Regulation of Human Blood Erythroid Burst-Forming Unit (BFU-E) Proliferation by T-Lymphocyte Subpopulations Defined by Fc Receptors and Monoclonal Antibodies

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To determine whether normal T-cell subpopulations influence human blood BFU-E proliferation variably, sheep erythrocyte rosettable cells (unseparated T cells) were fractionated into subpopulations based on their differential binding to IgM (Tμ cells) or IgG-coated ox erythrocytes (Tγ cells). For comparative purposes, T cells, Tγ cells, and T cells depleted of Tγ cells (T-non-γ cells) were further characterized by their reaction with the OK panel of monoclonal antibodies (OKT3, OKT4, OKT8, OKM1). Quantities of 2 × 10^5 Tμ, Tγ, or T-non-γ cells were mixed with 2 × 10^5 autologous BFU-E-enriched null cells in a methylcellulose culture system with 2.0 IU erythropoietin. Tμ cells or T-non-γ cells consistently increased BFU-E numbers above that observed with either unseparated T cells or Tγ cells (p < 0.005, n = 10). The differential burst-enhancing effect of Tμ and Tγ cells was evident in increasing cell numbers in culture and in mixing studies. Tγ cells in the absence or presence of mitogens did not consistently suppress BFU-E growth below that observed in cultures with null cells alone. Monoclonal antibody analysis of T-cell subsets indicated that Tγ cells were enriched for OKM1+ cells and reduced in OKT3+, OKT4+, and OKT8+ cells as compared to unseparated T cells or T-non-γ cells (p < 0.05). T-non-γ cells were enriched for OKT3+, OKT4+, and OKT8+ cells and were depleted of OKM1+ cells (p < 0.05). OKT8 positivity of these cell fractions was similar. Treatment of unseparated T cells with OKT4 antibody plus complement significantly reduced the BFU-E enhancing effects of these T cells compared to complement-treated controls (p < 0.005). These studies provide direct evidence for the concept of T-cell subset regulation of erythropoiesis. The variable burst-enhancing effects of Tμ, T-non-γ, and Tγ cells may be due to the presence of variable numbers of a burst-enhancing OKT4+ cell. The possibility that the differential BFU-E stimulatory effect of Tμ or Tγ cells is due to cellular interactions with monocytes is not excluded by these studies.

HUMAN AND ANIMAL STUDIES have firmly established that the proliferation and maintenance of early erythroid progenitor cells, termed burst-promoting units (BFU-E), do not require erythropoietin. Rather, these activities appear to be under the control of regulatory substances that are operationally referred to as burst-promoting activities (BPA). The exact physiologic role and biochemical nature of BPA is unknown. We and others have demonstrated that under a variety of conditions normal T lymphocytes (T cells) and/or adherent cells (monocyte-macrophages or fibroblasts) may be important sources of BPA. This concept is further supported by findings that both malignant T-cell and monocyte lines produce BPA. In view of the multiple physiologic activities mediated by T cells, we postulated that BPA may be elaborated by a specific subset of these cells. Other subsets could exert a negative influence. The importance of the latter is underscored by reports that have implicated cytotoxic/suppressor T cells either directly or indirectly in the pathogenesis of hypoplastic anemias.

To determine the specific influences of T-cell subsets on BFU-E proliferation, we separated E-rosette-positive cells (E+ = unseparated T cells) into two populations on the basis of differential Fc receptor binding to IgG- or IgM-coated ox erythrocytes. For comparative purposes, positively selected cells, i.e., Tγ or Tμ cells, or cells enriched by negative selection, i.e., T-non-γ cells, were further characterized by their ability to react with well defined hybridoma-derived monoclonal (OKT3, OKM1, OKT4, OKT8) antibodies. The effects of these defined populations on the proliferation of autologous human blood BFU-E from alpha-naphthyl esterase negative, nonadherent, E- surface immunoglobulin negative (SIg-) null cells is the subject of this article.

MATERIALS AND METHODS

Human Subjects

All blood samples were obtained by venipuncture from healthy adult volunteers who had given written consent according to the Human Studies Committee at Albany Medical College. The blood was collected into sterile heparinized (25 U/ml, preservative-free) syringes and diluted with equal volumes of isotonic saline prior to processing.

Cell Separation Procedures

The methods for procurement of unseparated T cells, T-cell subsets, and null cells have been previously described in detail.

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In brief, mononuclear cells (MNC) were obtained from diluted peripheral blood by sedimentation on a Ficoll-Hypaque (FH) gradient (density, 1.077 g/cc cm Pharmacia Fine Chemical Co., Piscataway N.J.). Subsequently, adherent cells were depleted to less than 3% alpha-naphthyl esterase positivity by two sequential incubations of MNC on plastic Petri dishes (25 × 10⁶/78 sq cm) at 37°C for 45 min. Nonadherent cells were then mixed with AET (2-aminoethylisothiouronium bromide) treated sheep erythrocytes (SRBC) to form E rosettes. E‘ cells were then subjected to a second sedimentation through FH. E‘ cells were recovered at the interface, then processed by incubation, over (Fab)₂ (Cappel Labs, Cochranville, Pa.) coated Petri dishes to remove cells containing surface immunoglobulin (Slg +, B cells). The nonadherent null cells were enriched for BFU-E. This null cell fraction was subsequently found to be less than 5% OKT³, 15%–25% OKM¹, and less than 5% Slg¹. Less than 2% of these cells were mature monocytes as judged by alpha-naphthyl esterase positivity.

After lysis of SRBC with 0.83% Tris-buffered ammonium chloride, pH 7.2, E‘ cells were further processed in two ways. In some experiments, E‘ cells were incubated at 37°C in 5% CO₂ atmosphere for 18–24 hr in Minimal Essential Medium (GIBCO, Grand Island, N.Y.) containing 20% fetal calf serum (FCS). Microbiological Associates, Walkersville, Md.) to develop Fc receptors for IgM. Aliquots of these preincubated cells were then mixed with ox erythrocytes coated with either anti-ox RBC IgG or anti-ox RBC IgM to form EA rosettes. Positively selected Tγ or Tµ cells were then retrieved by centrifuging EA rosettes through a FH gradient followed by ammonium chloride lysis of ox erythrocytes. Tγ and Tµ cells were >90% pure when checked by rerosetting with 1% EA IgG or IgM complexes, respectively. The remaining interface cells were recovered and are referred to as T-non-γ or T-non-µ cells. These fractions contained less than 5% Tγ of Tµ cells when checked by rerosetting with 1% EA IgG or IgM complexes. In other experiments where positive selection of Tµ cells was not required, the overnight preincubation step was omitted. E‘ cells were then directly separated into Tγ and T-non-γ populations after a 1-hr incubation with EA IgG complexes followed by sedimentation of EA rosettes through FH. The difference between the Tγ and Tµ cell populations was confirmed by their ability to differentially stain with α-naphthyl acid esterase (ANAE) and Wright-Giemsa stains as described. By these methods, Tγ cells were 5.6% ± 5% (mean ± SD, n = 5) ANAE positive (focal pattern) and greater than 80% had azurophilic granules. Tµ cells were 58.4% ± 10% ANAE positive (focal pattern) and fewer than 10% had azurophilic granules.

Monoclonal Antibodies

The OK panel of hybridoma-produced monoclonal antibodies (OKT3, OKT4, OKT8, OKM1) used in these studies were obtained from Ortho Pharmaceutical Corp., Raritan, N.J. Their production and characterization have been described in detail elsewhere. In brief, the OKT3 antibody identifies a T-cell antigen present on the majority of mature circulating T cells. The OKT4 antibody identifies the T-cell subset that provides helper/inducer function in T-T, T-B, and T-macrophage interactions. The OKT8 antibody identifies the T-cell subset that provides cytotoxic/suppressor functions in these cell–cell interactions. The OKM1 antibody identifies an antigen present on some monocytes, null cells, T cells, and natural killer cells. In two experiments, a fluorescein-conjugated pan-T-cell antibody, T101, obtained from Hybritech Corp, La Jolla, Calif., was used in place of the OKT3 antibody.

Immunofluorescence Procedures

Separated cell fractions (10⁶ cells) were suspended in RPMI1640 medium (GIBCO) supplemented with 5% v/v heat-inactivated FCS and 25 mM HEPES buffer. The cells were then mixed with 5.0 µl of reconstituted OK monoclonal antibodies at 0°C for 30 min, washed 3 times, and developed with a 1:20 dilution of fluorescein isothiocyanate labeled IgG fraction of goat anti-mouse IgG (Meloy Labs, Springfield, Va.). A minimum of 200 cells were then examined by visual inspection for presence of immunofluorescence using a Zeiss immunofluorescence scope. In some experiments, after washing, cell populations were suspended in RPMI 1640 media with 20 mM HEPES and 3 mM EDTA prior to automated flow cytometric analysis using the fluorescence-activated cell sorter (FACS IV, Becton-Dickinson Co, Rutherford, N.J.). Reagent controls run with FITC-labeled goat anti-mouse IgG were never more than 5% positive.

OKT4 Antibody T-Cell Lysis

A quantity of 5.0 × 10⁶ E-rosetted cells in 100 µl of RPMI 1640 media with 0.3% bovine serum albumin were reacted with 2.5 µg of OKT4 monoclonal antibody (Ortho Pharmaceutical) for 1 hr at 4°C, washed, then incubated for 2 hr at 37°C with a 1:10 dilution of fresh rabbit sera as a source of complement (C). Controls containing complement source only were handled similarly. Of unseparated T cells, 27% ± 9% (mean ± SD, n = 5) were lysed by OKT4 antibody + C as determined by Trypan blue staining; lysis of C’ controls was 3% ± 3%.

Mitogen Activation of T-Cell Subsets

In three experiments, phytohemagglutinin (PHA-P, Difco Labs, Detroit, Mich.) was added to culture dishes at a final concentration of 1.0 µg/ml. Alternatively, T-cell subsets were preincubated for 1 hr at 37°C in Hanks balanced salt solution with 100 µg PHA/10⁶ cells or 50 µg Concanavalin-A/10⁶ cells (Pharmacia Fine Chemical Co.) prior to removal by washing with 50 mM d-α-methylmannoside (Sigma Chemical Co., St. Louis, Mo.) or N-acetyl-d-galactosamine (Sigma), respectively, as described.

Erythroid Colony Assay

A quantity of 2.0 × 10⁶ null cells were cocultured with 2.0 × 10⁶ autologous T cells or T-cell subsets in a methylcellulose culture system with 2.0 IU erythropoietin (Connaught Labs, Ontario, Toronto) per 1.3-ml plate as described. Each culture was performed in triplicate. Cultures were incubated for 14 days in a 5% CO₂ atmosphere under high humidity, then stained in situ with benzidine. Only benzidine-positive colonies containing greater or equal to 50 cells/group were considered to arise from one BFU-E. Control cultures of unseparated T cells or T-cell subsets were always plated alone with erythropoietin to determine the number of BFU-E contributed by these cells. These fractions yielded a mean ± 1.0 SD of 3 ± 1.0 BFU-E/2 × 10⁵ cells plated (range 0–6 BFU-E/2 × 10⁵ cells). The results are expressed as number of BFU-E/2 × 10⁵ null cells in coculture studies.

Statistics

Comparisons between cohorts were made using Student’s t test.

RESULTS

The effects of adding unseparated T cells or T-cell subsets to autologous null cells on BFU-E proliferation in 10 separate experiments are shown in Table 1. Null cells alone yielded an average of 22 ± 6 BFU-E/2 × 10⁵ cells plated (column 1). The addition of an equal number of unseparated T cells (column 2) resulted in a
significant augmentation of erythroid colony formation (p < 0.005). As we had previously shown, the augmentation could not be attributed to the small numbers of BFU-E (0-6 BFU-E/2 x 10^5 T cells) that proliferated in control T-cell cultures. In comparison to unseparated T cells, Ty cells were slightly less effective in promoting growth on the average (column 5); however, the differences in burst-enhancing effects were not significant. By contrast, T cells (column 3) markedly augmented BFU-E proliferation. Similar results were obtained using the T-non-γ fraction (column 4). This latter observation indicated that the burst-promoting effect was not due to activation of cells by reaction with the IgM antibody. The burst-promoting effects of Tμ cells was significantly greater than that of unseparated T cells (p < 0.005) or Ty cells (p < 0.005).

When increasing numbers of Ty or Tμ cells were added to a constant number of null cells, their burst-promoting effects were magnified. A representative experiment is shown in Fig. 1. In mixing experiments in which total T-cell numbers were held constant (4.0 x 10^5 T cells) and Ty cells were added to equal numbers of Tμ cells, an intermediate value of BFU-E stimulation was observed (Fig. 2). This again sug-

![Graph](https://via.placeholder.com/150)

**Fig. 1.** The effect of increasing T-cell subset cell number additions on BFU-E proliferation from 2.0 x 10^5 null cells. Closed-circles, Tμ cells; open circles, Ty cells. Each point represents mean ± 1 SD of triplicate plates in presence of 2.0 IU erythropoietin/1.3 ml. Similar results were observed in three other experiments.

![Graph](https://via.placeholder.com/150)

**Fig. 2.** A typical experiment demonstrating the effects of mixing of 2.0 x 10^5 Ty cells to cultures containing 2.0 x 10^5 Tμ cells and null cells (total 6.0 x 10^5 cells/plate). The mean ± SD BFU-E/2 x 10^5 null cells is given for each coculture performed in triplicate. The burst-enhancing effect of the mixture of Ty and Tμ cells was between that observed in the presence of 4.0 x 10^5 Ty or Tμ cells. Similar results were observed in three other experiments.
suggested that the burst-promoting capacity of Tγ cells was not equivalent to Tμ cells. We asked whether Tγ cells may have provided a suppressive or inhibitory effect on BFU-E proliferation. However, we were unable to demonstrate suppression of BFU-E from null cells by either Tγ or T-non-γ cells after activation of these cells with PHA or Con-A (Table 2).

It was possible that variable burst-enhancing effects of Tγ and Tμ cells might be due to variable T-cell subset composition as defined by monoclonal antibodies. In order to test this possibility, the composition of T-cell subsets was determined by reaction with monoclonal antibodies (Table 3). Although the Tγ subset was significantly enriched for OKM1-bearing cells, the majority (69% ± 18%) of the cells bore T-cell antigens as defined by reactivity with (OKT3 or T101) antibodies. The Tγ fraction was not enriched for a cytotoxic/suppressor cells, as judged by percentage of cells reactive with OKT8 antibody. Although the percentage of OKT4 cells in the Tγ fractions was less than that in unseparated T cells, 29% ± 8% of these presumed helper/inducer cells were present. By contrast, removal of Tγ cells served to markedly enrich the total numbers of T cells, i.e. OKT3+ cells. In particular, the helper/inducer fraction (OKT4+ cells) were increased (p < 0.05). Removal of Tγ cells also lead to a pronounced reduction in OKM1+ cells. Considering these changes in subsets of T cells and OKM1+ cells, it appeared that the principal source of a burst-promoting stimulus was due to the OKT4+ helper/inducer cell population. In fact, when the percentage of OKT4+ cells was determined in Tμ or T-non-γ and Tγ fractions and the numbers of BFU-E were plotted against numbers of OKT4+ cells in culture, a strong correlation was found by linear regression analysis (Fig. 3). More direct evidence for this possibility was obtained by treating unseparated T cells with OKT4 antibody plus complement. A significant reduction (52% ± 22% of control, p < 0.005) in burst-enhancing effects in the OKT4-antibody-treated T-cell group compared to complement-treated control again suggested that the burst-enhancing cell was OKT4+ (Table 4).

DISCUSSION

These studies demonstrate for the first time that T cells with Fc receptors for IgM or IgG are functionally heterogeneous in their capacity to augment human blood BFU-E proliferation from null cells in vitro. Tμ cells consistently provided a greater burst-enhancing effect than either unseparated T cells or Tγ cells. Furthermore, the burst-enhancing effect of Tμ cells was not dependent on their prior exposure to IgM antibody, since T-non-γ cells were equivalent to Tμ cells. The increased burst-promoting capacity of Tμ cells was confirmed at higher cell numbers in culture and in mixing experiments with Tγ cells. These findings are consistant with reports that Tμ cells might provide inducer/helper effects under some conditions.

We sought possible explanations for the differential burst-promoting capacity of Tγ and Tμ cells with our correlative monoclonal antibody studies. As has been previously noted, it appears that all E+ cells are not reactive with the pan-T-cell antibody, OKT3. However, removal of Tγ cells serves to enrich for OKT3+ cells. This procedure also specifically serves to concentrate the OKT4+ subpopulation and deplete OKM1+...
cells. As noted, this separation procedure greatly enhances erythroid colony formation. As such, it was possible that the cells augmenting BFU-E proliferation probably belong to the OKT4+ subset. This thesis was further supported by the findings that, with whatever cell populations were mixed in cultures, the increment in BFU-E over that observed with null cells alone closely correlated with total numbers of OKT4+ cells plated. Moreover, when T cells were treated with OKT4 antibody plus complement, a significant decrease in burst-enhancing effect of these T cells was observed. The localization of burst-promoting effect to this fraction is consistent with reports showing that OKT4+ cells have helper/inducer effects in other cooperative systems. The recent report by Torok-Storb et al. seems to be consistent with our findings. These investigators demonstrated that T cells exposed to a monoclonal antibody (9.3) that includes the OKT4-positive subset in the presence of complement had reduced burst-enhancing effects.

Monocyte-macrophages and other cells have been suggested as possible sources of burst-promoting factor. Moreover, Zuckerman has presented evidence for cooperative effects between T cells and monocytes in the blood BFU-E system. Since low numbers of mature monocytes were present in our null cells, our studies do not exclude the possibility of selective Tμ or OKT4+ T-cell interaction with monocytes in the production of a BFU-E stimulatory effect. Whatever

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**Fig. 3.** Linear regression analysis of dose–response curve from Fig. 1 showing the relationship between OKT4+ cells in culture and BFU-E numbers. The numbers of OKT4+ cells in culture was determined by multiplying the number of Tμ or Tγ cells plated in culture by the percent OKT4+ cells found in the Tμ or Tγ cell fraction. In this experiment, Tμ = 55% OKT4 and Tγ = 20% OKT4, r = 0.879, y = 115.7, X = 1.9, p < 0.01.

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**Table 4. Burst-Promoting Effects of T Cells Treated With Complement Alone or OKT4 Antibody Plus Complement**

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Complement Alone</th>
<th>Antibody + Complement</th>
<th>Percent Control</th>
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<tr>
<td>1</td>
<td>115 ± 20</td>
<td>46 ± 11</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>122 ± 16</td>
<td>98 ± 5</td>
<td>80</td>
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<tr>
<td>5</td>
<td>40 ± 4</td>
<td>10 ± 6</td>
<td>25</td>
</tr>
<tr>
<td>Mean ± 1 SD</td>
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<td>52 ± 22</td>
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Values represent mean ± 1 SD BFU-E/2 x 10⁵ null cells in quadruplicate cultures with 2 x 10⁵ T cells treated with complement alone or OKT4 antibody plus complement as described in Materials and Methods. A significant (p < 0.005) decrease in BFU-E numbers found in the OKT4-antibody-treated T-cell group (expressed as percent complement control) compared to the complement-treated group suggests that a burst-enhancing OKT4+ T cell was lysed.
the mechanism for T-cell involvement in BFU-E stimulation, it is clear that under identical conditions, Tγ cells engage poorly or not at all in this process. Previous reports had indicated that Tγ cells expressed suppressor function in cell cooperative systems. In the B-cell system, exposure of Tγ cells to both immune complexes and mitogens are thought to be necessary to trigger (activate) Tγ suppressor activity. However, consistent suppression of BFU-E proliferation by Tγ cells was not observed in our studies either in the presence or absence of mitogens. It is possible that experimental conditions employed here, i.e., dose of mitogen or presence of low concentrations of monocytes in culture, were suboptimal for the activation of Tγ suppressor activity in all instances. Alternatively, the variable effect of Tγ cells on BFU-E proliferation might reflect switches in functional states in vitro. It is known, for example, that Tγ to Tμ phenotypic changes can occur. Our analyses of Tγ cell composition with monoclonal antibodies suggest a third possible explanation for these apparent discrepancies. The numbers of OKT4+ cells, in the Tγ cell fraction were roughly equivalent to the numbers of OKT8+ and OKM1+ cells. Thus, the presence of equal numbers of burst-promoting OKT4+ cells in these fractions may have masked any potential suppressor effects of OKT8+ or OKM1+ cells. It has been previously noted that the OKT8+ cell, which expresses suppressor function, is not enriched by the Tγ cell separation procedure. Direct isolation and coculture of OKT8+ or OKM1+ cells may be required to consistently demonstrate suppressor effects in the blood erythroid colony system. Some reports based on presence of OKM1 reactivity of Tγ cell fractions raised the possibility that the reduced burst-promoting function of Tγ cells might reflect presence of non-T-cells or monocytes. However, our histochemical stains did not indicate presence of mature monocytes. Moreover, although we also found increased numbers of OKM1+ cells in Tγ cell fractions, the majority of our Tγ cells expressed T-cell lineage antigens (Table 3). Using double labeling techniques and other T-cell monoclonal antibodies, Fox et al. have recently demonstrated the presence of both T-lymphocyte-associated and OKM1 antigens on the majority of Tγ cells. Although reactivity of Tγ cells to OKT3 antibody was variable (30%-50%), these authors concluded that Tγ cells were T cells. Therefore, it is unlikely that the reduced burst-promoting function of Tγ cells demonstrated here reflects presence of non-T-cells, but rather the presence of T-cell subsets within Tγ cells that have variable burst-promoting effects.

In conclusion, variable burst-promoting capacity is demonstrated by T-cell subsets isolated by differential Fc receptor binding. By these methods, burst-promoting cells are enriched in the Tμ and T-non-γ fractions, and this finding corresponds well with the enrichment of helper/inducer OKT4+ cells. The suboptimal burst-promoting activity of Tγ cells is probably reflected by the replacement of OKT4+ cells with OKM1+ T cells rather than by enrichment of OKT8+ suppressor cells. These findings lend credence to the idea that variations in T-cell subsets might influence the differentiation and/or proliferation of blood BFU-E by altering the release and/or availability of a burst-promoting activity. As we have previously noted, severe perturbations in T-cell subsets may contribute to the erythropoietic failure of some hypoplastic states by failing to provide an adequate differentiative/proliferative stimulus (BPA). However, further work will be needed to assess the importance of T-cell subset interactions with erythroid stem cells in the bone marrow. Alternatively, variations in T-cell subsets may potentially result in direct cytotoxicity/suppression of granulocyte or erythroid stem cells. Whatever the in vivo role of the T cell in the regulation of erythropoiesis, additional studies employing monoclonal antibody defined T-cell subsets should prove fruitful in further clarifying the cellular interactions of BFU-E in normals and disease states.

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