Isolated human T lymphocyte subpopulations were obtained by fluorescence-activated cell sorting using the murine monoclonal antibodies, OKT4 and OKT8. The capabilities of the isolated lymphocytes to produce granulocyte-monocyte colony-stimulating activity (CSA) in response to mitogen challenge were assessed by in vitro assays employing light density nonadherent bone marrow cells. Essentially, no CSA production was noted by any isolated T lymphocyte population [OKT4 positive (+) or OKT8 positive (+)] cultured alone or following the addition of 10^6 autologous monocytes/ml. When phytohemagglutinin (PHA) alone was added, OKT4+ lymphocytes elaborated small amounts of CSA. With the addition of concanavalin A (Con-A) alone, both OKT4+ and OKT8+ cells were able to produce modest amounts of CSA. Significantly enhanced CSA production was observed when either OKT4+ or OKT8+ lymphocytes were coincubated with autologous monocytes in the presence of mitogen. We conclude that highly purified T lymphocyte subpopulations, free of monocytes as assessed by nonspecific esterase staining, can elaborate small amounts of CSA in response to PHA or Con-A challenge. A synergistic augmentation of CSA production was noted with coincubation of sorted lymphocytes and autologous monocytes in the presence of mitogen. Finally, our results suggest that the ability of T lymphocytes to make CSA is not exclusively limited to either the OKT4+ or OKT8+ defined subsets.

**MATERIALS AND METHODS**

**Specimen Collection**

Peripheral blood was collected in dilute preservative-free heparin from healthy volunteers. Marrow cells were obtained from patients undergoing routine marrow aspirations. In all cases, marrow samples were determined to be free of morphological evidence of malignancy. All blood and marrow samples were collected following guidelines approved by the Institutional Review Board for Human Experimentation of University Hospital.

**Cell Separation Procedures**

Intact mononuclear cell suspensions were obtained by centrifugation on sodium metrizoate-Ficoll gradients by the method of Bøyum.

Suspensions enriched for T lymphocytes were collected by applying intact mononuclear cell fractions, isolated as described above, to

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**From the Section of Hematology, Department of Medicine, and the Evans Memorial Department of Clinical Research, Boston University Medical Center, and the Naval Blood Research Laboratory, Boston, MA.**

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Address reprint requests to Dr. Paul J. Hesketh, Section of Hematology, Boston University School of Medicine, University Hospital, 75 East Newton Street, Boston, MA 02118.

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sterile nylon wool columns by a modification\(^4\) of the method of Greaves et al.\(^1\)

Monocytes were isolated by adherence separation using a modification of the technique of Shaw et al.\(^3\). The percentage of monocytes, as determined by nonspecific esterase staining,\(^4\) consistently was \(\approx 90\%\).

T lymphocyte subpopulations were isolated by fluorescence-activated cell sorting, using the murine monoclonal antibodies OKT4 or OKT8.\(^1,4\) Cells (50–90 \(\times 10^6\)) from the T cell-enriched suspensions were incubated with 1.25 \(\mu\)g of OKT4 or OKT8 (Ortho Pharmaceuticals, Raritan, NJ) in 5 ml of Hank's balanced salt solution plus 50 IU/ml penicillin G, 50 \(\mu\)g/ml streptomycin, and 5% autologous human serum (HBSS 5% AHS) for 30 min on ice. The cells were washed twice with HBSS 5% AHS and then incubated with 450 \(\mu\)g of fluorescein-labeled rabbit anti-murine IgG [F(ab')\(_2\)] fragments, lot 17839, Cappel Laboratories, West Chester, PA in 5 ml of HBSS 5% AHS for 30 min on ice. Following 2 washes, the cells were resuspended in HBSS 5% AHS at a concentration of approximately 3–4 \(\times 10^6\) cells/ml.

The cells were then sorted using a fluorescence-activated cell sorter (Coulter Epics V, Coulter Electronics, Inc., Hialeah, FL) under sterile conditions into two lymphocyte subpopulations, those binding the monoclonal antibody [OKT4 positive (+) or OKT8 positive (+)] and those failing to do so [OKT4 negative (−) or OKT8 negative (−)]. At the end of the separation run, a sample of each isolated subpopulation of cells was analyzed on the fluorescence-activated cell sorter to determine the percentage of cells falling within the original respective sorting gates. These determinations were then expressed as percent purity for each subpopulation collected. For the seven experiments employing OKT4, purity for the OKT4+ and OKT4− cells was \(>97\%\) and \(>98\%\), respectively. With the six OKT8 experiments, purity for the OKT8+ and OKT8− cells was \(>95\%\) and \(>99\%\), respectively. Viability, as assessed by trypan blue exclusion,\(^5\) for the sorted lymphocytes was consistently \(\approx 92\%\) following sorting. For the other types of cell suspensions described above, this value was consistently \(\approx 95\%\). Using 500 cell differential counts, non-specific esterase staining (NSE) of the sorted lymphocytes revealed no NSE positive cells in any experiment.

**Conditioned Medium Preparation**

Intact mononuclear cell suspensions isolated as described above were suspended in McCoy's 5A medium containing 15% fetal calf serum, 50 IU/ml penicillin G, and 50 \(\mu\)g/ml streptomycin (McCoy's + FCS) at a concentration of 2 \(\times 10^6\) cells/ml, with varying concentrations of either phytohemagglutinin (PHA) (GIBCO Laboratories, Grand Island, NY) or concanavalin A (Con-A) (Pharmacia Fine Chemicals, Uppsala, Sweden). The cell suspensions were maintained in a humid tissue culture incubator containing 5% \(\mathrm{CO}_2\). At appropriate intervals, cell-free supernatant media were harvested and frozen at \(-8^\circ\mathrm{C}\) until assayed for CSA.

Unseparated T lymphocyte suspensions, prepared as described above, were incubated in McCoy's + FCS at varying cell concentrations for 7 days with either PHA at a final dilution of 1:10 of the stock reconstituted lopolyplylized powder or Con-A at a final concentration of 100 \(\mu\)g/ml. At the end of 7 days, the cell-free supernatants were harvested, frozen at \(-80^\circ\mathrm{C}\), and later assayed for CSA.

Isolated T lymphocyte subpopulations following sorting were resuspended in McCoy's + FCS supplemented with 100 \(\mu\)g/ml gentamicin, at a concentration of 2 \(\times 10^6\) cells/ml. These cells were then incubated either alone, with appropriate mitogen, with 10\(^4\) cells/ml autologous monocytes, or with both mitogen and monocytes for 4–5 days. At the end of these periods, the cell-free supernatant media were harvested and frozen at \(-80^\circ\mathrm{C}\) until assayed for CSA. Viability at the end of the cultures ranged from 80%–95%.

Control cultures consisted of unsorted enriched T cell fractions, maintained in an identical manner, at 2 \(\times 10^6\) cells/ml with or without mitogen. The percent of NSE positive cells in these preparations was 0%–4%. Additional controls consisted of autologous monocytes incubated in an identical manner at a concentration of 10\(^4\) cells/ml, with or without mitogen.

**CSA Assay**

CSA was quantified in bilayer agar cultures using a modification of the technique of Pike and Robinson,\(^6\) which we have described.\(^7\) Aliquots (0.2 ml) of the cell-free supernatant media to be tested for CSA were distributed into 35-mm petri dishes and resuspended in 1-ml volumes of supplemented modified Eagle's medium containing 0.5% agar. Buoyant nonadherent human marrow cells fractionated by soybean mitroziose-Coll centrifugation and nylon fiber chromatography were resuspended at a concentration of 2–3 \(\times 10^6\) cells/ml in the same medium containing 0.3% agar and then added in 1-ml volumes onto the previously prepared semisolid underlayers. CSA activity for each sample tested was expressed as "percent of control CFU-C proliferation" and was defined as the ratio of the number of CFU-C colonies that proliferated in the test cultures to the number that developed in 3–6 control cultures maximally stimulated by feeder layers containing 10\(^6\) human peripheral blood leukocytes. It was calculated according to the following formula:

\[
\% \text{Control CFU-C proliferation (CSA)} = \frac{\text{CFU-C per test sample}}{\text{CFU-C in control cultures}} \times 100
\]

Twelve of 13 assays were incubated for 13 days; the remaining assay was incubated for 12 days prior to scoring. In maximally stimulated control cultures, colony growth varied from 57 to 167 colonies/2 \(\times 10^6\) marrow cells. In 3 unstimulated cultures plated in each experiment as additional controls, no colony growth was ever observed. Colonies were defined as globular aggregates containing \(>40\) cells. After scoring the cultures the number of colonies present, selected cultures were fixed with formalin, mounted on glass slides using the technique of Salmon and Buick,\(^8\) and stained with hematoxylin-eosin,\(^9\) luxol fast blue\(^10\) or \(\alpha\)-naphthyl butyrate\(^14\) for the identification of granulocyte, eosinophil, or monocyte-macrophage colonies, respectively. In each experiment, each group was plated in triplicate.

**Statistics**

Standard error of the mean was used as an estimate of variance, and means were compared using the Student's t test.

**RESULTS**

**Mitogen Stimulation of Intact Mononuclear Cell Suspensions**

Effective mitogen concentrations for CSA production, using either PHA or Con-A, were defined. Figure 1 shows the results of two representative experiments. At the concentrations shown, the amount of CSA generated was found to increase with increasing mitogen concentrations. By 3 days, CSA elaboration comparable to the maximal control value was seen at a final Con-A concentration of 100 \(\mu\)g/ml (Fig. 1A) and final dilution of 1:10 with PHA (Fig. 1B). By 5 days,
the amount of CSA released was nearly comparable between cells stimulated with either 100 µg/ml or 50 µg/ml of Con-A.

Mitogen Stimulation of Cell Suspensions Enriched for T Lymphocytes

By adding mitogen to cell preparations enriched for T lymphocytes, progressively increasing CSA elaboration was noted at higher cell concentrations. As shown in Fig. 2, at a cell concentration below 5 x 10^4 cells/ml, no CSA production was noted. At higher cell concentrations, the amount of CSA progressively increased. When 2-3.5 x 10^5 cells/ml were incubated with either Con-A or PHA, >50% of the maximal control CFU-C proliferation was produced.

A cell concentration of 2 x 10^5/ml and final mitogen concentrations of 50 µg/ml for Con-A and a 1:10 dilution for PHA were chosen for the lymphocyte subpopulation investigations.

Fig. 2. The effect of cell concentration on CSA secretion. A cell suspension enriched for T lymphocytes prepared by centrifugation on a sodium metrizoate-Ficoll gradient, followed by nylon wool chromatography, was incubated at various cell concentrations in McCoy's + FCS with either Con-A (final concentration 100 µg/ml) (A), or PHA (final dilution 1:10) (B). After 7 days, the cell-free supernatants were harvested and assayed for CSA. Each point represents the mean of 3 cultures from 1 of 2 separate experiments.

CSA Production by Isolated T Lymphocyte Subpopulations

Following sorting, both the OKT4 and OKT8 labeled and unlabeled lymphocytes were divided into four fractions: lymphocytes alone, lymphocytes plus 10^5 autologous monocytes/ml, lymphocytes plus mitogen, and lymphocytes plus 10^6 autologous monocytes/ml plus mitogen.

OKT8 experiments. In all six experiments done on separate days using four different donors, sorted lymphocytes alone, either OKT8+ or OKT8−, elaborated no detectable CSA (Fig. 3). The addition of 10^6 autologous monocytes/ml to the sorted lymphocytes also resulted in negligible CSA production. When Con-A was added, OKT8+ (p < 0.001) and OKT8− (p < 0.001) cells produced more CSA than sorted cells alone or sorted cells containing autologous monocytes (Fig. 3). Monocytes incubated alone, with or without mitogen, at 10^6 cells/ml showed no CSA release.

When Con-A and monocytes were simultaneously added to suspensions of sorted lymphocytes, both OKT8 positive and negative cells secreted significantly more CSA than either the sorted lymphocytes incubated alone (p < 0.001), lymphocytes plus autologous monocytes (p < 0.001), lymphocytes treated with Con-A alone (p < 0.01), or isolated monocytes incubated with Con-A (p < 0.001).

OKT4 experiments. A similar pattern was seen in studies employing OKT4 as the labeling antibody. In
five experiments done on separate days using three different donors, sorted lymphocytes alone, either OKT4+ or OKT4−, produced no CSA (Fig. 4). The addition of 10⁴ autologous monocytes/ml to the sorted lymphocytes also resulted in no detectable CSA. When Con-A alone was added, OKT4+ (p < 0.01) and OKT4− (p < 0.01) cells produced more CSA than sorted cells alone or sorted cells containing autologous monocytes (Fig. 4). Monocytes alone, with or without Con-A, again showed no CSA release.

When Con-A and monocytes were simultaneously coincubated with sorted lymphocytes, both OKT4 positive and negative cells secreted significantly more CSA than either the sorted lymphocytes incubated alone (p < 0.01), lymphocytes plus autologous monocytes (p < 0.01), lymphocytes treated with Con-A alone (p < 0.05), or isolated monocytes incubated with Con-A (p < 0.01) (Fig. 4).

Two additional OKT4 sorting experiments were performed with PHA. Again, a pattern of CSA release similar to that produced with Con-A stimulation was noted. The only disparity lay in an inability to detect any CSA release by OKT4− lymphocytes incubated alone with PHA (Fig. 5).

**Colony Morphology**

Representative bilayer agar cultures produced in response to the various sources of conditioned media obtained were stained for identification of granulocyte, eosinophil, and monocyte-macrophage colonies (see Materials and Methods).

Table 1 details the types of colonies produced by unsorted T lymphocyte cell suspensions, as well as by the various fractions of sorted lymphocytes. Monocyte-macrophage, granulocyte, eosinophil, and mixed colonies were all present in varying proportions. Only one group of cells, OKT4+ lymphocytes combined with Con-A alone, produced a different pattern of colony growth. This fraction gave rise almost exclusively to monocyte-macrophage colonies.

**DISCUSSION**

In this study, we have demonstrated that two isolated highly purified T lymphocyte subpopulations, as defined by the murine monoclonal antibodies OKT4 and OKT8, can elaborate CSA in response to mitogen challenge, even in the absence of monocytes. The addition of a small number of autologous monocytes to the isolated T lymphocytes in the presence of mitogen resulted in a significant augmentation of CSA release. Our results amplify a number of prior studies, which have implicated the human T lymphocyte in the production of CSA.

Beginning with Cline and Golde in 1974, numerous investigators have described the production of CSA by human cell suspensions enriched for T lymphocytes in response to mitogen challenge. A variety of fractionation procedures were used to remove contaminating monocytes. In each of these reports, a small number of monocytes remained within the T lymphocyte suspensions following all separation steps. On calculation, it was assumed that too few monocytes were present to account for the observed CSA in these cell preparations, leading to the concept that most of the CSA derived from T lymphocytes rather than monocytes. However, prior analyses of CSA production and/or release by human monocytes have shown that as few as 45 cells could produce detectable CSA.
PRODUCTION OF CSA BY HUMAN T LYMPHOCYTES

Table 1. Morphology of CFU-C Colonies Stimulated by CSA From Sorted Lymphocyte and Control Cell Fractions

<table>
<thead>
<tr>
<th>CSA Source</th>
<th>Macrophage/Monocyte</th>
<th>Neutrophil</th>
<th>Mixed</th>
<th>Eosinophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsorted lymphocytes† + Con-A‡</td>
<td>59.0</td>
<td>28.5</td>
<td>3.0</td>
<td>9.5</td>
</tr>
<tr>
<td>OKT8 + lymphocytes§ + Con-A</td>
<td>55.6</td>
<td>20.0</td>
<td>4.4</td>
<td>20.0</td>
</tr>
<tr>
<td>OKT8 + lymphocytes + Con-A + MO</td>
<td>44.5</td>
<td>33.0</td>
<td>7.5</td>
<td>15.0</td>
</tr>
<tr>
<td>OKT4 + lymphocytes + Con-A</td>
<td>90.0</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OKT4 + lymphocytes + Con-A + MO</td>
<td>54.3</td>
<td>30.3</td>
<td>3.9</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Morphology of CFU-C colonies stimulated by CSA from sorted lymphocyte and control cell fractions was determined by fixing and then staining selected marrow cultures (see text). Percent of colonies produced by each CSA source was determined by counting 50–225 colonies from 4–8 cultures from 6 separate experiments.

*Combinations of macrophage-monocytes, neutrophils, and/or eosinophils.
†2 x 10^5/ml.
‡50 μg/ml.
§2 x 10^5/ml for all OKT8 and OKT4 cell fractions.

In addition, it remains conceivable that CSA production by the residual monocytes or macrophages could be augmented by coincubation with mitogen-stimulated lymphocytes through a direct lymphocyte-monocyte cellular interaction or by production of a soluble lymphokine(s).

Monoclonal antibody labeling of discrete cell populations followed by fluorescence activated sorting affords one means to refine the techniques of cell separation and thus obtain more highly purified cell populations. In this report, we have described the abilities of sorted T lymphocyte subsets, as defined by the murine monoclonal antibodies OKT4 and OKT8, to produce CSA in response to mitogen challenge. In the absence of mitogen, sorted lymphocytes, with or without autologous monocytes, produced no detectable CSA. When mitogen alone was added, sorted lymphocytes (OKT4+ and OKT8+) elaborated small but significant amounts of CSA. Nonspecific esterase staining (NSE) for the presence of monocytes revealed no NSE positive cells within the sorted lymphocyte fractions from any experiment. It remains conceivable that a small number of cells with monocyte function may fail to take up the NSE stain and thus escape detection. Mindful of the latter possibility, our results remain most consistent with the concept that T lymphocytes, both OKT8+ and OKT4+, can, under appropriate conditions, secrete CSA in response to mitogen challenge, even in the absence of monocytes.

The subsets of normal human T lymphocytes recognized by the monoclonal antibodies OKT4 and OKT8 are functionally distinct and nonoverlapping, together comprising between 80% and 90% of the peripheral T cell compartment.24 Our finding that the ability to produce CSA in response to mitogenic challenge is not exclusively limited to either T cell subset expands on an earlier report that alluded to this possibility. Employing monoclonal antibodies in combination with rabbit complement to produce selective cell depletion, Bagby et al.9 found that the CSA-producing T lymphocyte was eliminated with either the use of the OKT3 (pan-T cell) or anti-Ia antibodies, but not with either the OKT4 or OKT8 antibodies alone, suggesting that overlapping T cell subsets are capable of CSA release. The latter notion has been strengthened by our use of a positive means of viable cell isolation (fluorescence-activated cell sorting) as opposed to a cytotoxicity assay that at times is limited by incomplete cell depletion.

Various workers have demonstrated a clear-cut synergistic interaction between T lymphocytes and monocytes during the production of CSA, an observation that raises many questions on the nature of the cellular interactions as well as the roles of individual cells during CSA release. Verma et al.7 reported that far more CSA was elaborated by an autologous mixture of human monocytes and T lymphocytes than was expected on the basis of the amount of CSA produced by each of these classes of cells when incubated alone. Using murine thymocytes as target cells and staphylococcal enterotoxin B (SEB) as a stimulus, similar results were reported by Apte et al.8 These investigators felt that the enhanced amount of CSA noted in macrophage-lymphocyte cocultures was explained by the elaboration of a monokine that effected an increase in CSA production by lymphocytes. Bagby et al.9 have reported additional evidence pointing to the elaboration of a humoral substance by human monocytes that stimulates human T lymphocyte CSA production. Of note, when lymphocyte conditioned media was added to monocyte cell suspensions, no increase in CSA release was seen.9 We also observed a synergistic augmentation of CSA production when sorted T lymphocytes (OKT4+ and OKT8+) and small numbers of autologous monocytes were coincubated with mitogen, a finding consistent with these prior reports. Recently, Bagby et al.25,26 have suggested that, in addition to T lymphocytes, fibroblasts and endothelial
cells are also capable of enhanced CSA production when coincubated with monocyte conditioned medium.

Staining of representative bilayer agar cultures produced in response to the conditioned media from the Con-A stimulated, sorted and unsorted T lymphocyte populations revealed that monocyte-macrophage, granulocyte, eosinophil, and mixed colonies were all present in varying proportions. One fraction of sorted lymphocytes, however, OKT4+, combined with Con-A alone gave rise almost exclusively to macrophage-monoocyte colonies. This may relate to a lower degree of CSA production by this population of cells in comparison to the other groups that demonstrated CSA release in response to Con-A stimulation. This possibility is supported by prior studies of mitogenic stimulation of lymphoid cell suspensions. With reduced concentration of the conditioned medium, the proportion of macrophage colonies was noted to rise.25

In summary, we conclude that highly purified T lymphocyte subpopulations, free of monocytes as assessed by NSE staining, can elaborate CSA in response to PHA or Con-A challenge. When incubated with Con-A, OKT8+ lymphocytes showed a tendency to produce more CSA than their OKT4+ counterparts. A synergistic augmentation of CSA production was noted with coincubation of sorted lymphocytes and autologous monocytes in the presence of mitogen. Our results suggest that the ability of T lymphocytes to make CSA is not limited to the OKT4- or OKT8-defined subsets. This finding raises the possibility that many, if not the majority, of normal human peripheral blood T lymphocytes may share the capacity to produce CSA.

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