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IN VIVO ADMINISTRATION OF RECOMBINANT GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RESULTS IN A REVERSIBLE INHIBITION OF PRIMARY B LYMPHOPOIESIS¹

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To determine if *in vivo* administration of granulocyte-macrophage CSF (GM-CSF) affects production of B lymphocytes in the bone marrow, mice were treated with the cytokine and the kinetics of B cell production was analyzed. After 1 wk of GM-CSF treatment, the number of B cell progenitors that express the B220 Ag had fallen fivefold and surface IgM expressing B cells were barely detectable. Although cellularity in the spleen had increased twofold, due in part to an increase in the number of granulocyte-macrophage progenitor cells, splenic B cell levels were not affected by the GM-CSF treatment. Although numbers of marrow B cells and their immediate progenitors declined due to cytokine treatment, cells capable of rapidly restoring B lymphopoiesis were present in that tissue. After cessation of GM-CSF treatment, B lymphopoiesis resumed in the marrow of the mice and rebounded to supernormal levels at 1 wk after the last GM-CSF injection. This effect may be due to actions of GM-CSF on the B lymphopoietic support capability of the marrow environment, because the overproduction of B cells was not observed when marrow from the GM-CSF donors was used to reconstitute sublethally irradiated severe combined immunodeficient mice.

The CSF are a family of glycoproteins that can regulate the proliferation and/or differentiation of myeloid progenitor cells (1, 2). One of the CSF, GM-CSF³, has been shown to stimulate the production of granulocytes and macrophages in culture and has additional direct and indirect effects on the growth of eosinophil, megakaryocyte and erythroid progenitors (3). These *in vitro* results accurately reflect events that occur following *in vivo* administration of GM-CSF³ (4). In mice, GM-CSF treatment results in an increase in numbers of granulocytes and macrophages in the peritoneal cavity and spleen. In addition, levels of myeloid progenitors are elevated in the latter tissue (5, 6).

Although the myeloid stimulatory effects of GM-CSF *in vivo* are well documented, a fundamental issue is whether the high rate of cell production of all hemopoietic populations proceeds normally when stimulatory signals for one or a few lineages are elevated. This question is particularly relevant to marrow lymphopoiesis. The bone marrow is the site of primary B cell differentiation in adult mammals, and cells at various stages of development in that lineage can be identified based on their expression of Ig and other non-Ig phenotypic determinants (7-9). Previous results in which GM-CSF was added to long term bone marrow cultures indicated that the cytokine could interfere with normal B cell production (10), but whether these *in vitro* results predict what would occur *in vivo* has not been tested.

Inasmuch as GM-CSF has been used or is under consideration for use in a variety of clinical conditions (11-13), there are practical reasons, in addition to the basic issues noted above, for examining its effects on B cell production *in vivo*. Accordingly, mice were treated with GM-CSF for a 1-wk period and B cell production was assessed. The results indicate that primary B cell production in the bone marrow can be reversibly inhibited after GM-CSF treatment. In addition, indirect evidence suggests that GM-CSF can affect the lymphopoietic support capability of the marrow environment.

MATERIALS AND METHODS

Mice. Male or female BALB/cAn mice were bred and maintained in the vivarium of the Division of Biomedical Sciences, University of California (UCR), Riverside, CA and were used at 6 to 8 wk of age. SCID mice (14) were also bred at UCR under laminar flow conditions. Animals were housed in sterile cages, containing sterile bedding, food, and water at all times. All procedures using SCID mice were performed in a laminar flow hood. SCID mice were used at 6 to 8 wk of age.

Preparation of cell suspensions. Mice were killed by cervical dislocation and spleens and femurs removed and placed in α -MEM (GIBCO, Grand Island, NY) containing 5% FCS (HyClone, Logan, UT). Single cell suspensions of bone marrow were obtained by flushing the plug from bones with 3 ml of medium. Spleen cell suspensions were prepared by gently teasing the tissue apart with 18-gauge needles. Cell viability was determined by eosin dye exclusion and all cell counts were performed in a hemacytometer.

GM-CSF injection schedule. Murine rGM-CSF was purchased from Amgen, Thousand Oaks, CA. The *Escherichia coli* preparation was partially purified by the supplier by sequential chromatography and had a sp. act. of 10^6 U/mg. The rGM-CSF was diluted in PBS to produce a 2 μ g/ml working solution. Mice received an *i.p.* injection three times a day. Control animals received an injection of saline or PBS three times a day. Each injection was administered in a total volume of 0.2 ml.

Cell depletions. Cell *slg*⁺ cells were depleted from cell suspensions on antibody coated polystyrene petri dishes (Falcon 1001, Falcon Labware, Oxnard, CA) as described previously (15). For depletion of *slg*⁺ cells, petri dishes were incubated with 50 μ g of affinity-purified goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL) in 0.5

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³ Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; *slg*M, surface IgM-expressing cells; SCID, severe combined immunodeficiency disease.

Tris (hydroxymethyl) amino-methane buffer, pH 9.5, at room temperature for 70 min and washed three times with PBS pH 7.2 and once with PBS containing 5% FCS. Up to 2.5×10^7 bone marrow cells were added to the plates for 70 min at 4°C. Nonadherent cells were then collected and phenotypic analysis was used to confirm that sIgM expressing cells were depleted.

Immunofluorescence. Cells that express the B220 Ag were identified with a hybridoma supernatant made by collecting conditioned medium from the 14.8 cell line American Type Culture Collection (ATCC: TIB 164; Rockville, MD). Aliquots of 2×10^6 cells were suspended in 100 μ l of the conditioned medium for 30 min. After one wash in MEM at 4°C, cells were incubated with 10 μ g of a fluorescein-conjugated goat anti-rat Ig (Southern Biotechnology). After a 30-min incubation, cells were washed twice, fixed in 2% paraformaldehyde in PBS, and stored at 4°C until cell counts were performed on a Leitz Laborlux microscope equipped for epifluorescence. A fluorescein-conjugated goat-anti mouse IgM (Southern Biotechnology) was used to identify sIgM expressing cells. Labeling times, washes, and enumeration of cells were performed as described above.

Granulocyte-macrophage colony assay. Granulocyte-macrophage progenitor cells capable of forming colonies in semisolid medium were assayed by culturing 5×10^4 or 1×10^5 cells in 35-mm plastic petri dishes containing 1 ml of methylcellulose medium. This contained 40% methylcellulose (0.8% methylcellulose in Iscoves Modified Dulbeccos Medium; GIBCO), 30% fetal calf serum, 30% IMDM, 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO) and 50 U/ml of rGM-CSF (Amgen). Colonies were enumerated on day 11.

Reconstitution studies. In some experiments the ability of a bone marrow cell suspension to reconstitute B lymphocytes in SCID mice was measured. SCID mice received 300 rad of irradiation from an x-ray source (Pantak Ltd., Berkshire, England) at a rate of 110 rad/min to facilitate reconstitution (16). Immediately thereafter mice were administered an i.v. injection of bone marrow cells in 0.2 ml of PBS. At various times post reconstitution, mice were killed and total numbers of B220 and sIgM-expressing splenocytes and bone marrow cells were determined.

RESULTS

Effects of GM-CSF on B lymphopoiesis. Basic and clinical studies of GM-CSF administration in mice and humans have used doses ranging up to 1200 ng/day (6, 17). Inasmuch as the aim of this study was to examine the effects of elevated GM-CSF levels on B cell production in vivo, the high end dose was used throughout these experiments based on the rationale that any potential effects would be most demonstrable at such concentrations.

Figure 1 shows the results of experiments in which mice received 1200 ng/day of GM-CSF, administered as three injections of 400 ng each, on marrow lymphopoiesis and myelopoiesis at several time points during the week of treatment. Total marrow cellularity was not appreciably different between the GM-CSF and saline-treated mice (Fig. 1A). The total number of granulocyte-macrophage progenitors exhibited a twofold increase by day 2 after initiation of cytokine treatment but had declined by day 7 to levels present in saline-treated mice (Fig. 1B).

More dramatic differences were observed between the two groups of mice upon examination of B lineage cells. On day 4 after GM-CSF treatment, numbers of B cell progenitors that expressed the B220 Ag had declined by 50% as compared to age- and sex-matched control mice that received saline injections (Fig. 1C). Progenitor cell numbers continued to fall and were six times lower than in the control mice at the end of the week of treatment. A similar decline in the total number of sIgM⁺ B cells was also observed, and by day 8 they were rarely detected (Fig. 1D). A less marked decline in the number of B lineage cells in the saline treated mice was also observed and was most likely due to injection induced stress effects.

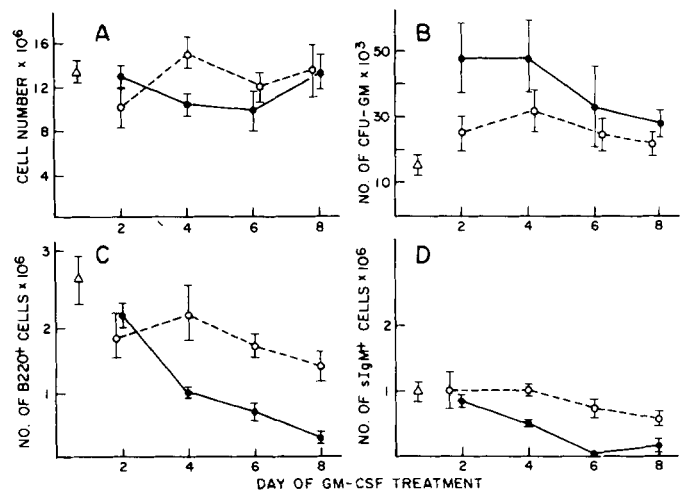


Figure 1. Effects of GM-CSF on lymphopoiesis in murine bone marrow. The different panels show the total cell number per two femurs (A), the total number of CFU-GM (B), the total number of B220 expressing B cell progenitors (C), and the total number of surface IgM expressing B cells (D) in the bone marrow of mice administered 400 ng of GM-CSF (●) or saline (○) three times a day for 1 wk. Each point is based on data from two separate experiments in which a total of four mice per time point were analyzed. The open triangle in each panel represents values obtained from four to six unmanipulated BALB/cAn mice. Error bars indicate SE.

Figure 2 shows the effect of GM-CSF treatment on the spleen of the animals described above. Consistent with previously reported findings (5, 6) was the observation that the total number of spleen cells increased after 1 wk of GM-CSF treatment. As shown in Figure 2A, after 7 days of treatment spleen cellularity in the GM-CSF-treated mice was twice that in the saline control animals. Granulocyte-macrophage progenitor cell numbers had also increased in the spleen of the GM-CSF mice (Fig. 2B). However, the absolute number of sIgM expressing B cells was comparable at all time points between the saline- and GM-CSF-treated animals (Fig. 2C).

Recovery of B lymphopoiesis after GM-CSF treatment. To determine how rapidly B lymphopoiesis returns to normal after termination of GM-CSF administration, mice were administered 400 ng of factor three times a day for 1 wk. The absolute number of B220⁺ B cell progenitors and surface IgM-expressing B cells was then examined at various times over the week after cessation of treatment. As shown in Figure 3, the effects of GM-CSF were still demonstrable at 1 day after termination of GM-CSF treatment, but a rapid recovery of B lineage cells occurred thereafter. A consistently observed phenomenon was that numbers of B lineage cells increased to levels up to 50% over those in saline-treated control animals by 1 wk after termination of GM-CSF treatment. In fact, even when the absolute number of B220 and sIgM-expressing cells in the day 7 post-GM-CSF-treated mice was compared to that in unmanipulated BALB/c animals, the levels were still up to 50% higher (data not shown).

Lack of elevated recovery of B cells after transplantation. To determine if the elevated recovery of B lymphocytes in GM-CSF-treated mice was dependent on the environment in the cytokine-treated animals, bone marrow cells were harvested from the mice after 1 wk of factor treatment and 3×10^6 cells were transplanted into SCID mouse recipients. In view of the above results dem-

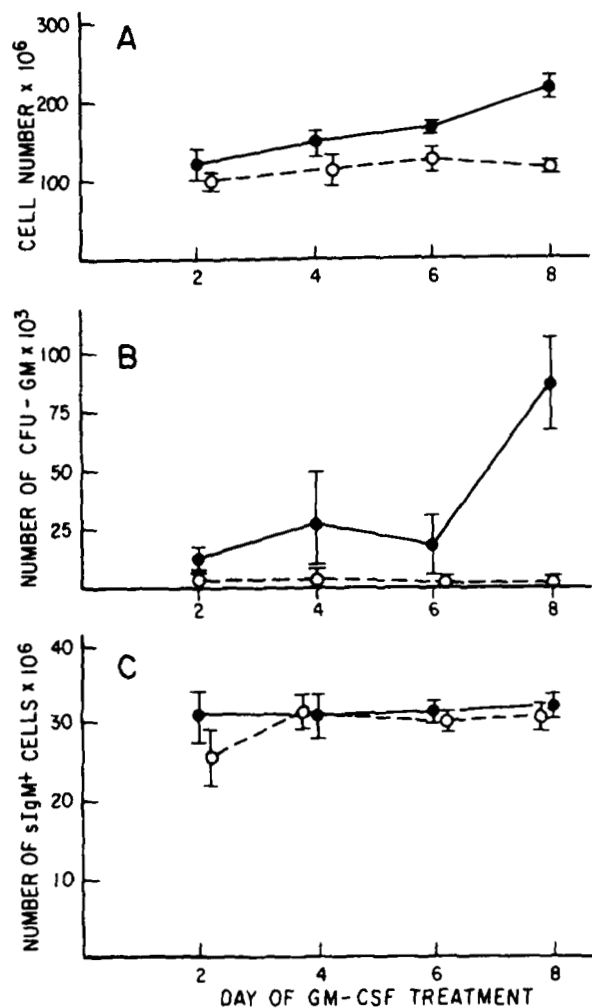


Figure 2. Effects of GM-CSF on the spleen. The different panels show the total cell number (A), the total number of CFU-GM (B), and the total number of surface IgM expressing B cells in the spleen of the mice described in the legend to Figure 1.

onstrating a marked elevation of B lineage cells at 1 wk after termination of GM-CSF treatment, B cell reconstitution in SCID mouse recipients was examined at 1 wk postrestitution. It is clear that there were no significant differences in the level of B cell reconstitution mediated by cells from GM-CSF- or saline-treated donors. Only minimal levels of B cell reconstitution were observed in the bone marrow and spleen of the SCID mice (Table I), a finding consistent with previous reconstitution studies that showed low levels of B cell repopulation at 1 wk postrestitution (18).

Bone marrow from GM-CSF-treated mice can repopulate B cells. To determine if longer periods of reconstitution would reveal differences in the B cell repopulating ability of marrow cells from the two donor sources, additional groups of immunodeficient recipients were examined at 2 and 4 wk posttransplantation. As shown in Table II, B220- and sIgM-expressing cells were easily detectable in the marrow and spleen of the SCID mice at these time points. In experiment 2, any remaining sIgM⁺ cells were removed from the donor marrow before transplantation to assure that reconstitution was mediated by lymphoid precursors and not expansion of mature B cells.

DISCUSSION

The simultaneous production of multiple cell lineages in the bone marrow is presumably the result of a highly

regulated process in which signals that control the growth and development of hemopoietic cells are in balance. The data in this report indicate that this balanced cell production can be altered when myeloepoietic stimulatory signals are increased above constitutive levels. Thus, after administration of rGM-CSF to mice, a marked marrow lymphopenia results due to a decline in the number of B cell progenitors and newly produced B cells. These observations are consistent with another *in vivo* study by Metcalf et al. (6) in which a fivefold decline in morphologically defined marrow lymphocytes occurred after a GM-CSF administration schedule similar to that used in this report. These data further corroborate findings observed *in vitro* using the Dexter to Whitlock-Witte switch system (19). After transfer of Dexter-type myeloid long term bone marrow cultures to the conditions described by Whitlock and Witte, myeloepoiesis ceases and B lymphopoiesis initiates. The first phenotypically identifiable B lineage cells that appear after the change in culture conditions express the B220 Ag. Subsequently, sIgM-expressing B lymphocytes can be detected. However, if GM-CSF is included in the culture medium, myeloepoiesis is sustained and lymphopoiesis does not initiate, even under the lymphoid conditions (10). Another similarity between the *in vivo* and *in vitro* results is that the inhibition of B lymphopoiesis by GM-CSF is reversible. After its removal from the long term cultures or at the termination of its *in vivo* administration, B lymphopoiesis initiated.

Bone marrow cells from GM-CSF-treated donors could repopulate B cells in SCID mice by 2 wk postrestitution. Inasmuch as committed B cell progenitors are believed to be responsible for B lymphocyte production at these relatively early times postrestitution (20), this result suggests that cytokine treatment did not eliminate all B lineage cells from the marrow. However, the data indicate that their number is decreased due to the GM-CSF treatment, and this finding is consistent with the observation that the average total number of B lineage cells in recipients of marrow from GM-CSF-treated do-

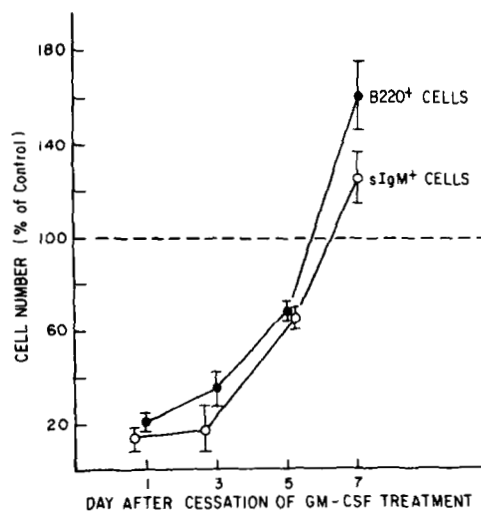


Figure 3. Recovery of B lymphopoiesis in the bone marrow of mice following one week of GM-CSF treatment. Total number of B220 and surface IgM expressing bone marrow cells in GM-CSF-treated mice as compared to levels in saline-treated control animals. Each point is based on data pooled from three separate experiments in which a total of six mice per time point were analyzed. Error bars indicate SE.

TABLE I
Numbers of B lineage cells in SCID mice at 1 wk postreconstitution (reconstitution parameters in SCID mice^a)

| Treatment ^b | Tissue | Cell No. × 10 ⁶ | Percent B220 | Total B220 × 10 ⁶ | Percent IgM | Total IgM × 10 ⁶ |
|------------------------|--------|----------------------------|--------------|------------------------------|-------------|-----------------------------|
| CSF | BM | 3.7 ± 0.8 | 8.4 ± 2.2 | 0.31 ± 0.1 | 0 | 0 |
| Saline | BM | 3.2 ± 0.7 | 11.5 ± 4.1 | 0.42 ± 0.13 | 0 | 0 |
| CSF | Spl | 2.5 ± 1.3 | | | 1.0 ± 0.7 | 0.02 ± 0.02 |
| Saline | Spl | 4.6 ± 2.5 | | | 3.3 ± 1.1 | 0.12 ± 0.07 |

^a Marrow from mice treated with GM-CSF or saline for 7 days was transplanted into two SCID mouse recipients per donor. Three GM-CSF and three saline treated donors were used. Recipient mice were processed 1 wk later. All animals were treated individually and data from the six recipient mice per donor group pooled and expressed as mean ± SD.

^b Treatment of BALB/cAn donors whose marrow was used to reconstitute SCID mice.

TABLE II
Repopulation of B lymphocytes in SCID mice (reconstitution parameters in SCID mice^a)

| Week ^b | Treatment ^c | Tissue | Cell No. × 10 ⁶ | Percent B220 | Total B220 × 10 ⁶ | Percent IgM | Total IgM × 10 ⁶ | |
|---------------------|------------------------|--------|----------------------------|-----------------------|------------------------------|-------------|-----------------------------|-------------|
| Exp. 1 ^d | 2 | CSF | BM ^e | 2.7 ± 1.3 | 21.6 ± 5.7 | 0.54 ± 0.25 | 5.3 ± 2.2 | |
| | | Saline | BM | 3.2 ± 0.42 | 33.7 ± 7.0 | 1.1 ± 0.30 | 9.6 ± 3.8 | |
| | 4 | CSF | Spl | 10.3 ± 5.2 | | | 34.5 ± 1.7 | 3.5 ± 1.7 |
| | | Saline | Spl | 17.8 ± 6.9 | | | 35.2 ± 1.0 | 6.1 ± 2.6 |
| | | CSF | BM | 4.8 ± 1.1 | 18.8 ± 10.4 | 0.9 ± 0.4 | 8.9 ± 3.8 | 0.87 ± 0.4 |
| | | Saline | BM | 4.2 ± 0.3 | 29.0 ± 10.4 | 1.3 ± 0.5 | 10.9 ± 5.5 | 1.3 ± 0.5 |
| | | CSF | Spl | 11.6 ± 12.6 | | | 32.0 ± 7.8 | 36.4 ± 12.9 |
| | | Saline | Spl | 73.8 ± 6.3 | | | 31.0 ± 5.9 | 23.2 ± 5.8 |
| Expt. 2 | 2 | CSF | BM | 3.3 ± 0.3 | 11.2 ± 0.7 | 0.37 ± 0.1 | 2.3 ± 0.4 | 0.08 ± 0.01 |
| | | Saline | BM | 3.6 ± 1.1 | 10.1 ± 0.4 | 0.36 ± 0.12 | 3.4 ± 1.9 | 0.12 ± 0.08 |
| | | CSF | Spl | 49.2 ± 12 | | | 9.9 ± 2.5 | 4.8 ± 1.6 |
| | | Saline | Spl | 46.2 ± 6.1 | | | 16.0 ± 4.2 | 7.5 ± 2.6 |
| | 4 | CSF | BM | 10.8 ± 0.8 | 15.9 ± 6.2 | 1.7 ± 0.4 | 3.7 ± 2.4 | 0.4 ± 0.2 |
| | | Saline | BM | 8.7 ± 1.6 | 31.2 ± 5.3 | 2.7 ± 0.9 | 11.6 ± 0.4 | 1.0 ± 0.14 |
| | | CSF | Spl | 214 ± 63 ^f | 23 ± 1.6 | 49.6 ± 18 | 20.1 ± 7 | 42.3 ± 2.2 |
| | | Saline | Spl | 97 ± 9 | 45.1 ± 0.3 | 43.1 ± 4 | 36.1 ± 4 | 34.7 ± 1 |

^a Marrow from BALB/cAn mice treated with CSF or saline was transplanted into SCID mice that were processed on wk 2 or 4 postreconstitution. In experiment 1, results represent marrow from three donor mice transplanted into three SCID mice per data point. In experiment 2, results represent marrow from two donor mice transplanted into two SCID mice per data point. All animals were treated individually and data pooled and expressed as mean ± SD.

^b Week postreconstitution of SCID mice.

^c Treatment of BALB/cAn donor animals whose marrow was used to reconstitute SCID mice.

^d In experiment 1, bone marrow cells harvested from donor mice and used to reconstitute SCID mouse recipients; in experiment 2, sIgM expressing cells were depleted from the donor marrow cell population before being transplanted into SCID recipients.

^e Per femur.

^f One of the reconstituted SCID mice had splenomegaly.

nors was often lower than in recipients of marrow from saline-treated mice.

GM-CSF would not be expected to bind directly to B lineage cells and directly affect their proliferation and/or differentiation. Therefore, indirect effects mediated by GM-CSF actions on the ability of the hemopoietic microenvironment to normally regulate lymphopoiesis may be a factor in the observed results. This possibility has been suggested previously by Billips et al. (21) and is consistent with a report indicating that GM-CSF can bind to and affect nonhemopoietic accessory cell populations (22). These indirect effects most likely affect primary B cell production, although the possibility that increased loss or accelerated discharge of B lineage cells from the marrow is also occurring must be considered.

The rapid and elevated recovery of B lineage cells in the marrow of the GM-CSF-treated mice would be concordant with an alteration of normal control mechanisms that regulate B cell production. A consistently observed phenomenon was that, approximately 1 wk after cessation of GM-CSF treatment, the absolute number of B220 and sIgM-expressing cells in the bone marrow of the GM-CSF-treated mice was elevated as compared to the saline control animals. A similar effect has been reported for postirradiation recovery of B lymphopoiesis by Park and Osmond (23). However, this effect was not observed when

the marrow cells were removed from the environment in the GM-CSF-treated animals and transplanted into SCID mice. This observation raises the possibility that conditions in the GM-CSF-treated animals, but not present in the "normal" SCID mouse recipients, contributed to the supernormal regeneration of B cells.

In agreement with previous results, the spleen in the GM-CSF-treated mice enlarged and the number of myeloid progenitor cells increased as compared to the saline-treated control animals (5, 6). However, the absolute number of sIgM⁺ B cells was not affected. This finding is consistent with a study in which GM-CSF was used therapeutically in patients with refractory aplastic anemia (17). In that report, GM-CSF actually increased numbers of circulating B lymphocytes in patients.

The observations in this report are consistent with a study by Fulop et al. (24) in which transplantation of a granulocytosis-inducing tumor into mice resulted in an inhibition of marrow B cell production. The tumor cell effects are believed to be mediated by soluble factors, providing further evidence that increases in myeloid stimulatory signals can severely compromise primary B lymphopoiesis. Conversely, if signals that normally regulate myelopoiesis are depressed, B cell production can be elevated. For example, Engelhard et al. (25) described a patient with cyclic neutropenia in whom pre-B cell

numbers cycled from normal to markedly elevated levels during periods of neutropenia.

These data may be of relevance to clinical use of GM-CSF. The cytokine has been used or is being considered for use in a variety of conditions, and complete knowledge of its effects could be important in planning therapeutic regimens. The dose used in these experiments was higher than in many other studies and was purposely chosen in order that inhibitory effects on B lymphopoiesis be marked and consistently observed. Even in this case, lymphopoiesis rapidly resumed after cessation of GM-CSF administration and B cell numbers in the periphery were not compromised. However, the observation that primary lymphopoiesis is depressed after 1 wk of treatment could be of relevance in considering long term use of the cytokine.

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