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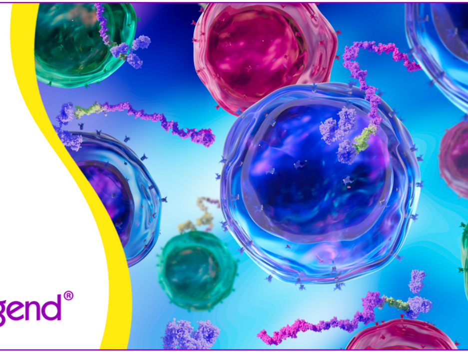

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T CELL MATURATION: THYMOCYTE AND THYMUS MIGRANT SUBPOPULATIONS DEFINED WITH MONOCLONAL ANTIBODIES TO THE ANTIGENS Lyt-1, Lyt-2, AND ThB[1]

ROLAND SCOLLAY[2] AND IRVING L. WEISSMAN[3]

From the Laboratory of Experimental Oncology, Department of Pathology, Stanford Medical School, Stanford, California 94305

The phenotypes of thymus cells for the antigens Lyt-1, Lyt-2 and ThB have been analyzed by using immunofluorescence techniques. Cells throughout the intrathymic maturation sequence have been tested, including the primitive subcapsular lymphoblasts, all size classes of cortical and medullary thymocytes, and thymus cell emigrants. ThB antigen is not detectable on migrant cells, but all subpopulations in the thymus are subdivided into two categories, bright and dull. Thus, it is possible that the bright and dull phenotypes represent a lineage specific rather than a stage-specific marker, at least inside the thymus. The Lyt-defined thymocyte subclasses Lyt 1^+2^- (Lyt-1) and Lyt 1^+2^+ (Lyt-1,2) are also both represented in all subpopulations, including subcapsular lymphoblasts. This suggests that they may represent two separate lineages, and that the Lyt-12(3) class is not a precursor of the Lyt-1 class, although the possibility of a very early Lyt-12(3) cell precursor common to both lines cannot be ruled out.

In the mouse, the genetically determined alloantigens Lyt 1,2, and 3 are useful as external markers for functionally defined T lymphocyte subsets (1). In peripheral lymphoid organs the Lyt 1^+2^- (Lyt-1) population includes several types of helper cells, whereas the Lyt 1^-2^+ (Lyt-2) population includes suppressor and cytotoxic cells (1). The class Lyt 1^+2^+ (Lyt-12) includes the precursors of Lyt-2 cells (1). It has been proposed that Lyt-12 cells are the precursors of both Lyt-1 and Lyt-2 cells, based partly on the report that a large majority of thymocytes throughout life and peripheral T cells in 1-week-old mice are Lyt-12 (2). Our observation that thymus migrants were already subdivided into the subclasses Lyt-1 and Lyt-12 suggested that the maturation step from Lyt-12 to Lyt-1 must occur, if at all, within the thymus (3). Mathieson *et al.* (4), who showed that there is a significant Lyt-1 population in the thymus, proposed that the Lyt-12 and Lyt-1 lines might be independent lines throughout thymic development residing in the cortex and medulla, respectively.

The question of whether functionally distinct T cell subsets share common precursors or are separate lineages throughout their ontogeny must be answered before one can outline the milestones of the T cell developmental program. In the former view, commitment to become a T cell precedes commitment to function, implying some plasticity in the program if external stimuli may influence the functional commitment (1). In the latter view functional commitment may precede or coincide with the acquisition of T cell characteristics, implying that subsequent external (microenvironmental) stimuli merely act as selective forces on already committed subpopulations (5, 6). To answer this question, one must examine the Lyt phenotype of thymocyte subclasses throughout their thymic stages of development, from the primitive subcapsular lymphoblasts to their thymic and peripheral T cell progeny (7, 8). In the following pages we will describe the Lyt phenotype of a number of thymocyte subpopulations, including subcapsular large cells. The data presented here support the contention that the classes Lyt-1 and Lyt-12 may be quite separate from the earliest definable stages of intrathymic development.

We also here present data on the T cell lineage expression of another marker, ThB, which is expressed on about 50% of thymocytes, on B cells, but not on most peripheral T cells (9–11). We have examined the phenotype of subcapsular cells and thymus migrants in order to define the relationship of this antigen to developmental stage. Here, too, the data suggest that ThB high and low cells are present in all thymic maturation stages.

MATERIALS AND METHODS

Animals. Mice were bred in our colonies at Stanford. Unless otherwise specified, animals were used at 3 to 6 weeks of age.

Cell preparation and *suspension staining* were done as previously described (12). *Thymus migrants* were done as previously described (12, 13) and *staining of subcapsular cells* was done as previously described (12).

Sera. Conventional anti-Lyt-1.2 and anti-Lyt-2.2 sera have been described (3). Monoclonal anti-Lyt-1 (53-7.2) and Lyt-2 (53-6.7) were prepared from hybrid cells kindly provided by Drs. J. Ledbetter and L. Herzenberg. Monoclonal anti-ThB was kindly provided by Dr. L. Eckhardt. All of these rat monoclonals have been described elsewhere (14, 15). The anti-Lyt reagents react with both known allotypic determinants at each locus and can be used in all strains.

Second stage sera were rabbit anti-mouse Ig, extensively absorbed with mouse thymocytes (which does not react with thymocytes or on T cells); and rabbit anti-rat Ig, eluted from a

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[2] Special Fellow of The Leukemia Society (N.Y.); current address is The Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia.

[3] Faculty Research Award, American Cancer Society (N.Y.); to whom correspondence should be addressed.

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rat Ig column and absorbed on a mouse Ig column. This serum does not react with any mouse lymphoid cells. Fluorescein and rhodamine conjugates were made with each serum.

Analysis of stained cells. Cells were analyzed on the fluorescence microscope or the fluorescence-activated cell sorter (FACS),[4] as previously described. FACS-analyzed cells were divided into large and small subpopulations on the basis of low angle light scatter characteristics (16). In the following discussion, "all cells" means all viable cells (85 to 95% of the total in most preparations), "small cells" means the smallest 30% of viable cells, and "large cells" means the largest 10% of viable cells.

Cortisone treatment. Mice were injected intramuscularly with 2 mg of hydrocortisone acetate 48 hr before sacrifice. Cell yield from the thymuses of treated animals was 5 to 8% of the normal controls.

RESULTS

Anti-Lyt-1 stains all thymocyte classes to the same extent. Anti-Lyt-1 monoclonal antibodies stain all thymocytes, which confirms the FACS analysis of thymocytes with anti-Lyt sera by Mathieson *et al.* (4). Analysis of different size classes shows increasing mean fluorescence with increasing cell size. Cortisone-resistant cells are also positive and twice as bright as whole thymocyte preparations (our data and 4). Staining of large subcapsular cells is shown in Table I. Again all cells are positive as might be predicted from the other data. Since these cells were counted visually, no real comparison of absolute brightness can be made.

We have previously published data on thymus migrants using conventional antisera (3). All or most migrants in spleen, lymph nodes, and blood were positive for Lyt-1.2. Staining with monoclonal sera give the same result.

Anti-Lyt-2 stains only a subpopulation of any thymocyte class. Data on staining of whole thymocyte preparations with conventional antibodies have been published (4). The staining pattern is biphasic and the distinction between negative or very dull cells and positive cells is clear-cut. Tables I and II show the staining of a variety of thymocyte subpopulations. The proportion of thymocytes stained varies from 70 to 90% in different experiments, but in our hands, the small thymic lymphocytes always had a higher proportion of Lyt-2 positive cells than the whole population, while the large cells had a somewhat lower proportion of Lyt-2 positive cells. Cortisone-resistant thymocytes (CRT) have the lowest fraction of Lyt-2 positive cells, usually in the range 35 to 40%, but as with Lyt-1, the positive cells are about twice as bright as whole thymocytes. This latter point is in disagreement with Mathieson *et al.* (4), who found CRT less bright than whole thymocytes.

Subcapsular cells are shown in Table I. There is clearly a proportion of subcapsular large cells that is Lyt-2 negative, although it is lower than for large cells elsewhere in the thymus. The same result was obtained with conventional sera (data not shown).

Migrants in blood, spleen, and lymph node, when examined 3 hr after intrathymic injection, were all about 30% positive for Lyt-2 with the monoclonal antibodies. Therefore, about 70% of migrants are Lyt-1, and about 30% Lyt-12, in agreement with our previously published data utilizing conventional antisera (3).

Lyt phenotype of migrants 30 min and 3 hr after intrathymic injection. In order to exclude the possibility that thymus migrants changed phenotype very rapidly in the periphery (i.e., within 3 hr), we compared the phenotype of migrants 30 min and 3 hr after intrathymic injection. Table III shows that there

[4] Abbreviations used in this paper: FACS, fluorescence-activated cell sorter; CRT, cortisone-resistant thymocytes; α, anti.

TABLE I

Staining of subcapsular cells with various antibodies microscopic: analysis

Antibody	Thymocyte Subpopulation	% of Total[a]	
		Undetectable[b]	Positive
α-Lyt-1 53-7.3	Subcaps large[c]	1 ± 1	99 ± 1
	All large[c]	4 ± 2	96 ± 2
	Small	1 ± 1	99 ± 1
α-Lyt-2 53-6.7	Subcaps large	15 ± 4	85 ± 4
	All large	28 ± 5	72 ± 6
	Small	10 ± 2	90 ± 1
α-ThB	Subcaps large	17 ± 2	83 ± 4
	All large	35 ± 2	65 ± 2
	Small	36 ± 1	64 ± 2
2nd Stage alone	Subcaps large	99 ± 1	1 ± 1
	All large	99 ± 1	1 ± 1
	Small	99 ± 1	1 ± 1

[a] Mean and S.E. of three individual animals.
[b] That proportion of cells that appeared negative by visual inspection.
[c] "Subcaps large" means the outer cortical subcapsular lymphoblasts stained by dipping the intact thymus into FITC (12); "all large" means fluorescein negative large cells.

TABLE II

Staining of various thymocyte subpopulations with anti-Ly 2.2 antiserum[a]: FACS analysis

Expt. No.	Cell Donor	Lyt-2 Allele	Thymocyte Subpopulation	% of total	
				Very dull or undetectable[b]	Positive
1	B6	2.2	All cells	15	85
			Small cells	10	90
			Large cells	30	70
			CRT[c]	60	40
	B6.Ly 2.1	2.1	All cells	99	1
2	B6	2.2	All cells	30	70
			Small cells	15	85
			Large cells	40	60
	B6.Ly 2.1	2.1	All cells	97	3

[a] Conventional anti-Lyt 2.2.
[b] Includes all the cells falling in the first peak on the FACS profile. Consists mainly of cells that fall within the range of the negative controls, but also a small proportion of very dull cells.
[c] Cortisone-resistant thymocytes.

TABLE III

Lyt-2 phenotype of thymus migrants 30 min and 3 hr after intrathymic injection

Time	% Positive with α-Lyt-2.2[a]	No. of Cells Counted
30 min	42 ± 5.8[b]	125
3 hr	37 ± 4.3[c]	360

[a] Conventional anti-Lyt-2.2 on B6 animals, 4 to 5 weeks old.
[b] Mean and S.E. of three individual animals.
[c] Mean and S.E. of four individual animals.

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was no difference. As in the previous experiments, the migrants were about 40% Lyt-2 positive.

The effect of stress on the Lyt phenotype of migrants. Although we have demonstrated (13) that stress did not markedly affect the rate of migration, the possibility remained that it may have affected the proportion of different cell types among migrants. Table IV shows that this is not the case, at least in terms of the Lyt antigens. Neither additional cortisone nor adrenalectomy affected the proportion of Lyt-2 positive and negative cells.

Lyt-1 and 2 staining of thymic migrants in young mice. Cantor and Boyse (17) reported that Lyt-123 cells were the predominant peripheral T cell population in 1-week-old mice; thus, it was important to test the phenotype of migrants in animals of this age. Table V shows that the phenotype of thymus migrants in 7-day-old mice (measured 3 hr after intrathymic injection) is the same as in adults. Lyt-1 cells make up 60 to 70% of all migrants, Lyt-12 make up most of the remainder, with Lyt-2 being quite rare.

Anti-ThB. Anti-ThB antibodies have been shown to separate thymocytes into two roughly equal populations of bright cells and dull cells (9–11). Larger cells are somewhat brighter, but still subdivided into the two categories (L. Eckhardt, personal communication). Data in Table I show that the subcapsular large cells are also subdivided. Visual analysis does not permit accurate separation of bright and dull categories, but there is nevertheless a population that is clearly ThB negative among the subcapsular cells.

Thymus migrants were stained for ThB in suspensions of pooled spleen and lymph node cells. Although slight spillover from the fluorescein into the rhodamine channel prevents identification of very dull positives, all migrants appeared negative, suggesting that none of them are in the ThB bright category.

TABLE IV

Effect of cortisone or adrenalectomy on the Lyt phenotype of thymus migrants

Expt. No.	Antiserum	Treatment	Organ	% Positive[a]
1	α-Lyt-1.2	Normal	Spleen	90 ± 4
		cortisone treated[b]	Spleen	95 ± 3
	α-Lyt-2.2	Normal	Spleen	21 ± 2
		cortisone treated[b]	Spleen	26 ± 4
2	α-Lyt-2.2	Normal	LN	29 ± 4
		adrenalectomized[c]	LN	39 ± 9
		Sham adrenalectomized[c]	LN	31 ± 13

[a] Mean and S.E. of values from three to four individual animals. Migrants were identified following intrathymic microinjection of FITC (3, 12).

[b] Animals received 2 mg of hydrocortisone acetate 45 min before intrathymic injections. Animals were killed 3 hr after intrathymic injection.

[c] Animals were adrenalectomized or sham operated 8 days before intrathymic injection, and maintained as described previously (13).

TABLE V

Lyt phenotype of thymus migrants in 1-week-old mice

Antiserum	Organ	% Positive[a]	Summary
α-Lyt-1.2[b]	Spleen	94 ± 3	All migrants
	LN	98	
			Lyt-1 = ~60%
α-Lyt-2.2[b]	Spleen	41 ± 5	Lyt-2 = <5%
	LN	44	Lyt-12 = ~40%
NMS[c]	Spleen	1 ± 1	
	LN	0	

[a] Mean and S.E. of 5 individual animals for spleen, value for a pool of 10 animals for lymph node. Migrants were identified 3 hr after intrathymic injection of FITC.

[b] Conventional antisera.

[c] Normal mouse serum, absorbed in the same way as the antisera.

DISCUSSION

We have recently shown that cells migrate out of the thymus of young adult mice at the rate of about 2×10^6 cells per day (13). This figure amounts to 1% of the resident thymocyte population per day. Since the bulk of thymocytes turn over every 5 to 7 days (18) this means that about 95% of thymocytes must die, presumably *in situ*. This also means that the subpopulation of thymocytes that represents the precursors of migrants is only a few percent of the total. Since such a minor subpopulation of cells could not be detected even if it were very different from the rest of the thymus, the phenotype of the migrant cells themselves is an important clue to the thymocyte lineages that give rise to peripheral T cells.

With regard to the Lyt antigens, we have previously shown that migrants, 3 hr after intrathymic injection, are approximately 70% Lyt-1 and 30% Lyt-12 (3). However, the possibility that they may have converted from Lyt-12 cells during that 3-hr period, or that these proportions were a result of a stress artifact was not excluded. In this paper we have excluded both of these possibilities. It would seem likely then that the phenotype of thymus migrants as they leave the thymus is about 70% Lyt-1 and 30% Lyt-12. (These numbers have ranged from 60/40 to 80/20 in a large number of different experiments.) It is of interest that although the original data on peripheral T cell phenotypes, based on cytotoxicity, suggested a 50/50 division of Lyt-1 and Lyt-12 (3), more recent work using immunofluorescence (15) has shown that peripheral T cells are in fact divided into 70% Lyt-1 and 30% Lyt-12, the same as the migrants. Taken together with the data in this paper on migrants in 1-week-old mice, and published data showing Lyt-2 negative cells in neonatal thymus (4), it seems likely that the phenotype of T cells (at least for Lyt) is decided within the thymus, and perhaps from the earliest stages of development. Thus, we looked for a stage of thymic maturation that might be composed of all Lyt-1,2 cells as the postulated common precursor. Subcapsular large thymic lymphoblasts, which probably represent one of the earliest cells in the intrathymic maturation sequence (8, 16), are also subdivided into the Lyt-1 and Lyt-12 classes. However, the large subcapsular cells almost certainly include several generations of dividing cells, from the earliest progenitors to cells undergoing their final division before differentiation (16). It is formally possible, therefore, that a common Lyt-1,2 progenitor cell exists, although it is no longer *necessary* to postulate its existence. Definitive proof of any lineage lies in the demonstration that a particular cell class does or does not give rise to another, and not in static demonstrations of phenotypic subgroups. Experiments of this kind have been done with peripheral T cells, and the independence of the Lyt-1 and Lyt-2 classes was demonstrated (3). Similar experiments are in progress in our laboratory with thymocyte subpopulations. The intriguing fact remains, however, that although the majority of cortical thymocytes (and therefore of the whole thymus) are

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Lyt-12, the majority of migrants are Lyt-1. The final answer to this paradox and to why the thymus produces so many Lyt-12 cells awaits further experimentation.

The situation with ThB antigen is complex, since cortical cells fall into two roughly equal categories of dull and bright, whereas medullary cells (cortisone resistant and in frozen sections [L. Eckhardt *et al.*, in preparation]) and peripheral T cells are negative. Our demonstration that subcapsular cells are also subdivided into bright and dull suggests two possible lineages of high and low ThB, and not one lineage of high to low to negative. However, all the migrants are low or negative for ThB, and whether high ThB cells can ever give rise to peripheral T cells remains to be investigated.

The major point of this study, and to some extent the companion study (12), is that for most of the cell surface markers we have examined, the thymocyte population is heterogeneous, and this is even true of the subpopulation considered to contain the progenitor cells (i.e., subcapsular lymphoblasts). If these markers are stable within a T cell lineage, several independent lines of T cell differentiation must occur throughout the intrathymic stage of T cell development. In that case, thymic differentiation may involve extensive selection from among many competing T cell subsets (5, 6) rather than a programmed course of instruction leading to phase-specific expression of well defined T cell and thymocyte cell-surface markers (1, 17). To resolve these competing hypotheses we need to clarify the extent of phenotypic heterogeneity in T cell hematopoietic precursors and/or in the most primitive thymic lymphocytes, and to test the stability of expression of these T cell markers by lineage analyses.

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