γ INTERFERON AND LYMPHOXIN, RELEASED BY ACTIVATED T CELLS, SYNERGIZE TO INHIBIT GRANULOCYTE/MONOCYTE COLONY FORMATION

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T lymphocytes exert both stimulatory and suppressive effects that regulate the activity of immune cells. Recent evidence suggests that they may also play a role in regulating growth and differentiation of other types of hematopoietic cells. T cells have been observed to enhance proliferation and differentiation of pluripotent (1, 2) as well as committed precursors from several different hematopoietic cell types through release of soluble factors (3–6). In addition, abnormalities in the numbers or state of activation of T cells are sometimes associated with inhibition of hematopoiesis. For example, certain cases of T cell chronic lymphocytic leukemia are associated with anemia or neutropenia (7–9), and increased numbers of activated T lymphocytes are found in the peripheral blood or bone marrow of patients with hematopoietic disorders such as aplastic anemia (10–12), Felty's syndrome (13, 14) or isolated neutropenia (15). In vitro colony formation by myeloid or erythroid precursor cells from the bone marrow of these patients is decreased. However, normal colony formation can be restored if bone marrow preparations are depleted of T cells (11, 16, 17). Moreover, colony formation by bone marrow cells from healthy donors is suppressed by the addition of patients' T cells, but not by T cells from healthy donors (12, 13, 16, 18, 19). Although the methods used to remove T lymphocytes in some of the discussed experiments also remove NK cells, which can mediate colony-inhibiting activity (20–22), a role for T cells in colony inhibition has been confirmed in some experiments in which treatment of patients' bone marrow cells with T cell-specific mAb and complement restored colony-forming ability (23).

The exact mechanism by which T cells suppress colony formation is unknown. Zoumbos et al. (12) have shown that mitogen-activated T cells from aplastic anemia patients produce levels of immune interferon (IFN-γ) higher than those produced by T cells from healthy donors, and that the titers of IFN-γ in the supernatants correlate with their ability to inhibit colony formation. The same authors (24) also reported the presence of IFN-γ in the bone marrow and peripheral blood of patients with aplastic anemia, and proposed that activated T cells suppress granulocyte precursors, in vivo as well as in vitro, by release of IFN-γ. Such a hypothesis is further supported by the observation that conditioned
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Medium (CM) from PHA-stimulated leukocytes from normal individuals contains colony-inhibiting activity (25, 26) that is neutralized by antibodies against IFN-γ (26). Indeed, recombinant preparations of IFN-γ have been shown (27-30) to inhibit colony formation by granulocyte and monocyte precursor cells (colony forming units of granulocytes and monocytes; CFU-GM). However, we have observed (31) that rIFN-γ, at concentrations corresponding to those normally present in PHA-CM, is capable of only low levels of inhibition of CFU-GM, and that it acts synergistically with recombinant tumor necrosis factor (rTNF) to inhibit CFU-GM (31), indicating that other inhibitory factors produced by activated leukocytes may act together with IFN-γ. TNF is a cytotoxin produced by activated myeloid or monocyte/macrophage cells (32, 33), and has biological and antigenic similarities with the colony-inhibiting activity that we reported (31) to be produced by NK cells upon interaction with bone marrow cells. Like its effect on bone marrow, antiproliferative and cytotoxic effects of TNF on cell lines are also synergistic with IFN-γ (34). Human lymphotoxin (LT) is a cytotoxin released by activated lymphocytes and present in PHA-CM (35, 36). LT has 28% homology in amino acid sequence to TNF, and the two cytotoxins share many biological activities (32). Analogous to the synergy of IFN-γ with TNF or NK colony-inhibiting activity the antiproliferative effect of LT on tumor cell lines is enhanced by the addition of IFN-γ (37-40).

In this paper, we discuss the effect of PHA-CM on colony formation, and show that PHA-activated purified T cells produce both IFN-γ and LT, which, together, inhibit colony formation by granulocyte and monocyte precursor cells.

Materials and Methods

Cell Lines. Murine L-929 cells (alpha subline), the human B lymphoblastoid cell line RPMI-8866 and the antibody-producing hybrid cell clones were maintained in RPMI-1640 culture medium (Flow Laboratories, Rockville, MD) supplemented with 10% FCS (Flow Laboratories).

Monoclonal Antibodies. Antibody OKT3, which reacts with all mature peripheral T cells (41), was used as supernatant from hybrid cells (American Type Culture Collection, Rockville, MD). Antibodies B133.1, B133.3, B133.5, and B133.7 (all IgG1) react specifically with human IFN-γ but not with IFN-α or -β (42). Ascites fluid from antibody B133.3, 1.5 × 10⁷ neutralizing U/ml, was diluted 1:200 for pretreatment (6 h, 4°C) of PHA-CM. Antibodies reacting with human LT (LTB, 5.5 × 10⁶ neutralizing U/μg) and human TNF (TNFD, 2.7 × 10⁵ neutralizing U/μg) were kindly provided by Dr. H. M. Shepard (Genentech, Inc., South San Francisco, CA) and used at concentrations of 5 μg/ml for pretreatment (6 h, 4°C) of PHA-CM.

Recombinant IFN-γ. Purified human rIFN-γ, produced in E. coli and having a titer of 7 × 10⁷ antiviral U/mg, was kindly provided by Dr. J. C. Sevastopoulos and H. M. Shepard (Genentech, Inc.). Antiviral titer was periodically assayed as previously described (31). rIFN-γ had no colony-stimulating activity on normal bone marrow cells nor LT activity on L-929 cells.

Bone Marrow Cell Preparations. Fragments of rib bone marrow, which are routinely removed during thoracic surgery (kindly provided by Dr. P. Addonizio, Dept. of Surgery, University of Pennsylvania, Philadelphia, PA), were collected in sterile RPMI-10% FCS. The specimen was sequentially flushed with medium through 18-, 23-, and 27-gauge

Abbreviations used in this paper: CM, conditioned medium; CSF, colony-stimulating factor; F/H, Ficoll/Hypaque; GCT, giant cell tumor; GM, granulocyte/monocyte; LT, lymphotoxin; TNF, tumor necrosis factor.
needles, and mononuclear cells were isolated on Ficoll/Hypaque (F/H) (1.077 ± 0.01 g/ml; Lymphoprep, Nyegaard and Co., Oslo, Norway) density gradient centrifugation.

**Bone Marrow Colonies.** The assay for CFU-GM was a modification of the method described by Pike and Robinson (43). Medium conditioned by the human monocyte-like cell line GCT (giant cell tumor) (Gibco, Grand Island, NY) was used as a source of colony-stimulating activity (44). GCT-CM contained neither IFN-γ, as measured by radioimmunoassay (RIA), nor LT, as measured on L-929 cells. Mononuclear bone marrow cells were suspended (10⁶ cells/ml) in supplemented McCoy's medium (Gibco) containing 15% FCS, 0.3% agar (Difco Laboratories, Detroit, MI), and 10% GCT-CM (unless indicated otherwise), and 1-ml aliquots were seeded into 35-mm Petri dishes (Flow Laboratories). Dishes were cultured at 37°C in a humidified 7% CO₂ incubator and the colonies (aggregates containing >40 cells) were counted at days 7 and 14 of culture. The colonies present after 7 d of culture are derived from more mature GM precursor cells (late CFU-GM), while those present after 14 days are derived from more primitive cells (early CFU-GM) (45). All experiments were performed in triplicate, and the same lots of FCS and GCT-CM were used throughout.

Inhibition of CFU-GM by PHA-CM, by its fractions prepared as described below, or by lymphokines was tested by adding, at the time of seeding, 100 μl of medium containing the inhibitors to 1-ml aliquots of bone marrow cell suspension, and comparing the number of day 7 and day 14 CFU-GM to control plates. The presence of colony-stimulating activity was assayed by adding 100 μl of PHA-CM or its fractions to bone marrow cell cultures in the absence of GCT-CM.

**Preparation of Peripheral Blood Cells.** Peripheral blood was obtained by venipuncture from healthy donors and anticoagulated with preservative-free heparin. Mononuclear cells were isolated using F/H density gradient centrifugation. Adherent cells were removed by two steps of adherence to plastic (45 min at 37°C) to obtain nonadherent PBL. Adherent cells were collected by scraping the plastic flasks with a rubber policeman. OKT3⁺ and OKT3⁻ lymphocytes were obtained by indirect rosetting of OKT3-sensitized PBL with CrCl₃-treated, goat anti-mouse Ig-coated sheep erythrocytes (E), followed by separation on an F/H gradient, as previously described (46, 47).

**Preparation of PHA-CM.** Peripheral blood mononuclear cells or purified lymphocyte subsets were cultured at a concentration of 2 × 10⁶ cells/ml in RPMI-1640 containing 1% FCS and 1% PHA (Wellcome Diagnostics, Dartford, England) for 4 d at 37°C, in a humidified atmosphere of 5% CO₂ in air. Autologous adherent cells were added at a final concentration of 10⁵ cells/ml, where indicated. Cell-free supernatants were collected and stored at 4°C until use.

**Absorption of IFN-γ from PHA-CM.** mAb B133.1, B133.3, B133.5, and B133.7, reacting with different epitopes of human IFN-γ (42), were conjugated to CNBr-activated Sepharose 4 B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) (50% ammonium sulfate precipitate, 5 mg/ml gel). IFN-γ was absorbed from PHA-CM by repeated mixing of PHA-CM with the antibody-coated Sepharose beads for 2 h at 4°C. In all experiments, the IFN-γ-depleted PHA-CM contained <1.5 U/ml of IFN-γ as measured with RIA described below.

**Chromatographic Fractionation of PHA-CM.** PHA-CM was concentrated 100-fold by an Amicon 8050 stirred cell equipped with a PM-10 membrane (Amicon Corporation, Danvers, MA). 2-ml samples were applied to a Sephadex G-100 (Pharmacia Fine Chemicals) column with a bed volume of 400 ml, and eluted with 0.1% PEG 8,000 (Fisher Scientific, Fair Lawn, NJ) in PBS, pH 7.4, at a flow rate of 40 ml/h. 24 10-ml fractions were eluted in the molecular weight range between 10,000 and 160,000, supplemented with 10% FCS, dialyzed twice against PBS, pH 7.4, once against RPMI-1640, and sterile-filtered. Molecular weight markers, run on the column under identical conditions, included IgG (158,000), BSA (67,000), ovalbumin (44,000), and myoglobin (17,000) (Bio-Rad Laboratories, Richmond, CA).

**Production and Purification of Human LT.** RPMI-8866 cells were suspended (10⁶ cells/ml) in serum-free RPMI containing 100 ng/ml of phorbol-12,13-dibutyrate (PdBu) (C.C.R., Inc., Eden Prairie, NM) and incubated at 37°C, 5% CO₂ for 48 h. The cell-free supernatants were harvested and concentrated 100-fold using a Diaflo hollow-fiber car-
tridge (HIP 10-20; Amicon Corp., Danvers, MA) and a stirred cell (PM 10 membrane; Amicon Corp.), and filtered through a 0.45 μm filter (Nalge Co., Rochester, NY). The protein concentrate was dialyzed against 20 mM Tris HCl buffer, pH 7.8. This buffer, and all other buffers used for purification, also contained 0.1% PEG 8,000 and 0.1 mM PMSF (Sigma Chemical Co., St. Louis, MO). After dialysis, the concentrate was applied to a DEAE-Sepharose (Pharmacia Fine Chemicals) column. Elution was carried out with 120 ml of a linear (0-0.4 M) NaCl gradient. LT-active fractions, determined by cytotoxicity on L-929 cells, were pooled and applied to a lentil-lectin Sepharose 4 B (Pharmacia Fine Chemicals) column. LT activity was eluted with 15 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl and 200 mM α-methylmannoside (Sigma Chemical Co.), dialyzed against 50 mM Tris-HCl buffer, pH 8.0, applied to a Blue-Sepharose CL-6 B (Pharmacia Fine Chemicals) column, and eluted with 50 mM NaCl. The eluate was concentrated 10-fold by ultrafiltration (PM 10 membrane; Amicon Corp.), applied to an Ultragel AcA44 (LKB Instruments, Gaithersburg, MD) column and eluted with 15 mM sodium phosphate buffer, pH 7.4, containing 135 mM NaCl, at a flow rate of 15 ml/h. LT-active fractions were dialyzed against 20 mM Tris-HCl buffer, pH 7.8, loaded on a Mono Q (Pharmacia Fine Chemicals) column equipped on a fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals), and eluted with 10 ml of a linear (0-0.25 M) NaCl gradient. The resulting protein was 200-fold purified with a 23% recovery of activity and had a final act of 8 x 10^5 U/mg protein.

Assay for LT Activity. Serial dilutions of PHA-CM or fractions from the G-100 column were added to 3 x 10^4 mouse L-929 cells (alpha subline) per well in 96-well flat-bottomed microtiter plates in the presence of 1 μg/ml actinomycin D (Calbiochem-Behring Corp., San Diego, CA). Cytotoxicity, identified by rounding-up and detachment of cells, was assessed microscopically after incubation for 24 h at 37 °C. The concentration (U/ml) of LT for each sample was defined as the reciprocal of the dilution that produced 50% cytotoxicity.

IFN-y RIA. Antibody B133.5 was labeled with Na^25I (sp act 13-17 mCi/μg; Amersham Corp., Amersham, United Kingdom) using the chloramine T method (48) (1 mCi ^25I per 20 μg protein). Antibody B133.1, (5 μg/ml in 0.1 M carbonate buffer, pH 9.5) was absorbed (100 μl/well, 48 h at 4°C) to 96-well vinyl plates (Serocluster; Costar, Cambridge, MA). The plates were washed three times with cold PBS containing 0.05% Tween 20 (Bio-Rad Laboratories) (PBS-Tween), and 100 μl of test sample was added to triplicate wells and incubated 18 h at 4°C. Plates were washed three times with PBS-Tween, ^25I-labeled antibody B133.5 was added (0.2 μg/ml, 100 μl/well) to each well, and the plates were incubated 18 h at 4°C. The plates were then washed six times with cold PBS-Tween, dried, and the well-bound radioactivity was measured in a Packard Auto-Gamma 800 automated gamma counter (Packard Instrument Co., Downers Grove, IL). The IFN-y concentrations in the test samples were determined on the basis of a standard curve, constructed in each experiment using a laboratory standard of purified natural human IFN-γ (Interferon Sciences, New Brunswick, NJ), previously titered against the NIH IFN-γ standard Gg-23-901-530. The sensitivity of this RIA is 0.5 U/ml of IFN-γ.

Results

 Supernatants from PHA-stimulated Lymphocytes or T Cells Contain Both Stimulatory and Inhibitory Activity on CFU-GM. As has been reported previously, PHA-CM contained both stimulatory (3) and inhibitory (25, 26) activity for CFU-GM. PHA-CM added to bone marrow cell cultures in the absence of GCT-CM displayed significant colony-stimulating activity for day 14 CFU-GM, but not for day 7 CFU-GM. PHA added at a concentration equal to that present in PHA-CM showed no stimulation of colonies under these conditions. Final concentrations of 10% PHA-CM, added to bone marrow cell cultures in the presence of GCT-CM as a source of colony-stimulating activity, inhibited day 7 CFU-GM by 42-75%, and significant inhibition was observed at concentrations of PHA-CM.
as low as 1%. Inhibition of day 14 CFU-GM ranged from 17 to 36% when PHA-CM was present at 10% final concentration.

To determine which cell types produce the inhibitory and stimulatory effects present in PHA-CM, nonadherent PBL from four donors were separated into T (OKT3+) and non-T (OKT3−) cells by indirect rosetting. The separated populations, with or without the addition of autologous adherent cells, were cultured in the presence or absence of PHA for 4 d, and the cell-free supernatants were assayed for stimulation or inhibition of day 7 and day 14 CFU-GM, and for titers of IFN-γ and LT (Table I). Supernatants from separated populations cultured for 4 d in the absence of PHA did not contain significant levels of colony-inhibiting or colony-stimulating activity, or of IFN-γ or LT (data not shown). Upon stimulation with PHA, lymphocyte populations produced all four of the activities measured, although there was variability in the amounts produced by different donors. Colony-stimulating activity, which was active only on day 14 CFU-GM, was produced by all lymphocyte populations. Adherent cells alone did not produce colony-stimulating activity, but when added to lymphocyte cultures, they enhanced its production. Supernatants from PHA-stimulated OKT3+ PBL mediated the highest levels of inhibition of both day 7 and day 14 CFU-GM. The addition of autologous adherent cells to cultures of PHA-stimulated OKT3+ or total PBL resulted in increased ability of the supernatant from the cultures to inhibit day 7 CFU-GM, but decreased its ability to inhibit day 14 CFU-GM. Supernatants from PHA-stimulated OKT3− PBL, either with or without added

Table I

| Cells cultured | Adherent cells added | Colony-inhibiting activity (percent inhibition of CFU-GM) on: | Colony-stimulating activity (CFU-GM per 10^5 cells) on day 14 | IFN-γ (U/ml) | LT (U/ml) |
|----------------|----------------------|---------------------------------------------------------------|-------------------------------------------------------------|--------------|-----------|
|                |                      | Day 7 | Day 14 |                                                   |               |           |
| Total PBL      | No                   | 38 ± 8 | 27 ± 9 | 25 ± 10                                      | 260 ± 57 | 12 ± 5 |
|                | Yes                  | 46 ± 7 | 2 ± 19 | 46 ± 16                                      | 469 ± 231 | 20 ± 8 |
| OKT3+ PBL      | No                   | 61 ± 7 | 45 ± 14| 52 ± 21                                      | 136 ± 128 | 32 ± 0 |
|                | Yes                  | 75 ± 14| 22 ± 23| 71 ± 30                                      | 281 ± 184 | 44 ± 24 |
| OKT3− PBL      | No                   | 18 ± 14| −4 ± 11| 22 ± 9                                       | 59 ± 75  | 3 ± 1   |
|                | Yes                  | 18 ± 5 | −5 ± 10| 33 ± 19                                      | 66 ± 64  | 4 ± 1   |
| Adherent cells | No                   | 5 ± 5  | −6 ± 5 | 2 ± 2                                        | 3 ± 4   | 1 ± 1   |

Cells were cultured (2 × 10^6 cells/ml) in medium containing 1% FCS and 1% PHA. After 4 d, supernatants were collected and tested for colony-inhibiting and colony-stimulating activity, IFN-γ, and LT. All results are expressed as the mean ± SD from four experiments.

* Autologous adherent mononuclear cells were added (10^5 cells/ml) to cell cultures, where indicated.

† Supernatants were added (100 μl/dish) to bone marrow cultures in the presence of 10% GCT-CM.

Percent inhibition was determined by comparing the number of colonies in test cultures to the number of colonies in control plates to which 100 μl/dish of culture medium was added. CFU-GM per 10^5 cells in control plates for day 7 and day 14 were 138 ± 40 and 60 ± 19, respectively.

‡ Colony-stimulating activity was determined by adding supernatants (100 μl/dish) to bone marrow cells in the absence of GCT-CM.
adherent cells, showed low levels of inhibition of day 7 CFU-GM and did not inhibit day 14 CFU-GM. IFN-γ was produced by all lymphocyte populations, but PHA-stimulated total PBL produced more IFN-γ than either OKT3+ or OKT3− PBL. LT activity was detected in supernatants from total PBL, and was two- to threefold enriched in supernatants from OKT3+ PBL. OKT3− PBL and adherent cells produced very low or insignificant levels of LT.

**IFN-γ Synergizes with Other Factor(s) Present in PHA-CM to Inhibit CFU-GM.** IFN-γ was absorbed by immunoaffinity chromatography from three different preparations of PHA-CM, and the IFN-γ-depleted PHA-CM preparations were assayed for inhibition of day 7 CFU-GM with or without the addition of 10³ U/ml rIFN-γ (Fig. 1). The final concentration of 10³ U/ml rIFN-γ is in excess of the concentration present in bone marrow cultures when 10% PHA-CM, not depleted of IFN-γ, is added. The inhibition of CFU-GM mediated by PHA-CM at 10% final concentration was completely abrogated after absorption of IFN-γ from the supernatants. When 10³ U/ml rIFN-γ were added to the cultures together with the IFN-γ-depleted PHA-CM, the inhibition of CFU-GM was significantly higher than that caused by 10³ U/ml rIFN-γ alone, suggesting that IFN-γ synergizes with another factor(s) present in PHA-CM to inhibit CFU-GM. Synergy between IFN-γ and IFN-γ-depleted PHA-CM is also observed at lower concentrations of IFN-γ, which show very little inhibition on their own (Fig. 2).

**The Colony-inhibiting Activity Present in PHA-CM Elutes from a Sephadex G-100 Column in Fractions Containing Both IFN-γ and LT Activities.** PHA-CM fractions obtained on a Sephadex G-100 column were assayed for inhibition and stimulation of CFU-GM, IFN-γ, and LT activity. Fig. 3 illustrates the results from one such fractionation, representative of four experiments. The highest levels of inhibition of day 7 CFU-GM eluted in fractions corresponding to a molecular weight range between 45,000 and 80,000 (Fig. 3A). Colony-stimulating activity
FIGURE 2. Synergy between IFN-γ and IFN-γ-depleted PHA-CM in CFU-GM inhibition. The indicated concentrations of rIFN-γ were added to bone marrow cells in the presence (●) or absence (○) of 100 μl/dish of IFN-γ-depleted PHA-CM. The data points represent the percent inhibition as compared to control plates, which received 100 μl/dish of culture medium. The number of day 7 CFU-GM per 10^5 cells in control plates was 167 ± 55 (mean ± SD from three experiments).

FIGURE 3. Sephadex G-100 gel filtration of PHA-CM. Each fraction was tested for (A) number of day 7 CFU-GM when added to bone marrow cultures containing 10% GCT-CM as a source of colony-stimulating activity (●), and number of day 14 CFU-GM colonies when added (100 μl/dish) to bone marrow cells in the absence of GCT-CM (●); (B) LT activity (△), as measured by cytotoxicity of L-929 cells, and IFN-γ activity (●), as measured by RIA. Mol wt markers (×10^5) are indicated by arrows. The number of day 7 CFU-GM in control plates in the presence of 10% GCT-CM was 195 ± 2 (mean and SD of triplicate cultures).

for day 14 but not for day 7 CFU-GM eluted in a molecular weight range between 10,000 and 30,000 (Fig. 3A). In some experiments, low levels of colony formation or cluster formation (<40 cells) was also observed when fractions eluting in higher molecular weight ranges were added to cultures (not shown). The peak of IFN-γ activity eluted at 45,000 mol wt, while most of the LT activity was eluted in fractions with a molecular weight of 85,000–120,000 (Fig. 3B). Thus, fractions exerting the highest levels of inhibition do not coincide with either the peak of IFN-γ or the peak of LT, but rather with fractions containing low levels of both activities.

To determine whether CFU-GM inhibition was due to synergism between the
two lymphokines, all fractions were tested in the presence of excess LT (10 U/ml), purified from RPMI-8866 supernatants, or excess IFN-γ (10³ U/ml rIFN-γ) (Fig. 4). If inhibition requires the presence of both factors, under such conditions, the inhibitory activity is expected to coelute with the lymphokine present at limiting levels. When excess LT was added to the cultures, the peak of colony-inhibition corresponded to the elution peak of IFN-γ (Fig. 4B), and likewise, when excess IFN-γ was added, the peak of colony inhibition corresponded to the elution peak of LT (Fig. 4C). Consistent with the results in Fig. 3, when fractions alone were added to the CFU-GM assay (Fig. 4A), the peak colony-inhibiting activity was present in fractions containing both IFN-γ and LT (Fig. 4D).

mAb against IFN-γ and LT, but not TNF, Block PHA-CM-induced Inhibition of CFU-GM. Table II shows the results from experiments where four PHA-CM preparations were treated with mAb against IFN-γ, LT, or TNF, and then tested for their ability to inhibit colony formation by day 7 and day 14 CFU-GM. Incubation of PHA-CM with antibody against either IFN-γ or LT abrogated its inhibitory activity, although a low level of inhibition of day 7 CFU-GM remained in some preparations after treatment with anti-LT antibody. Antibody against TNF did not abolish inhibition. Identical results were obtained using the gel filtration fractions of PHA-CM that showed the highest CFU-GM inhibiting
TABLE II

Effect of mAb against IFN-γ (B133.3), LT (LTB), and TNF (TNFD) on Colony-inhibiting Activity of PHA-CM

| Exp. | Day 7 CFU-GM per 10^5 cells | Day 14 CFU-GM per 10^5 cells | Day 7 CFU-GM per 10^5 cells | PHA-CM pretreatment |
|------|-----------------------------|-----------------------------|-----------------------------|--------------------|
|      | Exp.                        | PHA-CM pretreatment         | Exp.                        | PHA-CM pretreatment |
|      | Medium                       | None                        | Medium                       | None               |
|      | B133.3 LTB TNFD              | B133.3 LTB TNFD             | B133.3 LTB TNFD             | B133.3 LTB TNFD    |
| 1    | 156 47 156 120 50            | 61 43 75 62 48              | 156 47 156 120 50           | 61 43 75 62 48     |
| 2    | 126 69 127 117 75            | 54 55 58 56 37              | 126 69 127 117 75           | 54 55 58 56 37     |
| 3    | 179 104 161 171 ND          | 74 47 68 73 ND              | 179 104 161 171 ND          | 74 47 68 73 ND     |
| 4    | 175 45 181 ND ND           | 59 49 91 ND ND             | 175 45 181 ND ND           | 59 49 91 ND ND     |

Numbers are mean numbers of CFU-GM from triplicate plates. Standard deviations for triplicate plates were always less than five.
1 Control plates received 100 μl per dish of culture medium.
2 PHA-CM was incubated (6 h, 4°C) alone or with B133.3 (ascites fluid, 1:200), LTB (5 μg/ml), or TNFD (5 μg/ml) before being added (100 μl/dish) to bone marrow cells.

activity (not shown). The antibodies, when added alone to bone marrow cells, did not affect the number of colonies produced (not shown).

Purified LT and Recombinant IFN-γ Synergize to Inhibit Day 7 and Day 14 CFU-GM. To determine whether the synergy between LT and IFN-γ is sufficient to explain the strong inhibition of CFU-GM mediated by PHA-CM, we tested purified preparations of LT and rIFN-γ for their ability to inhibit colony formation. The purified LT preparation used in these experiments had a sp act of 8 x 10^5 U/mg, as measured by cytotoxicity on L-929 cells, and its activity was abolished by the same mAb (LTB) that abrogated LT activity in PHA-CM (Table II). Fig. 5 shows results of an experiment in which various concentrations of purified LT were added to bone marrow cells, either in the presence or absence of 10^3 U/ml rIFN-γ. LT alone at concentrations of 5 and 10 U/ml exerted modest inhibition on day 7 CFU-GM, but did not inhibit day 14 CFU-GM even at concentrations of 10 U/ml When rIFN-γ was present in addition to LT, inhibition was greatly enhanced. Treatment of bone marrow cells with 10^3 U/ml rIFN-γ alone produced 80% inhibition of day 7 CFU-GM; however, the combined effects of rIFN-γ and LT was more than additive for all LT concentrations tested. Neither 10^3 U/ml rIFNγ nor 10 U/ml of LT inhibited day 14 CFU-GM, but 65% inhibition was observed when the two factors were combined.

Discussion

Conditioned medium from mitogen-stimulated lymphocytes has been observed to contain colony-inhibiting (25, 26) and colony-stimulating activity (3, 49), IFN-γ (42, 50, 51), and LT (35). The cell separation experiments reported here show that OKT3+ lymphocytes (T cells), and not OKT3- lymphocytes (non-T cells) or adherent mononuclear cells are the most effective producers of these activities upon PHA stimulation. Mitogen-stimulated T cells produced colony-stimulating activity for day 14 but not for day 7 CFU-GM, whereas the colony-inhibiting activity present in supernatants of both T and non-T lymphocytes appeared to be most potent for the day 7 CFU-GM. The presence of granulocyte/macrophage colony stimulating factors (GM-CSF) has been observed in PHA-CM (49).
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Figure 5. CFU-GM inhibition by LT purified from RPMI-8866 cell supernatant. Purified LT was added to bone marrow cultures at the indicated final concentrations either in the presence (●) or absence (○) of 10³ U/ml rIFN-γ, and the number of colonies were counted at day 7 (A) and day 14 (B). The data represent the percent inhibition of colony formation determined by comparing the mean colony numbers of test cultures to those of control cultures containing 100 µl of culture medium. Error bars represent the standard deviation from three separate experiments, in which the number of colonies in control cultures were 231 ± 122 day 7 CFU-GM per 10⁵ cells, and 67 ± 20 day 14 CFU-GM per 10⁵ cells.

Attempts to define colony-stimulating activity in PHA-stimulated T-cell supernatants have also proven unsuccessful. This is indicative of the ability of PHA-stimulated T cells to mediate the inhibition of day 14 CFU-GM to a greater extent than colony-stimulating activity. The presence of both colony-stimulating activity and inhibitory substances in PHA-stimulated T-cell supernatants appears to suggest that T cells have a direct influence on hematopoiesis, and that the colony-stimulating activity is probably responsible for the colony-stimulating activity present in our supernatants. Despite the presence of colony-stimulating activity for day 14 CFU-GM, supernatants from PHA-stimulated T cells inhibited day 14 CFU-GM in the presence of 10% GCT-CM, suggesting that the inhibitory substances released by T cells override the influences of CSF, and are therefore the more potent regulators of GM colony formation contained in supernatants from PHA-stimulated T cells. Supernatants from mitogen-stimulated non-T cells (predominantly B and NK cells) contained very low levels of LT and approximately threefold less IFN-γ than did T cell supernatants; they did not inhibit day 14 CFU-GM, and only poorly inhibited day 7 CFU-GM. The IFN-γ present in the non-T lymphocyte supernatants may be a product of NK cells, which have been shown to produce IFN-γ upon stimulation with PHA (53) or IL-2 (42). Production of IFN-γ by NK cells after stimulation by PHA or T cell–derived IL-2 might also explain the higher titers of IFN-γ present in supernatants from total PBL than in supernatants from purified T cells.

The inability of IFN-γ-depleted PHA-CM to inhibit colony formation shows that IFN-γ is essential for PHA-CM-induced inhibition of CFU-GM. However, IFN-γ alone is not sufficient to explain the potent inhibition caused by PHA-CM. As shown in Figs. 1 and 2, 10³ U/ml of rIFN-γ, a concentration of IFN-γ ~20-fold higher than that present in cultures containing 10% PHA-CM inhibits colonies significantly less than either 10% PHA-CM or IFN-γ-depleted PHA-CM to which 10³ U/ml of rIFN-γ was added. These data suggest that IFN-γ acts together with other factor(s) present in PHA-CM to inhibit CFU-GM.

Our observation that IFN-γ alone mediates only minimal inhibition of CFU-
GM contrasts with other studies (26–30, 54) in which strong inhibition of CFU-GM by IFN-γ was reported. The purified preparations of natural IFN-γ used in early studies (26, 54) may have contained contaminating LT, as shown by Stone-Wolff et al. (38), in amounts sufficient to synergize with IFN-γ in inhibiting CFU-GM. The ability of antibodies against IFN-γ to completely block CFU-GM inhibition by IFN-γ preparations (54) does not rule out the possibility that contaminating LT is also responsible for inhibition, as we have observed complete abrogation of inhibition by LT-containing PHA-CM using an mAb against IFN-γ. More recent reports (27–30) show that purified, homogeneous rIFN-γ, at concentrations lower than those used in this report, is still capable of inhibiting CFU-GM, although to a lower degree than was shown using purified natural IFN-γ. We attribute the differences with our results to the different source and concentration of colony-stimulating activity, especially when using PHA-CM (28) or human placental–conditioned medium (29), which may also contain LT or TNF. The colony-stimulating activity (GCT-CM) used in our experiments did not contain IFN-γ or LT activity, as measured by RIA or cytotoxicity on L-929 cells, respectively. Differences in the methods used for purification of bone marrow cells have also been shown to influence the sensitivity of precursor cells to IFN-γ (55), and may explain the difference between our results and those of Griffin et al. (27) who report 58% inhibition of day 14 CFU-GM with 500 U/ml of rIFN-γ using partially purified progenitor cell preparations.

Fractionation of PHA-CM revealed that the fractions most active in inhibiting CFU-GM were those corresponding to mol wt between 45,000 and 80,000, in which both IFN-γ and LT activity were present. IFN-γ eluted in a broad peak of mol wt between 30,000 and 70,000, with the highest levels of activity eluting at mol wt of ~45,000. This mol wt is consistent with other measurements of IFN-γ by gel filtration (56). The peak LT activity eluted in the range of 85,000–120,000 mol wt. Analysis of LT by gel filtration has shown molecular weight heterogeneity of the LT molecule (57, 58), probably due to a strong tendency of a monomeric 25,000 mol wt form to aggregate into polymers (59). The elution pattern observed in our fractionations is consistent with that of α-LT described by Granger et al. (57). We also observed low levels of LT activity in the mol wt range of 50,000–65,000, which is similar to the LT partially purified from PHA-CM by Stone-Wolff et al. (38), or with the LT purified from supernatants of the B lymphoblastoid cell line RPMI-1788 (36, 60). Although variability among different PHA-CM preparations was also observed for the titers of IFN-γ produced, the peak of colony-inhibiting activity always eluted in fractions containing low levels of both IFN-γ and LT. This elution pattern suggested that both IFN-γ and LT are necessary for inhibition of CFU-GM. The results of the experiments in which excess LT or excess IFN-γ was added to PHA-CM fractions supports the conclusion that inhibition of CFU-GM is observed wherever adequate amounts of both activities are present. The ability of mAb against either LT or IFN-γ to almost completely abrogate PHA-CM-induced inhibition of CFU-GM establishes that both LT and IFN-γ are required for this activity. The low level of inhibition remaining in some supernatants after treatment with anti-LT antibody could be attributed to IFN-γ alone, which at high concentrations is capable of inhibiting day 7 CFU-GM (Figs. 1 and 2). Anti-TNF antibody had
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no effect on inhibition, indicating that LT and not TNF, a cytokine that we have previously shown to inhibit CFU-GM (31), and which may be produced by the monocytes or NK cells present in the mononuclear cell preparations (38, 61), is responsible for the CFU-GM inhibition mediated by PHA-CM. The negative results obtained with the anti-TNF antibody cannot be attributed to inactivity of the antibody, because the same preparations of antibody were effective in abrogating the colony-inhibiting activity of NK cell–derived supernatants (31).

We used rIFN-γ and purified LT preparations to show that these two factors together are capable of strong inhibition of CFU-GM in the absence of other factors that might be present in PHA-CM. Like IFN-γ, LT is capable of only minimal inhibition of day 7 CFU-GM at concentrations (10 U/ml) in excess of those present when 10% PHA-CM is added to bone marrow cells. However, LT, at concentrations as low as 0.6 U/ml, when added together with 10^5 U/ml of rIFN-γ, showed significant inhibition of both day 7 and day 14 CFU-GM. Unfractionated PHA-CM preparations did not strongly inhibit day 14 CFU-GM, probably due to colony-stimulating activity for day 14 CFU-GM contained in PHA-CM. This interpretation is supported by the observations that supernatants from PHA-stimulated lymphocytes produced in the presence of adherent cells contain more colony-stimulating activity (6, 62) and less inhibitory activity for day 14 CFU-GM than supernatants produced without added adherent cells. Conversely, gel filtration fractions containing LT and IFN-γ, but not colony-stimulating activity strongly inhibit both day 7 and day 14 CFU-GM. Thus, the low levels of LT contained in PHA-CM, acting together with IFN-γ, are sufficient to explain the PHA-CM-induced inhibition of both day 7 and day 14 CFU-GM.

The mechanism by which IFN-γ and LT act together to inhibit CFU-GM is unknown. Studies of the synergistic interaction between these two factors on the cytotoxicity of cell lines have shown that the cytostatic or cytotoxic effects of LT are enhanced when target cells are pretreated with IFN-γ (39), suggesting that IFN-γ may act by priming the target cells to the effect of LT. The increased sensitivity of IFN-treated target cells to LT or TNF may be due to the ability of IFN-γ to increase the number of receptors for these cytokines (63, 64). A similar mechanism may be operative on granulocyte and monocyte precursor cells. Our data, however, do not exclude the possibility that IFN-γ and/or LT act indirectly on precursor cells through other bone marrow cell types that might regulate precursor cell growth.

LT and TNF share the ability to induce tumor necrosis and cytotoxicity of cell lines (60); however, our data indicate that they may not behave identically in their colony-inhibiting activities. We have previously reported (31) that rTNF, at the same low concentrations used for LT in these experiments, is capable of significant inhibition of day 14 CFU-GM in the absence of IFN-γ. In the present experiments, purified LT had very little colony-inhibiting activity in the absence of IFN-γ. Equivalent concentrations of LT and TNF, however, are capable of synergizing with IFN-γ to inhibit day 7 CFU-GM. These distinct properties of LT and TNF probably do not depend on variability in sensitivity to cytokines among bone marrow donors, because the same effects were observed when purified LT and rTNF were simultaneously tested for colony-inhibiting activity.

We have shown that activated T cells produce a variety of soluble factors that,
together, influence the growth and differentiation of granulocyte and monocyte precursor cells. We have also identified a mechanism whereby activated T cells, associated with certain forms of bone marrow failure, might suppress hematopoiesis through the simultaneous release of IFN-γ and LT.

Summary

We have shown that lymphocytes stimulated by PHA produce colony-forming unit of granulocyte/monocyte (CFU-GM)-stimulating and -inhibiting activities, IFN-γ, and lymphotoxin (LT). IFN-γ is necessary for inhibition of CFU-GM by PHA-conditioned medium (CM), as shown by experiments in which removal of IFN-γ from PHA-CM abrogated inhibition. However, experiments in which rIFN-γ was added to IFN-γ-depleted PHA-CM revealed the presence, in PHA-CM, of other factors that act in synergy with IFN-γ to inhibit CFU-GM. Fractionation of PHA-CM on a Sephadex G-100 column was used to separate IFN-γ and LT. Colony-inhibiting activity was eluted in fractions that contained both IFN-γ and LT activities, identifying LT as a factor present in PHA-CM that synergizes with IFN-γ to inhibit CFU-GM. Treatment of PHA-CM with mAb against either IFN-γ or LT completely abrogated the colony-inhibiting activity, demonstrating a requirement for both lymphokines in PHA-CM-induced inhibition of CFU-GM. Experiments using rIFN-γ and preparations of purified LT confirmed that neither lymphokine alone, when added to bone marrow cells at the concentrations present in PHA-CM, strongly inhibited day 7 or day 14 CFU-GM, but that the two lymphokines, added together, behaved synergistically to inhibit CFU-GM by up to 70%. The inhibition observed using purified preparations of lymphokines shows that synergy between IFN-γ and LT is sufficient to explain PHA-CM-induced inhibition of CFU-GM. Our findings suggest that activated T cells regulate hematopoiesis through the release of inhibitory as well as stimulatory factors, and that the simultaneous production of IFN-γ and LT may represent a mechanism of suppression of hematopoiesis in the cases of bone marrow failure associated with the presence of activated T cells.

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