Production of Colony-Stimulating Activity by Normal and Neoplastic Human B Lymphocytes

By V. Pistoia, R. Ghio, S. Roncella, F. Cozzolino, S. Zupo, and M. Ferrarini

Normal human B cells were purified from peripheral blood or tonsils and tested for their ability to release colony-stimulating activity (CSA) in short-term cultures. The target cells used in the CSA assays were from peripheral blood or bone marrow. Unstimulated B cells produced CSA in amounts similar to those present in the GCT-conditioned medium used as a positive control. The B-cell derived CSA predominantly promoted the growth of colonies that contained macrophages alone or macrophages and granulocytes. CSA eluted in a single peak from a G-75 Sephadex column with an approximate molecular weight (mw) of 65 to 70 kilodaltons (kd). Fractionation of tonsil B lymphocytes on Percoll density gradients showed that large B cells, probably already activated in vivo, were the main source of CSA. By contrast, small, resting B cells recovered from a different fraction of the Percoll gradient released minimum amounts or no CSA. However, these B cells became CSA producers following stimulation with Staphylococcus aureus Cowan (SAC) in vitro. B cells purified from the peripheral blood of nine out of 12 patients with B-cell chronic lymphocytic leukemia (B-CLL) also released CSA in vitro in the absence of stimuli. These findings suggest that by releasing CSA, B cells may have a role in the regulation of hematopoiesis and in the control of the inflammatory process.

Materials and Methods

Cell fractionation procedures. Peripheral blood mononuclear cells (MNC) from normal donors were separated on Ficoll-Hypaque (F-H) density gradients. Monocytes were removed partially by adherence. T cells were isolated from the suspensions by rosetting with neuraminidase-treated sheep erythrocytes (E), as previously reported, followed by fractionation on two subsequent F-H density gradients. Indicator cells were removed from E rosetting cells by hypotonic lysis. When indicated, T cells were further depleted of monocytes by treatment with an anti-HLA-DR monoclonal antibody (PTF 29/12) and complement. B cells were purified as previously described.

Briefly, non-T cells (E rosette-negative cells) were depleted of monocytes by treatment with the OKM1 monoclonal antibody (Ortho Pharmaceutical, Raritan, NJ) and complement. They were subsequently sensitized with the B1 monoclonal antibody (Coulter, Hialeah, FL). Then 3 x 10^5 sensitized cells were rosetted with 2 mL of 5% ox erythrocytes coated with a purified rabbit antimmune Ig antibody. Rosetting cells (B1" cells) were separated from nonrosetting cells (B1" cells) on F-H density gradients. Adherent monocytes were recovered from plastic with a rubber policeman. Suspensions of tonsil lymphocytes were prepared by gentle mincing. Separation of MNC on F-H density gradients and depletion of E rosetting cells was carried out as above. Non-T cells were subsequently treated with the OKM1 antibody and complement. In order to separate large B cells from small B cells, tonsil non-T cells were fractionated on a Percoll density gradient. Cells (5 x 10^5) were resuspended in 2 mL of 100% Percoll (Pharmacia, Uppsala, Sweden) solution in a 15-mL conical test tube. Four additional Percoll concentrations (diluted in medium supplemented with 10% FCS) of 60, 50, 40, and 30% were layered over the 100% fraction starting from the bottom of the tube.

Cells were spun at 3,000 rpm for 15 min. Four cell fractions were obtained: the first fraction contained low density cells, responding predominantly of large B cells; the fourth was enriched for small B cells. The second and third fractions comprised cells of intermediate characteristics.

Cells from the first and fourth fractions only were washed twice and used for further tests. Cells from twelve B-CLL patients were also studied. Peripheral blood samples from B-CLL patients were obtained after informed consent. The diagnosis of B-CLL was established by clinical, morphologic, and immunological criteria. MNC suspensions were depleted of cells forming E rosettes, as
above, and treated with the OKM1 monoclonal antibody and complement.

**Cell surface marker analysis.** Cells with receptors for sheep erythrocytes or surface immunoglobulin (slg) were detected as previously reported. The murine monoclonal antibodies used in this study were the pan-T reagent, OKT3 (Ortho Pharmaceutical); the B cell specific antibody, B1 (Coulter Electronics); the natural killer (NK) cell specific reagent, Leu 11 (Becton Dickinson, Sunnyvale, CA); and the anti-monocyte-macrophage, OKM1 (Ortho Pharmaceutical), which also stains NK cells. The anti-Tac monoclonal antibody that identifies IL-2 receptors was kindly provided by Dr T. Waldman. The anti-HLA-DR monoclonal PTF 29/12 was kindly donated by Dr G. Damiani. All of the above reagents were used in indirect immunofluorescence with a fluorescein-isothiocyanate (FITC)-conjugated goat F(ab')2 antirabbit immunoglobulin. Wet cell preparations were examined with a Leitz-Orthoplan fluorescence microscope. The percent of positive cells was calculated on at least 200 cells per preparation.

**Cell cultures.** Cell suspensions enriched for T cells, monocytes, or B cells were cultured for 48 hours at the concentration of 1 x 10⁷/mL in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) (RPMI-FCS) or, alternatively, with 2% endotoxin-free human albumin (ISM, Milano, Italy).

In some experiments, small resting B cells were cultured for 48 hours at a concentration of 1 x 10⁶ in RPMI-FCS, with killed *Staphylococcus aureus* of the I Cowan strain (SAC), kindly donated cell specific antibody, BI (Coulter Electronics); the natural killer and stained with Giemsa for morphological examination. Slides were twice, with Hank's agar.

*Subsequently isolated on F-H density gradients, washed, and cultured in ability to support myeloid colony formation by murine bone marrow suspensions were also counted. A colony was defined as an aggregate air and 5% CO₂ at 37°C and scored for colonies after 14 days. On a -supernatants from the above cultures were harvested following production by human B cells CSA above, and treated with the OKM1 monoclonal antibody and complement.

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In some experiments, small resting B cells were cultured for 48 hours at a concentration of 1 x 10⁶ in RPMI-FCS, with killed *Staphylococcus aureus* of the I Cowan strain (SAC), kindly donated by Dr S. Romagnani. In some experiments, B-cell supernatants were absorbed onto Concanavalin A (Con A) bound to Sepharose beads (PharMacia, Uppsala, Sweden) or on human albumin coupled to Sepharose beads. Three ml of supernatants were admixed with 150 µL of Sepharose beads and incubated for six hours at room temperature in a rotating mixer apparatus. Beads were subsequently removed by centrifugation. All of the supernatants from the above cultures were harvested following centrifugation, filtered, and stored at −20°C until tested.

**CSA assay.** The 1 x 10⁶ bone marrow cells or 2 x 10⁶ peripheral blood MNC, isolated on F-H density gradients and depleted of both adherent cells and E rosetting cells, as well as control targets for the assay. Cells were resuspended in a final volume of 1 mL of a mixture containing α-medium (Gibco) 10% heat-inactivated FCS and 0.3% agar and supplemented with non-essential amino acids, L-glutamine, and sodium pyruvate. Test supernatants were included in the culture medium at the final concentration of 10%. Control plates contained the conditioned medium from the GCT cell line (Gibco) as a source of CSA. Cultures were incubated in 95% air and 5% CO₂ at 37°C and scored for colonies after 14 days. On a few occasions, seven-day colonies formed by bone marrow cell suspensions were also counted. A colony was defined as an aggregate of more than 50 cells.

In some experiments, B-cell supernatants were tested for the ability to support myeloid colony formation by murine bone marrow cells (from the Balb/c mouse strain) collected from individual femoral shafts by flushing the marrow cavity. Cells were subsequently isolated on F-H density gradients, washed, and cultured in agar.

Cell types within colonies were identified by morphological and cytochemical criteria. Each dish was washed twice, with Hanks' balanced salt solution (HBSS). Agar was subsequently removed by floating in distilled water and was dried onto glass slides under filter paper. Dried cultures were fixed with acetone-methanol fixative (60% acetone, 10% methanol, 30 mmol/L sodium citrate, pH 7.4) and stained with Giemsa for morphological examination. Slides were also stained for naphtho-ASD-chloroacetate esterase to detect cells of the granulocyte lineage, and for alpha-naphthyl-acid esterase to identify monocytes/macrophages.
human and murine bone marrow cells. A higher number of colonies was consistently obtained in the human assay, as assessed by counting the colonies at both seven and 14 days (data not shown).

The purity of the B-cell suspensions isolated from peripheral blood or tonsils was tested by surface marker analysis. These suspensions were comprised almost exclusively of B cells, as shown in Table 2, with minor contaminants of monocytes or T cells.

Additional control experiments were carried out to determine whether contaminant T cells or monocytes were responsible for CSA production. Purified T cells and monocytes were cultured alone or in combination, with or without phytohemagglutinin (PHA), and their supernatants were subsequently tested for CSA (Fig 1).

Unstimulated T cells, cultured with or without monocytes, did not release CSA (Fig 1A). When stimulated with PHA, the mixture of T cells and monocytes as well as the T cells alone produced CSA (Fig 1B). However, treatment of purified T cells with an anti–HLA-DR monoclonal antibody and complement, which eliminated the few contaminant monocytes, virtually abrogated CSA production (Fig 1B). Monocytes alone never released CSA (Figs 1A and 1B). These findings indicate that under the experimental conditions used T cells and monocytes had to interact in order to release CSA; furthermore, they had to be stimulated with PHA. Taken together, all of these data argue against the possibility that in unstimulated, purified B-cell suspensions CSA was produced by contaminant monocytes or T cells.

Supernatants from B cells purified from two tonsil cell suspensions were ammonium sulphate–precipitated and gel-filtered over a G-75 column. CSA eluted in a single peak with an apparent mw of 65 to 70 kd (Fig 2).

Aliquots of the same supernatants were absorbed onto Sepharose-bound Con A or Sepharose-bound human albumin (control). Treatment with Con-A reduced the capacity of B-cell supernatants to support myeloid colony formation by 50% to 60%, whereas treatment with albumin caused a 10% reduction of colony growth. These findings suggest the glycoprotein nature of the B-cell derived substance(s) responsible for CSA.

**CSA is released predominantly by activated B cells.** The experiments reported above could be interpreted as indicating that unstimulated B cells release CSA spontaneously. The alternative hypothesis was that the B cell resting nature of this particular cell fraction, as opposed to their ability to produce CSA in vitro in the absence of

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<th>Table 1. CSF Activity in B Cell Culture Supernatants</th>
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<td><strong>Supernatants</strong></td>
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<tr>
<td>Peripheral blood B cells*</td>
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<tr>
<td>Tonsil B cells*</td>
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<td>GCT cell line*</td>
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<td>Medium (control)</td>
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*Tested at a final concentration of 10%.
†Results are mean values ± SE from triplicate cultures.
‡Colonies were counted after 14 days.
§CSA produced by 1 x 10⁶ cells cultured for 48 hours in RPMI-FCS.
¶CSA produced by 1 x 10⁶ cells cultured for 48 hours in RPMI supplemented with 2% human serum albumin.

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<th>Table 2. Surface Markers of Purified B Cells</th>
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<td><strong>Surface Marker</strong></td>
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<td>slg</td>
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<tr>
<td>B1</td>
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<td>HLA-DR</td>
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<td>OKT3</td>
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*Mean ± SE from three different experiments.
†B cells were isolated from peripheral blood MNC by indirect rosetting according to the expression of B1 antigen (see Materials and Methods).
‡Normal tonsil B cells and B-CLL B cells were obtained by depleting E rosetting cells followed by treatment with the OKM1 monoclonal antibody and complement (see Materials and Methods).
§Not done.
¶Detected by rosetting during the separation procedure.
exogenous stimuli. Figure 3 shows that CSA was released predominantly by low density, activated B cells. In order to confirm a correlation between CSA release and B-cell activation, lymphocytes from the fourth Percoll fraction (small B cells) were cultured with and without SAC for 48 hours. CSA was detected only in culture supernatants of SAC-stimulated B cells (Fig 4).

**B cells from B-CLL patients release CSA in vitro.** B cells from twelve B-CLL patients were tested for CSA production. B cells were isolated from peripheral blood MNC by removal of E rosetting cells followed by treatment with the OKM1 monoclonal antibody and complement. This method yielded cell preparations containing almost exclusively B cells (Table 2).

B cells from nine cases consistently produced CSA in unstimulated cultures when tested on different occasions, whereas cells from the remaining cases proved consistently negative for CSA production.

Figure 5 shows one determination per case tested and demonstrates variations in the amount of CSA produced by the various cases.

CSA from B-CLL cells induced the growth of two types of colonies, ie, colonies that comprised monocytes-macrophages exclusively and colonies that contained neutrophils and
Fig 2. Elution pattern of CSF activity produced by normal B cells. Culture supernatants were ammonium sulphate-precipitated and filtered on a Sephadex G-75 column: fractions were collected and aliquots of each fraction were assayed for CSF activity.

Fig 3. CSA production by small and large tonsil B cells. Purified tonsil B cells were fractionated on a Percoll density gradient. Large and small B cells were cultured for 48 hours in RPMI-FCS; their supernatants were tested using peripheral blood target cells. Results are mean ± SE of triplicate cultures/2 x 10⁶ cells seeded.

Fig 4. CSA production by small tonsil B cells stimulated with SAC. Small, resting B cells were cultured for 48 hours in the presence or in the absence of SAC. Culture supernatants were subsequently tested on peripheral blood target cells. Results are mean ± SE of triplicate cultures/2 x 10⁶ cells seeded.
PATIENT CSF ACTIVITY (COLONIES / 2x10^5 CELLS)

A

B

C

D

E

F

G

H

I

J

K

L

macrophages. Colonies of the former type were, however, predominant in 14-day cultures of peripheral blood precursors.

DISCUSSION

This study demonstrates that highly purified normal B cells release CSA in culture without apparent stimulation. When B cells were fractionated on Percoll density gradients, only large, low-density cells that expressed activation markers were found to release CSA, thus suggesting that CSA production was a property of in vivo preactivated B cells. Since activation of B cells in vivo requires exposure to antigen, an intriguing implication of these results would be that CSA release is under the control of specific (antigen-driven) recognition mechanisms. In this respect the mode of CSA production by B cells would not differ from that of T cells, which also need to be stimulated. T cells require the additional help of accessory cells, which present the antigen and supply IL-1. Consistent with the notion that activated B cells produce CSA is the finding that stimulation of resting B cells with SAC induced CSA production. Upon infection with Epstein-Barr virus (EBV) normal B cells release different hemopoietic factors, including CSA. There is ample evidence that EBV-infected cells are in a state of activation; therefore, our findings agree with these data.

The low number of monocytes and T cells that contaminated the B-cell suspensions makes it unlikely that they were responsible for CSA production in our experimental system. This hypothesis is also ruled out by additional control experiments. Thus, when monocytes and T cells were tested, CSA was detected only in the supernatants of cultures that contained mixtures of T cells and monocytes. Furthermore, these cells failed to release CSA spontaneously and had to be stimulated with PHA.

Purified B cells from nine out of 12 B-CLL patients consistently produced CSA in vitro in the absence of any stimulus. Recent studies have demonstrated that, in the individual B-CLL clones, there are a number of maturing, possibly activated cells, the proportion of which varies from case to case. One explanation for the differences in CSA production by cells from the various patients may be related to the different content in the number of activated B cells within each malignant clone. In the individual cases, however, no relationship could be established between CSA release and other features, such as lymphocyte count, treatment, and stage of disease.

The available data do not permit assessment of the type of CSA produced by B cells. Although the apparent mw of B-cell derived CSA (65 to 70 kd) is similar to that reported for CSF-1, other features argue against this possibility. For example, unlike CSF-1, CSA released by B cells supports the growth of both macrophage and mixed colonies and is much more effective on human than on mouse bone marrow cells. Furthermore, the biochemical characterization should be
considered as preliminary; therefore, comparisons are not possible on these grounds.

B-cell supernatants were tested for CSA mainly on peripheral blood target cells largely depleted of T cells and monocytes. The experimental system used suggests two interpretations for the data, namely, (a) B cells could release a factor acting directly on myeloid precursors, ie, a bona fide CSA, or (b) B cells could release a factor that triggers other accessory cells present in the suspensions to produce CSA. For example, some of the remaining monocytes or the large granular lymphocytes could be good candidates for this accessory function. In this connection it is worth mentioning that monocytoid-derived soluble factors stimulate CSA release by endothelial cells.14

Besides promoting proliferation and maturation of myeloid progenitors, CSFs also exert a regulatory activity on some effector functions of fully differentiated myeloid cells. CSFs potentiate the cytotoxic capacity of macrophages21 and of granulocytes,4,7 induce macrophage proliferation,3 and promote release of prostaglandins,38 plasminogen activator,39 and of IL-1.4 By demonstrating the release of CSA by B cells, our results suggest that B cells may play a role in promoting hematopoiesis and in the initiation of the inflammatory process. The latter hypothesis is also in line with the previous findings that both normal18,40 and malignant18 B cells release IL-1, a potent mediator of the inflammatory and of the acute phase responses. IL-1 and CSA activities found in B-cell conditioned media are apparently mediated by different molecules, separated on the basis of their mw (IL-1 mw = 20 to 22 kd14,40; CSA mw = 65 to 70 kd). Interestingly, however, B-CLL cells, when capable of releasing CSA, also produce IL-1 (Pistoia et al, unpublished results), suggesting, perhaps, that the two factors are often needed synergistically.

ACKNOWLEDGMENT

We thank Drs R. Adami and P. Strada for the supply of normal blood samples; Dr A. Marmont and his group for providing blood samples from B-CLL patients; Dr G. Damiani for the PTF 29/12 monoclonal antibody; Dr T. Waldmann for the anti-Tac monoclonal antibody; Dr S. Romagnani for the SAC supply; Drs Maria Torcia and Anna Rubartelli for the help in some experiments; Dr C. E. Grossi for critical reading of the manuscript; and Susanna Caprile for secretarial help. This study has been approved by the authorities of our University.

REFERENCES

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