IMMUNOLOGIC PROPERTIES OF MOUSE THYMUS CELLS

IDENTIFICATION OF T CELL FUNCTIONS WITHIN A MINOR, LOW-DENSITY SUBPOPULATION*

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In the first paper of this series¹ it was shown that the mouse thymus cell population is heterogeneous. Two general cell subpopulations were identifiable on the basis of average density and membrane antigen representation. The majority cell subpopulation was of high density in albumin gradients and had high TL, G₁X, θ, and Ly antigen content but was low in H-2. A minor subpopulation, comprising less than 10% of all cells, was of low density and low thymus antigen content but high in H-2. Evidence was developed that this latter population is similar to that shown by others to be cortisone- (1-8) or irradiation-resistant (2). In this report the minor, low-density subpopulation is examined with respect to primary alloantigen recognition, both in vitro and in vivo, and to other attributes which appear to identify it as a relatively “pure” population of T cells.

Materials and Methods

Animals.—Female CBA, A/J, C57BL/6, and various F₁ hybrid mice employed ranged from 6-12 wk of age. These animals were either obtained directly from Jackson Laboratory, Bar Harbor, Maine, or derived from inbred lines maintained in this laboratory, originating from Jackson stocks. Congenic C57BL/6-TL⁺ and C57BL/6-TL⁻ were generously supplied by Dr. E. A. Boyse of the Sloan-Kettering Institute for Cancer Research, New York.

Preparation of Cells.—Thymus or spleen cells were taken after exsanguination via the abdominal aorta. As described previously,¹ special care was taken to exclude lymph nodes adjacent to thymus. Suspensions were prepared by gently pressing small fragments, suspended

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in RPMI-1640 (Grand Island Biological Company, Grand Island, N. Y.), through 60-mesh, stainless steel screens and passing the resultant suspension through 23- and 25-gauge needles.

**Bovine Serum Albumin Gradient Separation.**—Bovine serum albumin (BSA) gradients were prepared by the method described in the previous paper. Concentrations of the various gradients for thymus cells were as follows: A layer, that above 23%; B layer, above 27%; C layer, above 29%; D layer, above 35%. The gradients for spleen cells were: A layer, that above 23%; B layer, above 26%; C layer, above 29%; D layer, above 35%.

**Preparation of Alloantiserum.**—Anti-0 C3H serum was prepared by repeated intraperitoneal injection of female CBA thymus cells into AKR females. Anti-H-2\(b\) was prepared and tested as described. Anti-TL serum, prepared by injecting ASLI, and A-strain, TL\(\pm\), spontaneous leukemia, into congenic TL\(\pm\) A-strain mice, was also obtained from Dr. E. A. Boyse.

**Treatment of Thymus Cells with Various Antisera and Complement.**—2 \(\times\) 10^6 thymus cells, suspended in 10 ml of RPMI-1640, were incubated with 10 ml of antiserum, diluted with RPMI-1640, and 10 ml of complement at 37°C for 45 min. After washing three times with RPMI-1640 the cells were resuspended in medium. Viability was ascertained by trypan blue exclusion after incubation and washing. Lyophilized guinea pig serum (Grand Island Biological Company) was used as a source of complement. This serum was diluted 1:5 with RPMI-1640 and absorbed with 80 mg/5 ml special agar-Noble (Difco Labs., Detroit, Mich.) for 1 hr on ice, according to the method of Cohen and Schlesinger (9). The mean survival of C57BL/6 cells in these experiments was 12.6% (range: 10.5-16.5%) after treatment with anti-0 C3H, diluted 1:100. Treatment of C57BL/6 thymus cells with anti-H-2\(b\), diluted 1:20, gave survival ranging from 60 to 63%. Survival of C57BL/6-TL \(\pm\) cells ranged from 25 to 28% after treatment with anti-TL, diluted 1:100.

**Cortisone Treatment.**—Cortisone treatment consisted of an intraperitoneal injection of 2.5 mg of cortisone acetate (Cortone; Merck, Sharpe & Dohme, West Point, Pa.) per 20 g body weight 2 days before collection of thymus and spleen cells.

**Graft-versus-host Reactions.**—Adult female C57BL/6 thymus cells or CBA thymus or spleen cells were injected intraperitoneally into newborn (CBA X C57BL/6)F1 or (CBA X A/J)F1 mice. 8 days after cell injection the recipients were sacrificed, and spleen weights were determined and expressed as milligrams per 100 g body weight. Controls consisted of littermates injected with 0.1 ml of RPMI-1640. The spleen index was calculated as the ratio of spleen weight/100 g body weight in experimental to that of controls. An index over 1.30 was taken as evidence of a positive graft-versus-host (GVH) reaction (10).

**In Vitro Assays.**—RPMI-1640 culture medium, to which 100 units of penicillin and 100 \(\mu\)g of streptomycin/ml with 5% heated (56°C, 30 min) human serum obtained from a single donor had been added, was employed throughout these experiments. The technique, slightly modified from that previously reported from this laboratory (11), was as follows: 1-2 \(\times\) 10^6 reacting cells were distributed to each tube (12 \(\times\) 75 mm polypropylene tubes; No. 2063 Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) containing 0.5 ml of culture medium, incubated at 37°C, loosely capped, in a 5% CO\(_2\) atmosphere at 80% humidity for varying periods of time.

For PHA or LPS stimulation 1.0 \(\mu\)l of phytohemagglutinin (PHA-P; Difco Labs.) or 10 \(\mu\)g of *Salmonella typhimurium* type-7 endotoxin (LPS) (obtained from Dr. J. W. Shands, Department of Immunology and Medical Microbiology, University of Florida College of Medicine), prepared according to the method of von Westphal et al. (12), was added to each tube containing 2 \(\times\) 10^6 cells and cultured for 48 hr. For mixed leukocyte cultures target cells were prepared by incubating 15 \(\times\) 10^6 spleen cells/0.5 ml with 50 \(\mu\)g mitomycin-C/0.1 ml (Nutritional Biochemicals Corporation, Cleveland, Ohio) at 37°C for 30 min. The cells were then washed three times with RPMI-1640 and resuspended in culture medium at a

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2 **Abbreviations used in this paper:** BSA, bovine serum albumin; GVH, graft-versus-host; LPS, *Salmonella typhimurium* type-7 endotoxin (lipopolysaccharide); PHA, phytohemagglutinin.
concentration of $4 \times 10^6$/ml. 1 million target cells in 0.25 ml were added to each tube (containing $1 \times 10^6$ reacting cells/0.25 ml) before incubation and incubated for 72 hr.

Assay of thymidine-$^3$H incorporation was made by adding 0.5 $\mu$Ci of tritiated thymidine (Schwarz Bio Research, Orangeburg, N. Y.; specific reactivity, 1.9 Ci/mole, 1 $\mu$Ci/2 $\mu$l) to each tube for the final 24 hr of culture. At the end of the appropriate incubation period the tubes were centrifuged at 1500 rpm, and the cell pellet was washed twice with 0.15 $\mu$NaCl and twice with trichloroacetic acid at 4°C. The resultant precipitate was washed once with cold absolute methanol, air dried, and allowed to dissolve in 0.25 ml of Nuclear-Chicago solubilizer (Nuclear-Chicago Corporation, Des Plaines, Ill.) for 2 hr at 45°C. 12 ml of scintillation fluid, $\beta$-bis-2(5-phenyloxazolyl)benzene 100 mg/ml, and 2,5-diphenyloxazole 5 g/liter in toluene, were then added to the soluble precipitate. The solution was transferred to screw-top vials and counted in a Beckman Model LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) after 24 hr accommodation. The data obtained are presented as mean values ± standard error (SE) for at least four replicate tubes. Controls for mixed leukocyte cultures included tubes containing reacting cells plus syngeneic mitomycin-treated target cells.

RESULTS

Functional Attributes of Thymus Subpopulations Separated on BSA Density Gradients.—Separation of thymus cells in discontinuous BSA gradients has been shown to yield a low-density, low $\theta$-antigen-bearing subpopulation in which the cooperative recognition effect attributable to thymus cells and necessary to an antibody response to sheep erythrocytes is concentrated (13). The antigenic properties of this minor subpopulation have been characterized as being high in H-2 but low or completely deficient in the thymus-associated TL and Grx antigens. This subpopulation contained some $\theta$-antigen and Ly-A.2 and thus resembled, except in density, the T lymphocyte population in the periphery, rather than the majority of cells in the thymus. It was of interest to examine this subpopulation further with respect to established T cell functions. Primary alloantigen recognition was, therefore, measured in vivo by the GVH reactions evoked by various fractions of cells and in vitro by one-way mixed lymphocyte cultures in which cells from the various fractions were incubated with allogeneic target cells. Data illustrating the results of in vivo experiments are given in Table I.

Cells from CBA whole thymus cell populations gave positive GVH reactions in very few instances. Regardless of the cell dose administered to the (CBA $\times$ A/J)F$_1$ hybrids, mean values were below significance according to Simonsen standards. The slightly lower incidence of GVH reactivity in those receiving larger rather than smaller numbers of whole thymus cells is unexplained. Significant GVH occurred when whole C57BL/6 thymus cells were given to (CBA $\times$ C57BL/6)F$_1$ hybrids. Graft-versus-host reactions in (CBA $\times$ A/J)F$_1$ hybrids which received BSA gradient subpopulations of CBA thymus cells were positive only in those groups which received the B layer. This was evident in the degree of positivity, the proportion of positive reactions, and the average values at a high level of significance as compared with those of the other fractions. Whole spleen cells evoked strong GVH reactions, as would be anticipated.
# THYMUS T CELL FUNCTIONS

## TABLE I

| Source of injected cells | Mean % ± se cells in fraction | No. of cells injected (X 10^6) | Spleen index | No. positive/No. tested |
|--------------------------|--------------------------------|--------------------------------|--------------|-------------------------|
| Whole thymus             | 16.3 ± 2.5                     | 1.43, 1.24, 1.13, 1.01, 0.98, 0.97, 0.94, 0.90 | 1.07 ± 0.06 | 34                       |
| Thymus cell fractions    |                                |                                |              |                         |
| B                        | 35.6 ± 2.9                     | 1.96, 1.81, 1.48, 1.44, 1.33, 1.32 | 1.55 ± 0.10 | 24                       |
| C                        | 36.9 ± 3.7                     | 1.27, 1.25, 1.14, 1.05, 1.04 | 1.15 ± 0.04 | 24                       |
| D                        | 1.26, 1.14, 1.12, 1.09, 1.05, 1.02 | 1.11 ± 0.03 | 24                       |
| Whole spleen             | 10.40, 2.10, 1.88, 1.87, 1.83, 1.79, 1.71, 1.70, 1.43, 1.28, 1.19 | 1.69 ± 0.09 | 1013                      |
| Spleen cell fractions    | 12.5 ± 2.8                     | 1.48, 1.42, 1.41, 1.29, 1.09, 1.07, 0.80 | 1.22 ± 0.09 | 34                       |
| A                        | 42.3 ± 3.8                     | 1.46, 1.32, 1.19, 1.17, 1.02, 0.72 | 1.14 ± 0.10 | 36                       |
| B                        | 39.3 ± 3.6                     | 1.62, 1.54, 1.46, 1.34, 1.32, 1.27, 1.19 | 1.39 ± 0.05 | 24                       |
| C                        | 5.8 ± 1.2                      | 1.60, 1.56, 1.53, 1.46, 1.45, 1.30, 0.86 | 1.39 ± 0.09 | 24                       |

* CBA whole spleen or thymus cells, or BSA gradient cell fractions thereof, were injected i.p. into newborn (CBA × A)F₁ hybrid mice, and the spleen index was assayed 8 days later. Those indices greater than 1.30 are considered as positive in this assessment.

† A layer (1.3 ± 0.3) and pellet (8.5 ± 2.1) were not tested for GVH reactivity.

This capacity, however, was contained in the denser gradient fractions, C and D, and in this respect differed from the low-density, reactive thymus subpopulations.

The various density subpopulations of the thymus were also tested in vitro.
for susceptibility to alloantigen stimulation by mitomycin-treated target cells as previously reported from this laboratory (14). Table II illustrates results of such studies, in which C57BL/6 thymus cells were examined for in vitro reactivity to mitomycin-blocked CBA spleen cells and the T-dependent mitogen PHA. The data clearly indicate that thymic responsiveness to both PHA and alloantigens is highly concentrated and in some experiments limited to cells in the low-density B layer; reactivity was nearly absent from cells in the C and D layers.

It would appear, then, that the limited capacity of whole thymus to react to alloantigens, both in vivo and in vitro, is related to the representation of a minor subpopulation of B layer-type cells diluted by the more numerous but nonreactive cell populations. The data in Tables II and III also show that the degree of PHA responsiveness in the B layer, although exceeding that of the other fractions and of whole thymus, is less than that found here and previously (15, 16) in whole spleen populations taken from C57BL/6 mice. This suggests

### Table II

**PHA and Primary Alloantigen Stimulation of Whole and BSA Gradient-Separated Subpopulations of C57BL/6 Thymus Cells**

| Experiment | Cells tested | Unstimulated Tdr-3H incorporation – mean ± se | PHA added (μl) | Increment of Tdr-3H incorporation | Ratio: PHA/control | C57BL/6 (mito) target cells | CBA (mito) target cells | Increment of Tdr-3H incorporation | Ratio: CBA/C57BL/6 |
|------------|--------------|-----------------------------------------------|----------------|---------------------------------|-------------------|-----------------------------|-----------------------------|---------------------------------|-------------------|
| a | Whole thymus | 960 ± 34 | 857 ± 74 | 0.9 | 140 ± 16 | 4,117 ± 254 | 4,369 | 30.3 |
| | B layer1 | 665 ± 27 | 1243 ± 117 | 576 | 1.9 | 256 ± 20 | 14,374 ± 447 | 14,098 | 52.0 |
| | C layer | 228 ± 6 | 216 ± 7 | 48 | 1.2 | 195 ± 25 | 951 ± 68 | 756 | 4.8 |
| | D layer | 170 ± 7 | 130 ± 7 | | 0.9 | 123 ± 20 | 144 ± 21 | 21 | 1.1 |
| b | Whole thymus | 3135 ± 143 | 1743 ± 81 | | 0.6 | 350 ± 135 | 6,500 ± 301 | 6,110 | 16.7 |
| | B layer2 | 1371 ± 85 | 5305 ± 239 | 3943 | 3.9 | 663 ± 96 | 27,610 ± 1,711 | 27,007 | 45.8 |
| | C layer | 486 ± 155 | 430 ± 113 | | 0.9 | 202 ± 73 | 872 ± 42 | 670 | 4.3 |
| | D layer | 293 ± 52 | 260 ± 14 | | 0.9 | 114 ± 15 | 1,154 ± 201 | 973 | 8.0 |
| c | Whole thymus | 2007 ± 385 | 1119 ± 133 | | 0.6 | 362 ± 76 | 5,72 ± 152 | 5,590 | 15.9 |
| | B layer3 | 1203 ± 210 | 3599 ± 237 | 2036 | 2.6 | 738 ± 136 | 10,838 ± 1,196 | 10,043 | 21.3 |
| | C layer | 425 ± 91 | 736 ± 109 | 301 | 1.7 | 801 ± 120 | 1,642 ± 144 | 1,539 | 5.6 |
| | D layer | 471 ± 77 | 292 ± 83 | | 0.6 | 249 ± 51 | 1,005 ± 72 | 764 | 4.2 |

* C57BL/6 thymus, either whole or separated in discontinuous BSA density gradients, was cultured with either 1 μl of PHA or 1 × 10⁶ allogeneic mitomycin-treated cells, and the resultant stimulation was assayed in terms of Tdr-3H incorporation after 48 or 72 hr, respectively. The data are given as mean values ± se for four replicate tubes using 1 or 2 × 10⁶ cells in the PHA reactions and 1 × 10⁶ reacting cells in the mitomycin reactions. In experiments b and c 2 × 10⁶ reacting cells were employed for PHA responses.

The distribution of thymus cells in BSA gradients in the three experiments shown was: A layer (above 83%), B layer (above 27%), 16.3 ± 4.6%; C layer (above 29%), 31.3 ± 4.1%; D layer (above 33%), 38.2 ± 3.6%; pellet, 16.3 ± 2.2% (no data given).
that PHA receptors in the membranes of thymus cells may have more limited representation than in similarly reactive cells in the periphery. It was concluded from these experiments that two established thymus-dependent functions of lymphoid cells are found in the minor, low-density thymus subpopulation which has, on the average, low representation of thymus-unique membrane antigens.

TABLE III

PHA and Alloantigen Stimulation of C57BL/6 Spleen Cells and BSA Gradient Subpopulations

| Experiment | Cells tested | Proportion of cells in fraction | Tdr-{superscript}3H incorporation - mean ± SE (1 × 10^6 reacting cells) | Tdr-{superscript}3H incorporation - mean ± SE (1 × 10^6 reacting cells) |
|------------|--------------|---------------------------------|-------------------------------------------------|-------------------------------------------------|
|            |              | Unstimulated | PHA added (1 μl) | Unstimulated | PHA added (1 μl) | Ratio: PHA/CBA |
| 4          | Whole spleen | 626 ± 98     | 9,277 ± 1,916    | 8,651        | 4,379 ± 84     | 10,834 ± 964 |
|            | A layer      | 13           | 2,906 ± 205      | 0.3          | 11,938         | 10,622 ± 659 |
|            | B layer      | 156          | 3,302 ± 193      | 0.3          | 10,622 ± 659  | 10,622 ± 659 |
|            | C layer      | 18           | 11,938 ± 990     | 7.3          | 19,903 ± 831  | 19,903 ± 831 |
| 5          | Whole spleen | 4,379 ± 84   | 16,314 ± 964     | 11,938       | 5,300 ± 147   | 17,640 ± 1,458 |
|            | A layer      | 8            | 13,138 ± 293     | 3.1          | 3.1            | 3.1            |
|            | B layer      | 33           | 3,902 ± 193      | 0.3          | 11,938 ± 990  | 11,938 ± 990 |
|            | C layer      | 47           | 11,938 ± 990     | 7.3          | 19,903 ± 831  | 19,903 ± 831 |
|            | D layer      | 12           | 19,903 ± 831     | 6.5          | 19,903 ± 831  | 19,903 ± 831 |
| 6          | Whole spleen | 6,503 ± 112  | 19,903 ± 831     | 13,400       | 6,503 ± 112   | 19,903 ± 831 |
|            | A layer      | 3            | 14,775 ± 210     | 4.7          | 14,775 ± 210  | 14,775 ± 210 |
|            | B layer      | 22           | 4,436 ± 128      | 8.5          | 4,436 ± 128   | 4,436 ± 128 |
|            | C layer      | 59           | 12,873 ± 475     | 7.3          | 12,873 ± 475  | 12,873 ± 475 |
|            | D layer      | 14           | 23,737 ± 938     | 6.5          | 23,737 ± 938  | 23,737 ± 938 |

* In these experiments the reactions of C57BL/6 spleen cells, or those of pooled A + B and C + D BSA gradient fractions thereof, were tested with either 1 μl of PHA per culture or mitomycin-treated target cells. The PHA culture period totaled 48 hr, and the alloantigen culture period was 72 hr.

and relatively great representation of membrane antigens found on T lymphocytes in the peripheral lymphoid system.

Functional Attributes of Thymus Cell Subpopulations Resistant to Treatment with Various Antisera and Complement.—In the previous paper it was found that a variable population of thymus cells remained after treatment of whole thymus with relatively dilute anti-β, anti-TL, or anti-H-2. In each case a residual population retained reactivity to the other antisera, indicating some inde-
pendence in representation of susceptible antigenic structures in the membranes of the various thymus cell subpopulations. The functional characteristics of alloantigen recognition in vitro and in vivo and PHA responsiveness in sub-populations derived in a similar fashion were then examined.

Tables IV, V, and VI illustrate experiments in which whole C57BL/6 thymus cells were treated with anti-TL, -θ, or -H-2b in various experiments. The functional capacity of varying numbers of the resultant cell populations was then evaluated in terms of the ability to evoke GVH reactions in (CBA × C57BL/6)F1 hybrids, to recognize mitomycin-C-treated CBA alloantigens in vitro, or to be stimulated by PHA.

Table IV shows that GVH reactivity evoked by $2 \times 10^7$ C57BL/6 whole cells.
TABLE V

PHA and Primary Alloantigen Responses of Cells Surviving Treatment of C57BL/6 Thymus Cells with Anti-0 or Anti-H-2b and Complement*

| Experiment | Treatment of thymus cells tested | Survival after antiseraum treatment | Tdr-3H incorporation — mean ± SE | Tdr-3H incorporation — mean ± SE |
|------------|----------------------------------|-----------------------------------|---------------------------------|---------------------------------|
|            |                                  | Unstimulated | PHA added (1 μL) | Increment of incorporation | Ratio: PHA/control | C57BL/6 (mito) target cells | CBA (mito) target cells | Increment of incorporation | Ratio: CBA/C57BL/6 |
| a          | Untreated Anti-0 (1:100)         | 18%          | 916 ± 34        | 2260 ± 970         | 875 ± 74          | 10,785 ± 802       | 8,327 ± 4,8        | 4.8                      | 1488 ± 1809         | 4,517 ± 254       | 4369 ± 29,529     | 9.5 |
|            | Anti-H-2b (1:20)                | 61.5%        | 117 ± 7          | 260 ± 7            | 71 ± 3            | 220 ± 7           | 143 ± 9            | 6.7                      | 141 ± 19            | 1,150 ± 177       | 2,018 ± 27,81     | 27.8 |
| b          | Untreated Anti-0 (1:100)         | 6%           | 460 ± 21        | 200 ± 26          | 851 ± 181        | 1,685 ± 99        | 1,415 ± 6.7        | 1.9                      | 141 ± 19            | 6,609 ± 1,009     | 6371 ± 28,13     | 27.8 |
|            | Anti-H-2b (1:20)                | 60%          | 124 ± 2          | 333 ± 11          | 9 ± 1.1           | 184 ± 33          | 139 ± 18           | —                        | —                   | 9.53 ± 0.8        | —               | —               |
| c          | Untreated Anti-0 (1:100)         | 10%          | 490 ± 61        | 1,356 ± 102       | 33,138 ± 2,953   | 866 ± 17.7        | 96 ± 5             | 2.8                      | 997 ± 74            | 15,010 ± 1,839    | 17,103 ± 20.1     | 20.1 |
| d          | Untreated Anti-0 (1:100)         | 11.5%        | 348 ± 23        | 737 ± 168         | 437 ± 28          | 8,965 ± 533       | 8,240 ± 12.4       | 1.3                      | 221 ± 121           | 661 ± 63          | 440 ± 3.0         | 7.2 |
|            | Anti-H-2b (1:20)                | 66%          | 141 ± 21        | 98 ± 3            | 98 ± 3            | 123 ± 6           | 171 ± 12           | —                        | —                   | 131 ± 15          | 762 ± 98          | 631 ± 5.8 |
| e          | Anti-0 (1:200)                  | 20.5%        | 206 ± 23        | 703 ± 106         | 503 ± 5.5         | 131 ± 15          | 762 ± 98           | 1.4                      | —                   | —               | —               | —               |

* C57BL/6 thymus cells were either treated with the indicated dilutions of anti-0 C3H or anti-H-2b plus complement (1:5), washed, and the surviving subpopulation stimulated either by PHA (1 μL/tube) or mitomycin-treated syngeneic or allogeneic (CBA) cell preparations. Tdr-3H incorporation was assayed after 48 or 72 hr, respectively, and the data are expressed as mean incorporation values ± SE for four replicate tubes.

thymus cells was strong and significant, as compared with insignificant reactions after 5 × 10⁶ cells. After treatment with anti-TL and complement the residual subpopulation evoked significant GVH reactions in the F₁ host at cell doses one-fifth to one-fourth those required for whole thymus. After administration of as few as 2 × 10⁶ of the survivors of anti-0 treatment, GVH reactions were highly significant.

Thus, it may be roughly calculated that after treatment with anti-TL the residual cell population had approximately the same GVH-inducing powers as 2 × 10⁶ whole thymus cells, and anti-0 treatment gave residual populations having the GVH equivalent of 5 × 10⁶ whole thymus cells. In contrast, 3 × 10⁷ H-2b-treated cells failed to evoke any GVH reactions. These data strongly suggest that the subpopulation effective in evoking GVH reactions was rela-


**TABLE VI**

PHA and Primary Alloantigen Responses of Cells Surviving Treatment of C57BL/6/TL+ Thymus Cells with Anti-TL and Complement*

| Treatment of thymus cells tested | Survival after anti-TL treatment | TdR-3H incorporation = mean ± se | TdR-3H incorporation = mean + se |
|----------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                  |                                 | 2 × 10^6 reacting cells          | 1 × 10^6 reacting cells          |
|                                  | Unstimulated                    |                                 |                                 |
| a Un-treated                    | 336 ± 10                        | 183 ± 8                         | 0.5 106 ± 2 165 ± 52             | 1.1 |
| Anti-TL                         | 24 406 ± 39 2918 ± 152 2512 7.2 | 97 ± 8 1,454 ± 152 1,357 15.01 |
| b Un-treated                    | 366 ± 15 380 ± 22                | 1.0 134 ± 9 1,866 ± 160 1,738 13.0 |
| Anti-TL                         | 25 218 ± 16 2002 ± 146 1744 7.8 | 108 ± 8 2,057 ± 76 1,949 19.1 |
| c Un-treated                    | 2396 ± 98 638 ± 53               | 0.3 328 ± 35 2,882 ± 218 2,554 8.8 |
| Anti-TL                         | 28 2016 ± 163 3110 ± 177 3054 2.5 | 341 ± 60 14,100 ± 407 13,359 26.1 |
|                                 | 28 1973 ± 186 4738 ± 241 2763 2.4 | 607 ± 53 13,719 ± 480 13,112 22.6 |
|                                 | 28 1697 ± 117 4652 ± 156 1705 2.6 | 347 ± 111 14,254 ± 129 13,707 20.0 |

* Thymus cells taken from C57BL/6-TL+ mice were incubated with anti-TL (1:100) plus 1:5 guinea pig complement for 45 min at 37°C, and the indicated proportions of surviving cells were washed and tested for stimulation by PHA or mitomycin-treated allogeneic (CBA) or syngeneic cells. In each experiment shown the number of reacting cells was 1 or 2 × 10^6 per tube as indicated. TdR-3H incorporation was assayed after 48 (PHA) or 72 hr (alloantigen) incubation, and the data are expressed as mean values ± se per tube for four replicate tubes.

By a similar approach the effects of anti-θ, -H-2b, and -TL on the capabilities of residual thymus cells to be stimulated by PHA and by alloantigens were assessed. These experiments are illustrated by data given in Tables V and VI. As with GVH reactions, PHA and alloantigen stimulation of whole thymus, whether expressed as absolute incorporation per 10^6 cells, increment of incorporation, or ratio, was increased by treatment with 1:100 anti-TL or anti-θ. In contrast, the subpopulation remaining after treatment with anti-H-2b and complement had no residual function.

Anti-θ, diluted 1:100, appeared to have the effect of concentrating PHA- and alloantigen-reactive cells in the thymus by eliminating 90% of cells, the high-θ majority cell subpopulation. At 1:200 dilution, however, anti-θ was much less effective in concentrating these functional activities above the activity in whole thymus, even though 70% of the treated population was killed. At high concentrations of anti-θ (1:20–1:40) the low-θ functional cells did not survive to be tested for their activity. These data show that θ is probably necessary to T cell functions but that it is a quantitative requirement.
**PHA and Primary Alloantigen Responses of Thymus or Spleen Cells Taken from Cortisone-Treated Animals.**—Membrane antigen patterns found in B layer density gradient subpopulations closely resemble those of the population which remains after cortisone treatment or irradiation. It was of interest to determine whether functional characteristics of these subpopulations were also identical. The reactivity of thymus cells from cortisone-treated and control animals was compared with respect to PHA and to alloantigen stimulation; similar determinations were made on spleen cells from the same animals. In addition, LPS reactivity, a non-thymus-dependent function, was also examined. Data illustrating findings in these experiments are shown in Tables VII and VIII.

PHA and alloantigen responsiveness of thymus cells was concentrated by cortisone treatment, whether judged by absolute incorporation per 10⁶ cells, increment of incorporation, or the ratio. By comparison, spleen cells from the same animals were unaffected in their capacity to respond to PHA or to be stimulated by alloantigens.

**TABLE VII**

**PHA and Primary Alloantigen Responses of Thymus Cells Taken from Cortisone-Treated C57BL/6 Mice**

| Experiment | Group tested | Mean No. of thymus cells per mouse (× 10⁶) | Tdr-³H incorporation — mean ± se | Tdr-³H incorporation — mean ± se | Ratio | Ratio |
|------------|--------------|----------------------------------------|---------------------------------|---------------------------------|-------|-------|
| a          | Un-treated   | 224                                    | 1548 ± 118                      | 472 ± 48                        | 0.3   | 950 ± 114 |
|            | Cortisone    | 231                                    | 1537 ± 68                       | 10,387 ± 848                    | 9.00  | 16,606 ± 114 |
| b          | Un-treated   | 133.7                                  | 510 ± 17                        | 1,356 ± 102                     | 2.8   | 24,499 ± 114 |
|            | Cortisone    | 10.6                                   | 707 ± 21                        | 25,206 ± 3,325                  | 32.9  | 16,135 ± 114 |
| c          | Un-treated   | 153                                    | 2129 ± 56                       | 716 ± 36                        | 0.3   | 303 ± 107 |
|            | Cortisone    | 5.23                                   | 862 ± 103                       | 4,647 ± 804                     | 5.4   | 6,650 ± 426 |
| d          | Un-treated   | 148                                    | 782 ± 141                       | 1,722 ± 107                     | 3.8   | 204 ± 20 |
|            | Cortisone    | 8.2                                    | 2169 ± 112                      | 18,280 ± 2,617                  | 8.7   | 1157 ± 215 |

* C57BL/6 mice were given 2.5 mg cortisone/20 g body weight; 2 days later the number of thymus cells was counted, and the cells were assayed in comparison to cells from untreated controls for response to PHA (1 μl) or mitomycin-treated allogeneic (CBA) and syngeneic target cells. Stimulation was estimated by incorporation of Tdr-³H after 48 or 72 hr of incubation and expressed as mean values ± se for four replicate tubes. In experiment a, the data represent parallel assays in duplicate or triPLICATE of a single pool of thymus cells.
TABLE VIII
LPS, PHA, and Primary Alloantigen Responses of Spleen Cells Taken from Cortisone-Treated C57BL/6 Mice

| Experiment | Group tested | Mean No. of spleen cells per mouse (X 10^6) | Tdr[3H] incorporation – mean ± se 2 X 10^6 reacting cells | Tdr[3H] incorporation – mean ± se 1 X 10^6 reacting cells |
|------------|--------------|--------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
|            |              | Unstimulated | LPS added (10 μg) | Increment of incorporation | Ratio: PHA/ control | PHA added (1 μl) | Increment of incorporation | Ratio: CBA/ control | CSBL/6 (mito) target cells | CBA (mito) target cells | Increment of incorporation | Ratio: CBA/ CSBL/6 |
| a          | Untreated    | 122.0        | —                | —                          | —                  | —                | —                          | —                  | 2529 ± 920                | 14,551 ± 2,184           | 12,022 ± 5,895           | 5.8                          |
|            | Cortisone    | 42.2         | —                | —                          | —                  | —                | —                          | —                  | 3522 ± 204                | 20,767 ± 2,042           | 10,935 ± 5,422           | 5.4                          |
| b          | Untreated    | 109.0        | 2771 ± 358       | 29.550 ± 1,443            | 30,829             | 10.8             | 2,832 ± 529                | 111                | 1.0                       | —                          | —                          | —                          |
|            | Cortisone    | 43.6         | 1711 ± 134       | 16,646 ± 1,142            | 14,935             | 9.7              | 6,384 ± 1,505              | 467                | 3.7                       | —                          | —                          | —                          |
| c          | Untreated    | 98.0         | 2507 ± 282       | 22.370 ± 764              | 19,863             | 8.9              | 7,321 ± 517                | 4814               | 2.9                       | —                          | —                          | —                          |
|            | Cortisone    | 37.2         | 4865 ± 282       | 34,666 ± 696              | 29,821             | 7.1              | 10,277 ± 1,052             | 5412               | 2.1                       | —                          | —                          | —                          |
| d          | Untreated    | 92.0         | 4662 ± 190       | 31,606 ± 3,166            | 26,774             | 6.6              | 9,346 ± 843                | 4484               | 1.9                       | 1395 ± 93                 | 17,322 ± 1,619            | 15,927 ± 12.4           |
|            | Cortisone    | 107.0        | 4132 ± 485       | 28,160 ± 1,328            | 24,028             | 6.8              | 7,007 ± 472                | 3855               | 1.9                       | 1531 ± 207                | 8,155 ± 1,014             | 6,924 ± 5.3             |
|            |              | 25.5         | 5266 ± 476       | 29,570 ± 1,169            | 26,341             | 9.1              | 10,485 ± 391               | 7229               | 3.2                       | 1822 ± 86                 | 14,980 ± 669              | 12,566 ± 7.9            |
|            |              | 22.0         | 5488 ± 408       | 35,795 ± 3,907            | 30,307             | 6.32             | 13,031 ± 1,016             | 7432               | 2.8                       | 1987 ± 93                 | 10,987 ± 5,823            | 9,000 ± 5.5             |

* Spleen cells from C57BL/6 mice used in the experiments shown in Table VI were counted and assayed for stimulation by LPS (10 μg/tube), PHA (1 μl/tube), or 1 X 10^6 mitomycin-treated allogeneic (CBA) or syngeneic target cells. Tdr[3H] incorporation was measured after 48 (LPS, PHA) or 72 hr (alloantigen) incubation, and the data are expressed as mean values ± se for four replicate tubes.
These data are consistent with the interpretation that reactive cells within the thymus are concentrated by cortisone treatment, perhaps the result of selective destruction of the nonreactive majority of thymus cells. The PHA- and alloantigen-reactive cell populations present in the spleens of cortisone-treated animals were unaffected under these conditions. The non-thymus-dependent, LPS-reactive spleen subpopulation was slightly decreased in its capacity to be stimulated after cortisone treatment. These data are consistent with antigenic patterns indicating that the B layer, low-density population of the thymus is both functionally and antigenically equivalent to that remaining after steroid therapy.

**DISCUSSION**

The data presented in this and the preceding paper add to evidence of the heterogeneity of the mouse thymus cell population. Here it was shown that at least two subpopulations can be identified in terms of immunocompetence toward alloantigens and receptivity to PHA stimulation.

The major subpopulation, which showed evidence of neither primary alloantigen recognition in vivo or in vitro nor PHA stimulation in vitro, comprised 80–90% of thymic lymphocytes. This subpopulation is of high average density in BSA gradients, consists mostly of cells highly sensitive to anti-0 and anti-TL cytotoxicity, and disappears after cortisone treatment. This fraction also corresponds to that shown to be highly sensitive to anti-G{sub}IX and anti-Ly sera and relatively insensitive to anti-H-2.

The minor subpopulation was identified as having most or all of the properties of primary alloantigen recognition and PHA reactivity present in whole thymus. This fraction was of relatively low average density, contained little or no TL or G{sub}IX, was relatively insensitive to anti-0 or anti-TL, and complement, and resisted cortisone treatment. The functional attributes examined in this population and those of whole thymus were concordantly abrogated by treatment with anti-H-2 and complement, whether the subpopulation was derived on the basis of average density (i.e. the B layer), its insensitivity to cortisone treatment in vivo, or by anti-0 and anti-TL treatment in vitro. This subpopulation probably corresponds to the fractions having GVH reactivity described by Dicke et al. (17) and to that having the T cell cooperative effect for an anti-SRBC response (13).

The various means used for deriving functional subpopulations from the thymus appear to depend upon concentrating the responsive cells by eliminating the majority which are inactive and which thereby dilute the active cells. An alternate possibility, that the various treatments destroyed inhibitory cells in the major subpopulation, seems less likely, since the various functions were detected in in vivo tests as well as in vitro. However, in view of a possible suppressor role ascribed to thymus cells recently (18), the possibility cannot be categorically eliminated.
The size of the thymus cell population recognizing alloantigen, as in the case of peripheral lymphoid cells, is a matter of considerable theoretical interest (19). Limiting dilution data are not reported here; however, estimates can be made with respect to maximum numbers of alloantigen-reactive cells. Calculating from the GVH capability of CBA thymus B layer cells, the active population consists of less than 10% of thymus cells, or less than $10 \times 10^8$ per thymus. This test for immunocompetence was across a relatively weak histocompatibility barrier (CBA reacting to [CBA × A/J]F1 hybrids). When a C57BL/6 thymus subpopulation reacted to the (CBA × C57BL/6)F1 hybrid in the GVH model or in vitro to CBA, the responsive population was contained in less than 1.6% of all thymus cells. In another context, less than 12% of the 13% of cells surviving anti-θ plus complement gave strong GVH reactivity. This is consistent with the interpretation (14) that the representation of cells responding to stronger H-2 differences is larger than that responding to weak ones Reactivity toward a strong alloantigenic mosaic is contained in less than 1% of thymus cells. At a practical level the minor thymus cell subpopulation yielded by treating whole thymus with 1:100 anti-θ and complement has proven to be the most pure and convenient alloantigen-reactive T cell source employed experimentally.

Because average properties were studied, the individual properties of specific cells from this functional subpopulation may only be inferred. Within these limits the characteristics of the low-density, immunocompetent cell can be adduced as (a) significant but low representation of θ-antigen, (b) mandatory presence of the H-2 antigen, (c) relatively few PHA receptors, and (d) resistance to cortisone. TL is ostensibly not required, but the known propensity of this antigen to modulate in the presence of specific antibody (20) limits the validity of this conclusion.

Peripheral T cells are found in fractions of both low and high density (15); those of higher density are the more numerous, however. Therefore, the immunocompetent subpopulation of the thymus differs from a portion of the peripheral T cell subpopulation, chiefly with respect to average density and cell size.

Since θ- and Ly antigen representation is greater in the high- than the low-density components in the spleen, it has been suggested that the minor competent subpopulation of the thymus is poised for entry into the peripheral pool (21) and there increases in density and associated characteristics as it accommodates to this environment. Memory cells, defined as a numerically augmented, specifically reactive subpopulation resulting from immunization, have not been identified in the mouse thymus. The one class of mouse peripheral T cells known to represent memory cells, that population responding in vitro to purified protein derivative after bacille Calmette Guérin immunization (15, 22), was found to have high average density in the spleen. On the other hand, the only known class of peripheral T cells currently considered to be primary recog-
nition cells is that responding to alloantigen in vitro. These cells were found in both high- and low-density fractions of the spleen; actually, they were more prevalent, calculated as incorporation per $10^6$ cells, in the low-density subpopulation (Table III) (15).

These data suggest that a significant representation of primary antigen-reactive cells emerging from the thymus may actually retain low-density characteristics in the peripheral lymphoid tissues for some time or until specific antigenic encounter stimulates their expansion into a memory population. The recent report of Pelet et al. (23) provides some direct evidence favoring this hypothesis. They found that mice responding to alloantigen immunization initially had splenic cytotoxic lymphocytes of low density, but these were of higher density after prolonged stimulation. Dyminski and Argyris⁶ have found bone marrow T cells in C57BL/6 mice which have significant $\theta$-markers; these are also apparently of low density.

These considerations lead to the concept that the T cell class includes at least three or, perhaps, four subpopulations of lymphoid cells defined on the basis of geography, density, antigenic characteristics, and functional attributes. Some properties of these tentative subpopulations are suggested in Table IX. $T_h$ is proposed as a notation to cover all cells found within the thymus; $T_p$ describes peripheral T cells. $T_h$ (thymus incompetent) is the dominant cell subpopulation in the thymus, antigenically endowed but immunologically incompetent and of uncertain significance in the internal cellular ecology of the organ. Evidence exists that the $T_h$ population provides precursor cells for the $T_h$ subpopulation (25, 26). $T_h$ (thymus competent) is the minor subpopulation of the thymus which is, by most criteria, immunologically competent, of low average density, low in thymus antigens, and requires the H-2 component. It is virgin with respect to antigen encounter and, therefore, has no memory augmentation. Two subpopulations of $T_p$ are proposed: $T_{p_v}$, the virgin, antigen-reactive T cell, probably of low density and apparently identical to the $T_h$, except for distribution; and $T_{p_m}$, the peripheral high-density, memory T cell population. These two cells probably also differ in $\theta$ representation, $T_{p_m}$ being higher on the basis of over-all subpopulation susceptibility to anti-$\theta$.¹

It seems probable, on the basis of these data and those published recently, that $T_h$ is the equivalent of the $T_{es}$ population of Cohen and Claman,⁴ and that $T_h$ is equivalent to their $T_{es}$ subpopulation. The experiments reported here do not reveal any $T_{es}$ class of spleen T cells as described by Cohen and Claman.

The $T_p$ and $T_h$ subclasses appear identical to the $T_1$ class of Raff and Cantor (25), and $T_{p_m}$ seems to include their $T_2$ class. Bone marrow T cells are of low $\theta$ content, low density, are cortisone resistant, and are thus, probably, of the $T_p$ subclass.

While operationally useful, these arbitrary categories will doubtless be

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¹ Dyminski, J., and B. Argyris. Personal communication to the authors.
⁴ Claman, H. N. 1972. Cell. Immunol. In press.
TABLE IX

Summary of Characteristics of Tentative Mouse T Cell Subpopulations

| Property of subpopulation | Th
|---------------------------|---|
| Distribution              | Thymus cortex | Uncertain | Thymus-dependent areas of peripheral lymphoid tissues |
| Size, appearance          | Small, lymphoid cell | Large, lymphoid cell | Small lymphocytes |
| Relative density in BSA gradient | High | Low | High |
| Percent of cells          | 80-90% of thymus | 2-10% of thymus; unknown proportion of peripheral T cells | 20-30% of spleen or lymph node cells |
| Cortisone effect in vivo  | Sensitive | Insensitive | Insensitive |
| Relative irradiation      | Sensitive | Less sensitive | Less sensitive |
| Membrane antigens         | High | Low, but required | Low |
|                          | High | Low or absent | Absent |
|                          | High | Low or absent | Absent |
|                          | High | Low | Low |
|                          | Low or absent | Intermediate | High |
|                          | Absent | Absent | Absent |
| Functional attributes     | No | Yes | Probable yes |
| Primary alloantigen        | No | Yes | Yes |
| recognition               | No | Yes | Untested |
| PHA stimulation           | No | No | No |
| Cooperative effect -- SRBC | No | No | No |
| LPS stimulation*          | No | No | No |

* From reference 24.

changed as population heterogeneity is further defined with regard to those cells which are, will be, or have been under thymus influence. The data presented here allow for experimental focus upon the relatively pure Th subpopulation of T cells as one easily obtained, having a high degree of alloantigenic responsiveness, antigen sensitivity, defined membrane antigens, and free from contamination by B cells and other cells found in lymphoid tissues. This should permit identification of specific antigen receptors and clarification of their origin, their differentiation history within the thymus, and the mechanism of their emergence and distribution.

SUMMARY

The functional attributes of minor subpopulations of mouse thymus cells derived by bovine serum albumin density gradient centrifugation, cortisone
treatment, or selective depletion by anti-TL or anti-θ treatment have been examined. A subpopulation derived in each fashion contains the cells required to evoke graft-versus-host reactions in neonatal F₁ hybrid recipients and to be stimulated by alloantigens in vitro in one-way mixed lymphocyte cultures and by phytohemagglutinin. The functions of this subpopulation are abrogated by treatment with anti-H-2 plus complement and by high concentrations of anti-θ. A tentative ordering of the various thymus cell subpopulations, on the basis of these and other data, is described.

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