Brief Definitive Report

HEMATOPOIETIC THYMOCYTE PRECURSORS:
IV. Enrichment of the Precursors and Evidence for Heterogeneity*

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The hematopoietic precursor of thymocytes (prothymocyte) is a specialized cell, committed to thymocyte differentiation and discrete from both the already characterized precursors of B cells and the pluripotential stem cell (1–3). We have developed a quantitative assay for this cell and have previously used it to explore the kinetics of repopulation of the murine thymus after γ-irradiation (4) and to establish that prothymocytes are found among the null cell population of both the spleen and bone marrow. They may be distinguished from other cells in this population since they bear surface antigens reactive with rabbit anti-mouse brain antisera and can be induced to display characteristic thymocyte differentiation antigens (5, 6). The physical and surface properties of these cells have permitted us to obtain populations which are enriched 40-fold. Preparations of precursors from bone marrow contain the enzyme terminal deoxyribonucleotidyl transferase (Tdt) while those from spleen lack this activity.

Materials and Methods

Mice—Source and Care. Female mice, 5–6 wk old, were obtained from The Jackson Laboratory, Bar Harbor, Maine. (hereafter designated as AKR/J) and Cumberland View Farms, Clinton, Tenn. (AKR/Cumberland, hereafter designated as AKR/C). AKR/J mice bear the Thy 1.1 (θAKR) alloantigen, while AKR/C anomalously bear Thy 1.2 (θC_3H) (7).

Antisera and Complement. Anti-Thy 1 antisera were produced by the reciprocal immunization of AKR/J and AKR/C mice with thymocytes. The preparation of these sera as well as rabbit antisera to mouse brain and mouse Ig has been described previously (4).

Rabbit serum selected for low toxicity to mouse thymocytes was used as a source of complement in alloantiserum-mediated cytotoxicity; guinea pig serum was used as a complement source in heteroantiserum-mediated killing.

Origin of Cells Repopulating the Thymus. The assay for cells capable of repopulating the thymuses of irradiated mice has been described in detail (2). Thymocytes derived from the injected cells are identified by a cytotoxic test making use of the difference in Thy 1 antigens in AKR mice purchased from different sources. AKR/C (Thy 1.2) mice are injected with hematopoietic cells from AKR/J (Thy 1.1) mice. The proportion of cells in the regenerating thymus which bear the donor type Thy 1 antigen is determined by selectively killing the thymocytes bearing one of the two Thy 1 alleles and measuring residual [^{3}H]TdR incorporation.

To facilitate comparison of the activity of the various populations tested, their repopulation

* Supported by U. S. Public Health Service grants AI 11326 and HD 09130 and contract NCI CE 43927.

‡ Recipient of Career Development Award HD 39968.

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activity was measured. This was defined as the number of cells required to produce 10% repopulation of AKR/C thymus with ALR/J derived thymocytes 14 days after transfer and was calculated by dividing the number of cells injected by the percentage of cells of donor origin found ($\times$ 10).

Cell Preparation. The procedure for inducing the appearance of thymic alloantigens with thymopoietin and the techniques for separating cells on discontinuous gradients of bovine serum albumin (BSA), eliminating Thy 1 or mouse immunoglobulin-positive cells and depleting complement receptor-jpositive lymphocytes have all been described in detail (2, 6, 8).

Terminal Deoxyribonucleotidyl Transferase. (EC 2.7.7.31 deoxyribonucleoside triphosphate: DNA nucleotidyl exotransferase; terminal transferase) was assayed in preparations after homogenization and partial purification by phosphocellulose chromatography as described by Kung et al. (8), with 2.5 μg oligo (dA 12–18) obtained from P-L Biochemicals, Inc., Milwaukee, Wis. as an initiator. The polymerization was followed by measuring the incorporation of [^{3}H]dG (supplied as the triphosphate, sp act 12 Ci/mM) into trichloroacetic precipitable material. [^{3}H]dGTP was purchased from New England Nuclear Corp., Boston, Mass. 1 U of enzyme activity is the amount which catalyzes the incorporation of 1 nmol of deoxynucleotide monophosphate into acid insoluble material in 1 h.

The soluble material obtained between 4×10^7 and 10^9 nucleated cells was applied to each phosphocellulose column. The activity in each cell population was calculated by integrating the activity found in all enzymatically active fractions. The two peaks of activity described by Kung et al. were not calculated separately.

Results

Isolation of Prothymocytes. Spleen cells or bone marrow cells, suspended in RPMI-1640 and 7 mg/ml BSA are initially separated by flotation on either a single step BSA or Ficoll-Hypaque density gradient. The least dense third of the initial spleen cell preparation is used for the subsequent purification. The population accumulating at the interface is removed, suspended at a concentration of 10^7/ml, and incubated sequentially with rabbit antiserum to mouse immunoglobulin and guinea pig complement, and then anti-Thy 1 antiserum and rabbit complement. The cells are then pelleted by centrifugation and resuspended in warm (37°C) isotonic Tris-buffered ammonium chloride (ACT). This step was originally introduced to remove erythrocytes (10). We find that it consistently removes a population of nucleated cells and increases the efficiency of the next step in removing dead or damaged cells. After 10 min in ACT the cells are diluted five-fold with cold (4°C) medium and passed through a glass wool column. The eluted cells are again pelleted and resuspended at 10^7/ml. Complement receptor (CR) positive cells which escaped the anti-Ig treatment are removed and a further separation on the basis of density achieved by flotation in a discontinuous gradient of BSA. The cells accumulating at the 25 and 27% interfaces are pooled, washed, and tested for their prothymocyte activity.

Examples of the results achieved by using this procedure are shown in Table I. Also included are the proportion of cells in each fraction which become Thy 1 positive after thymopoietin treatment. The degree of enrichment of the inducible cells directly paralleled that of the thymus-repopulating activity. The starting materials used here were suspensions of cells from 10 wk old female AKR/J mice. The results are representative of many other experiments with young adult mice. The degree of enrichment achieved does vary with the age of the mice used for the experiments and the greatest repopulation activity was obtained when spleen cells from weanling mice were used (0.08×10^6 cells required to achieve 10% repopulation after 14 days).

TABLE I
Enrichment of Prothymocytes

Fraction		Recovery* of cells	Repopulation‡ activity	Degree of enrichment	Tp§ inducible
					%
S-I	Unfractionated spleen	5.0×10^8	7.0×10^6	1	ND‖
S-II	Hypaque-Ficoll	1.5×10^8	3.4×10^6	2.1	ND
S-III	Anti-Ig + Anti-Thy 1	4.0×10^7	1.1×10^6	6.4	(1)¶
S-IV	ACT Treated	2.6×10^7	0.81×10^6	8.8	(5)
S-V	Glass wool filtered	1.0×10^7	0.39×10^6	17.5	15
S-VI	CRL Depleted‖ BSA Gradient	2.3×10^6	0.23×10^6	30.4	27
M-I	Unfractionated BM	1.8×10^8	1.20×10^6	1	ND
M-II	Hypaque-Ficoll	9.2×10^7	0.66×10^6	1.8	ND
M-III	Anti-Ig and anti-Thy 1	4.8×10^7	0.58×10^6	2.1	(6)
M-IV	ACT Treated	4.1×10^7	0.52×10^6	2.3	11
M-V	Glass wool filtered	1.3×10^7	0.25×10^6	4.8	21
M-VI	CRL Depleted BSA Gradient	4.0×10^6	0.11×10^6	10.9	46

* Viable cells corrected for sampling losses.

‡ Number of cells required to produce 10% repopulation of AKR/C thymus with AKR/T derived thymocytes 14 days after transfer.

§ The percentage of cells rendered susceptible to α-Thy 1.1 antiserum by incubation with thymopoietin. This is calculated by subtracting the percentage killed by this serum in a control sample of cells (not treated with thymopoietin) from the percentage of cells killed in a thymopoietin-treated sample. A total of 200 cells was counted for each determination. The results shown are the average of duplicates. In similar experiments the coefficient of variation for the determination of inducible cells is 22%. Due to the small number of inducible cells present no attempt was made to estimate these cells until the Thy 1.1-positive cells present in the bond marrow and spleen cell preparations had been eliminated by treatment with anti-Thy 1.2 serum.

‖ CRL, complement receptor lymphocyte; ND, not done

¶ Because of the statistical problems inherent in counting small numbers of cells we regard only values greater than 10% as significant. The parentheses indicate results which do not meet this criterion.

Terminal Deoxyribonucleotidyl Transferase in Prothymocyte Enriched Fractions. Bollum (10) has described an enzyme with the unusual property of adding deoxyribonucleotides to a DNA primer in the absence of a template. The principal source is the thymus, where it is found in cortical thymocytes (12, 13). Peripheral T cells lack the enzyme. Low concentrations are found in bone marrow (14, 9). No other tissue has detectable activity. Although no function is known for this enzyme, Baltimore (14) has pointed out that it is a potential somatic mutator and could thus be implicated in the generation of immunologic diversity.

Table II shows that the population of bone marrow cells obtained as a consequence of preparing fractions enriched in prothymocytes is substantially (12-fold) enriched in terminal transferase. The degree of enrichment is proportional to the extent of the purification of the precursor cells. This prothymocyte-rich fraction consists of Thy 1-negative cells. The majority of the Tdt-containing cells in the fractions are killed by rabbit antiserum against mouse brain (Table II, line 3). We have previously shown this serum to be cytolytic for thymocyte precursors (2). In three separate experiments with unfractionated bone marrow the reduction in the specific enzymatic activity of the surviving cells varied from 68 to 83%. Neither increasing the antibody concentrations nor subjecting the surviving cells to a second antibody treatment eliminated the residual activity.

No transferase was found in any preparation from spleen cells including one 40-fold enriched in prothymocyte activity (Table II, line 5), nor was any activity detectable in 18-day fetal liver or spleen (data not shown). Mixing spleen cells

TABLE II
Terminal Deoxyribonucleotidyl Transferase Activity of Prothymocyte Fractions

Fraction	Activity
	units/10⁶ cells
Untreated bone marrow	0.16
Prothymocyte-rich fraction*	1.91
Anti-mouse brain treated‡ prothymocyte-rich fraction	0.35
Normal rabbit serum treated§ prothymocyte-rich fraction	1.78
Prothymocyte-rich fraction‖ from spleen	0.01

* Cells obtained from a BSA gradient after the prior removal of both glass wool adherent Thy 1.1 and Ig-bearing cells. 3.6×10^8 bone marrow cells from 105 AKR/J mice were fractionated to yield 1.6×10^8 cells in the prothymocyte-enriched fraction. (Equivalent to fraction M-VI in Table I).

‡ Cells from the prothymocyte-rich fraction treated with rabbit anti-mouse brain and guinea pig complement. Dead cells were removed by centrifugation over Hypaque-Ficoll. Live cells, which accumulated at the interface were washed twice before homogenization.

§ Cells from the prothymocyte-rich fraction treated with normal rabbit serum and guinea pig complement and prepared as above.

‖ Cells obtained from a BSA gradient after the removal of glass wool adherent, Thy 1.1, and Ig bearing cells. 8.3×10^8 spleen cells were fractionated to yield 4.3×10^7 cells in the prothymocyte-enriched fraction. This fraction had a thymic precursor activity 37 times that of the starting population. (Equivalent to fraction S-VI in Table I).

with thymocytes did not interfere significantly with the assay of the enzyme in the thymocytes.

Discussion

The procedures described here permit the partial purification of the cells responsible for the repopulation of the thymus after irradiation. They are based on the facts that the precursors are relatively low density cells, nonadherent to glass wool, and resistant to the cytotoxic effects of anti-Thy 1 and anti-Ig antisera (2). The entire sequence requires about 4 h and results in a 30-40-fold enrichment of the precursors from spleen. The procedure can also be applied to bone marrow cells but only a 10-15-fold enrichment is obtained with these cells. As expected, both precursor cells and thymopoietin-sensitive cells are purified together. Previous work had indicated that thymopoietin inducible cells can serve as thymocyte precursors (16). The lesser degree of purification achieved with bone marrow is a consequence of both the paucity of Ig-positive and Thy 1-positive cells and the greater number of low density cells in the marrow.

The procedures which lead to the enrichment of the bone marrow precursor also lead to the partial purification of cells which contain the enzyme deoxyribonucleotidyl transferase. This enzyme is present in high concentrations in thymic lymphocytes and it is reasonable to assume that it is also present in the precursor cells. Such an association is also suggested by the susceptibility of both Tdt containing cells and prothymocytes to cytolysis by antisera against mouse brain. Tdt has also been found in the precursor cells which display thymic alloantigens after treatment with thymopoietin or endotoxin (17). Although this is strong evidence that terminal transferase is associated with a prothymocyte in the bone marrow, difficult and intriguing questions remain

unanswered. If the enzyme is present in prothymocytes, why can no enzyme be found in populations obtained from spleen or fetal liver? The splenic precursor is otherwise indistinguishable from the bone marrow-derived cell and the assay for transferase is sufficiently sensitive to detect 1% of the amount found in bone marrow. Furthermore, irradiated mice repopulated with Tdt-negative spleen cells have Tdt-positive thymocytes. R. S. Basch, A. V. Hoffbrand, and K. Ganeshaguru, unpublished results.

We have considered a variety of possibilities to explain this apparent heterogeneity. In the simplest, the assumption is made that the precursors only express the enzyme in the bone marrow environment. Either the induction of previously negative cells by the marrow environment, or the accumulation of enzyme-positive cells by that environment, would account for the apparent heterogeneity. In either case we would argue that the precursors are Tdt negative for most of their existence and only acquire the enzyme in the last stages of their prethymic development.

The distribution could also result from a negative influence exerted in the spleen, suppressing enzyme expression in cells which would otherwise contain it. The splenic environment of normal mice does appear to be inimical to the expression of the thymus leukemia (TL) antigen which, like Tdt, is essentially restricted to thymocytes (18).

More complicated models are required if the heterogeneity in Tdt content actually reflects specialization of the precursor pool. It is plausible that some of the diverse T-cell subsets could arise from progenitors who differed from each other in the time at which they acquired or lost Tdt. Furthermore, some Tdt containing bone marrow cells cannot be induced to display thymic alloantigens (17) and are thus presumably not in the T-cell lineage. If terminal transferase-mediated somatic mutation is to be considered as a source of immunologic diversity, the enzyme should appear at some stage of the development of B-cell progenitors. Perhaps these are the other Tdt containing cells of the bone marrow.

Summary

A method has been developed for the enrichment of the hematopoietic precursors of thymocytes from spleen and bone marrow cells. Up to 40-fold enrichments were obtained resulting in preparations in which as few as 10^5 cells produced prompt repopulation of the thymus of an irradiated mouse.

Precursor cells from bone marrow appear to contain the enzyme terminal deoxyribonucleotidyl transferase (Tdt), an agent suggested as a potential somatic mutator. This enzyme (Tdt) was not detectable in any spleen cell preparation examined, including one in which a 40-fold enrichment of thymocyte precursors had been produced. This is the first difference reported between the splenic and bone marrow precursors of thymocytes.

Discussions with Doctors Allen Silverstone and David Baltimore have been invaluable. The excellent technical assistance of Bohumila Fahmy, Thomas Panagiotatos, and Shirley Carchi are gratefully acknowledged.

Received for publication 21 February 1978.

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