Modulation of Murine Erythropoiesis In Vitro by Syngeneic Thymocytes: Interactions of Enhancing and Suppressing Subpopulations With Fluorescent Anti-Theta Antibody and Polyamino Acids

By Fritz Sieber and Saul J. Sharkis

SEVERAL LABORATORIES have presented evidence that in vitro colony formation by erythroid progenitor cells is, in part, controlled by accessory cell populations.1,6 However, the exact identity of these regulatory cells, their function, their molecular mode of action, and their physiologic relevance are still controversial. Data published by Nathan and collaborators2,7 suggest that T lymphocytes, but not monocytes, are required for optimal colony formation by human peripheral blood. Other authors contend that monocytes inhibit colony formation by BFU-E from human peripheral blood, whereas T lymphocytes have no effect.1,8,9 Much of this controversy may be due to different culture conditions and the use of poorly defined populations of regulatory cells and target cells. Sharkis and collaborators3 have shown that anti-Thy-1.2-sensitive thymocytes (“anti-theta sensitive regulatory cells” or “TSRC”) can both inhibit and enhance colony formation by early (BFU-E) and late (CFU-E) erythroid progenitors. This dual effect depends on the relative concentration of TSRC in coculture with marrow cells. When the ratio of thymocytes:bone marrow cells is kept below 1, erythropoiesis is suppressed. When the ratio is above 1, a significant enhancement of erythroid colony formation is observed. Several possible hypotheses have been suggested to explain the concentration-dependent effects of thymocytes.3,10 One of them postulates two subpopulations of thymocytes: an inhibitory population that requires cell proliferation and dominates at low concentration and a stimulatory population that does not require cell proliferation and whose influence dominates at high concentration. The differential sensitivity of enhancing and suppressing TSRC to radiation and the alkylating agent, 4-hydroperoxycyclophosphamide, suggests that this hypothesis is correct.11 In this article, we present additional experimental evidence in support of this model. Thymocytes were fractionated on the basis of theta antigen density and negative surface charge using fluorescein conjugates of antitheta antibody and poly-L-ornithine as optical probes for antigen density and negative surface charge, respectively. Both separations yielded distinct subpopulations that expressed markedly different capacities for enhancement and suppression of erythroid colony formation.

MATERIALS AND METHODS

Materials

Poly-L-ornithine HBr (molecular weight 120,000), poly-L-lysine HBr (molecular weight 90,000), fluorescein isothiocyanate (FITC), bovine serum albumin, citrated bovine plasma, and bovine thrombin were obtained from Sigma (St. Louis, Mo.). Fluoresceinated poly-L-ornithine was prepared according to the procedure of Valet et al.,12 except that only 50 μg of FITC were added per milligram of polyamino acid. Any lot of polyamino acid that depressed erythroid colony growth when added directly to the plasma clot in concentrations of ≤50 μg was rejected. A selected lot of fetal calf serum, medium NCTC 109, and McCoy’s medium were obtained from GIBCO (Grand Island, N.Y.). Sheep plasma erythropoietin step III was obtained from Connaught Laboratories (Willowdale, Ontario, Canada), and monoclonal anti-Thy-1.2 antibody13 (IgM; F705) was from the Clayton Laboratories of the Department of Medicine, The Johns Hopkins University School of Medicine, and the Experimental Hematology Laboratory of The Johns Hopkins Oncology Center, Baltimore, Md.

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a gift of Dr. P. Lake (Washington, D.C.). This antibody abolished helper T-cell activity for IgM and IgG responses in both in vivo and in vitro assays. It also abolished cytotoxic alloantigen responses (H-2) and suppressor cell activity. The 50% cytotoxicity titer against thymocytes and peripheral T cells was 1:500,000. A fluorescein-conjugated IgG fraction of rabbit anti-mouse IgM was obtained from Cappel Laboratories (Cochranville, Pa.).

Cells
Male WBB6F1 (WB/ReJ x C57BL/6J, F1) mice (Jackson Laboratories, Bar Harbor, Me.), 5–7 wk old, were used as a source for both bone marrow cells and thymocytes. Single cell suspensions of thymocytes were prepared as described by Jedrzejczak et al. The viability of our thymocyte preparations was consistently >95%, as indicated by the exclusion of trypan blue. Bone marrow cells were flushed from the femoral shaft with a small amount of medium.

They were then cultured in the plasma clot system of McLeod et al. as detailed. Briefly, each clot contained 5 × 10^6 bone marrow cells with or without 10^5 or 10^6 added thymocytes. The erythropoietin concentration was 0.1 U/ml. Clots were fixed with glutaraldehyde after 2 days in culture and stained with benzidine and hematoxylin. Erythroid colonies were then enumerated under the microscope at a magnification of 150x.

Flow Cytometric Analysis and Flow Sorting of Thymocytes
A FACS II cell sorter (Becton, Dickinson, Sunnyvale, Calif.) was used for the flow cytometric analysis and flow sorting. FITC-labeled probes were excited with the 488 nm band of the 5 W argon laser. The fluorescence emission was measured through a 530 nm cut-off filter. Size estimates were based on the forward light scatter signal. For preparative cell sorting, the light scatter windows were set to exclude most dead cells from deflection into the collection tubes. Sheath fluid, cell reservoir and collecting tubes were cooled with ice water to maintain cell viability and to minimize cell aggregation.

Thymocytes (2 × 10^6/ml) were suspended in McCoy's medium supplemented with 2% fetal calf serum, incubated on ice with FITC-poly-L-ornithine (10 μg/ml) for 10 min and then transferred to the cell sorter.

When thymocytes were labeled with anti-theta antibody, the cells were suspended at the concentration of 10^7/ml in serum-free McCoy's medium to which anti-theta antibody was then added to a final dilution of 1:20. After 20 min at 0°C, the cells were washed twice with McCoy's medium and then reacted with the FITC-labeled rabbit anti-mouse developing antibody for 10 min at 0°C. After 2 additional washes with McCoy's medium, labeled thymocytes were transferred to the cell sorter. During the sorting process, the cells were suspended in McCoy's medium supplemented with 2% fetal calf serum.

RESULTS

Interactions With Anti-Theta Antibody
Flow sorting fractionated anti-theta-labeled thymocytes into two distinct subpopulations. The major fraction (approximately 90% of sorted cells) consisted of relatively large cells whose surfaces had absorbed a small amount of anti-theta antibody (low theta density cells). The minor fraction (approximately 10% of sorted cells) consisted of smaller cells with high amounts of anti-theta antibody absorbed to their surfaces (high theta density cells) (Fig. 1). The high and low density of surface theta antigen was confirmed when wet mounts of the two sorted populations were examined with the fluorescence microscope. Wright-Giemsa-stained cytospin preparations of sorted cells displayed characteristic lymphoid morphology.

When low theta density cells were cocultured with bone marrow cells, erythroid colony formation was suppressed by at least 50% at both high and low thymocyte:bone marrow cell ratios (Fig. 2). Conversely, high theta density cells stimulated erythroid colony formation at high thymocyte concentrations and only marginally suppressed at low concentrations. These results suggested that the suppressor function is associated with the subpopulation of thymocytes that is characterized by a large cell size and a low density of cell surface theta antigen. By contrast, the enhancing effect appeared to be attributable to a subpopulation of small thymocytes characterized by a high density of surface theta antigen.

Interactions With Polyamino Acids
Valet and collaborators have shown that the amount of fluorescent poly-L-ornithine or poly-L-lysine bound to the surfaces of intact or neuraminidase-treated erythrocytes is directly proportional to the electrophoretic mobility of these cells. This indicated that polyclation binding can be used as a quantitative probe for negative surface charge.

When FITC-poly-L-ornithine-treated thymocytes were subjected to flow cytometric analysis, a broad
Fig. 2. Cocultures of bone marrow cells with sorted and unsorted thymocytes. Bone marrow cells (5 x 10⁴) were cocultured with high (10⁶) or low (10³) numbers of thymocytes. Thymocytes were added as (A) unfraccionated, anti-Thy-1.2-labeled cells (shown), (B) unfraccionated, unlabeled cells (now shown), or (C) after fractionation into high and low theta density cells by flow sorting (shown). Unfraccionated, labeled (A) and unfraccionated, unlabeled (B) thymocytes were indistinguishable with regard to their enhancing and suppressing effects on erythroid colony formation. All data are expressed as mean colony numbers in 4 replicate clots ± SEM.

The most striking result of this experiment was that enhancing cells, but not suppressing cells were recovered after the sorting of FITC poly-L-ornithine-labeled thymocytes. Both high and low concentrations and strongly fluorescent cells caused a marked stimulation of erythropoiesis. The stimulatory effect of weakly fluorescent thymocytes was less pronounced but noticeable at both high and low concentrations of sorted cells. The control experiment with labeled but unsorted cells (Fig. 4) demonstrated that the loss of the suppressive function was a consequence of the incubation with FITC poly-L-ornithine and not an artifact of the sorting process. Thymocyte preparations that had been treated with FITC poly-L-ornithine contained about 20% more trypan-blue-positive cells than untreated thymocyte preparations. When we examined treated thymocytes with the phase contrast microscope, we noticed an approximately 20% increase in cells with the typical appearance of dead cells, and a determination of lactate dehydrogenase activity in the cell supernatant confirmed that about 20% of the total cellular content of this cytoplasmic enzyme had been released into the medium.

The experiments summarized in Table 1 show that an incubation of thymocytes with nonderivatized polycations was as effective in abrogating the suppressor effect as an incubation with FITC-labeled polycations. However, when poly-L-ornithine was replaced by another basic polyanino acid, poly-L-lysine, the suppressor function was only partially suppressed.
Fig. 4. Coculture of bone marrow cells with sorted and unsorted thymocytes. 5 x 10⁶ bone marrow cells were cocultured with high (10⁶) and low (10³) numbers of thymocytes. Thymocytes were added either as untreated and unsorted cells, as FITC-poly-L-ornithine-labeled but unsorted cells, or as cells that had been labeled with FITC poly-L-ornithine and sorted into intensely and weakly fluorescent (more and less negatively charged) cells. All data are expressed as mean colony numbers in 4 replicate clots ± SEM.

DISCUSSION

During T-cell differentiation, the expression of the theta antigen undergoes characteristic changes.¹⁷,¹⁸ No theta antigen is detectable on prethymic precursor cells. High amounts of theta are found on postthymic precursor cells and low amounts of the antigen are found on competent T cells. Our experiments with anti-theta antibody support the hypothesis that the inhibitory and stimulatory effects of syngeneic thymocytes on erythroid colony formation in plasma clots are caused by at least two distinct subpopulations that may represent different maturational stages of thymocytes. Stimulatory cells were only found in the high theta density fraction, whereas inhibitory cells appeared to be present in both the high and the low theta density fractions (Fig. 2). Sorted high theta density thymocytes stimulated erythropoiesis somewhat less effectively than unFractionated thymocytes. This may have been due to the presence of suppressing cells in the high theta density fraction or an incomplete recovery of enhancing cells in the high theta density fraction.

The results obtained with basic polyamino acids were complex. Poly-L-ornithine and poly-L-lysine did not act only as probes for negative surface charge, but also selectively abrogated the suppressor function.

Polycations have been shown to exert a wide range of biologic effects when applied to intact cells, cell-free extracts, or artificial membranes. They modulate bilayer/nonbilayer transitions¹⁹ and they stimulate the transport of ions and macromolecules across plasma membranes.²⁰,²¹ Polycations can also stimulate the release of histamine by mouse mast cells²² and phagocytosis by polymorphonuclear leukocytes.²³ They have been shown to inhibit the calmodulin-stimulated cyclic nucleotide phosphodiesterase by combining with the calcium-binding protein and to modify the activity of several other soluble and membrane-bound enzymes.²⁴,²⁶ Other effects include the induction of specific intracellular adhesion in Simian virus-transformed 3T3 cells,²⁷ a temporary block of DNA, RNA, and protein synthesis, and a marked antineoplastic activity against certain ascites tumor cells.²⁸ In many instances, a clear correlation was demonstrated between the size of the basic polymer, its primary

<table>
<thead>
<tr>
<th>Thymocytes per 5 x 10⁶ Marrow Cells</th>
<th>Treatment of Thymocytes*</th>
<th>Colony Formation By CFU-E in Coculture With Syngeneic Thymocytes (Colonies Formed per 5 x 10⁶ Marrow Cells)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>None</td>
<td>FITC-poly-orn</td>
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<tr>
<td>0</td>
<td>39.0 ± 1.5</td>
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<tr>
<td>10³</td>
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<tr>
<td>10⁶</td>
<td>85.3 ± 5.0</td>
<td>92.0 ± 4.5</td>
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*All data are expressed as mean number of colonies in 4 replicate clots ± SEM. Thymocytes cultured alone produced no erythroid colonies.
structure, the stereoisomerism of its monomeric constituents, and its biologic potency. Differences in primary structure and/or degree of polymerization were probably responsible for the reduced ability of poly-L-lysine to abrogate the suppressor function.

Enumeration of trypan-blue-positive cells and a quantitative determination of lactate dehydrogenase release indicated that poly-L-ornithine was cytotoxic for about 20% of all thymocytes present. It is conceivable that suppressing cells were among this polycation-sensitive subpopulation. Alternatively, treatment with polyamino acids may have abrogated the suppressor function by altering a metabolic function of the suppressor subpopulation.

In conclusion, our studies on the differential sensitivity of the enhancing and suppressing function of TSRC to gamma radiation and treatment with 4-hydroperoxycyclophosphamide indicate that enhancing and suppressing functions are mediated by separate mechanisms. The results presented in this paper support this view and offer direct evidence for the existence of at least two distinct subpopulations of thymocytes, one enhancing and the other suppressing erythroid colony growth in plasma clot cultures. The definitive identification of the two subpopulations and the clarification of their relation to T cells that act as helper (i.e., Ly-1+, 2, 3−) and suppressor (i.e., Ly-1−, 2, 3+) cells in the immune response will require the examination of additional markers. A characterization of TSRC on the basis of surface Ly antigen is in progress. Preliminary experiments indicate that novel approaches will be required for the efficient isolation of Ly phenotypes because only 3% of the total population express the Ly-1+, 2, 3− phenotype. Additional in vivo studies will also be required to clarify the physiologic significance of our in vitro findings.

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F Sieber and SJ Sharkis