Human Spleen Cell Generation of Factors Stimulating Human Pluripotent Stem Cell, Erythroid, and Myeloid Progenitor Cell Growth

By Ina Fabian, Dan Douer, Lee Levitt, Yehudith Kletter, and Peter L. Greenberg

Mitogen-stimulated murine spleen cells produce humoral substances capable of supporting murine hematopoiesis and pluripotent stem cell proliferation in vitro. Thus, we evaluated conditioned media generated by human spleen cells (SCM) in the presence or absence of mitogens for factors stimulatory for human pluripotent (CFU-GEMM), erythroid (BFU-E), and myeloid (CFU-GM) precursors. Two and one half percent to 10% SCM stimulated proliferation of all three types of precursor cells from nonadherent buoyant human marrow target cells. Mitogen-stimulated SCM augmented CFU-GM (175% to 225%), whereas CFU-GEMM and BFU-E growth was essentially unchanged. Cell separation procedures used to determine which cells provided these microenvironmental stimuli indicated that nonadherent mononuclear spleen cells provided the bulk of the CSF-GM, whereas adherent cells (95% nonspecific esterase + monocyte-macrophages) and nonadherent cells provided similar proportions of CSF-mix and erythroid burst-promoting activity (BPA). The nonadherent cells generating high levels of CSF-mix, BPA, and CSF-GM were predominantly Leu-1-negative, ie, non-T, cells. In the presence or absence of mitogens, SCM was a more potent source (1.3- to 3.8-fold) than peripheral leukocyte CM of the growth factors for the three progenitor cell types. Specific in situ cytochemical stains for analyzing morphology of myeloid colonies demonstrated that SCM stimulated the proliferation of the same types and proportions of colonies as human placental CM, suggesting that these CMs may contain similar CSF-GMs. These data show the contribution of spleen cell subsets to the generation of hematopoietic growth factors and the responsiveness of these cells to various mitogenic stimuli.

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STUDIES in experimental animals and humans have demonstrated that spleen cells provide microenvironmental influences and humoral substances capable of stimulating hematopoiesis in vivo and in vitro. 1-5 Human spleen cells were found to provide CSF-GM, 6 the humoral substance necessary for granulocyte-monocyte progenitor cells to undergo proliferation and differentiation in vitro. 6,7 Mitogen-stimulated murine spleen cells produce humoral substances capable of supporting murine hematopoiesis and pluripotent stem cell proliferation in vitro. 8,9 Thus, we have undertaken studies to investigate whether the human spleen is a potent source of hematopoietic regulatory factors for human cells. Conditioned media generated by human spleen cells (SCM) in the presence or absence of mitogens were evaluated for factors stimulatory for hematopoietic progenitors with characteristics of pluripotential stem cells (CFU-GEMM), 9 BFU-E, and CFU-GM in vitro. In order to evaluate factors involved in providing local cellular potential for modulating splenic hematopoiesis in humans, we used fractionated suspensions of spleen cells to determine the cellular source of the humoral factors. Specific cytochemical staining of colonies was performed to evaluate whether SCM contained myeloid stimuli other than those present in human placental CM (PCM). The potential of SCM to stimulate the growth of hematopoietic precursor cells was compared with that of peripheral mononuclear cell conditioned medium (LCM) and conditioned medium from a human T lymphoblast cell line (MoCM) 10-11—two major sources found to stimulate the growth of human CFU-GEMM. 9,11

MATERIALS AND METHODS

Preparation of SCM

Immediately after splenectomy, a portion of the spleen was placed in modified McCoy’s medium containing 15% fetal calf serum (FCS) and was finely minced, producing a single cell suspension. Supernatant cells were obtained after Ficoll-Hypaque gradient centrifugation as previously described. 6 SCM containing CSF-mix, burst-promoting activity (BPA), and CSF-GM was obtained by placing the buoyant mononuclear cells at a concentration of 1 to 10 x 10^6 cells per milliliter of McCoy’s medium with FCS and 0.5 mmol/L 2-mercaptoethanol (2-ME) in tissue culture dishes (Falcon Plastic). These media were harvested after five days’ incubation at 37 °C in a humidified air-5% CO2 incubator, and stored at −20 °C until use. (Preliminary studies [data not shown] indicated that day 5 supernatants of spleen cultures showed optimal CSF-mix, BPA, and CSF-GM activity.)

Preparation of LCM

Mononuclear cells were obtained from heparinized peripheral venous blood of healthy volunteers by centrifugation with Ficoll-Hypaque at 400 g for 30 minutes. Cells were plated at a concentra-
tion of 5 × 10⁶ cells per milliliter of McCoy’s medium with 15% FCS and 0.5 mmol/L 2-ME in tissue culture dishes. These media were harvested after incubation for five days at 37 °C in a humidified air-5% CO₂ incubator.

Mitogens

Mitogens used to stimulate spleen cells and peripheral blood leukocyte cultures were phytohemagglutinin-M ([PHA] Gibco, Grand Island, NY), 50 μL of a 1:15 dilution per milliliter of cell suspension; pokeweed mitogen ([PWM] Gibco), 50 μL of a 1:15 dilution per milliliter; concanavalin A ([Con A] Difco Laboratories, Detroit), 5 μg/ml; and lipopolysaccharide-W (LPS) from Escherichia coli (0.11I:B4; Difco), 10 μg/ml.

Separation of Spleen Cells

Adherence separation. Our previously described adherence separation procedure was used. A suspension of 10⁶ spleen cells was placed in 2 mL medium with 7.5% FCS in 35 × 10-mm plastic tissue culture dishes for 60 minutes at 37 °C in an air-CO₂ incubator.

Following incubation, nonadherent and loosely adherent cells were separately removed by gently rinsing the plates three times with phosphate-buffered saline (PBS) and processed as described below. The adherent cells were then removed by flushing the plates with ice-cold PBS lacking Ca²⁺ and Mg²⁺, resuspended in McCoy’s medium containing 0.5 mmol/L 2-ME and cultured at a concentration of 5 × 10⁶ cells per milliliter for five days to provide regulatory factors. The purity of the adherent cells, determined by staining with α-naphthylacetate esterase (ANA esterase) stains, was generally shown to be 95% ANA esterase positive. In four normal spleens, the percentage of ANA esterase positive was generally >96% lymphoid and <4% monocytes (ANA esterase positive). In the remainder of this paper, these cell populations are designated as nonadherent buymonuclear cells and they served as target cells in part of the experiments.

In other experiments, the nonadherent buymonuclear cells were depleted of T lymphocytes by using the indirect panning technique (see above regarding separation of splenic T lymphocytes). Briefly, 2 × 10⁶ nonadherent buymonuclear bone marrow cells were coated with a pan-T lymphocyte mononuclear antibody for 20 minutes at 4 °C. The cells were washed, then suspended in 3 mL 5% FCS in PBS, poured onto antibody-coated plates, and incubated at 4 °C for 70 minutes. Nonbound cells were removed, washed with 1% FCS in PBS, and resuspended in IMDM. These suspensions contained 2% ± 1% lymphocytes (as determined by indirect immunofluorescence). These cells served as target cells in some experiments and were designated as nonadherent T cell-depleted bone marrow cells.

Culture System for CFU-GEMM, BFU-E, and CFU-GM

The previously described clonogenic assay system was used. Briefly, nonadherent buymonuclear bone marrow cells (1.0 to 1.5 × 10⁶/mL) or nonadherent, T-depleted cells (5 × 10⁵/mL) were cultured in IMDM, 10% SCM, 5% LCM, or 1% MoCM were added to the plates. The cultures were incubated for 14 days at 37 °C in humidified atmosphere with air-5% CO₂. One unit of human urinary erythropoietin (EP) was added on day 4 of culture. Erythropoietin (1,140 U/mg protein) was kindly provided by the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Md. On days 12 to 14, using an inverted microscope, CFU-GEMM, BFU-E, and CFU-GM were counted. The mixed nature of colonies derived from CFU-GEMM was confirmed by cytocrit analysis after removal from the dish by micropipette and staining with Wright’s stain.

Bone Marrow Agar Culture

The agar culture system was also used for the detection of CFU-GM as described previously. Briefly, 1.5 × 10⁶ nonadherent...
buoyant normal human marrow cells in 1 mL McCoy's 5A medium containing 15% FCS and 0.3% agar were added to 35-mm Petri dishes containing 13% SCM, mitogen-stimulated SCM, or PCM. PCM was prepared as previously described. Cultures were incubated at 37 °C in a humidified atmosphere of air-5% CO2 for seven days.

Cytochemistry

A recently developed in situ triple staining technique was used17 to determine the type of colonies generated by SCM. Staining for ANA esterase (monocytes/macrophages), naphthol AS-D chloroacetate esterase (neutrophils), and Luxol fast blue (eosinophils) in sequence permitted the typing of myeloid colonies on the same culture dish.

Patient Material

Specimens of bone marrow were obtained from normal volunteers and consenting patients undergoing diagnostic or therapeutic procedures. All samples were aspirated into heparinized syringes.

Seven spleens were obtained from patients undergoing splenectomy for traumatic rupture.

Statistical analyses were performed using the Student's t test. Results were considered significant when the P value was <.05.

RESULTS

SCM (5% concentration) generated the growth of CFU-GEMM, BFU-E, and CFU-GM (ie, possessed CSF-mix, BPA, and CSF-GM) using nonadherent buoyant bone marrow as target cells. Dose–response curves (Fig 1) indicated cell concentration dependence for CSF-GM and BPA production within SCM. At lower spleen cell concentrations, no CSF-mix was noted. In the absence of CM, control cultures contained 1.0 ± 0.2 BFU-E, 2.0 ± 0.2 CFU-GM, and no CFU-GEMM per 10^5 nonadherent mononuclear cells.

We investigated the responsiveness of nonadherent buoyant bone marrow cells to various concentrations of SCM and CM prepared from 5 × 10^6 mitogen-stimulated spleen cells per milliliter. A representative experiment is shown in Fig 2. Apparently increased

Fig 1. Growth of CFU-GEMM, BFU-E, and CFU-GM in the presence of humoral factors produced by buoyant mononuclear spleen cells. Dose–response curves show means ± SE of four cultures. Nonadherent cells served as target cells. ■—■ CFU-GEMM, ●—● BFU-E, ▲—▲ CFU-GM.

Fig 2. The effect of conditioned medium from unstimulated and mitogen-stimulated spleen cells on the growth of CFU-GEMM (A), BFU-E (B), and CFU-GM (C). Colony formation was determined using 10^5 buoyant nonadherent mononuclear bone marrow cells. Each datum point represents the mean of five replicate cultures in a single representative experiment. SE generally ± 10% to 15%. Error bars have been omitted for clarity.

amounts of CSF-mix were found at SCM concentrations of 2.5% to 10.0% (25 to 100 µL/mL). PWM-SCM at 2.5% to 5.0% showed slight increments of CSF-mix compared with (unstimulated) SCM (Fig 2A). Decrements in these stimulated values occurred at 10% concentration. (Because of low numbers of
CFU-GEMM, the changes in CSF-mix production were not statistically significant.) PHA-SCM stimulated increased BFU-E at 5% and 10% concentrations. The other mitogens did not augment BFU-E at 5% and generated decrements at 10% concentration (26% to 77% growth, \( P < .05 \)) (Fig 2B).

In contrast to these findings, marked increases in the number of CFU-GMs was noted (Fig 2C) with increasing concentrations of SCM or mitogen-stimulated SCM, from 2.5% to 10.0% (128% to 200%) \( (P < .05) \). In view of the decremental effects of high (10%) concentrations of the conditioned media on the growth of CFU-GEMM, BFU-E, and CFU-GM, further experiments were performed with 5% CM. In control cultures, mitogens alone did not augment the number of colonies that grew in the absence of CM.

Subpopulations of Spleen Cells

The buoyant spleen cells were separated into adherent and nonadherent fractions. The morphology of the cell fractions was assessed by Wright's-Giemsa and ANA esterase stains. Adherent cells (95% ± 6%) were ANA esterase positive. Nonadherent cells contained 4% ANA esterase-positive cells (Table I).

We examined the production of humoral factors by separated and unseparated spleen cells. Bone marrow cells depleted of T cells and of adherent cells (see purity under Bone Marrow Cell Separation) served as target cells and were seeded at a concentration of 5 \( \times 10^4 \) cells per milliliter. Results are summarized in Fig 3 and demonstrate:

1. Relatively equal amounts of CSF-mix and BPA were produced by unstimulated and mitogen-stimulated adherent and nonadherent spleen cells (Fig 3A and B).

2. High BPA levels were produced by unseparated cells. In general, all four mitogens did not alter BPA production by these cells (the increase in the presence of PHA was not significant). Adherent and nonadherent unstimulated and mitogen-stimulated spleen cell production of BPA was relatively equal (Fig 3B).

3. Unstimulated spleen cells showed low levels of CSF-GM (Fig 3C), with all four mitogens increasing these values (175% to 225%) \( (P < .05) \). Nonadherent unstimulated spleen cells showed high levels of CSF-GM production. Adherent and unseparated cells responded to mitogens for CSF-GM production to a greater extent than did nonadherent cells.

Prostaglandin E, which is produced by adherent cells, is capable of inhibiting granulocytic colony formation. However, addition of indomethacin (10 \( ^7 \) mol/L) to the incubation mixture did not alter CSF-GM, BPA, or CSF-mix production (data not shown).

The nonadherent spleen cells were separated by panning into Leu-1-bound (T lymphocytes) and non-bound (non-T cell) fractions (ie, containing B lympho-

<table>
<thead>
<tr>
<th>Cell Fraction</th>
<th>Percentage of Recovery From Nonadherent Cells</th>
<th>Purity of Fractions (%)</th>
<th>ANA Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonadherent spleen cells</td>
<td>-</td>
<td>42% ± 7% (3)</td>
<td>45% ± 6% (2)</td>
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<tr>
<td>Leu-1 bound</td>
<td>36 ± 3 (4)</td>
<td>85% ± 4% (2)</td>
<td>5% ± 2% (2)</td>
</tr>
<tr>
<td>Leu-1 nonbound</td>
<td>56 ± 8 (4)</td>
<td>5% ± 2% (2)</td>
<td>81% ± 2% (2)</td>
</tr>
</tbody>
</table>

Indirect (for Leu-1, detecting T lymphocytes) and direct (for anti-human slg, antibody detecting B lymphocytes) immunofluorescence techniques were used as described in Materials and Methods. ANA esterase staining techniques may be found in the same section.
cytes and null cells). Table 1 shows 92% recovery of the nonadherent spleen cells and the enrichment of the fractions, as determined by immunofluorescence and ANA esterase staining. Using spleen cells obtained by panning with Leu-1 antibody on a per-cell basis, the vast majority of CSF-mix, BPA, and CSF-GM was produced by the non-T cell population (Fig 4).

Morphology of Bone Marrow Colonies Stimulated by PCM and SCM

We used an in situ triple-staining technique to identify the type of myeloid colonies formed in soft agar cultures. Table 2 indicates that, with PCM and SCM, the highest percentage of colonies was macrophage in type (37% and 31%, respectively). Similar growth of granulocyte, granulocyte-macrophage, eosinophil-granulocyte, and eosinophil-granulocyte macrophages was noted in the presence of unstimulated SCM and PCM. We also examined the morphology of the myeloid colonies following use of CM prepared from mitogen-stimulated spleen cells. The distribution of the various myeloid colonies was similar to that with unstimulated SCM (data not shown).

Comparison of the Potency of SCM and LCM

We tested the potency of SCM, LCM, and MoCM, to stimulate the growth of CFU-GEMM, BFU-E, and CFU-GM from nonadherent bone marrow cells. As shown in Fig 5 and Table 3: (1) In the presence or absence of mitogens, SCM was more potent than LCM in generating all three humoral factors (CSF-mix, BPA, and CSF-GM). (2) SCM and MoCM had similar potency stimulating the growth of CFU-GEMM and BFU-E (Fig 5). (3) MoCM was more potent in stimulating CFU-GM growth than was stimulated SCM (2.4-fold) (Fig 5).

DISCUSSION

Our studies indicated that unstimulated spleen cells from normal individuals generated factors enhancing the proliferation and differentiation of human CFU-GEMM, BFU-E, and CFU-GM. Dose–response experiments revealed that 5% SCM contained the optimal concentration of CSF-mix, BPA, and CSF-GM. We examined the effect of mitogens on the production/release of humoral factors by spleen cells. Our findings that the mitogens PHA, PWM, Con A, and LPS enhanced the release of CSF-GM from human cells are similar to data reported from investigations performed with mice.

We investigated the cellular source of the regulators of primitive progenitor cells and demonstrated that both adherent (95% monocyte-macrophages) and nonadherent spleen cells produced CSF-mix. The present study also showed that nonadherent and adherent spleen cells provided similar levels of BPA. We found that among the nonadherent cells, regarding BPA, CSF-GM, and CSF-mix production, there was more...
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and peripheral blood cells per milliliter were used. Values represent a comparison of colony formation stimulated by SCM or LCM. The numbers in parentheses represent P values. NS, not significant.

Table 3. The Effect of Mitogens on Mononuclear Spleen and Peripheral Blood Cell Production of Humoral Factors

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>CFU-GENM</th>
<th>CFU-GM</th>
<th>CFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>2.0 ± 0.5 (NS)</td>
<td>1.7 ± 0.2 (0.04)</td>
<td>2.2 ± 0.3 (0.04)</td>
</tr>
<tr>
<td>PWM</td>
<td>2.1 ± 0.2 (0.01)</td>
<td>2.1 ± 1.0 (0.02)</td>
<td>1.9 ± 0.2 (NS)</td>
</tr>
<tr>
<td>Con A</td>
<td>3.5 ± 0.6 (0.04)</td>
<td>3.8 ± 0.6 (0.04)</td>
<td>3.3 ± 0.7 (0.04)</td>
</tr>
<tr>
<td>LPS</td>
<td>1.9 ± 0.3 (0.03)</td>
<td>1.5 ± 0.2 (NS)</td>
<td>1.3 ± 0.1 (NS)</td>
</tr>
</tbody>
</table>

NAB cells (1.5 x 10^6) served as target cells (n = 3); 5 x 10^6 spleen and peripheral blood cells per milliliter were used. Values represent a comparison of colony formation stimulated by SCM or LCM. The numbers in parentheses represent P values. NS, not significant.

activity on a per-cell basis in relatively purified nonadherent non-T cells than in T cell populations. This confirms prior studies with mitogen-stimulated murine spleen cells and with nonstimulated human spleen cells, which also demonstrated splenic CSF-GM production by non-T lymphocytes. However, the minor degree of contamination of our cell fractions with T cells and macrophages may contribute to the provision of these humoral factors. BPA, which is a proposed regulator of the proliferation and initial stages of differentiation of BFU-E, has previously been detected in media conditioned by unstimulated, mitogen-stimulated, or antigen-stimulated cells, and has been produced by monocytes, macrophages, or T lymphocytes. Others have shown that macrophages and lymphocytes interact and control the elaboration of BPA production.

In the present study, we compared the stimulatory potential of SCM to that of peripheral blood LCM. We found that in the presence or absence of mitogens, SCM is more potent than mitogen-stimulated LCM in providing the humoral factors needed for growth of the three hematopoietic precursors assayed. Recent investigations have shown the inconsistent production of CSF-mix from peripheral blood LCM. In further contrast to nonadherent spleen cells, nonadherent marrow cells are a poor source of CSF-GM. These findings are likely related to qualitative or quantitative differences of the cell populations capable of generating the growth factors in the spleen compared with those in peripheral blood (PB) and marrow. The higher concentration of these two cell populations in the spleen as compared with PB (macrophages comprise approximately 17% [see Materials and Methods] of mononuclear cells in the spleen and 10% in PB; B lymphocytes comprise approximately 45% [Table 1] of nonadherent cells in the spleen and 25% in PB) may explain the relatively high activity of SCM. Of interest in this regard is the splenic derivation of the cell line producing MoCM.

Our cytochemical studies of myeloid colonies revealed the presence of colonies that differentiated into unipotent myeloid cells (eg, neutrophil, macrophage, or eosinophil lineages) and several bi- or tripotent myeloid progenitors (eg, eosinophil-monocyte). Our experiments demonstrated that SCM caused the proliferation of myeloid colonies similar to those stimulated by PCM, suggesting that these CMs may contain similar myeloid colony-stimulating factors.

In conclusion, we have shown that SCM generates CSF-mix, BPA, and CSF-GM, and have described stimuli and cell subsets involved in their production. These features may explain differing hematopoietic stimulatory potencies of various sources (spleen, PB, marrow) of CM, likely related to altered ratios or types of microenvironmental support cells from these disparate hematopoietic organs.

ACKNOWLEDGMENT

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Human spleen cell generation of factors stimulating human pluripotent stem cell, erythroid, and myeloid progenitor cell growth

I Fabian, D Douer, L Levitt, Y Kletter and PL Greenberg