HEMATOPOIETIC THYMOCYTE PRECURSORS*

II. Properties of the Precursors

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Repletion of the thymic lymphoid compartment after irradiation depends upon the existence of the stem cells in the hematopoietic tissues (1–3). Neither the origin nor the state of differentiation of these precursor cells has been firmly established. Their ultimate progenitor appears to be a pluripotential stem cell (CFUs)[1] among whose progeny are counted T and B lymphocytes as well as the erythroid and myeloid elements (4, 5). The subsequent development of this pluripotent stem cell appears to involve several discrete stages of differentiation.

Indirect evidence suggests that the thymus precursor or prothymocyte is a specialized cell, committed to thymocyte differentiation and discrete from both the precursors of B cells and the pluripotential stem cell (6–8). 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 (9). We now use this assay to examine the state of differentiation of the hematopoietic precursor. Prothymocytes are found amongst the "null cell" population of both the spleen and bone marrow but bear surface antigens reactive with rabbit anti-mouse brain antisera.

Materials and Methods

Mice—Source and Care. Female mice, 5- to 6-wk old, were obtained from Texas Inbred, Houston, Texas (AKR/Texas, hereafter designated as AKR/T), and Cumberland View Farms, Clinton, Tenn. (AKR/Cumberland, hereafter designated as AKR/C). AKR/T mice bear the Thy-1.1 (θAKR) alloantigen while AKR/C anomalously bear Thy-1.2 (θC3H) (10). All animals were housed three to four per cage, fed commercial mouse food, and maintained on acidified chlorinated water.

Antisera and Complement. Antisera were produced by the reciprocal immunization of AKR/T and AKR/C mice with thymocytes of the appropriate strains as previously described (9).

Rabbit antiserum to mouse immunoglobulin (RaMIG) was obtained from rabbits immunized

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[1] *Abbreviations used in this paper:* B cells, Ig-positive peripheral lymphocytes; BM, bone marrow; BSA, bovine serum albumin; CFUs, spleen colony-forming pluripotential stem cells; CR, complement receptor; EAC, sheep cells coated with 19S antibody and complement components; Fc receptor, a receptor for the portion of the Ig molecule bearing isotypic specificities; GaRIG, goat antiserum to rabbit immunoglobulin; [^{3}H]TdR, tritiated thymidine; KLH, keyhole limpet hemocyanin; RaMIG, rabbit antiserum to mouse Ig; T cells; Thy-1-positive peripheral lymphocytes; TL, thymus leukemia antigen (a differentiation antigen of mouse cortical thymocytes).

with insoluble keyhole limpet hemocyanin (KLH); mouse anti-KLH antibody (antigen-antibody) complexes in complete Freund's adjuvant and absorbed with mouse thymocytes before use. Rabbit antisera to mouse brain were prepared by injecting AKR/C brain emulsified in complete Freund's adjuvant as described by Golub (11). The sera were absorbed with an equal volume of packed mouse erythrocytes and had a cytotoxic titer of 1:1,000 against mouse thymocytes when tested with guinea pig complement (C). In some experiments the serum was also absorbed with a liver cell membrane fraction.

Goat anti-rabbit immunoglobulin (GaRIG) was purchased from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Rabbit serum selected for low toxicity to mouse thymocytes was used as a source of C in alloantiserum-mediated cytotoxicity; guinea pig serum was used as a C source in hetero-antiserum-mediated killing.

Cell Suspensions. Cell suspensions were prepared in tissue culture medium RPMI1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 50 U/ml penicillin and 50 μg/ml streptomycin and supplemented with bovine serum albumin (BSA, 7 mg/ml obtained from Miles Laboratories, Inc., Miles Research Div.) as previously described (9), and maintained in this medium throughout the subsequent manipulations.

Irradiation. A ^{137}Cs source (Model M Gammator; Radiation Machinery Corp., Parsippany, N. J.) delivering 630 rads/min was used to irradiate the mice used in these experiments.

Origin of Cells Repopulating the Thymus. The assay for cells of donor origin has been described in detail previously (9). It is a cytotoxic test making use of the differences in Thy-1 antigens in AKR mice purchased from different sources. AKR/C (Thy-1.2) mice are irradiated (760 rads) and injected with hematopoietic cells from AKR/T (Thy-1.1) mice. At various times after irradiation and cell transfer, the proportion of replicating thymocytes bearing the donor type Thy-1 antigen is determined by selectively killing the cells bearing one of the two Thy-1 alleles. The viability of the remaining cells is assessed by measuring their incorporation of ([^{3}H]TdR) into DNA and this is compared to the incorporation by the whole population. The proportion of cells bearing the donor Thy-1 allele is calculated as follows:

$$\% \text{ Cells of donor origin} = \frac{([^3\text{H}]\text{TdR incorporation after elimination of host cells} - \text{background})}{(\text{total } [^3\text{H}]\text{TdR incorporation} - \text{background})}.$$

Isopycnic Density Gradient and Isokinetic Velocity Gradient Centrifugations. Discontinuous gradients of BSA were prepared and harvested as previously described (7).

For velocity sedimentation, 10^8 spleen cells in 2 ml of Puck's saline were separated in 50-ml centrifuge tubes containing shallow BSA gradients as described by Pretlow and Boone (12). They were centrifuged for 18 min at 120 rpm in a model PR2 Centrifuge (IEC). 2-ml fractions were collected from the bottom of the tube. When necessary fractions were pooled to provide sufficient cells for a transfer.

CFUs. CFUs were determined as described by Till and McCulloch (13). Macroscopic colonies were counted on the 8th day after 890 rads in spleens fixed in Bouin's solution.

Antibody-Mediated Cytotoxicity. A two-stage assay in which the cells are incubated with antiserum for 60 min at 4°C, washed, and then incubated with C for 45 min at 37°C was used. Anti-Thy-1 and RaMIG sera were used at a concentration of 0.1 ml/10^7 cells. Rabbit antisera to mouse brain absorbed with erythrocytes had residual toxicity for all mouse cells when used at greater than 0.02 ml/10^7 cells. When further absorbed with liver cell membranes it could be used at the same concentration as the other sera. After treatment with C the cells were washed twice in medium and then adjusted to the appropriate concentration on the basis of viable cells as determined by trypan blue exclusion.

Differential Adhesiveness. Spleen cells were fractionated on glass wool and nylon fiber columns according to the method of Trizio and Cudkowicz (14) in RPMI 1640. 10^7 cells from each fraction were injected into irradiated AKR/C mice and the extent of thymic repopulation measured 2 wk later.

Rosette Techniques. Three different erythrocyte preparations were used to prepare rosettes with different populations of lymphocytes. First, sheep erythrocytes coated with 19S antibody and C (EAC) prepared by the method of Bianco et al. (15) were used to remove C receptor-positive (CR+) lymphocytes. Rosettes were made by incubating nucleated cells with EAC at 37°C for 45 min with continuous agitation. When velocity sedimentation was used to separate the rosettes from the nonrosetting cells EAC and nucleated cells were mixed at a ratio of 1 erythrocyte (RBC)

per nucleated cell, but when density gradients were used 50–100 RBC were added per nucleated cell.

Sheep RBC coated with the globulin fraction of sheep anti-rabbit serum were used to form rosettes with RaMIG-treated cell suspensions as described by Parrish et al. (16). 100 RBC were added per nucleated cell. This technique selectively produces rosettes among cells with high concentrations of immunoglobulin on their surface.

Finally, ox RBC heavily coated with mouse 7S anti-ox RBC antibodies were used to prepare rosettes with cells having a receptor for the Fc portion of the immunoglobulin molecule as described by Ramasamy and Munro (17). 50–100 RBC were added per nucleated cell.

In most experiments cells forming rosettes were separated from those that did not by centrifugation at 2,000 *g* for 20 min over a layer of Ficoll-Hypaque (density 1.075) which had been prewarmed to 20°C (18). The cells to be tested for rosette formation were first layered onto a Ficoll-Hypaque gradient and centrifuged as described to remove dense cells and RBC. These had been shown to be devoid of repopulating activity and were discarded. The cells that accumulated at the interface were washed and then used to prepare rosettes. Rosettes, plus a few residual dense nucleated cells pellet in the Ficoll-Hypaque leaving the nondense, nonrosetting cells at the interface. The RBC in the rosettes were lysed with Tris-buffered isotonic ammonium chloride and the remaining nucleated cells injected.

Self-sterilization via [^{3}H]TdR Incorporation. AKR/T spleen cells were prepared as a single cell suspension and incubated for 3 h at a concentration of 6.25×10^6 cells/ml in medium containing 1.6 μCi/ml [^{3}H]TdR (43 Ci/mmol; New England Nuclear, Boston, Mass.). The cells were washed three times in large volumes of medium and injected into irradiated AKR/C mice. Controls were incubated in the same medium containing a 1,000-fold excess of unlabeled thymidine. Aliquots were removed for counting and autoradiography performed as previously described.

Results

Physical Properties of the Thymocyte Precursor

RELATIVE DENSITY. A single cell suspension of AKR/T spleen cells was fractionated according to buoyant density by centrifugation on a four-step discontinuous BSA density gradient. Cells from each interface band were injected into irradiated AKR/C mice and 12 days later their thymuses were assayed for AKR/T-type thymocytes. The results are shown in Table I. Layers 2 and 3 (those cells that did not pass through 25 and 27% BSA, respectively) are enriched for the thymocyte precursor compared to unfractionated spleen cells. Layers 1 and 4 are partially depleted of these cells. Spleen cells dense enough to pellet 29% BSA (nearly 40% of the cells recovered) are totally depleted of the thymocyte precursor. Identical results are found with bone marrow (BM) cells.

Similar results could be obtained using a continuous BSA gradient. In this medium the model density of the thymocyte progenitor is 1.068 ± 0.003. Significant enrichment of prothymocytes could also be achieved by floatation above a dense (sp gr 1.075) layer of Ficoll-Hypaque. The interface layer contains 30% of the nucleated cells of the spleen and 80% of the thymocyte precursor activity.

SIZE OF THE PRECURSOR. An estimate of the size of the prothymocyte was made on the basis of its sedimentation behavior in isokinetic velocity gradients of BSA in Puck's saline. Fig. 1 shows the size-distribution analysis obtained using low angle light scattering of cells from a normal AKR/T mouse spleen. The peak at the left is produced by RBC, while the major peak is produced by small and medium sized lymphocytes. The most rapidly sedimenting fractions were pooled to provide sufficient cells for a transfer. The median size in each fraction was determined and these are indicated on the figure. The ability of

TABLE I

Relative Density of the BM-Derived Thymocyte Precursor: Separation on a Discontinuous BSA Gradient

Source of cells	% of cells* recovered	% Donor type thymocytes (at 12 days ± SD)
Unfractionated normal BM cells	–	6.6 ± 2.1
Layer 1‡	7.8	5.1 ± 0.6
Layer 2	9.6	11.4 ± 2.3
Layer 3	13.6	9.5 ± 3.8
Layer 4	29.6	3.4 ± 0.7
Pellet	39.7	0.0 ± 1.1

* 63.8% of the cells applied to the gradient were recovered.

‡ Layer 1 was obtained at the interface above a 23% BSA layer. Layers 2–4 were found at the interface of a 23–25, 25–27, and 27–29% BSA, respectively. 5×10^6 cells were injected per mouse.

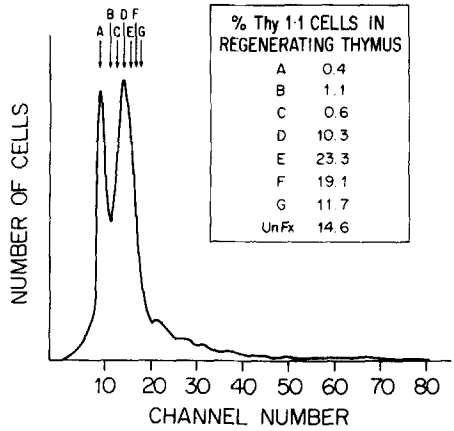

FIG. 1. Relative size of the hematopoietic thymocyte precursor. The figure illustrates the relative size distribution of normal spleen cells as measured by low angle light scattering in a Cytograph (Bio/Physics Systems Inc., Mahopac, N. Y.). The abscissa is an arbitrary scale representing the magnitude of the light scattering produced by the cells. The arrows indicate the median size of the cells found in the various fractions of an isokinetic velocity sedimentation experiment and the insert indicates the extent of thymic repopulation achieved 14 days after the injection of 10^7 cells from each fraction.

the cells in fraction to serve as a source of thymocyte precursors was tested. The results are shown in the insert to the figure. Both the small and very large fractions are depleted of thymic precursors. Fractions E and F are significantly enriched in precursors. The majority of prothymocytes are thus similar in size to medium to large lymphocytes (10–15 μm in diameter).

Biologic Properties of Hematopoietic Thymic Precursors

CORTISONE SENSITIVITY. The sensitivity of the thymocyte precursor to a large dose of hydrocortisone succinate was tested by two methods: by treatment of the recipients with the drug after injection of the donor cells, and by treatment of the donor animals before the cells were removed. In mice receiving 5 or 10 mg of hydrocortisone immediately after the injection of 10^7 bone marrow cells

TABLE II
Cortisone Sensitivity of the Thymocyte Precursor

Test animal treatment*	% Donor type thymocytes (± SD) on day 14 after irradiation and grafting
Experiment 1	
10^7 Normal AKR/T BM cells	47.1 ± 11.0
10^7 BM cells plus 5 mg HC‡	38.1 ± 1.5
10^7 BM cells plus 10 mg HC‡	34.6 ± 7.8
Experiment 2	
5×10^6 normal AKR/T spleen cells	4.7 ± 2.3
5×10^6 Cortisone-treated§ AKR/T spleen cells	6.6 ± 1.3

* All recipients were AKR/C mice irradiated with 760 rads before injection of cells.

‡ HC, hydrocortisone succinate (Upjohn Co., Kalamazoo, Mich.) injected S.C. 2 h after injection of BM cells.

§ AKR/T donors were treated with 5 mg hydrocortisone succinate subcutaneously 16 h before the spleen cells were prepared.

(see Table II, top) thymic size at 14 days is not altered and the percentage of dividing cells of the donor type is only slightly less than in the untreated control. Pretreatment of the donors with cortisone (Table II, bottom) produces a modest enrichment of the thymocyte precursors. 16 h after the administration of 5 mg of hydrocortisone succinate spleen size is reduced by 50%. If prothymocytes are insensitive to cortisone, the cell suspension prepared from such cortisone-treated animals should be approximately 100% enriched in this activity. A 40% enrichment was observed. Both experiments, therefore, indicate that the precursors are relatively but not completely resistant to hydrocortisone.

MITOTIC ACTIVITY. The sensitivity of the thymocyte precursor to self sterilization by high specific activity [^{3}H]TdR ("thymidine suicide") was used as a measure of their turnover rate. AKR/T BM cells were incubated with [^{3}H]TdR for 3 h in vitro. The cells were washed and injected into irradiated AKR/C recipients. 2 wk later the thymuses of these animals were examined for the presence of cells bearing the Thy-1 antigen characteristic of the donor cells. The results are shown in experiment 1 of Table III. The effect of this treatment of CFUs is shown for comparison. The proportion of donor-type cells was reduced by only 19% indicating that few of these cells are dividing [doubling time(T_D) 50–60 h]. As expected CFUs were unaffected by this treatment (19).

SENSITIVITY TO γ-IRRADIATION. The ability of AKR/T BM cells, irradiated in vitro, to serve as thymocyte precursors was tested. The results are shown in the second experiment of Table III. The lethal dose for 90% (LD_{90}) of the precursor, estimated from a semilogarithmic plot of the data is 400 R but this is greatly influenced by the large threshold effect observed. Calculation of D_{37} in the exponential portion of the curve gives a value of 75 R. Under similar conditions an LD_{90} of 120 R and a D_{37} of 47 R have been calculated for cortical thymocytes (20).

ADHERENCE TO GLASS WOOL AND NYLON FIBER COLUMNS. The adherence of prothymocytes to glass wool and washed nylon fibers was examined by sequen-

TABLE III
Sensitivity of thymocyte precursors to ^{3}HTdR "Suicide" and to γ-Irradiation

	CFU* (± SD)	% Thymocytes with donor‡ type Thy-1 antigen (± SD)
Control BM	28.3 ± 2.6	32.9 ± 1.2
BM incubated with [^{3}H]TdR	26.2 ± 2.5	26.5 ± 1.2
Irradiation dose to donors§ marrow (in vitro) in rads		
0	–	25.9 ± 0.8
70	–	28.5 ± 2.7
140	–	24.7 ± 2.3
210	–	21.6 ± 1.9
350	–	14.7 ± 1.1
420	–	7.3 ± 0.9
490	–	2.4 ± 0.6

* CFUs were counted 9 days after the transfer of 2.5×10^5 cells to AKR/T mice which had received 820 rads on day 0. (Six mice per group.)

‡ Measured 14 days after the transfer of 5×10^6 BM cells to AKR/C mice which had received 720 rads on day 0. (Five mice per group.)

§ Incubated as described in text. During the incubation the cells incorporated 1.4×10^5 dpm of [^{3}H]TdR per 10^6 cells into TCA precipitable material. Autoradiographs made from aliquots of the preparation showed that 6.2% of the cells had been labeled. Each labeled cell therefore incorporated an average of 2.3 dpm.

tially passing a suspension of AKR/T spleen cells through a glass wool column and then a nylon fiber column (14). The results of these experiments are shown in Table IV. Approximately half of the cells applied to the glass wool columns are recovered in the nonadherent fraction. All thymocyte precursor activity present in the original suspension is recovered in this nonglass-adherent fraction. The cells in this fraction were then applied to a nylon wool column. Slightly less than one-third of the cells eluted in the nonadherent fraction. 85% of these cells are Thy-1 positive as judged by their sensitivity to alloantisera to Thy-1.1. The concentration of thymocyte precursors in this fraction is significantly less than the concentration in the sample applied to the column, indicating that many precursors are retained by the column.

In a separate experiment the possibility of recovering these adherent stem cells was tested and these results are also shown in Table IV. The distribution of cells on the two columns was essentially the same as in the previous experiment. Nylon wool-adherent cells were recovered by removing the fibers from the column and "combing" them in the presence of a large volume of Puck's saline G. After several washes nearly three-quarters of the adherent cells are recovered. These cells are not enriched in their content of prothymocytes. Thus, although nonadherent to glass wool, the precursors are found in both the adherent and nonadherent fractions after passage through nylon fibers.

Surface Membrane Properties of Prothymocytes

THY-1 (θ) ANTIGEN. Although not a uniquely thymocyte antigen, the presence of Thy-1 is a consistent and well-characterized property of thymic lymphocytes (21, 22). Since thymopoietin and other thymic factors induce the appear-

TABLE IV
Adherence of Thymocyte Precursors from Spleen to Glass and Nylon Fiber Columns

Cells transferred*		% Repopulation (cells bearing donor type Thy-1 antigen ± SD)	% of Original sample recovered
Number	Treatment		
10^7	Unfractionated	6.7 ± 2.1	100
10^7	Nonadherent to glass wool	11.7 ± 1.2	49.6
10^7	Nonadherent to both glass and nylon wool	7.1 ± 1.0	16.4
2×10^7	Nonadherent to glass wool	39.5 ± 3.3	61.8
2×10^7	Nonadherent to both glass and nylon wool	39.2 ± 4.6	18.3
2×10^7	Eluted from nylon fiber	28.1 ± 2.2	29.5

* AKR/T spleen cells were transferred to irradiated (760 rads) AKR/C recipients and the proportion of donor-type cells in the thymus estimated 14 days later.

ance of this antigen in hematopoietic cells, the possibility that thymocyte precursors possess significant quantities of Thy-1 before being subject to thymic influence seems remote. Nevertheless it was investigated by exposing the AKR/T donor spleen and marrow cells to high concentrations of anti-Thy-1.1 antisera and C before injecting them into irradiated AKR/C mice. The results are shown in the first experiment in Table V. As a control, other spleen and BM cells were exposed to antisera specific for the Thy-1.2 antigen which is never expressed on these cells. The extent of proliferation of thymocytes derived from precursors treated with either antiserum is essentially identical, indicating that prothymocytes do not express significant quantities of Thy-1 antigen on their surface. This is in agreement with previously published data (6, 7).

MOUSE BRAIN-ASSOCIATED THYMIC ANTIGENS. Golub and Day (11, 23) have demonstrated that the immunization of rabbits with mouse brain leads to the production of antibodies against a variety of antigens found on hematopoietic cells. The predominant antibody is one reactive with thymocytes. Appropriately absorbed sera retain their cytotoxicity for thymocytes and T cells but are unreactive with B lymphocytes. Thymic repopulation is almost totally abolished when spleen or bone marrow suspensions are treated with absorbed rabbit anti-mouse brain (exp. 2, Table V). Thymocytes, splenic T cells, and prothymocytes are equally susceptible to this antiserum.

BRAIN-ASSOCIATED STEM CELL ANTIGEN. Golub (24) has also reported that absorption of anti-brain sera with thymocytes produces a reagent capable of reacting specifically with the colony-forming stem cell normally present in hematopoietic tissues. A thymocyte-absorbed anti-brain serum (i.e., an anti-stem cell antiserum) was prepared as described by Golub and tested for its ability to eliminate that thymocyte repopulating capacity of spleen and BM cells. The results are included in experiment 2 of Table V. At the dilution used, this antiserum is unreactive with thymocytes or splenic lymphocytes in the cytotoxic test yet it retains its capacity to react with the thymocyte precursor. Dilutions of this antiserum were tested for their anti-prothymocyte activity and compared with similar dilutions of antiserum to mouse brain which had not

TABLE V

Antigenic Properties of the Hematopoietic Thymocyte Precursor: Sensitivity to Antisera Against Thy-1, Mouse Brain, and Immunoglobulin

Antiserum	% Repopulation produced by:	
	Spleen cells	Bone marrow cells
Anti-Thy-1.2	7.2 ± 2.1*	5.1 ± 2.7‡
Anti-Thy-1.1	6.5 ± 1.9	6.3 ± 0.7
Normal rabbit serum	7.0 ± 1.2§	12.4 ± 1.7‖
Rabbit anti-mouse brain	0.4 ± 0.2	2.4 ± 1.0
Rabbit anti-mouse brain (thymus absorbed)	2.1 ± 1.7	1.0 ± 0.8
Normal rabbit serum	8.9 ± 0.8*	5.8 ± 0.7‡
RaMIG	8.3 ± 1.6	6.1 ± 1.2

All results are given as the arithmetic mean of quadruplicate determination ± SD.
* Measured 14 days after 10×10^6 spleen cells.
‡ Measured 12 days after 5×10^6 BM cells.
§ Measured 12 days after 50×10^6 spleen cells.
‖ Measured 12 days after 10×10^6 BM cells.

been absorbed with thymocytes. Absorption with thymocytes reduces the titer against prothymocytes by four- to eightfold but cytotoxic activity against these cells is clearly retained by dilutions of sera which are totally devoid of cytotoxicity for more mature numbers of the Thy-1-positive peripheral lymphocyte (T-cell) lineage.

Surface Immunoglobulin. Although cortical thymocytes and peripheral T cells lack readily detectable surface immunoglobulin the status of the precursor cell is unclear. Neonatal thymocytes have been claimed to be strongly Ig positive (25) and murine T-cell precursors have been reported to be retained by anti-Ig columns (26). The presence of Ig on prothymocytes would have profound implications for our understanding of the development of differentiated immune function. Mouse thymocyte precursors were tested for the presence of surface Ig by two different methods. First, an attempt was made to deplete spleen and BM suspensions of prothymocytes by treating them with a polyvalent rabbit anti-immunoglobulin serum and C. The results are shown in experiment 3 of Table V. No reduction in repopulating activity was produced by treatment with RaMIG although more than 90% of the Ig-positive cells (as measured by direct immunofluorescence) were killed in the procedure. An additional attempt to demonstrate Ig-positive thymocyte precursors was made using the "rosetting" method of Parish et al. (16) in which Ig-bearing mouse cells are coated with RaMIG and then allowed to form rosettes with sheep RBC coated with anti-rabbit immunoglobulin. The rosettes are separated from other lymphocytes by sedimentation through a dense layer of Ficoll-Hypaque. BM cells depleted of Ig-positive cells by this method are also enriched for, rather than depleted of, prothymocytes (Table VI), strongly suggesting that these cells lack significant quantities of surface immunoglobulin.

CR and Fc Receptor. Lymphocytes of the B series and macrophages bear on

TABLE VI

Surface Properties of the Hematopoietic Thymocyte Precursor: Ability of Cells Expressing IG, CR, or Fc Receptors, to Repopulate the Thymus of Irradiated mice

	% Donor type cells* found after the injection of:	
	Spleen cells‡	BM cells§
Unseparated	5.3 ± 0.7	3.0 ± 0.8
CR+	1.2 ± 0.6	1.3 ± 0.5
CR−	5.4 ± 0.2	4.5 ± 0.6
Fc+	0.5 ± 1.2	ND‖
Fc−	8.6 ± 1.7	ND
Ig+	ND	0.9 ± 0.6
Ig−	ND	3.9 ± 0.7

* Results are the mean % as determined in quadruplicate determinations ± SD.

‡ Measured 14 days after the transfer of 5×10^6 cells.

§ Measured 12 days after the transfer of 2×10^6 cells.

‖ ND, not done.

¶ All of the separations used in this experiment were performed with cells separated by the rosetting techniques described under Materials and Methods.

their surfaces a receptor for the third component of C (15). This receptor is absent from T cells and thymocytes and as shown in Table VI is also absent from their hematopoietic precursor. As indicated, both BM and splenic CR-positive cells fail to serve as thymocyte precursors. Conversely, the CR-negative population is enriched in precursor activity.

B lymphocytes and macrophages (27–29) also bear a receptor that enables them to bind 7S antibodies via the Fc portion of the Ig molecule. A receptor with similar binding properties has been found on a subset of T cells (30). This receptor is not detectable on prothymocytes. The data (Table VI) show that "Fc-negative" cells are an enriched source of prothymocyte precursors.

Discussion

The cells that repopulate the thymus of an irradiated mouse are of hematopoietic origin. These prothymocytes are relatively large cells with a low buoyant density and do not adhere to glass. Unlike their progeny they are relatively resistant to the cytolytic effects of corticosteroids and γ-irradiation. The majority of the population does not appear to be mitotically active when prepared from normal mice. Neither the Thy-1 alloantigen characteristic of thymocytes and thymus-derived cells, nor a variety of markers generally associated with Ig-positive peripheral lymphocytes (B cells) (including surface Ig, Fc receptor, and the CR) can be detected. The hematopoietic precursors do bear on their surface, antigens that react with antisera against mouse brain. At least two specificities appear to be involved; one of these is found on mature thymocytes and can be removed by absorption with cortical thymocytes. The other remains after absorption with thymocytes.

Cells that act as thymocyte precursors or prothymocytes have been described in other contexts. The cells that populate the embryonic thymic rudiment are generally believed to be immigrants from a hematopoietic source (31, 32). We believe the migratory cells which we are studying are the adult equivalent of these embryonic cells. The lymphoid cells which appear in the embryonic thymus lack the differentiation antigens characteristic of adult thymus and therefore resemble cells described herein (33). Direct comparison of the properties of the cells colonizing the embryonic thymus with those serving this role in the adult is technically difficult.

Loor and Roelants and their associates (34–36) have presented indirect evidence for the existence of a "pre-thymic T cell." The cells that they described react with antisera to mouse brain (and are called "weak" theta positives by them) and anti-TL antisera but not with antisera against immunoglobulins. They are present in neonatally thymectomized mice and in congenitally athymic mice homozygous for the nude gene, born of homozygous nude mothers. No formal evidence for the precursor role of these cells has been adduced but the authors argue that their thymic cell characteristics and their presence in athymic mice "makes it tempting to consider them a prethymocyte." We think it highly probable that the cells that we have characterized are identical with those described by Loor and Roelants. Questions about the presence of low levels of Thy-1 and thymus leukemia (TL) antigens on their surface are difficult to resolve. The weak theta-positive cells of Roelants et. al. (35, 36) were generally detected by indirect immunofluorescence using an anti-mouse brain serum as the primary reagent. Despite extensive absorption, reagents of this type almost certainly contain many specificities including gangliosides (37), T25 protein (38), and the uncharacterized antigen(s) present on B cells (39) and stem cells. The question is in part moot since all agree that the thymic precursor reacts with anti-mouse brain sera. Furthermore, the presence of small amounts of theta alloantigen and TL antigen on the precursor population cannot be excluded by our methodology. The efficiency of C-mediated cytolysis in eliminating a specific population depends upon many factors including the concentration of antigen present on the cell (40).

Komuro and Boyse (6) and Basch and Goldstein (7) have used the term "prothymocyte" to designate the cells that respond to the presence of thymic hormones or nonspecific inducing agents, by expressing thymic differentiation antigens. Scheid et al. (8) have presented some, and we will shortly offer extensive evidence, that the inducible cells can serve as thymic progenitors in a repopulation assay.

The assay used in the experiments presented in this paper measures the precursors of Thy-1-positive cortical thymocytes. It does not measure the development of functional T cells. El-Arini and Osoba (41) have used a functional test (mixed leukocyte reactivity) to describe a low density, Thy-1-resistant, nonreactive cell found in BM which could develop into a Thy-1-positive mixed leukocyte-reactive cell in an irradiated host. Although their data did not indicate that thymic passage was necessary for the maturation of those cells, we believe them to be the same as the cells measured in our assay. Rubin et al. (26) have reported that passage of bone marrow cells through

columns of glass beads coated with the mouse immunoglobulin alone or in combination with RaMIG, selectively removed T-cell precursors as indicated by depressed graft-vs.-host and/or helper activity in irradiated and repopulated mice. Although the effect was not seen in all experiments they concluded that the thymic precursor was either Ig or Fc positive. We cannot explain the discrepancy between their results and our own except to note that different assays were used and that they did not rule out the possibility that the functional activities observed were due to the presence of cells descended from Fc-positive T cells in the original marrow samples (41).

Our assay does not distinguish between functionally or antigenically discrete subsets of thymocytes or T cells (42–44). Although all of these cells presumably arise from a common precursor there is little evidence as to when in their life history commitment to their ultimate fate occurs. It is possible that this occurs early in their development, perhaps during their nomadic prethymic existence. While this would not vitiate the general description which we have attempted, it would add the further complexity that our data may be describing the aggregated properties of an already heterogeneous population.

The data presented and the work cited above clearly indicate the existence of cells capable of serving as the progenitors of cortical thymocytes. We believe that the majority of the cells measured in the assay are committed cells, destined for no other role but maturation into thymocytes and thymus-derived cells. It is not possible at this time to eliminate totally the possibility that these cells are "pro-immunocytes," capable of differentiating into either T or B cells depending on the environment in which they find themselves. Although they have been the subject of much discussion, little evidence has been presented which indicates that such cells actually exist. It is true that the precursors of both T and B cells are both found amongst the "null" cells of the spleen (45) and share many physical properties (46). It is also true that cells simultaneously expressing both T- and B-cell markers do exist (36, 47, 48) indicating that the developmental programs are not mutually exclusive. On the other hand, B-cell progenitors have a different tissue distribution than prothymocytes (46). They also appear to become committed to immunoglobulin production before they express it on their surface (49, 50), while prothymocytes bear receptors and antigens associated with the T-cell lineage. The question remains, but the possibility of producing highly enriched populations of thymocyte precursors which respond to the thymic peptide, thymopoietin (7), should facilitate its resolution.

The relationship of the prothymocyte to the multipotential hematopoietic stem cell also remains to be explored. Although minor differences in density distribution and susceptibility to self-sterilization with [^{3}H]TdR can be demonstrated, no physical separation of these cells has been achieved. We do not believe them to be identical. The data is best explained by assuming that they are related to each other, either as parent and progeny, or as the offspring of a common progenitor. The fact that both can be eliminated by anti-mouse brain antisera which have been absorbed by thymus is intriguing, but given the complexity of that reagent it is premature to conclude that the same antigen is

present on both cells. A major distinguishing feature between prothymocytes and CFUs is the fact that the former can be induced to express thymocyte differentiation antigens without influencing the number or distribution of the latter (51).

We have already noted that thymic hormone-responsive cells are capable of repopulating the thymus. It is not certain that all cells capable of repopulating the thymus are inducible with thymic hormones. If the prothymocytes in spleen or BM cell preparations are induced to express the Thy-1 antigen by thymopoietin and then eliminated with anti-Thy-1 antisera, thymic repopulation is retarded but not eliminated (reference 8; and Basch and Goldstein, unpublished results). There thus appears to be another stem cell compartment capable of either replacing or replenishing the prothymocyte pool. This population can be detected in our repopulation assay. The kinetics of repopulation indicate that they make a negligible contribution to the early phases of repopulation, but their role in other aspects of thymic development remains to be assessed.

Summary

The properties of hematopoietic cells which serve as the precursors of cortical thymocytes in irradiated reconstituted mice have been described. These cells have been termed "prothymocytes." They are 10- to 15-μm diameter cells of low buoyant density. They are nonadherent to glass wool and more resistant to the lytic effects of steroids and γ-irradiation than their progeny. They lack detectable amounts of the surface markers associated with either B or T cells but do bear at least two antigens recognized by antisera to mouse brain.

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References

1. Harris, J. E., D. W. H. Barnes, C. F. Ford, and E. P. Evans. 1964. Cellular traffic of the thymus experiments with chromosome markers. Evidence from parabiosis for an afferent stream of cells. *Nature (Lond.).* **201**:886.
2. Ford, C. E., H. S. Micklem, E. P. Evans, J. G. Gray, and D. A. Ogden. 1966. The inflow of bone marrow cells to the thymus studies with part body irradiated mice injected with chromosome marked bone marrow and subjected to antigenic stimulation. *Ann. N. Y. Acad. Sci.* **129**:283.
3. Gengozian, N., I. S. Urso, C. C. Congdon, A. D. Conger, and T. Makinodan. 1957. Thymus specificity in lethally irradiated mice treated with rat bone marrow. *Proc. Soc. Exp. Biol. Med.* **96**:714.
4. Wu, A. M., J. E. Till, L. Siminovitch, and E. A. McCulloch. 1968. Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. *J. Exp. Med.* **127**:455.
5. Nowell, P. C., B. E. Hirsch, D. H. Fox, and D. B. Wilson. 1970. Evidence for the existence of multipotential lympho-hematopoietic stem cells in the adult rat. *J. Cell. Physiol.* **75**:151.
6. Komuro, K., and E. A. Boyse. 1973. In vitro demonstration of thymic hormone by the mouse by conversion of precursor cells into lymphocytes. *Lancet.* **1**:740.

7. Basch, R. S., and G. Goldstein. 1974. Induction of T cell differentiation in vitro by thymin, a purified polypeptide hormone of the thymus. *Proc. Natl. Acad. Sci. U. S. A.* 71:1474.
8. Scheid, M., G. Goldstein, U. Hammerling, and E. A. Boyse. 1975. Lymphocyte differentiation from precursor cells in vitro. *Ann. N. Y. Acad. Sci.* 249:531.
9. Kadish, J. L., and R. S. Basch. 1976. Hematopoietic thymocyte precursors. I. Assay and kinetics of the appearance of progeny. *J. Exp. Med.* 143:1082.
10. Acton, R. T., E. P. Blankenhorn, T. C. Douglas, R. D. Owen, J. Hilgers, H. A. Hoffman, and E. A. Boyse. 1973. Variations among sublines of inbred AKR mice. *Nat. New Biol.* 245:8.
11. Golub, E. S. 1971. Brain-association θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* 2:353.
12. Pretlow, T. G., and C. W. Boone. 1969. Separation of mammalian cells using programmed gradient sedimentation. *Exp. Mol. Pathol.* 11:139.
13. Till, J. E., and E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14:213.
14. Trizio, D., and G. Cudkowicz. 1974. Separation of T and B lymphocytes by nylon wool columns: evaluation of efficiency by functional assays in vivo. *J. Immunol.* 113:1093.
15. Bianco, C., R. Patrick, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterisation. *J. Exp. Med.* 132:702.
16. Parrish, C. R., S. M. Kirov, N. Bowern, and R. V. Blanden. 1974. A one step procedure for separating mouse T and B lymphocytes. *Eur. J. Immunol.* 4:803.
17. Ramasamy, R., and A. J. Munro. 1974. Surface T and B cell markers on murine lymphomas and plasmacytomas. *Immunology.* 26:563.
18. Boyum, A. 1968. Ficoll-Hypaque separation of blood mononuclear cells. *Scand. J. Clin. Lab. Invest. Suppl.* 21:51.
19. Becker, A. J., E. A. McCulloch, L. Siminovitch, and J. E. Till. 1965. The effect of differing demands for blood cell production on DNA synthesis by hematopoietic colony-forming cells of mice. *Blood.* 26:296.
20. Kadish, J. L., and R. S. Basch. 1975. Thymic regeneration after lethal irradiation: evidence for an intrathymic radioresistant T cell precursor. *J. Immunol.* 114:452.
21. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distibution in leukemias and nervous tissues. *J. Exp. Med.* 120:413.
22. Raff, M. C., and H. Wortis. 1970. Thymus dependence of θ-bearing cells in the peripheral lymphoid tissues of mice. *Immunology.* 18:929.
23. Golub, E. S., and E. D. Day. 1973. Brain associated erythrocyte antigen: an antigen shared by brain and erythrocytes. *Exp. Hematol. (Copenh.).* 1:105.
24. Golub, E. S. 1972. Brain associated stem cell antigen: an antigen shared by brain and hematopoietic stem cells. *J. Exp. Med.* 136:369.
25. Jones, V. E., H. E. Graves, and E. Orlans. 1976. The detection of $F(ab')_2$ related surface antigens on the thymocytes of children. *Immunology.* 30:281.
26. Rubin, B., L. Trier, B. Hertel-Wulff, and B. Rumle. 1975. Selective retention of T cell precursors in bone marrow cell populations after filtration through anti-immunoglobulin columns. *Cell. Immunol.* 16:315.
27. Dickler, H. B., and H. G. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* 136:191.
28. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1972. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. *J. Exp. Med.* 135:610.
29. Huber, H., S. D. Douglas, and H. H. Trudenberg. 1969. The IgG receptor: an immunological marker for the characterisation of mononuclear cells. *J. Immunol.* 17:7.

30. Anderson, C. L., and H. M. Grey. 1974. Receptors for IgG on mouse lymphocytes, their presence on thymocytes, thymus-derived, and bone marrow-derived lymphocytes. *J. Exp. Med.* **139**:1175.
31. Moore, M. A. S., and J. J. T. Owen. 1967. Experimental studies on the development of the thymus. *J. Exp. Med.* **126**:715.
32. Le Douarin, N. M., and F. V. Jotereau. 1975. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *J. Exp. Med.* **142**:17.
33. Owen, J. J. T., and M. C. Raff. 1970. Studies on the differentiation of thymus-derived lymphocytes. *J. Exp. Med.* **132**:1216.
34. Loor, F., and G. E. Roelants. 1975. Immunofluorescence studies of a possible prothymic T cell differentiation in congenitally athymic (nude) mice. *Ann. N. Y. Acad. Sci.* **254**:226.
35. Roelants, G. E., F. Loor, H. von Boehmer, J. Sprent, L. B. Hagg, K. S. Mayor, and A. Ryden. 1975. Five types of lymphocytes ($Ig^-\theta^-$, $Ig^-\theta^+$ weak, $Ig^-\theta^+$ strong, $Ig^+\theta^-$ and $Ig^+\theta^+$) characterised by double immunofluorescence and electrophoretic mobility organ distribution in normal and nude mice. *Eur. J. Immunol.* **5**:127.
36. Roelants, G. E., K. S. Mayor, L. B. Hagg, and F. Loor. 1976. Immature T lineage lymphocytes in athymic mice. Presence of TL, lifespan and homeostatic regulation. *Eur. J. Immunol.* **6**:75.
37. Esselman, W. J., and H. C. Miller. 1974. Brain and thymus lipid inhibition of antibrain-associated θ-cytotoxicity. *J. Exp. Med.* **139**:445.
38. Letarte-Muirhead, M., A. N. Barclay, and A. F. Williams. 1975. Purification of the Thy-1 molecule, a major cell surface glycoprotein of rat thymocytes. *Biochem. J.* **151**:685.
39. Santana, V., and J. L. Turk. 1976. Binding of heterologous anti-brain antibodies to mouse B cells. *Immunology.* **30**:859.
40. Basch, R. S. 1974. Effects of antigen density and non-complement fixing antibody on cytolysis by alloantisera. *J. Immunol.* **113**:554.
41. El-Arini, M. O., and D. Osoba. 1973. Differentiation of thymus-derived cells from precursors in bone marrow. *J. Exp. Med.* **137**:825.
42. Cantor, H., E. Simpson, V. L. Sato, C. G. Fathman, and L. A. Herzenberg. 1975. Subpopulations of T cells distinguished by different amounts of Thy-1.2 (theta) antigen. I. Separation and functional studies of peripheral T cells binding different amounts of fluorescent anti-Thy-1.2 (theta) antibody using a fluorescence activated cell sorter (FACS). *Cell. Immunol.* **15**:180.
43. Stobo, J. D., and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subsets. *J. Immunol.* **110**:362.
44. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141**:1376.
45. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface θ or immunoglobulin determinants. *J. Exp. Med.* **138**:71.
46. Lafleur, L., R. G. Miller, and R. A. Phillips. 1972. A quantitative assay for the progenitors of bone marrow-associated lymphocytes. *J. Exp. Med.* **135**:1363.
47. Greenberg, R. S., and M. M. Zatz. 1975. Spontaneous AKR lymphoma with T and B cell characteristics. *Nature (Lond.).* **257**:314.
48. Dickler, H. B., N. F. Adkinson, Jr., and W. D. Terry. 1974. Evidence for individual human peripheral blood lymphocytes bearing both B and T cell markers. *Nature (Lond.).* **247**:213.

49. Nossal, G. J. V., and B. L. Pike. 1972. Differentiation of B lymphocytes from stem cells. *Adv. Exp. Med. Biol.* **29**:11.
50. Raff, M. C., M. Megson, J. J. T. Owen, and M. D. Cooper. 1976. Early production of intracellular IgM in B lymphocyte precursors in mouse. *Nature (Lond.)*. **259**:224.
51. Basch, R. S., and G. Goldstein. 1975. Antigenic and functional evidence for the in vitro inductive activity of thymopoietin (thymin) on thymocyte precursors. *Ann. N. Y. Acad. Sci.* **249**:290.