e^{-0.0753 \times 24} = 1.7$, a loss of 8.8×10^9 cells/l. Net production is, therefore, equal to $(5.0 + 8.8) \times 10^9$/l $= 13.8 \times 10^9$/l. By comparison, taking $t_{1/2} = 8$ h ($\lambda = 0.0866$) for the controls, over 24 h, a PMN count of 1.8×10^9/l, as seen in these animals, would become 0.225×10^9/l, a loss (net production) of 1.575×10^9/l. The amplification factor for SD/01 over control is therefore given by $13.8 \times 10^9/1.575 \times 10^9 = 8.76$, and this is achieved by incorporating 3.1 extra amplification divisions into the neutrophil maturation sequence.

In neutropenic mice, the equivalent calculations indicate 3.9 extra divisions for SD/01 and 3.4 for G-CSF; all these results compare well with the 3.2 and 3.8 determined previously for G-CSF in humans (3) and mice (4), respectively. It appears, therefore, that SD/01 stimulates the kinetics of granulocytopoiesis in the same way and to the same extent as G-CSF. Table 2 summarizes the characteristics of neutrophil production with SD/01 and G-CSF.

As with G-CSF (4, 9), monocyte production was also stimulated by SD/01 both in normal and leukopenic mice. The emergence of labeled monocytes after stimulation by G-CSF or SD/01 was not followed as closely in this study as in the previous one (4), and the curves shown in Figs. 2*B* and 6*B* are not well defined. However, it was clear that in both normal and leukopenic mice, monocytes were released into the peripheral circulation much more rapidly than in control animals, peaking at 12–24 h, and similarly, they were very quickly removed. An estimate from these curves suggests peripheral half-lives of 12–15 h and 9–10 h in normal and leukopenic mice, respectively, compared with a half-life of 46–60 h in humans and control mice (4, 11). In our previous study with G-CSF (4), a comparably short half-life of 6 h was indicated, but the absolute level of this factor probably depends quite acutely on the current health status of the animals.

It is equally difficult to define the production stimulus for monocytes from these data, but with SD/01 monocytes increased from 11 to 14×10^8/l over 24 h in a normal animal that initially carried 4×10^8 monocytes/l. On the basis of an average $t_{1/2}$ of 12.3 h for stimulated monocytosis and 60 h in controls, production levels of 11.3×10^8 monocytes/l compared with 1.0×10^8 monocytes/l were estimated. These values suggest an additional 3.5 amplification divisions, which again corroborates the degree of stimulation seen with G-CSF. The mechanism of this effect on monocytopoiesis remains unclear, as it does for G-CSF. Direct stimulation of monocyte progenitors with G-CSF has not been demonstrated. It remains an open question whether this amplification results from direct stimulation of monocytopoiesis or is in an attempt to reestablish monocytosis in the face of accelerated monocyte destruction.

In conclusion, therefore, it is clear that sustained-duration SD/01 is comparable to conventional G-CSF in its capacity to stimulate granulopoiesis and in its effect on the cellular kinetics involved in that process. The ability to replace a protracted course of G-CSF treatments with a single application of SD/01 would clearly be beneficial for the comfort and convenience of both operator (the physician) and subject (the patient) alike.

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