Evidence for the Separate Human T-Lymphocyte Subpopulations That Collaborate With Autologous Monocyte/Macrophages in the Elaboration of Colony-Stimulating Activity and Those That Suppress This Collaboration

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We investigated the interaction of monocyte/macrophages and autologous T lymphocytes in the methanol extraction residue (MER) of BCG-induced production of granulocyte-macrophage colony-stimulating activity (CSA). Coincubation of monocyte/macrophages and T lymphocytes at a 1:3 ratio produces an optimum collaboration; a change to a 1:9 ratio diminished this collaboration. Coincubation of monocyte/macrophages and T lymphocytes primed with lithium carbonate (2 meq/liter) for 40 hr synergistically increased CSA elaboration and prevented the decline in CSA noted for the 1:9 monocyte/macrophage:T lymphocyte ratio. In contrast, concanavalin-A primed T lymphocytes did not enhance CSA elaboration at any monocyte/macrophage:T lymphocyte ratio except, occasionally, at 1:9. However, this was overcome if the T lymphocytes were primed with both concanavalin-A and lithium carbonate before their coincubation with monocyte/macrophages. Further cell-mixing experiments revealed that concanavalin-A-primed T lymphocytes contained a subpopulation that suppressed monocyte/macrophage and T-lymphocyte collaboration. Activation of suppressor T lymphocytes could be effectively prevented by lithium carbonate and, in a dose-dependent manner, by irradiation. Also, suppressor T lymphocytes not only diminished the elaboration of colony-stimulating factor(s), but also elaborated an inhibitor of granulocyte-macrophage colony-forming cells. We further demonstrated that the respective hemopoietic helper and suppressor T-lymphocyte activities could be enriched with OKT8+ (or OKT4+) and OKT8− subpopulations.

COLONY-STIMULATING FACTORS (CSF), operationally termed colony-stimulating activity (CSA), are the glycoprotein macromolecules essential for in vitro growth of granulocyte-macrophage colony-forming cells (GM-CFC). These cells are committed to divide and differentiate into mature granulocytes and monocytes.1–3 CSA is primarily elaborated by monocyte/macrophages and T lymphocytes.4–12 Recently, we reported that human peripheral-blood-derived monocyte/macrophages and T lymphocytes, incubated together in the presence of an antigen, collaborate in elaborating CSA.13–16 We have also produced circumstantial evidence that concanavalin-A-treated T lymphocytes, incubated with autologous monocyte/macrophages, suppress CSA elaboration.13–16 The mechanisms of this phenomenon, however, remain unknown. In this article, we present evidence (1) for helper and suppressor T-lymphocyte subpopulations that, respectively, augment and suppress CSA elaboration in response to MER (methanol extraction residue) of BCG, (2) that the concanavalin-A-primed T lymphocytes contain a subpopulation that, when incubated with monocyte/macrophages, not only suppresses CSA elaboration, but also elaborates an inhibitor of GM-CFC growth, and (3) that helper and suppressor T-lymphocyte activities can be enriched with OKT8− (OKT4+) and OKT8+ subpopulations.

MATERIALS AND METHODS

Specimen Collection

Peripheral blood, a source of monocyte/macrophages and T lymphocytes, was obtained from normal human volunteers. The marrow cells used for assaying CSA were from normal volunteers or solid tumor patients without bone marrow involvement or previous chemotherapy, radiotherapy, or immunotherapy. Before acquiring any specimens for experimentation, we obtained written, signed consent from the volunteers, as required by the surveillance committee on human experiments at the University of Texas System Cancer Center. Specimens were collected in polystyrene tubes (Corning Glass Works, Corning, NY) containing 0.3 ml preservative-free heparin (1,000 U/ml) in 1.7 ml phosphate-buffered saline (PBS).

Cell Separation Procedures

T Lymphocytes

Mononuclear cells were obtained by diluting peripheral blood in an equal volume of PBS and then centrifuging it at 400 g for 35 min over Ficoll-Hypaque (density 1.077 g/ml) columns in 15-ml plastic tubes. T lymphocytes were collected using a modification of the method of Weiner et al.17 Briefly, 1 ml of 5% sheep erythrocyte suspension was added to 0.03 ml neuraminidase solution (15.0 U/ml) and washed 3 times with alpha-modified minimum essential medium (α-MEM, K.C. Biological, Inc., Kansas City, KS) after incubation for 1 hr at 37°C. The mononuclear cells were then incubated with neuraminidase-treated erythrocytes (NE) and the
rosettes pelletized by centrifugation over a Ficoll-Hypaque column (density 1.080 g/ml). The NE were lysed with ammonium chloride containing Tris buffer. The remaining cells were subject to carbonyl iron phagocytosis and plastic adherence to remove contaminating phagocytic and adherent cell populations. The final preparation contained > 99% NE-rosetting cells.

Monocyte/macrophages

The T-lymphocyte-depleted interface cells were aspirated. Less than 1% of these cells were NE-rosetting cells. To obtain monocyte/macrophages, the interface cells (2 × 10^6/ml) were subjected to active adherence in 35-mm polystyrene culture dishes (Corning Glass Works) by incubation for 3 hr in α-MEM plus 15% heat-inactivated fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO₂, 12% O₂, and 83% N₂ (Analyzed Gas, Instrumentation Laboratory, Inc., Lexington, MA). The number of interface cells incubated was adjusted according to the proportion of phagocytic, nonspecific esterase (NSE) stain positive cells (monocyte/macrophages) present in the interface fraction to obtain the required number of monocyte/macrophages in the dish.

The dishes were flushed, and supernatant aspirated twice with α-MEM plus 15% FCS to remove nonadherent cells. The number of adherent cells remaining in the dish was determined by subtracting the number of nonadherent cells obtained per dish from the total number of interface cells subjected to adherence. The adherent cells were added in appropriate numbers to the dishes containing autologous, freshly prepared adherent cell monolayers.

B Lymphocytes

B lymphocytes were isolated from T-lymphocyte-depleted fraction as described by Wysocki et al.18

Separation of Helper (OKT4+ or OKT8−) and Suppressor (OKT8+ or OKT4−) Subpopulations of T Lymphocytes.

Since we found that pretreatment of T lymphocytes with complement alone diminished subsequent T lymphocyte and monocyte/macrophage collaboration in CSA elaboration and complement-enhanced monocyte/macrophage CSA elaboration, we opted not to use complement-lysis to produce separate OKT4+ or OKT8+ subpopulations. Instead, α RBC (ORBC) rosetting technique was developed to separate OKT4+ and OKT8+ subpopulations.

Purified T lymphocytes (4 × 10^6/ml) suspended in PBS plus 0.01% sodium azide were treated with either OKT4 or OKT8 mouse monoclonal antibodies (final titer 1:25; Ortho Diagnostic Systems Inc., Raritan NJ) at 4°C for 1 hr. The cells were then washed twice with a PBS-sodium azide solution and resuspended in 1 ml of 0.9% saline solution.

ORBC were washed and suspended in normal saline (50% suspension); 0.6 ml of this suspension was mixed with 1.06 mg of protein (goat anti-mouse IgG). Eight-tenths milliliter of chromium chloride (0.05%) solution was added drop-wise while the suspension was mixed continuously. The anti-mouse IgG-coated ORBC suspension was then washed 3 times, suspended in 40 ml of normal saline, and kept at 4°C until used.

To form rosettes, T lymphocytes with OKT4 or OKT8 monoclonal antibody were mixed with a 1% anti-mouse IgG-coated ORBC suspension at a final concentration of 10^6/ml. The combination was centrifuged at 400 g for 10 min, and the supernatant aspirated. The cells were resuspended gently in the residual fluid and the percentage of rosettes determined. To separate rosetted T lymphocytes, Ficoll-Hypaque density (1.080 g/ml) gradient centrifugation was used. Since these monoclonal antibodies identify T-lymphocyte subpopulations that are mutually exclusive,20,20 we used either OKT8 (4 experiments; 40%-48% of TL) or OKT4 (1 experiment; 49% of TL) antibodies to separate the two subpopulations. The data presented in this paper are from the experiments that used OKT8 antibody. No difference resulted if we used either antibody. The OKT8 subpopulation obtained was contaminated with <0% OKT8+ T lymphocytes, whereas the OKT8+ subpopulation was contaminated with <7% OKT4+ T lymphocytes.

Light-Density, Nonadherent Marrow Cells

Light-density, nonadherent cells (<1.077 g/ml), obtained by Ficoll-Hypaque gradient centrifugation, were subjected to the plastic adherence procedure. The nonadherent cells were aspirated, washed once, and resuspended in α-MEM plus 15% FCS at a cell concentration of approximately 10^6/ml. Since T-lymphocyte depletion of marrow cells did not affect the results (unpublished observations), all experiments, unless specified, utilized light-density, nonadherent marrow cells for the CSA assay. No differences in the pattern of results were noted when the same batch of conditioned media (CM) was tested against the marrow cells from a normal volunteer or from a solid tumor patient, as described above.

Experimental Design

An increasing number of T lymphocytes was incubated with a constant number of monocyte/macrophages in a constant volume of α-MEM plus 15% FCS. MER (50 μg/ml; a gift from Mr. Paul Vilk, NCI, Bethesda, MD) was used to stimulate CSA elaboration. To change T-lymphocyte function, they (3–5 × 10^6/ml) were treated with lithium carbonate (2 meq/liter; Fisher Scientific Co., Pittsburg, PA), concanavalin-A (10–40 μg/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), or both (lithium added 5–10 min before concanavalin-A) for 40 hr at 37°C in an atmosphere of 5% CO₂, 12% O₂, and 83% N₂ (henceforth referred to as primed T lymphocytes). Subsequently, the primed T lymphocytes were washed thoroughly with α-MEM plus 15% FCS and used for CM preparation by incubation alone or with autologous fresh monocyte/macrophages in the presence of MER. Washing with a-methyl mannoside to remove concanavalin-A did not change our results compared to washing with α-MEM plus 15% FCS. Control T lymphocytes were primed with α-MEM plus 15% FCS (culture medium) alone. The effect of radiation on helper and suppressor T-lymphocyte function was investigated by irradiating purified T lymphocytes or their two subpopulations with either 900 R or 3,000 R from a cesium-137 source before coincubation with autologous monocyte/macrophages.

Preparation of Conditioned Media

After 96-hr incubation of various cell populations in the presence of MER, CM were harvested, centrifuged at 1,200 g for 10 min to remove cell debris, and dialyzed against α-MEM for 3 days to remove any low molecular weight (<12,000 daltons) inhibitors, such as prostaglandins. These CM were filtered and then assayed for CSA.

Colonizing-Stimulating Activity Assay

To assay CSA in various CM, a semisolid agar culture system was used, as described.21 One-milliliter underlayens of a mixture of 0.5% Bactoagar (Difco Laboratories, Detroit, MI) and α-MEM plus 15% FCS were prepared in 35-mm Petri dishes. The CM were always incorporated in these underlayens at a 10% concentration (v/v), unless dose–response was to be tested. After the underlayens solidified, they were overlaid with 10^4 light-density (<1.077 g/ml).
nonadherent human marrow cells or light-density, nonadherent, T-lymphocyte-depleted marrow cells suspended in a 1-ml mixture of 0.3% Bactoagar and α-MEM plus 15% FCS. The cultures were incubated in triplicate for either 8 or 14 days at 37°C in sealed plastic boxes (Freezette, Republic Molding Corp., Chicago, IL) that had been flushed with a mixture of 5% CO₂, 12% O₂, and 83% N₂. The culture dishes were then scored for colonies (aggregates of ≥40 cells) under an Olympus dissecting microscope. The data were expressed as the mean ± 1 standard deviation. Since the results for day 8 (mainly granulocytic colonies) and day 14 (mainly granulocyte-macrophage and eosinophil colonies) followed a similar pattern, only day 8 data have been presented.

The results described in this article have been reproduced in 3 or more consecutive experiments. Cultures without CM did not grow colonies; also, concentrations of ≤0.5 meq/liter of lithium carbonate alone did not induce colony formation.

Statistical Methods

The mean numbers of colonies were compared by two-sided two-sample t tests. The dose–response were analyzed by analysis of covariance. Synergistic increase in CSA was defined as a significantly higher CSA than the combined CSA from the two constituent cell populations in the coincubations cultured separately.

RESULTS

MER-Induced CSA Elaboration Following Coincubation of T Lymphocytes With Monocyte/Macrophages

To investigate the various aspects of monocyte/macrophage and T-lymphocyte interaction for MER-induced CSA elaboration, we kept the monocyte/macrophage concentration constant (0.5 x 10⁶/ml) and varied the number of T lymphocytes. As shown in Fig. 1A, a synergistic increase in CSA elaboration was observed at a monocyte/macrophage:T lymphocyte ratio of 1:1.5 (p < 0.01). This increase was most pronounced, however, at a 1:3 ratio (except in one experiment in which it occurred at a 1:6 ratio). Changing the monocyte/macrophage:T lymphocyte ratio to 1:4.5 or higher progressively reduced CSA in the CM in 4 of 6 such experiments. A marked decline at a 1:4.5 ratio followed by stabilization was observed in the other two experiments. This reduction was not due to nutritional depletion or cell crowding, since incubation of increasing numbers of T lymphocytes alone (up to 4.5 x 10⁶) progressively increased CSA elaboration. Increasing numbers of monocyte/macrophages also progressively increased CSA elaboration, until it plateaued at 4 x 10⁶/ml (data not shown). Cell viability at the end of coincubation at different ratios did not differ.

MER-Induced CSA Elaboration Following Coincubation of Lithium-Primed T Lymphocytes With Monocyte/Macrophages

Using a reverse hemolytic plaque-forming cell assay system, Gelfand et al. previously demonstrated that lithium can block activation of antigen-specific suppressor T lymphocytes. Therefore, we used lithium-primed T lymphocytes in the experiment described in Fig. 1A. As shown in Fig. 1B, coincubation of lithium-primed T lymphocytes with monocyte/macrophages again synergistically increased CSA elaboration at a monocyte/macrophage:T lymphocyte ratio of 1:1.5, which was not significantly different from that described in Fig. 1A. However, increasing the lithium-
primed T lymphocytes further to produce monocyte/macrophage:T lymphocyte ratios of 1:4.5 or higher did not significantly diminish CSA elaboration.

**MER-Induced CSA Elaboration Following Coincubation of a B-Lymphocyte-Enriched Cell Population With Monocyte/Macrophages**

To determine whether monocyte/macrophages specifically interacted with T lymphocytes and not another cell population in elaborating CSA, we coincubated monocyte/macrophages with an autologous B-lymphocyte-enriched (82%) cell population at various ratios (Table 1). Even at the ratios of 1:1.5 and 1:3, only an additive interaction was observed. The T lymphocytes from the same blood donor, however, interacted with monocyte/macrophages in a manner similar to that described in Figure 1A (data not shown).

**MER-Induced CSA Elaboration Following Coincubation of Concanavalin-A-Primed T Lymphocytes With Monocyte/Macrophages**

As shown in Fig. 2A, the CSA resulting from the incubation of monocyte/macrophages with T lymphocytes primed with concanavalin-A (30 μg/ml) at a 1:3 ratio was significantly less (p < 0.001) than that resulting from the incubation with T lymphocytes primed with culture medium alone (Fig. 1A). There was no significant difference in the extent of this depression in CSA at 10.0 μg/ml or 40.0 μg/ml of concanavalin-A priming; also, increasing the duration of concanavalin-A priming from 40 hr to 84 hr did not produce any significant difference (data not shown). Increasing the concanavalin-A-primed T-lymphocyte numbers (1:6 and 1:9 ratios) resulted in no significant further suppression. However, CSA remained higher (p < 0.001) than that with culture-medium-primed T lymphocytes at these ratios (Fig. 1A). This may either be related to the concomitant provision of a higher number of activated CSA-producing T lymphocytes or helper T lymphocytes.

**MER-Induced CSA Elaboration Following Coincubation of Lithium Plus Concanavalin-A-Primed T Lymphocytes With Monocyte/Macrophages**

Concanavalin-A activates both helper and suppressor subsets of T lymphocytes; however, at the T-lymphocyte numbers used, suppression remains dominant. To further explore the findings illustrated in Fig. 1, we primed T lymphocytes with lithium and concanavalin-A simultaneously prior to coincubation with monocyte/macrophages (Fig. 2B). At all the monocyte/macrophage:T lymphocyte ratios tested (1:1.5, 1:3, 1:4.5, 1:6, and 1:9), CSA elaboration was significantly higher (p < 0.01) than that obtained from T lymphocytes primed with lithium alone (Fig. 1B). Increasing the proportion of such T lymphocytes did not reduce the resultant CSA below that observed with lithium-primed T lymphocytes. These data further suggest that concanavalin-A-primed T lymphocytes contain a dominant suppressor subset that suppresses the helper T lymphocyte and monocyte/macrophage collaborative interaction for CSA elaboration. If lithium is incorporated during priming, however, an unopposed synergistic interaction between such T lymphocytes and monocyte/macrophages markedly increases CSA elaboration. To confirm this, we performed the following type of experiments.

**Effect of Concanavalin-A-Primed T Lymphocytes on CSA Elaboration by Coincubated T Lymphocytes and Monocyte/Macrophages**

To confirm that concanavalin-A-primed T lymphocytes contain a cell population that suppresses the

### Table 1. Effect of Coincubating Monocyte/Macrophages With B-Lymphocyte-Enriched Cell Population on MER-Induced CSA Elaboration

<table>
<thead>
<tr>
<th>Cell Source of CSA</th>
<th>MØ</th>
<th>BL-Enriched Cells</th>
<th>Ratio</th>
<th>Number of Colonies* (\times 10^3) Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MØ</td>
<td>0.5</td>
<td></td>
<td></td>
<td>49 ± 7</td>
</tr>
<tr>
<td>BL-enriched cells</td>
<td>0.75</td>
<td>1.5</td>
<td>1.15</td>
<td>4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>3.0</td>
<td>1.9</td>
<td>15 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td></td>
<td></td>
<td>29 ± 3.5</td>
</tr>
<tr>
<td>MØ + BL-enriched</td>
<td>0.5</td>
<td>0.75</td>
<td>1:1.5</td>
<td>55 ± 4.6</td>
</tr>
<tr>
<td>cells</td>
<td>0.5</td>
<td>1.5</td>
<td>1:3</td>
<td>56 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.0</td>
<td>1:6</td>
<td>64 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4.5</td>
<td>1:9</td>
<td>85 ± 6.5</td>
</tr>
</tbody>
</table>

*Aggregates of 40 cells scored after 8 days of incubation; mean of triplicate cultures ± 1 standard deviation. Light density, nonadherent, TL-depleted marrow cells.

MØ, monocyte/macrophages.

BL, B lymphocytes.
monocyte/macrophage and helper T-lymphocyte collaboration in elaborating CSA, we added different proportions of concanavalin-A-primed T lymphocytes to either T lymphocytes alone or mixtures of monocyte/macrophages and culture-medium-primed T lymphocytes coincubated at 1:1.5 or 1:3 ratios. The resulting data are illustrated in Fig. 3. Compared with the sum of CSA elaborated from monocyte/macrophages alone and from T lymphocytes alone, coincubation at the above ratios augmented CSA elaboration (p < 0.004). Further increases in the proportion of T lymphocytes progressively suppressed the resultant CSA (Fig. 3). Also, use of concanavalin-A-primed T lymphocytes instead of culture-medium-primed T lymphocytes coincubated at 1:1.5 or 1:3 ratios significantly diminished (p < 0.001) CSA elaboration at the optimal monocyte/macrophage:T lymphocyte ratio. Addition of concanavalin-A-primed T lymphocytes to cell mixtures of monocyte/macrophage plus culture-medium-primed T lymphocytes suppressed CSA elaboration (p < 0.02). These data confirm that concanavalin-A-primed T lymphocytes contain a cell population that suppresses interaction between monocyte/macrophages and culture-medium-primed T lymphocytes in elaborating CSA.

**Effect of Lithium Plus Concanavalin-A-Primed T Lymphocytes on MER-Induced CSA Elaboration by Coincubated Monocyte/Macrophages and T Lymphocytes**

To further substantiate the results of the above experiment, we simultaneously added lithium-concanavalin-A-primed T lymphocytes to coincubated cell mixtures containing monocyte/macrophages and culture-medium-primed T lymphocytes at 1:1.5 and 1:3 ratios. The concentration of lithium-concanavalin-A-primed T lymphocytes added was either equivalent to...
or double that of culture-medium-primed T lymphocytes. As shown in Fig. 4, addition of concanavalin-A-primed T lymphocytes abrogated the synergistic enhancement of CSA elaboration. However, addition of lithium-concanavalin-A-primed T lymphocytes markedly enhanced CSA elaboration over that from cell mixtures containing monocyte/macrophages and culture-medium-primed T lymphocytes ($p < 0.001$). Furthermore, larger proportions of lithium-concanavalin-A-primed T lymphocytes did not suppress CSA; rather, they further enhanced CSA elaboration, probably on account of providing an unopposed and enhanced helper T-lymphocyte activity. These data provided further evidence for the existence of helper and suppressor T-lymphocyte subsets for CSA elaboration.

**Mechanisms of Concanavalin-A-Primed T Lymphocyte-Induced CSA Suppression**

Figure 5 is a representative dose–response curve from one of three experiments investigating the effect of coincubating concanavalin-A-primed T lymphocytes and monocyte/macrophages on subsequent MER-induced CSA elaboration. Conditioned media from different cell sources described in the figure were tested for CSA at different concentrations (1.25% to 30% per dish; v/v). Synergistically increased CSA was noted at as low a CM concentration as 2.5% from monocyte/macrophage plus culture-medium-primed T lymphocytes. Also, the CSA in CM from monocyte/macrophages plus culture-medium-primed T lymphocytes was significantly higher ($p < 0.05$) than in the CM from monocyte/macrophages plus concanavalin-A-primed T lymphocytes from 2.5% to 30% concentrations. The curve for CM from monocyte/macrophages plus concanavalin-A-primed T lymphocytes was shifted to the right but was parallel up to a concentration of 20%, indicating its low colony-stimulating potency as determined by an analysis of covariance; at higher concentrations (30%), CSA leveled off, unlike that noted with the CM from coincubation of monocyte/macrophages and culture-medium-primed T lymphocytes. This leveling of CSA at high CM concentration suggested that the CM from coincubation

<table>
<thead>
<tr>
<th>Cell Source of CSA</th>
<th>Cell Concentrations (1 x 10^6)</th>
<th>Mer Total TL Ratio</th>
<th>Number of Colonies per 15 Marrow Cells</th>
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</thead>
<tbody>
<tr>
<td>MO</td>
<td>0.5</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>0.75</td>
<td>1.15</td>
<td>40</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>1.3</td>
<td>50</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>1.8</td>
<td>60</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>1.3</td>
<td>70</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>1.45</td>
<td>80</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>90</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>100</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>120</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>140</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>160</td>
</tr>
<tr>
<td>MO plus TL</td>
<td>1.5</td>
<td>0.75</td>
<td>180</td>
</tr>
</tbody>
</table>

**Fig. 4.** Effect of adding Con-A or lithium (Li) plus Con-A-primed TL to the coincubation mixtures of MO and culture-medium-primed TL on the subsequent MER-induced CSA elaboration for day-8 granulocyte-macrophage colonies. It is evident that the addition of Con-A-primed TL suppressed the synergistic CSA elaboration by the MO plus culture-medium-primed TL mixtures. In contrast, the addition of Li plus Con-A-primed TL led to much higher quantities of CSA elaboration.

**Fig. 5.** Dose–response curves for various conditioned media. The right-shifted curve with a leveled response at higher CM concentration suggests that the low colony-stimulating capacity of CM for MO plus Con-A-primed TL is due to both low amounts of CSF and the presence of some GM-CFC inhibitor.
of concanavalin-A-primed T lymphocytes with monocyte/macrophages also contained an inhibitor of GM-CFC growth.

To investigate this further, we mixed various concentrations of CM prepared from monocyte/macrophages plus concanavalin-A-primed T lymphocytes with a constant, supraoptimal concentration (20%) of human placenta-conditioned medium prior to incorporation in culture dishes (Fig. 6A). Human placenta-conditioned medium was used as a partially purified potent source of CSF, since it was available in the larger quantities required for this type of experiment. CM prepared by coincubating monocyte/macrophages and concanavalin-A-primed T lymphocytes progressively diminished human placenta-conditioned medium-induced colony stimulation, suggesting the presence of an inhibitor in the CM. The CM from monocyte/macrophages plus culture-medium-primed T lymphocytes neither suppressed nor increased in the human placenta-conditioned medium induced colony formation (Fig. 6B).

MER-Induced CSA Elaboration
Following Coincubation of Irradiated T Lymphocytes With Monocyte/Macrophages

To investigate whether helper and suppressor T lymphocyte subpopulations required proliferation for their interaction with monocyte/macrophages, we irradiated the T lymphocytes with either 900 R or 3,000 R and then coincubated them with monocyte/macrophages at varying ratios in the presence of MER. Nonirradiated T lymphocytes were used as a control. As in previous experiments, monocyte/macrophage concentration was kept constant in all cell combinations (0.5 x 10^6/ml). As can be seen in Fig. 7A (representative data from one of three such experiments), a synergistic increase (p < 0.001) in CSA was noted with nonirradiated T lymphocytes at 1:1.5 monocyte/macrophage:T lymphocyte ratio; CSA increased further at the 1:3 ratio. Increasing the ratio to 1:6 markedly decreased CSA (p < 0.001); but, CSA did not decline further at a 1:9 ratio. The reason for this remains unclear. Use of 900 R irradiated T lymphocytes significantly (p < 0.001) reduced CSA elaboration at 1:1.5 and 1:3 ratios compared with that using nonirradiated T lymphocytes. However, at 1:6 and 1:9 ratios, a progressively increasing CSA elaboration occurred. The use of 3,000 R irradiated T lymphocytes resulted in a significantly higher synergism, which peaked at a ratio of 1:6. These data indicate a presence of at least two subsets of helper T-lymphocyte subpopulations: (1) a radiosensitive subset, responsible for collaborating with monocyte/macrophages at lower T-lymphocyte numbers, and (2) a radioresistant subset, whose function becomes apparent when higher T-lymphocyte numbers are used for coincubation with monocyte/macrophages.

Coincubation of nonirradiated, concanavalin-A-primed T lymphocytes with monocyte/macrophages again did not increase CSA elaboration at any monocyte/macrophage:T lymphocyte ratio. In contrast, 900-R-irradiated concanavalin-A-primed T lymphocytes synergistically increased (p < 0.001) CSA elaboration at ratios of 1:1.5, 1:3, 1:6, and, to a lesser extent, 1:9 (Fig. 7B). A radiation dose of 3,000 R markedly enhanced synergistic CSA elaboration at all ratios, although it was highest at 1:1.5 ratio. This may be related to the presence of some 3,000 R resistant suppressor T lymphocytes in the concanavalin-A-treated T lymphocytes.

Fig. 6. Effect on HPCM-induced colony formation of varying concentrations of MØ plus Con-A-primed TL (A) or of varying concentrations of MØ plus culture-medium-primed TL (B). MØ plus Con-A-primed TL CM progressively diminished the HPCM-induced colony formation with increasing concentration. However, the MØ plus culture-medium-primed TL CM caused neither augmentation nor suppression.
MER-Induced CSA Elaboration
Following Coincubation of OKT8- and OKT8+ Subpopulations of T Lymphocytes With Monocyte/Macrophages

We attempted to separate the two putative subpopulations (OKT8- and OKT8+) of T lymphocytes into those that collaborated with monocyte/macrophages for CSA elaboration and those that suppressed it. Figure 8 shows representative data from one of three such experiments. The subpopulation of T lymphocytes alone did not elaborate any detectable CSA (therefore not shown in Fig. 8). Synergistic CSA elaboration from coincubation of monocyte/macrophages and OKT8- subpopulation was significantly higher at all the ratios than that from monocyte/macrophages and whole T lymphocytes \((p < 0.01)\), with a progressive increase in CSA up to a ratio of 1:6. Coincubation of monocyte/macrophages with OKT8+ subpopulation did not augment CSA elabora-
tion, suggesting that OKT8+ subpopulation either (1) consisted of functionally inert T lymphocytes or (2) contained T lymphocytes that suppressed the collaboration between monocyte/macrophages and the helper T lymphocytes contaminating the OKT8+ fraction. To delineate this further, we performed the following experiments.

Either an unirradiated or an irradiated OKT8+ subpopulation was added to the coincubation mixture of monocyte/macrophages and OKT8− cells at the beginning of the CM preparation. The data from two such experiments are shown in Table 2. It is clear from these data that the OKT8+ subpopulation functioned by suppressing the collaboration between monocyte/macrophages and the OKT8− subpopulation.

MER-Induced CSA Elaboration Following Coincubation of Irradiated OKT8− and OKT8+ Subpopulations With Monocyte/Macrophages

Again, in these experiments, T-lymphocyte subpopulations alone did not elicit detectable CSA. Since we could not obtain a sufficient number of various T-lymphocyte subpopulations, only one (1,500 R) irradiation dose was used. Irradiation of the OKT8− subpopulation significantly decreased CSA elaboration (p < 0.05) at ratios of 1:1.5 to 1:6 (Fig. 9). However, at the 1:9 ratio, the CSA elaborated was comparable to that elaborated by the monocyte/macrophage plus nonirradiated OKT8− subpopulation. Irradiation of the OKT8+ subpopulation before coincubation with monocyte/macrophages markedly increased subsequent CSA elaboration.

Table 2. Effect of OKT8+ Subpopulation of T Lymphocytes on Monocyte/Macrophage and OKT8− Collaboration for CSA Elaboration

<table>
<thead>
<tr>
<th>Cell Source of CSA</th>
<th>Cell Concentrations During CM Preparation (×10^6/ml)</th>
<th>Ratio</th>
<th>Type of OKT8+ Subpopulation Used</th>
<th>Number of Colonies/10^4 Light Density, TL-Depleted Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Source of CSA</td>
<td>M0</td>
<td>OKT8−</td>
<td>OKT8+</td>
<td>M0-OKT8−:OKT8+</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>M0</td>
<td>0.5</td>
<td>OKT8−</td>
<td>1:1.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.75</td>
<td>OKT8+</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.5</td>
<td>OKT8+</td>
<td>1:6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.0</td>
<td>OKT8+</td>
<td></td>
</tr>
<tr>
<td>M0 + OKT8−</td>
<td>0.5</td>
<td>1.5</td>
<td>1:3</td>
<td>Unirradiated</td>
</tr>
<tr>
<td>M0 + OKT8− + OKT8+</td>
<td>0.5</td>
<td>1.5</td>
<td>1:3:3</td>
<td></td>
</tr>
<tr>
<td>Exp. 2</td>
<td>M0</td>
<td>0.5</td>
<td>OKT8−</td>
<td>1:1.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.75</td>
<td>OKT8+</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.5</td>
<td>OKT8+</td>
<td>1:6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.0</td>
<td>OKT8+</td>
<td></td>
</tr>
<tr>
<td>M0 + OKT8−</td>
<td>0.5</td>
<td>0.75</td>
<td>OKT8+</td>
<td>1:1.5:3</td>
</tr>
<tr>
<td>M0 + OKT8− + OKT8+</td>
<td>0.5</td>
<td>0.75</td>
<td>1:1.5:3</td>
<td></td>
</tr>
</tbody>
</table>

M0, monocyte/macrophages.
OKT8−, OKT8 antigen-negative T lymphocytes.
OKT8+, OKT8 antigen-positive T lymphocytes.
T LYMPHOCYTE MODULATION OF CSA

DISCUSSION

CSF are essential for the in vitro growth of GM-CFC. There is also some evidence that CSF may function as "granulomonopoietin" in vivo. In fact, CSF or similar humoral factors could effectively and specifically modulate granulomonopoiesis when so desired, e.g., in response to bacterial infections. The mechanisms by which the humopoietic system recognizes these messages, however, remain unknown. Since phagocytic cellular elements are a part of the total defense mechanism, which in addition comprises the cellular and humoral immune systems, we hypothesized that for a harmonious functioning of the whole defense mechanism, it would be vital for the host that the functions of the two systems (myelopoietic and phagocytic cellular elements) are a part of the total defense mechanism. Therefore, it seemed logical to postulate that the same category of regulatory cells governing an immune response might also be responsible for modulating CSF elaboration in a coordinated manner.

To investigate this possibility, we coincubated T lymphocytes with autologous monocyte/macrophages at varying ratios and thus showed that there are helper T lymphocytes that collaborate with monocyte/macrophages to enhance CSA in CM and suppressor T lymphocytes that suppress this collaboration. We have further demonstrated that, in response to MER, two types of helper T lymphocytes are produced: (1) radiosensitive and (2) radioresistant. Only with more T lymphocytes could the radiosensitivity be overcome. Recently, similar findings have been reported by Morimoto et al., who investigated T-lymphocyte—T-lymphocyte interaction for B-lymphocyte differentiation to Ig-synthesizing cells. In contrast, the radiosensitivity of suppressor T lymphocytes seems to be dosee-dependent (Fig. 7B). Similarly, we found that concanavalin-A-treated T lymphocytes contained both helper and suppressor T lymphocytes. Helper T-lymphocyte activity appeared only if the T lymphocytes were coincubated with monocyte/macrophages in high numbers (in one donor, Fig. 7B) or if they were irradiated prior to their coincubation with concanavalin-A treatment. Thus, concanavalin-A-induced suppressor T lymphocytes were also radiosensitive in a dose-dependent manner. We also demonstrated that, similar to the lithium abrogation of suppressor T-lymphocyte-mediated suppression of B-lymphocyte immunoglobulin synthesis, lithium could block the development of MER-induced or concanavalin-A-induced suppressor T-lymphocyte activation.

Using mouse monoclonal antibodies, OKT4 and OKT8, which identify human helper and suppressor T lymphocytes, respectively, for B-lymphocyte differentiation and Ig synthesis, we further demonstrated helper T-lymphocyte activity for CSA elaboration with the OKT8− subpopulation and suppressor T-lymphocyte activity with OKT8+ subpopulation. The decline in CSA at a monocyte/macrophage:OKT8− ratio of 1:9 may be due to the contaminating OKT8+ cells; this decline in CSA is also likely to be due in part to an unknown OKT4+ suppressor subset. Similarly, some CSA increase observed with monocyte/macrophage and OKT8+ combinations could be due to the contaminating OKT4+ cells. Although the two T-lymphocyte subpopulations were not completely pure, in our experiments, the significant finding remains: we could significantly concentrate helper and suppressor T-lymphocyte activities for CSA elaboration by using the OKT8− and OKT8+ subpopulations, respectively. We have also identified a helper subpopulation in T lymphocytes lacking Fc receptors for IgG (Fcγ) and suppressor subpopulation in T lymphocytes with Fcγ receptors (unpublished data). The experiments investigating the effects of irradiation on the isolated T-lymphocyte subpopulations showed a consistent and significant decline in CSA elaboration on coincubating monocyte/macrophages with radiated OKT8− subpopulation at 1:1.5, 1:3, and 1:6 ratios compared to the coincubation with uniradiated OKT8− subpopulation. This again indicated that two subsets of helper T lymphocytes existed: one radiosensitive and the other radioresistant. The markedly increased CSA elaboration that occurred on irradiating the OKT8+ subpopulation prior to their coincubation with monocyte/macrophages again pointed to the radiosensitivity of suppressor T-lymphocyte subpopulation.

We further investigated the suppression of monocyte/macrophage and T lymphocyte collaboration by concanavalin-A-primed T lymphocytes by analyzing dose responses for the various CM and by mixing CM from monocyte/macrophage plus concanavalin-A-primed T lymphocytes with a supraoptimal dose of human placenta-conditioned medium. The curve for CM from monocyte/macrophage plus concanavalin-A-primed T lymphocytes was biphasic: right-shifted but parallel to the curve for CM from monocyte/macrophages plus culture-medium-primed T lymphocytes at lower concentrations, and leveling at higher concentrations. Since this leveling could not be due to saturation of CSF-receptors on marrow target cells (as colony stimulation progressively increased with increasing concentrations of CM from monocyte/macrophage plus culture-medium-primed T lymphocyte coincubation), it can only be due to an inhibitor. However, analysis of covariance showed that an inhibitor alone cannot explain the right-shifted but parallel...
part of the curve at low concentrations. Therefore, we suggest that the decreased potency of CM from monocyte/macrophages plus concanavalin-A-primed T lymphocytes is due to both decreased CSF and the presence of an inhibitor in the CM. That the CM prepared from monocyte/macrophage plus culture-medium-primed T lymphocytes coincubation did not increase human placenta-conditioned medium stimulated colony formation in cultures when human placenta-conditioned medium was used at a supraoptimal dose suggests that the increased CSA was not due to an augmentor of CSF activity. However, the possibility cannot be ruled out completely, since the human placenta-conditioned medium was only partially purified. Absolute proof may come only when a CSA radioimmunoassay becomes available.

Thus, we have demonstrated that MER-induced CSA elaboration is controlled by two separate T-lymphocyte subpopulations; helper T lymphocytes interact with autologous monocyte/macrophages to augment CSA elaboration, while suppressor T lymphocytes suppress this interaction. The diminution in the colony-stimulating capacity of the CM caused by suppressor T lymphocytes is due to both (1) an elaboration of diminished quantities of CSF, and also, (2) the elaboration of a GM-CFC inhibitor evident by the fact that our CM inhibited HPC-induced colony formation (Fig. 6A). Furthermore, helper and suppressor T lymphocytes, respectively, can be enriched with OKT8+ and OKT8− T lymphocytes, the subpopulations that also regulate the elaboration of factors responsible for B-lymphocyte differentiation to Ig-synthesizing cells.

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Evidence for the separate human T-lymphocyte subpopulations that collaborate with autologous monocyte/macrophages in the elaboration of colony-stimulating activity and those that suppress this collaboration

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