Regulation of Normal Human Blood Neutrophilic, Macrophagic, and Eosinophilic Committed Stem Cell Proliferation by Autologous Blood T Lymphocyte Subsets

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The influence of T lymphocyte (T cell) subsets on the proliferation of the blood neutrophilic, macrophagic, and eosinophilic committed stem cells (CFC) was evaluated. The T cells and the CFC-enriched Bnull cells (mononuclear cells depleted of monocytes and T cells) and null cells (Bnull cells depleted of B lymphocytes) were separated from the blood of normal individuals. The population enriched for OKT4 (T4 cells) mononclonal antibody-binding T helper/inducer cells and for OKT8 (T8 cells) mononclonal antibody-binding T suppressor/cytotoxic cells were separated from the T cells or mononuclear cells depleted of monocytes by negative enrichment using an immunoconformation "panning" method. These separated cell populations were cultured separately, and the Bnull or null cells were cocultured with the T, T4, and T8 cells in agar medium in the presence and absence of an exogenous source of colony-stimulating activity (CSA). The cultures were evaluated at day 14. The Bnull and null cells, but not the T cells or subsets, contained CFC. The number of CFC proliferated from the Bnull or null cells increased significantly (p < 0.001) in cocultures with unseparated T and T8 cells in a dose-dependent manner. The T4 cells neither promoted nor inhibited the CFC growth. The T8 population was a better promoter (p < 0.01) of CFC growth than the unseparated T cells. This suggests that the CFC promoting effect of the unseparated T cells is probably due to the influence of the T8 subset present within the T cells. The proportion of the neutrophilic, macrophagic, and eosinophilic CFC that proliferated in these cultures was comparable under the influence of the T and T8 cells. The results of these studies indicate that the T8 population, which is the suppressor in the classical immune system, promotes the growth of the blood CFC. We speculate that the T cells are involved in the regulation of granulopoiesis in vivo.

NORMAL HUMAN BLOOD contains neutrophilic, macrophagic, and eosinophilic committed stem cells (CFC). Their growth is promoted in vitro cocultures with autologous blood thymus-dependent lymphocytes (T cells). There are at least two well-defined subpopulations of T cells, which are appropriately referred to as inducer/helper and suppressor/cytotoxic cells on the basis of their capacity to help or suppress B lymphocyte differentiation. Whether the observed influence of the T cell on the CFC resides in the T helper or suppressor cell is not known. This study was undertaken to evaluate the interactive influences of the T cell subsets on the growth of normal human blood CFC. Highly purified populations of T cells and of T helper and suppressor cells were obtained from the blood of normal humans, and their influence on the growth of autologous blood CFC was evaluated in an in vitro culture system. The results of this study indicate that the T suppressor population is responsible for the CFC growth promotion.

MATERIALS AND METHODS

Subjects

The blood samples were obtained from healthy adults who were not taking any drugs, and preservative-free heparin (3–5 U/ml of blood) was used as an anticoagulant. Informed written consent was obtained from each subject, as required by the Institutional Human Studies Committee.

Separation of Cells

The T cells, monocytes (Mo), Bnull cells (mononuclear cells depleted of Mo and T cells), and null cells (Bnull cells depleted of B lymphocytes) were separated as described previously; the steps are shown in Fig. 1. Briefly, the blood sample was layered over Isolymp, specific gravity 1.077 (Gallard-Schlesinger, Carle Place, NY) and centrifuged at 400 g for 40 min. The light density interface mononuclears (MNC, lymphocytes, monocytes, and null cells) were obtained. The MNC were depleted of Mo by incubating them overnight in Petri dishes at 37°C with 5% CO2 flow. The nonadherent MNC-Mo were decanted and then incubated with sheep erythrocytes (SRBC) to rosette the T cells present in the MNC-Mo fraction. The T rosettes were then pelleted through the Isolymp by centrifugation. The interface populations containing B lymphocytes and null cells (Bnull cells) and the pelleted T rosettes were collected separately. The SRBC were removed from the T cells by lysing them with Tris-buffered ammonium chloride or by hypotonic lysis. The Bnull population was incubated in Petri dishes coated with the F(ab)2 fragment of human IgG, which results in binding of the B cells to the plate. The free-floating null cells were then collected. In most studies, the Bnull was used as the CFC-enriched population instead of the null cells, as initial experiments revealed that the CFC growth pattern was similar in cocultures of these cells with T cells.

Separation of T-Cell Subsets

The monoclonal antibodies OKT4 and OKT8 were obtained from Ortho Pharmaceutical Corp. (Raritan, NJ). The T cells reacting with OKT4 are defined as helpers and those reacting with OKT8 as suppressors. The OKT4- and OKT8-reacting cells were separated by the "panning" technique. In brief, IgG-coated Petri dishes were prepared by adding 5 ml of affinity-purified sheep anti-mouse IgG (Cappel Labs., Cochranville, PA) in phosphate-buffered saline (PBS) to each dish (100-mm diameter). They were incubated overnight at 4°C. The unbound IgG was aspirated, and the plates...
were washed gently 4 times with cold PBS. For obtaining T cell subsets, the T cells separated by SRBC rosetting method were used in two studies, and the MNC-Mo fraction was used in the others. They were suspended in PBS containing 0.25% human serum albumin (HSA). A portion of them was treated with OKT4 and the other with OKT8, each at a dose of 500 U. They were incubated on ice for 45 min and then washed twice with PBS. They were suspended in RPMI 1640 medium containing 25 mM HEPES buffer with 0.25% HSA and added into sheep anti-mouse IgG plates. After incubating for 1 hr at 5°C, the plates were centrifuged at 250 g for 7 min. The free-floating population depleted of OKT4-reacting cells (or enriched for OKT8 antibody binding, T8 cells), and the population depleted of OKT8-reacting cells (or enriched for OKT4 antibody binding, T4 cells), respectively, were decanted, pelleted and resuspended in McCoy's medium containing 5% heat-inactivated fetal bovine serum (FBS).

**Statistics**

A paired or two-sample t test was applied to determine the significance (p value) of differences between the results of cohort cultures. A p value of <0.05 is considered significant. Linear regression analysis was done to determine the fitness of the cell dose CFC response data. All these statistics were done by Hewlett-Packard Model 85 programs.

**RESULTS**

The purity of the Bnull, null, T, T4, and T8 cells is shown in Table 1. The T cells had negligible contamination with other cells. The Bnull and null cells were minimally contaminated with T cells (<6%), but the Mo contamination was considerable, with 25% in the Bnull and 12% in the null cell fractions. The T4 and T8 cells had very small numbers of neutrophils (<1%), and the Mo contamination was comparable in them, about 7%. The T4 cells were enriched (88.0% ± 4.2%) for OKT4 binding cells and contained 15.0% ± 1.4% OKT8-positive cells. The T8 cells were enriched (99.5% ± 7%) for the OKT8 binding cells and had 4.9% ± 1.6% OKT4-positive cells. The unseparated
Lymphocytes (MNC-Mo) had 45.5% ± 5.4% OKT4-reacting and 25.5% ± 2.1% OKT8-reacting cells. The purity of the T4 and T8 populations was about the same, whether they were separated from MNC-Mo or SRBC-rosetted T cells.

Cultures of a constant number of Bnull or null cells with increasing amounts of HPCM resulted in formation of increasing numbers of CFC in a dose-related manner. No such growth was observed when the T cells were cultured with increasing amounts of HPCM. These results indicate that the CFC are present in the Bnull and null fractions and not in the T cells.

The numbers of CFC proliferated from cocultures of Bnull and null cells with T cells are shown in Fig. 2. For comparison, data from separate cultures of these cells are also shown. In the separate cultures, 10⁵ cells/per dish, and each dish contained 0.1 ml HPCM. In the cocultures, 10⁵ Bnull or null cells were incorporated per dish, and the number of T cells added varied as shown. These cultures also contained 0.1 ml of HPCM per dish. Numbers of CFC proliferated from cocultures of Bnull or null with T cells, at all ratios, are better (p < 0.05) than those from separate cultures of Bnull or null cells alone. These results were similar when MoCM was substituted for HPCM. When HPCM or MoCM was not added, the CFC growth from the cocultures was zero or very poor (results not shown). These results indicate that the T cells promote CFC proliferation.

The numbers of CFC proliferated from separate cultures of Bnull, T, T4, and T8 cells and cocultures of Bnull with T, T4, and T8 cells are shown in Fig. 3. In the separate cultures, 10⁵ cells/dish were incorporated and the same numbers of Bnull cells were added into

| Table 1. Purity of Cells [Percent (Mean ± SD) of Total] |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Wright-Giemsa   | Special Methods |                  |                  |
|                  | Lymphs*         | SRBC' (T)       | Slg' (B)         | ANAE' (Mo)       | OKT4*           | OKT8*           |
| Bnull            | 69.0 ± 20.0     | 6.0 ± 2.0       | 24.0 ± 3.0       | 25.0 ± 8.0       | NT              | NT              |
| Null             | 76.0 ± 13.0     | 4.5 ± 4.0       | 1.9 ± 2.7        | 12.0 ± 3.0       | NT              | NT              |
| T                | 94.5 ± 5.7      | 93.0 ± 5.0      | 1.4 ± 1.4        | 6.9 ± 8.0        | NT              | NT              |
| T4               | 87.3 ± 7.0      | 0.2 ± 0.0       | 0.3 ± 0.2        | NT              | NT              | 6.7 ± 7.5       |
| T8               | 75.3 ± 11.0     | 0.7 ± 0.5       | 0.2 ± 0.2        | NT              | NT              | 6.6 ± 8.1       |

*Lymphs, lymphocytes; Mo, monocytes; Neutro, segmented and band forms of neutrophils; Eos, segmented and band forms of eosinophils; SRBC' (T), sheep erythrocyte receptor positive T lymphocytes; Slg' (B), surface immunoglobulin receptor positive B lymphocytes; ANAE' (Mo), acid alpha-naphthylacetate esterase positive monocytes; OKT4*, T inducer/helper cells; OKT8*, T suppressor/cytotoxic cells; NT, not tested.

Fig. 2. Numbers (mean and SD of 3 plates) of neutrophilic, macrophagocytic, and eosinophilic cluster and colony-forming cells (CFC) proliferated in separate cultures of Bnull or null and T cells, and in cocultures of Bnull + T cells or null + T cells in various ratios are shown. The crosshatched bars represent colonies and the open bars clusters.

Fig. 3. Numbers (mean and SD of 3 plates) of neutrophilic, macrophagocytic, and eosinophilic clusters and colony-forming cells (CFC) proliferated in separate cultures of Bnull, T, T4, and T8 cells and in cocultures of Bnull + T, T4, or T8 cells at 1:4 ratio. The crosshatched bars represent colonies and the open bars clusters.
the cocultures. The number of cocultured T, T4, or T8 cells added was 4 times the number of Bnull cells, and 0.1 ml HPCM/dish was added to these cultures. The number of CFC proliferated increased significantly ($p < 0.001$) in cocultures of Bnull cells with T and T8 cells in all studies. In each case, T8 cells promoted the CFC growth better ($p < 0.01$) than did the unseparated T cells. The pattern of these results was the same whether the T4 and T8 cells were obtained from MNC-MO or from T cells separated by the SRBC-rosetting method.

Types of cells proliferated in these cultures are shown as percent of total aggregates in Table 2. The data of three separate studies are pooled and HPCM was used as a source of CSA in these studies. Although the numbers of various cell types of aggregates formed varied between the groups, the differences are not significant ($p > 0.05$). These results reflect that the growth of all types of cells is promoted approximately to the same extent under the influence of the T and T8 cells. The lack of mixed neutrophilic and macrophagic aggregates in our cultures may reflect the property of the CSA source used in this study.

The results obtained from cocultures of increasing numbers of T, T4, and T8 cells with a constant number, $10^7$/dish, of Bnull cells are shown in Fig. 4. Each dish also contained 0.1 ml HPCM. The results of cocultures are expressed as percentages by taking the number of aggregates formed from the Bnull cell cultures as 100%. Data from three separate studies are pooled for the figure. The results indicate that both the unseparated T cells and the T8 cells promoted the CFC growth from the Bnull cells in a dose-dependent manner. This effect was greater with T8 cells than with unseparated T cells or T4 cells ($p < 0.01$ for Bnull + T8 versus Bnull + T; $p < 0.001$ for Bnull + T8 versus Bnull + T4) at 1:4 and 1:8 ratios. The growth promotion was nonexistent by the T4 cells at Bnull:T4 cell ratio of 1:4 and was minimal ($p < 0.05$) at 1:8 ratio when compared with the number of CFC proliferated from Bnull cells alone.

To test the possibility of modulation of CFC growth by the monoclonal antibodies themselves, reconstituted OKT4 and OKT8 were added directly to Bnull cultures in the presence and absence of HPCM. The amount of antibodies used was the same as that for panning, i.e., 5 μl/10^6 cells. These additions did not influence the CFC growth, which indicated that the observed CFC growth-promoting effect by the T8 cells is not due to the OKT4 antibody, and the lack of growth-promoting effect by the T4 cells is not due to the OKT8 antibody.

To test whether T4 cells modulated the CFC growth-promoting effect of T8 cells, equal numbers of T4 and T8 cells were added to cocultures with Bnull cells in the presence of HPCM. The results (not shown) indicated neither promotion nor inhibition by the T4 cells of the CFC growth induced by the T8 cells.

**DISCUSSION**

The T cells are heterogeneous, in that multiple functions are carried out by separate subpopula-

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**Table 2. The Cell Types of Aggregates Proliferated in Cultures of Bnull Cells and in Cocultures of Bnull Cells With T, T4 or T8 Cells**

<table>
<thead>
<tr>
<th></th>
<th>Macrophagic</th>
<th>Neutrophilic</th>
<th>Eosinophilic</th>
<th>Mixed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bnull</td>
<td>74 ± 3</td>
<td>6 ± 0</td>
<td>25 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>Bnull + T(1:4)</td>
<td>74 ± 13</td>
<td>8 ± 6</td>
<td>22 ± 13</td>
<td>0</td>
</tr>
<tr>
<td>Bnull + T4 (1:4)</td>
<td>57 ± 12</td>
<td>3 ± 4</td>
<td>29 ± 15</td>
<td>0</td>
</tr>
<tr>
<td>Bnull T8 (1:4)</td>
<td>54 ± 29</td>
<td>8 ± 2</td>
<td>30 ± 27</td>
<td>3 ± 2</td>
</tr>
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</table>

* Mixed, aggregates with greater than two types of cells.
The roles of T helper and suppressor cells in B cell differentiation have been defined by functional assays, and the populations involved have been characterized by their interactions with monoclonal antibodies directed against surface markers unique to each population. While the T cell subpopulations have distinct functions in the immune system, they seem to serve as regulators of other hemopoietic cells as well. Unseparated T cells have been shown to promote the growth of the pluripotent stem cell (CFU-S) of the mouse, and we have demonstrated that normal human blood T helper cells promote proliferation of the autologous and allogeneic blood erythroid burst-forming unit (BFU-E). In this study, we show that the T suppressor cell-promoting growth of the normal human autologous blood CFC. Further, we postulate that the growth-promoting activity demonstrated by the unseparated T cells probably resides in the T8 population present in them. The slight augmentation observed in cocultures of Bnull with T-helper-enriched cells, at a higher Bnull to T4 ratio, is probably due to the influence of the T8 subset contamination present in the T4 population.

The blood Mo and T cells have been shown to produce CSA in vitro cultures. However, the Mo by themselves are not responsible for the observed differences in the growth of CFC from Bnull cells in cocultures with T, T4 and T8 cells, as their concentration in the various cohort cocultures was comparable (Table I). The CFC growth promotion occurred in cocultures of Bnull cells with T8 cells only in the presence of a CSA source. It is not clear whether the CSA source stimulated the release of additional quantities of CSA and/or a CSA-potentiating factor from the T8 population which resulted in the CFC growth promotion. Also, the possibility cannot be excluded that an interaction occurred between Mo and T cells in the presence of a CSA source, which lead to the production of more CSA and/or a potentiating factor. Whatever the mechanism may be, it is the T8 and not the T4 cells in our culture setting that possess the capacity to promote the growth of the blood CFC.

The cell dose-dependent augmentation of CFC growth in cocultures of Bnull cells with T cells, which were negatively enriched for suppressor cells in this study, supports the findings of a recent report. In that study, mouse bone marrow CFC growth was found to be promoted by "lymphokine" produced primarily from Lyt-2 (cytotoxic/suppressor) and not the Lyt-2+ (inducer/helper) spleen cells. While our studies did not investigate the possibility of a humoral factor released from T cell subsets, it is clear that stimulatory activity resides with the T-cytotoxic/suppressor cells.

There are conflicting reports concerning the influence of the T cell on the bone marrow CFC. Some investigators report stimulation, others inhibition, and still others report lack of uniform effect. Bagby found no consistent pattern of influence of T cells depleted of either OKT4 or OKT8 antibody-binding populations on the growth of the marrow CFC. We also found no consistent pattern of the marrow CFC growth in cocultures with autologous blood or marrow T cells. This article deals with the influence of the blood T cells on blood CFC. There is evidence from the literature that the blood and marrow CFC are intrinsically different. The differences include: (A) the blood CFC are of lighter density cells than the marrow CFC; (B) none or very few blood CFC are in DNA synthesis, as compared with higher fractions in the marrow CFC; (C) the blood CFC have a longer lag period before initiation of proliferation than do the marrow CFC; (D) the clusters and colonies produced from the marrow peak at days 7-9 and those from the blood at or after 14 days; and (E) the blood CFC grow very poorly in the absence of an exogenous CSF source, whereas a modest growth occurs from the marrows in the absence of a CSF source. These differences suggest that the blood CFC are more primitive than the majority of the marrow CFC, especially of those that form aggregates at days 7-9, in a manner similar to the proposed relationship between the blood BFU-E and marrow CFU-E in the erythroid system. Therefore, it is possible that the nature of the T cell influence on the blood CFC is different from that on the marrow CFC. Also, there is an analogy between the human granulocytic and erythroid progenitors in terms of their growth response to T cells. The unseparated T cell promotes the growth of the blood erythroid (BFU-E) and granulocytic (CFC) progenitors. However, a dichotomy exists in the T cell subsets involved in the promotion of growth of the two progenitors—the T helper promotes the BFU-E growth and the T suppressor the CFC growth, as demonstrated in this study. This involvement of two distinct T-cell subsets in the regulation of erythropoiesis and granulopoiesis may have some physiologic significance. In cyclic hemopoiesis of man and the grey collies, the most intense marrow erythropoiesis is in a phase opposite to granulopoiesis and vice versa. We speculate that such a fluctuation may be a consequence of the fluctuation in the T cell subsets. Additional and more definitive studies are to be performed to establish the in vivo role of the T cell subsets in the regulation of granulopoiesis.

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REFERENCES

Regulation of normal human blood neutrophilic, macrophagic, and eosinophilic committed stem cell proliferation by autologous blood T lymphocyte subsets

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