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T CELL MATURATION: THYMOCYTE AND THYMUS MIGRANT SUBPOPULATIONS DEFINED WITH MONOCLONAL ANTIBODIES TO MHC REGION ANTIGENS[1]

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The maturation sequences of thymocytes is known to some extent: A generative layer of subcapsular large lymphoblasts gives rise to a major population of small cortical thymocytes and a minor population of midsize medullary thymocytes. The relative contribution of these three populations to the peripheral T cell populations is not yet known.

In this study, subcapsular lymphoblasts, cortical small cells, medullary cells, and thymic emigrant cells have all been analyzed by immunofluorescence for expression of the antigens H-2D, I-A, H-2K, and TL. H-2D is expressed brightly on all subcapsular large cells, dimly on cortical small cells, and brightly on all migrants, cortisone-resistant thymocytes (CRT), and peripheral T cells. I-A can be detected at low levels on 30 to 50% of cells in all the thymic subpopulations, and on 30 to 50% of migrants and peripheral T cells. Fifty to 80% of small cortical cells do not express detectable H-2K, but all the other subpopulations, both inside and outside the thymus, stain uniformly quite brightly. TL.3 is expressed on 70 to 80% of subcapsular and cortical thymocytes, 30 to 40% of CRT, is undetectable on migrants but can be seen at low levels on 10 to 20% of spleen and lymph node T cells. The possibility that some or all of these antigens represent stable markers of separate lineages rather than unstable, stage-specific markers is discussed.

The thymus contains many lymphocyte subpopulations, and a variety of techniques have been used to isolate and characterize them (reviewed in 1). At least three distinct populations may be distinguished by size and architectural location: subcapsular lymphoblasts, cortical small lymphocytes, and medullary medium sized cells (2, 3). In addition, thymus cell migrants may be considered as a fourth subpopulation, and can be readily marked by intrathymic microinjection of unequivocal markers (4–6). Some understanding of the lineage relationship of these various subpopulations is necessary in order to determine which ones represent key points in the pathway(s) of T cell differentiation. Topical labeling of the thymus with radioactive nucleosides results in rapid accumulation of label in the large subcapsular lymphocytes of the outer cortex (2, 3). These rapidly repopulate the small nondividing lymphocyte pool of the inner cortex and appear to repopulate the medullary pool more slowly (2, 3, 7). At least some thymus cell emigrants are also eventually derived from these labeled outer cortical lymphoblasts, but their penultimate precursor subpopulation is still a matter of speculation.

The division of cells into large, small, and medullary is a useful one, and these populations have been shown to express different levels of Thy 1 (3, 8). But large cells do exist throughout the thymus, including the medulla, and the category "large" therefore lacks specificity. We, therefore, developed a technique for labeling subcapsular cells with fluorescein so that the category of subcapsular large cells can be defined unequivocally for surface phenotype and lineage analysis.

It has been known for many years that thymocytes express as a population lower surface concentrations of H-2 antigens than peripheral lymphocytes. Gorer and Boyse in 1959 (9) and Winn in 1960 (10) made the observation based on the susceptibility of cell populations to the cytotoxic activity of antisera. In 1967 (11), the same observation was made by using immunofluorescent techniques. Most determinations, however, were based on techniques that averaged the H-2 levels of the whole thymocyte population (or in some cases whole organ homogenates (12)), sometimes compared with cortisone resistant thymocytes (CRT)[5] (reviewed in 13).

It is the purpose of this paper to report studies on the distribution of major histocompatibility complex mouse (MHC) region antigens on thymic lymphocyte and thymic emigrant subpopulations, by using well defined polyclonal sera and monoclonal antibodies to H-2K, H-2D, I-A, and TL3 alloantigens in fluorescence cytophotometric assay.

MATERIALS AND METHODS

Animals. Mice were bred in our colonies at Stanford or were kindly provided from the colony of Dr. H. McDevitt. Unless otherwise specified, animals were used at 3 to 6 weeks of age.

Cell preparations. Animals were etherized, various organs were removed and gently pressed through 200-gauge stainless steel screen. Cells were washed once and aliquoted for staining.

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[5] Abbreviations used in this paper: α, anti; CRT, cortisone-resistant thymocytes; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex.

Medium was 5% fetal calf serum (FCS) in a mixture of equal parts of medium 199 (Grand Island Biological Co., Grand Island, N. Y.) and phosphate-buffered saline (PBS).

Suspension staining. All staining manipulations and serum dilutions were done in PBS containing 5% FCS and sodium azide (5×10^{-3} M). Two to 3 million cells were placed in 2-ml glass conical tubes, spun down, and resuspended in 25 μl of the 1st stage antiserum. After 20 min on ice, 1 ml of medium was added and underlayed with 0.5 ml FCS. The cells were spun down and resuspended in 25 μl of the second stage reagent. After 20-min staining on ice, the cells were washed as before, and after an additional wash were fixed in 1% formaldehyde (in PBS) for analysis. No difference was observed between fixed and unfixed cells with any of the antisera used in this paper.

Thymus migrants and intrathymic injections. These techniques have been described (5, 6), but briefly were as follows. Anesthetized mice received injections, directly into each thymic lobe, of 10 μl of a solution of fluorescein isothiocyanate (FITC) in PBS. Three to 4 hr later the animals were killed and cell suspensions were made from thymus, mesenteric lymph node, and spleen. Thymus migrants could be identified in cell suspensions of lymph node and spleen by the presence of bound fluorescein. The technique labels about 30% of thymocytes, and the frequency of labeled migrants in lymph node and spleen 3 hr after injection is about $300/10^6$ nucleated cells. These cells can be counter stained with rhodaminated reagents.

Staining subcapsular cells in the thymus. Thymuses were removed without damage to the capsule from anesthetized mice and placed in a solution of FITC at 200 μg/ml in PBS (with 5×10^{-3} M azide), at room temperature for varying periods of time (5 sec to 2 min). The thymuses were then rinsed in two consecutive cold FCS baths, and the cell suspension was prepared in medium containing 50% FCS.

Sera. The following monoclonal antibodies were kindly provided by Dr. G. J. Hammerling and Dr. U. Hammerling. Anti H-2K^k [(100-SR28; H-2.m3 similar to H-2.11)], anti H-2d^b [(B22 249R1; H-2.m2 similar to H-2.2)], anti I-A^k [(17/227; Ia-m4 similar to IA.15)] and anti TL.3 [(18/20)]; their preparation and specificity are discussed elsewhere (14–16). Monoclonal anti-I-A^k (11-5.2) and anti-H-2K^k (11-4.1) were kindly provided by Dr. L. A. Herzenberg and colleagues (17). All of these antibodies are of the IgG2a class. As a control in all experiments we used an IgG2a anti-IgD (anti-δ) allotype monoclonal. In no case was this distinguishable from controls for second stage alone, so second stage alone controls will not be shown. Staining with anti-δ will be called the serum control. Staining cells from congenic animals of a different haplotype will be called the specificity control.

Conventional antisera were kindly provided by Dr. H. McDevitt (anti-D^d) and Dr. J. Stimpfling (anti-K^k and anti-K^{d}IA^d). These sera were all absorbed before use with appropriate negative congenic strains, and have been characterized in our laboratory (18). The specified activity of each serum could be absorbed with appropriate positive congenic strains.

Second stage reagents were prepared in our lab. Rabbit anti-mouse Ig was absorbed on and eluted from mouse Ig columns, and then rhodamine or fluorescein conjugated. The serum gave completely negative staining on mouse thymocytes.

All the antibodies were fully titrated and used at just supersaturating concentrations. It should be noted that some monoclonals have marked prozones, so extensive titration is always necessary.

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.

Analysis of stained cells: a) Microscopic analysis. Subcapsular cells or migrants were located by fluorescence with a 25 × objective, and checked for rhodamine positivity with 25 and/or 40 × objectives. Large and small thymocytes and live and dead cells could easily be distinguished by using the phase optics. *b) Fluorescence activated cell sorter (FACS).* Analyses were carried out on FACS II and FACS III machines (Becton Dickinson Electronics, Rutherford, N. J.). The use of low angle scatter to distinguish live and dead and large and small thymocyte subpopulations has been described previously (3). In the following discussion, "all cells" means all viable cells (85 to 90% of the total in most preparations), "small cells" means the smallest 30% of the viable cells, and "large cells" means the largest 10% of the viable cells.

Preparation of peripheral T cells. T cells were prepared from spleen and lymph node cell suspensions by passage through nylon wool columns, according to established techniques (19). Staining these nonadherent cells with anti-Thy 1 and anti-Ig reagents showed the lymph node cells to be 90 to 97% T cells (Thy 1 positive, Ig negative), whereas the spleen cell preparations contained 85 to 90% T cells.

RESULTS

General

The following data are all based on fluorescence of cells stained in two stages with antibodies to cell surface molecules. Negative controls include a) specificity controls on cells of animals that do not express or do not carry the gene for the marker involved, b) serum controls in which the first stage is a monoclonal antibody of the same Ig subclass as the test antibody, at the same concentration, which does not (or should not) see a molecule on the cells being tested, and c) control of the second, fluoresceinated, stage alone.

Two methods of analysis were used. Flow microfluorometry using the FACS, and visual analysis on a fluorescence microscope. The FACS has the advantages of large sample sizes, precise analysis, and separation of size and brightness categories, which are reproducible from day to day, extreme sensitivity, and complete objectivity. Disadvantages include an inability to distinguish patterns of staining on individual cells, and ineffectiveness when very infrequent cells (e.g., thymic migrants) require analysis. Both these disadvantages can be overcome through use of the fluorescent microscope, but the sensitivity to dim cells, the absolute discrimination between classes, and the objectivity are lost.

In the following tables, we have called undetectable any cells that on the FACS fall in the same brightness range as 97 to 98% of the negative control cells. Undetectable cells remain in this range even at the highest FACS III photomultiplier gains. Dull positive cells gradually move away from negative controls as the gain is increased. On the microscope, some cells will be counted as negative even though on the FACS they would be dull positives. This discrepancy will vary somewhat depending on a variety of factors, in particular the cell-surface staining pattern observed with each antigen. Microscope discrimination is more difficult with diffuse staining patterns than in patterns that show varying numbers of clearly defined specks.

Two qualifications of the data should also be borne in mind. 1) Undetectable is a relative term and this category may include some cells that bear the antigen in a masked form or at extremely low levels. 2) Positive cells are not necessarily syn-

thesizing cells. Positivity, especially faint positivity, could represent passively acquired antigen. Our experiments provide no information on this point.

MHC distribution on thymocyte subpopulations separated by size

a) All thymocyte subpopulations express H-2D. Figure 1*a* shows the fluorescence profiles of total thymocytes, large thymocytes, and small thymocytes stained with monoclonal anti-H-2D^b (a typical control can be seen in Fig. 2*a*). Table I shows the quantitative data. As with all the sera we have tested, there is little difference between the curve for all cells and the curve for small cells since small cells make up about 80% the total. Practically all cells are positive (even more apparent at higher gain settings) but there is a distinct peak of dull small cells,

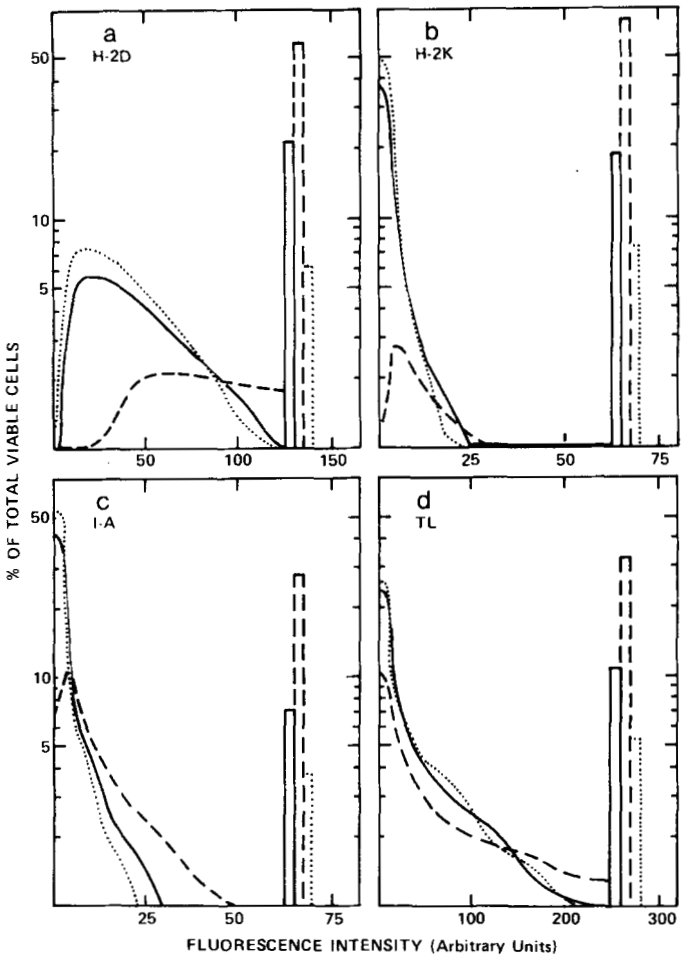

Figure 1. Figures 1, *a* to *d*, represent the FACS profiles of the whole thymocyte subpopulation (——), small thymocytes, defined by scatter (. . . .) and large thymocytes defined by scatter (-----). Antibodies were monoclonals as follows: *a*, anti-H-2D^b, B22 249RI; *b*, anti-H-2K^k, 100-SR28; *c*, anti-I-A^k, 17/227; and *d*, anti-TL.3, 18/20. Note that the vertical axis is logarithmic and that the horizontal scale varies from box to box. Each profile represents a smoothed curve from 25 histogram bars. The last histogram bar is shown, and represents that percentage of cells that accumulated in the last FACS channel; that is, any cells brighter than the intensity level shown on the scale (e.g., in box *a*, any cells of brightness greater than 125 fluorescence units). Quantitative data can be seen in the tables, where "undetectable" means fluorescence intensity 0 to 10, "dull" means 11 to 100, and "bright" means greater than 100. Thymus donors were B6(a), B6.H-2^k (*b* and *c*), and B6.TL+ (*d*). In all cases, greater than 98% of the serum control cells fall in the fluorescence intensity range 0 to 10 (see Fig. 2).

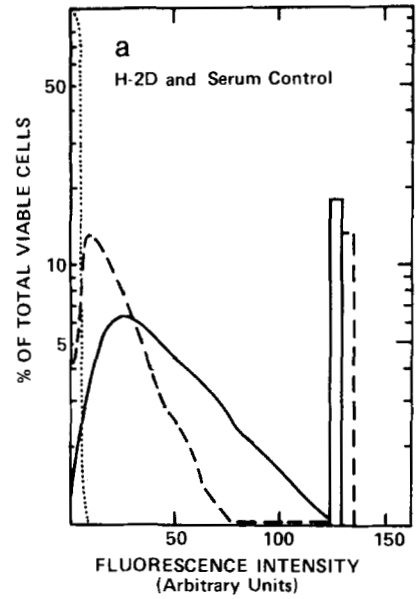

Figure 2. FACS profiles as described in Figure 1. Staining with monoclonal anti-H-2D^b on B6 thymocytes (——) or B6.TL+ thymocytes (-----). Serum control of an anti-Delta monoclonal on B6 thymocytes is also shown (. . . .).

whereas large cells are considerably brighter. On the dot plot (which is a two-dimensional plot of size *versus* fluorescence) three distinct populations can be seen; small, dull positive cells, large bright cells, and extremely bright medium sized cells (Fig. 3). The latter populations almost certainly represents CRT, which, like spleen and lymph node T cells (data not shown) are all brightly positive (3).

A number of other H-2D^b strains were also tested with monoclonal B22.249R1. Figure 2 shows a comparison of B6 and B6.TL+ mice. The shape of the curves was very similar for all the H-2D animals tested (B6, B6.TL+, 129, C3H.SW, B10A[4R]). The data in Figure 2 agree with the finding by Boyse *et al.* (20) that TL+ strains appear to have decreased expression of H-2D relative to TL-strains; that is, the TL+ strain expresses about one-half as much H-2D per cell as the congenic TL-strain (the brightness of CRT was no different in these two strains). However, we found considerable differences between individual animals, and this variation was common to all the experiments that compared TL+ and TL− strains. Some TL+ animals (e.g., Fig. 2*a*) showed a 2-fold decrease, whereas others (shown to be TL positive in the same test), showed little or no decrease relative to TL− animals. This was also true of other TL+ strains such as 129.

Staining with a conventional anti-D^d antiserum on BALB/c and A/J also gave curves of the same shape as those in Figure 1*a* and 2*a*; this pattern of staining may be typical of all H-2D antigens. In our hands, all spleen and lymph node T cells are brightly positive for H-2D^b.

b) Most small thymocytes express undetectable levels of H-2K. It is clear from Figure 1*b* and Table II that H-2K molecules are less well represented on small thymocytes than H-2D molecules. Thus, whereas large cells are generally bright and CRT are very bright (as bright as peripheral cells), the population of small cells is mainly negative or very dim. Again these three populations (small dull, large bright, medium very bright) can be clearly seen on the dot plot (Fig. 3). Increasing the gain moves the dim positives out, but still leaves behind the population with undetectable levels of H-2K.

In order to test whether this was a peculiarity of this partic-

TABLE I

Staining of thymocyte subpopulations with anti-D^b monoclonal

Antibody	Strain	H-2D Allele	TL Allele	Subpopulation	% of Total		
					Undetectable[a]	Dull positive[b]	Bright positive[c]
	B6	b	0	All	4	75	21
α-D^{bd}		b	0	Small	6	88	6
		b	0	Large	0	40	60
	B6.TL+	b	1, 2, 3	All	19	63	18
	129	b	2	All	20	68	12
	B10.A(4R)	b	0	All	4	81	15
α-Delta	B6	b	0	All	98	2	0
α-D^b	B6.H-2^k	k	0	All	91	8	1
α-D^b	B6		0	CRT[e]	2	8	90
α-Delta	B6	b	0	CRT	93	6	1

[a] Background level staining by FACS. This number does not change, relative to negative controls, with increasing gain.

[b] Dull positive consists of cells that are clearly above serum control levels on the FACS, namely fluorescence intensity 10 to 100 (Figs. 1 and 2). Some of these cells could not be distinguished as positive under the microscope, some would be distinguished as doubtful or dim positives.

[c] Bright positives are mainly those cells accumulating in the last channel, and would all be easily distinguished on the microscope.

[d] Monoclonal B22.249R1.

[e] Cortisone-resistant thymocytes.

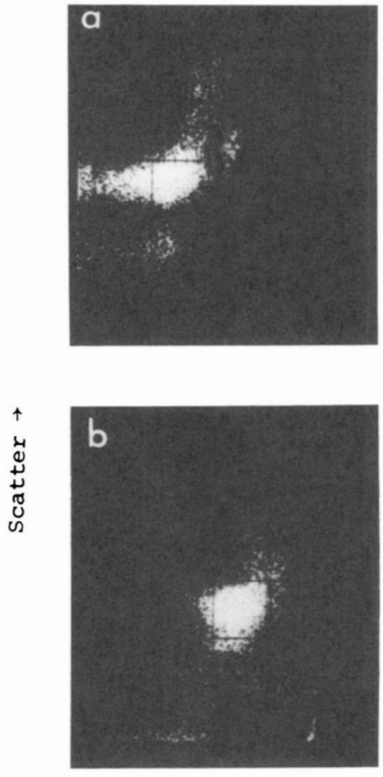

Figure 3. FACS profiles in which fluorescence is expressed on a log scale. *a*) Monoclonal anti-H-2K^k (100-SR28) or serum control on B6.H-2^k thymocytes; *b*) monoclonal anti-H-2D^b (B22 249RI) or serum control on B6 thymocytes. In both cases the serum control curve is hatched.

ular monoclonal antibody or of the B6.H-2^k strain, we tested B6.H-2^k with another anti-K^k monoclonal (11-4.1). In addition, we tested a number of different K^k strains with 100-SR28 (Table II) and we tested a number of strains with conventional anti-K^k and anti-K^d sera (Table III). In all cases the fluorescence profiles were almost indistinguishable from those shown in

TABLE II

Staining of thymocytes from different strains with anti-K^k monoclonal

Anti-body	Strain	H-2K Allele	Thymocyte Subpopulation	% of Total[a]		
				Undetectable	Dull positive	Bright positive
	B6.H-2^k	k	All	59	25	16
α-K^{kb}	B6.H-2^k	k	Small	72	21	7
	B6.H-2^k	k	Large	13	39	48
	BALB/k	k	All	55	38	17
α-K^k	CBA/J	k	All	32	40	28
	AKR/Cu	k	All	51	32	17
	B10.A(4R)	k	All	56	31	13
α-Delta	B6.H-2^k	k	All	98	1	1
α-K^k	B6	b	All	93	6	1
α-K^k	B6.TL+	b	All	87	11	2
α-K^k	B6.H-2^k	k	CRT[c]	1	3	96
α-Delta	B6.H-2^k	k	CRT	86	11	3

[a] As in Table I.

[b] Monoclonal 100-SR28.

[c] Cortisone-resistant thymocytes.

Figure 1*b*, and in all cases more than 50% of the small cells were negative (with the possible exception of CBA/J, which shows a slight difference).

Staining of spleen and lymph node T cells with anti-H-2K reagents is uniformly bright and comparable with CRT.

This difference between expression of H-2D and H-2K is shown very clearly in Figure 4. Here the fluorescence intensity is expressed on a logarithmic scale, which facilitates comparison of negative controls and dull cells with bright cells. The fact that all cells stained positively with anti-H-2D is obvious and the extensive overlap between the negative control and the H-2K stained cells is also clear. The highest intensity fluorescence-positive cells to the right in Figure 4*a* and 4*b* are medium sized cells, which, as expected, have the same staining pattern as CRT. This can be seen on the dot plots in Figure 3.

c) Some thymocytes of all size subclasses express I-A^k. Surprisingly, both anti-I-A^k antibodies stain about 30% of B6.H-2^k thymocytes, with larger cells somewhat brighter (Fig. 1*c*, Table IV). In fact, some of these cells stained with an intensity that could be seen on the microscope, as the data in Tables VI and VII indicate. Both the serum and specificity controls for the two different anti-I-A^k monoclonals show negligible staining above background.

We found that 40 to 50% of lymph node T cells were also faintly positive for I-A^k. Splenic T cells appeared to have fewer positive cells, although higher background with nylon wool-purified spleen cells made precise quantitation impossible (data not shown).

d) Not all cortical thymocytes express TL3. Only about 70% of thymocytes are stained above background with TL.3 antibodies, with large cells somewhat brighter (Fig. 1*d* and Table V). Serum and specificity controls again show negligible staining above background. Both staining and cytotoxicity with conventional anti-TL1, 2, 3 sera give similar results (data not shown). Surprisingly, a high proportion of CRT were positive (about 40%) although mostly fairly dull. Thus, about 30% of all thymocytes and 60% of CRT did not express detectable levels of TL.

We have also noted that this monoclonal anti-TL.3 antibody stains a significant proportion of peripheral T cells (10 to 20% of spleen and lymph node T cells) faintly but significantly above the background seen in serum and specificity controls.

TABLE III

Staining of thymocytes from different strains with conventional anti-H-2K sera

Serum	Strain	H-2K Allele	Thymocyte Subpopulation	% of Total[a] Undetectable	Dull positive	Bright positive
Conventional	BALB/c	d	All	82	16	2
α-K^k	BALB/k	k	All	31	42	27
	B6.H-2^k	k	All	49	36	15
Conventional	BALB/c	d	All	45	52	3
α-K^{d}I-A^d	BALB/K	k	All	87	12	1
	B6.2H-2^k	k	All	90	9	1

[a] Categories as in Table I.

TABLE IV

Staining of thymocyte subpopulations with anti-I-A^k monoclonals

Antibody	Strain	I-A Allele	Thymocyte Subpopulation	% of Total[a] Undetectable	Dull positive	Bright positive
	B6.H-2^k	k	All	57	36	7
α-I-A^{kb}		k	Small	71	26	3
		k	Large	24	49	27
α-I-A^{kb}	B6	b	All	92	7	1
α-I-A^{kc}	B6.H-2^k	k	All	66	30	4
		k	Small	77	21	2
		k	Large	31	51	18
α-I-A^{kc}	B6	b	All	93	6	1
α-Delta	B6.H-2^k	k	All	98	1	1

[a] See Table I. In this instance, data was collected at 2 times the gain used in Tables I to III, so the bright category in fact includes some cells that are quite dull, but still clearly positive.

[b] Monoclonal 17/227.

[c] Monoclonal 11-5.2

TABLE V

Staining of thymocyte subpopulations with anti-TL monoclonal

Antibody	Strain	TL Phenotype	Thymocyte Subpopulation	% of Total[a] Undetectable	Dull positive	Bright positive
α-TL.3[b]	B6.TL+	1, 2, 3	All	26	47	27
			Small	29	51	20
			Large	15	37	48
	B6	0	All	97	3	0
α-Delta	B6.TL+	1, 2, 3	All	96	3	1
α-TL.3[b]	B6.TL+	1, 2, 3	CRT[c]	60	33	7
α-Delta	B6.TL+	1, 2, 3	CRT	95	3	2

[a] As in Table I.

[b] Monoclonal 18/20.

[c] Cortisone-resistant thymocytes.

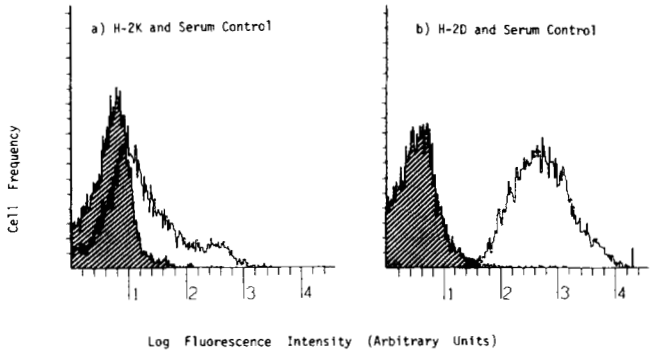

Figure 4. Figure 4 shows the FACS-generated dot plot of the two curves shown in Figure 3 (controls not shown). Each dot represents a single cell. Note in each case the prominent subpopulation is the small cells. The small distinct subpopulation slightly above and to the right of these consists of bright, medium-sized (medullary) cells that are indistinguishable from CRT. Points below the main small cell peak represent red cells, dead cells, and small debris.

MHC expression of subcapsular cells

a) The technique. Subcapsular cells were stained by dipping the whole thymus in a solution of 200 μg/ml FITC in PBS for 45 sec. These conditions were chosen so that 2 to 5% of cells were fairly dimly stained. The labeled cells contain 30 to 60% large cells, as compared to 10% in the whole thymus. The proportion and brightness of stained cells can be changed by varying the FITC concentration and the duration of the dip.

Figure 5 shows a frozen section of a thymus stained for 15 sec with a solution of 600 μg/ml FITC in PBS. Only the very outside layer of cells is positive. On the microscope, the high porportion of fluorescein-positive large cells can easily be distinguished on the sections and in cell suspensions.

b) Counterstaining subcapsular cells with monoclonal antibodies. Suspensions of thymocytes were prepared from FITC-dipped thymuses and stained with monoclonal antibodies (as

above). In this case, a rhodaminated second stage was used. The suspension was scanned for large fluorescein-positive cells (or any other category), the filters were switched, and the cell was checked for the presence of rhodamine.

The results of these stainings are shown in Table VI. In the table, "subcaps. large" means fluorescein-positive large cells; all large means large cells regardless of fluorescein, and "small cells" means all small cells. Fluorescein-positive small cells were also separately scored, but were always the same as all small cells, so were not included. With visual counting, some dull positives will be counted as negatives, so the proportion of negative cells will be somewhat higher than the proportion estimated with the FACS. (Note that since different sera were tested at different times, the dull/bright distinction is imprecise.)

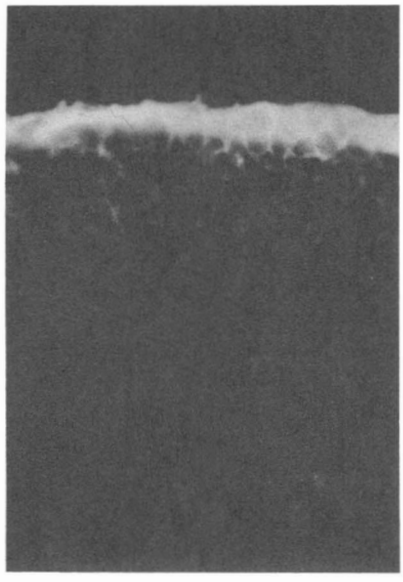

Figure 5. Figure 5 is a photomicrograph of a frozen section of mouse thymus that had been dipped in a solution of FITC for 15 sec. A layer two to three cells deep can be seen stained at the top of the figure, whereas cells deeper into the thymic cortex (*lower part of figure*) remain unstained (× 1600).

TABLE VI

Staining of subcapsular cells with monoclonal antibodies: microscopic analysis

Antibody	Strain	Allele	Thymocyte Subpopulation	% of Total[a]		
				Undetectable	Dull positive	Bright positive
100-SR28	B6.H-2^k	k	Subcaps large	1 ± 1	59 ± 4	40 ± 6
α-K^k		k	All large	2 ± 1	66 ± 3	32 ± 7
		k	Small	69 ± 7	28 ± 4	3 ± 1
17/227	B6.H-2^k	k	Subcaps large	60	26	14
α-I-A^k		k	All large	57	26	17
		k	Small	76	19	5
B22.249R1	B6.TL+	b	Subcaps large	0	15	85
α-D^b		b	All	50	17	33
18/20	B6.TL+	1, 2, 3	Subcaps large	33	nd[b]	67
α-TL.3		1, 2, 3	All	48	nd	52
2nd Stage alone	B6.TL+		Subcaps large	98	1	1
			All	99	1	0

[a] Each value is the mean of determination from three individual animals. Usually a total of 300 cells counted. For simplicity, standard errors are indicated only for the K^k antibody, since these are typical for all sera.

[b] nd, This category not discriminated during counting.

The table shows clearly that all large subcapsular cells are H-2K^k and H-2D^b positive, but many are I-A and TL negative (in about the same proportion as whole thymus).

MHC distribution on thymus migrants

a) General. This technique has been previously described (5, 6) but two points should be made. First, it is more difficult in this system to control the brightness of migrants and in general they are fairly bright. This means that fluorescein spillover into the rhodamine channel is more of a problem. Although the pattern of rhodamine staining can usually be distinguished for the pattern of spillover, some faintly rhodamine-positive cells could be missed. Second, since our second stage was an anti-mouse Ig, it did see B cells. The absence of positive migrants in the anti-δ control is confirmation of our previous finding (5) that all the "migrants" are T cells. The control antibody is against the Ig-5^a allotype. B6 animals are Ig-5^b.

b) Thymus cell migrants are all H-2D and H-2K positive; a portion are I-A positive. Table VII shows the staining of migrants with various monoclonal antibodies. Some other populations are included for comparison of the criteria used here with those used on the FACS. Migrants all have a phenotype comparable with peripheral cells. They are all bright for K^k and D^b, and a proportion are positive for I-A^k. It is of interest to note that visual counting confirms the FACS data that fewer spleen cells seem to be I-A positive than lymph node cells, but that the difference is not obvious among the migrants. No migrants have detectable TL.3, but because TL.3 staining of peripheral T cells has a diffuse, poorly defined staining pattern, the fluorescein marker spillover could mask dull TL.3 staining. Thus, it is possible that 20% (or all) of the migrants could be in the dull TL.3-positive category.

DISCUSSION

In the following discussion, three points should be reemphasized. First, when we say undetectable, we mean below the levels of detectability with our reagents. This does not mean the cells were completely negative, although immunofluorescence analysis on the FACS at high gains is fairly sensitive. Second, positive cells may have passively acquired the antigen being measured. Another surprising finding is that some monoclonals show pronounced prozones in staining studies. For example, the anti-K^k monoclonal (100-SR28) did not reach maximum brightness (on lymph node cells) until a dilution of 1/100 was reached. At 1/25, the staining was less bright. In such a situation careful and complete titration is essential. Such titrations with both first and second stage reagents were carried out in this study.

A summary of the antigenic phenotypes of the known subclasses of thymic and T lymphocytes is given in Table VIII.

With regard to the staining with the various monoclonals, a number of points warrant discussion: First, a majority of small thymocytes have undetectable levels of H-2K. This was confirmed with two different monoclonal antibodies, on several H-2K^k strains and with conventional antisera to two different haplo types (H-2K^k and H-2K^d). Even in the latter cases where backgrounds are higher and some anti-I-A activities may exist (H-2K^d)—both factors that would reduce the proportion of negative cells—a substantial proportion of thymocytes (and a majority of small cells) still do not stain above background

TABLE VII

Staining of thymic migrants with monoclonal antibodies microscopic analysis

Antibody	Strain	Allele	Subpopulation	% of Total[a]			
				Undetectable	Dull positive		Bright positive
100-SR28	B6.H-2^k	k	All thymocytes	51	38		11
α-K^k		k	All LN cells	0		[100][b, c]	
		k	All Spl cells	0		[100][c]	
		k	LN migrants	0		[100]	
		k	Spl migrants	3		[97]	
17/227	B6.H-2^k	k	All thymocytes	82	18		0
α-I-A^k		k	All LN cells	41	30		29[c]
		k	All Spl cells	42	8		50[c]
		k	LN migrants	53	47		0
		k	Spl migrants	68	32		0
B22.249RI	B6	b	All thymocytes	40		[60]	
α-D^b		b	All LN cells	0		[100][c]	
		b	All Spl cells	0		[100][c]	
		b	LN migrants	0		[100]	
		b	Spl migrants	0		[100]	
18/20	B6.TL+	1, 2, 3	All thymocytes	31		[69]	
α-TL.3			LN migrants	100		[0]	
			Spl migrants	100		[0]	
α-Delta	B6.H-2^k	k	All thymocytes	100	0		0
		k	All LN cells	67	3		30[c]
		k	All Spl cells	46	2		52[c]
		k	LN migrants	100	0		0
		k	Spl migrants	99	1		0

[a] Each value is the mean of determination from two to three individual animals, with the exception of α-TL, which was a pool. Standard errors are comparable with those in Table VI. Each value represents 50 to 150 total cells.

[b] Bracket indicates cells only counted as positive or negative.

[c] Note that in all cases of "all LN cells" and "all Spl cells", the bright category includes B cells, since the second stage reagent is anti-mouse Ig.

TABLE VIII

Summary of the phenotypes of various T cell subpopulations

Subpopulation	Antigen[a]			
	H-2K	I-A	H-2D	TL
Subcaps large[b]	100 b/d	40 d	100 b/d	70 b
All large[b]	85 b/d	75 b/d	100 b/d	85 b
Small[b]	30 d	30 d	100 d	70 b
CRT[b]	100 b	n.d.[c]	100 b	20 d
Migrants in LN[d]	100 b	50 d	100 b	0[e]
Migrants in Spl[f]	100 b	30 d	100 b	0[e]
LN T cells	100 b	50 d	100 b	20 d
Spl T cells	100 b	10 d	100 b	20 d

[a] Figures represent the rounded % of cells positive for each antigen within each subpopulation; b, mostly bright cells (among positives); d, mostly dull cells (among positives); b/d, a mixture.

[b] Thymocytes.

[c] n.d., not done.

[d] LN, mesenteric lymph node.

[e] Visual analysis which could not detect FACS-defined dim cells.

[f] Spl, spleen.

levels. It is not likely that the difference in staining patterns with anti-H-2D and anti-H-2K resulted from an artifact of the reagents involved, since both antibodies stain all peripheral T cells brightly. This finding, although surprising to us, is in fact not in disagreement with published data. It is well known that thymocytes are not well endowed (as a population) with H-2 antigens, but the techniques used to make this point, namely absorption of cytotoxicity (21, 22) or binding of labeled antibodies (23) measured only the total and/or average H-2 levels and would not have discriminated heterogenous populations. Furthermore, U. Hammerling has recently shown that a cytolytic anti-K^k monoclonal lyses only 30 to 35% of thymocytes in the presence of a source of complement (personal communication).

Since normal thymocytes may be H-2K positive or negative, it is possible that previous reports of induction of high H-2K levels in thymocytes of preleukemic mice (24, 25), or loss of H-2K in some thymic lymphomas (25, 26) might result from subpopulation selection rather than antigen change within a population.

An additional point remains, which applies to the discussion of both H-2K and TL. Most conventional antisera contain antiviral (GP70) activities, which in some cases can be extremely strain specific (18). This sort of activity may possibly account for higher levels of staining seen by others, although, as mentioned above, even conventional sera in our hands leave a substantial negative population.

The anti-H-2D monoclonal stained most thymocytes relatively dimly, as expected, but stained medullary lymphocytes and peripheral T cells quite brightly. In addition, our data could be interpreted to agree with the published data on the influence of TL on the levels of H-2D expression (i.e., TL$^+$ cells are said to express less H-2D than TL- cells) (20). However, this effect was quite variable in our experiments and in some cases no difference in H-2D expression was detected between B6 and

B6/TL+ thymocytes. Neither increasing or decreasing the amount of antibody used affected the variability of this phenomenon.

These data (obtained with the TL-3 monoclonal) demonstrate that TL3 is expressed on most, but not all cortical thymocytes, and is expressed dimly but definitely on 30 to 40% of medullary cells and 10 to 20% of peripheral T cells. These results contradict the impression that TL is expressed on all cortical thymocytes, but not on medullary thymocytes or peripheral T cells (27, 28), but are nonetheless consistent with the published data. The original experiments using direct cytotoxicity to define the distribution of TL antigens would not have detected these low levels of TL on CRT or peripheral cells (29, 31). Although more recent experiments suggested that Qa antigens were the targets when TL sera showed reactivity against peripheral T cells (28, 32), at least one report of TL 1, 2, 3 on peripheral spleen cells of young mice (33) is corroborated by our findings. In the thymus it is possible that TL 3 is expressed less extensively than TL 1, 2, but we have similar data using conventional anti-TL 1, 2, 3 sera. There are also published data that suggest that TL is not expressed on all cortical thymocytes. Thus, anti-TL sera (34) and monoclonal anti-TL 3 antibodies (U. Hammerling, personal communication) killed only 75 to 80% of thymocytes; and cytotoxic treatment of thymocytes with anti-TL sera (35) resulted in considerably less increase in GVH reactivity than did cortisone treatment (36), suggesting that anti-TL treatment either left a substantial number of non-CRT intact, or killed some of the reactive CRT.

The presence of low levels of I-A on thymocytes (37) and peripheral T cells (16, 38) has been reported. This low level of I-A on a large minority of cells seems to be true of all the subpopulations examined, but whether these represent synthesized antigens or passively acquired antigens remains to be seen.

Finally, the question of lineage relationships between these phenotypically defined subpopulations should be considered. The argument is the same for all the antigens, but let us consider H-2K as an example. There are two main possibilities. 1) H-2K-positive subcapsular precursors give rise to H-2K positive and H-2K negative progeny as a result of differential selection or differentiation signals. These sublines are stable and only the H-2K-positive cells (in this case a minority) ever give rise to migrants. 2) Alternatively, the H-2K-positive subcapsular cells may also differentiate into H-2K-negative small cells, which, when they receive an appropriate signal will reexpress H-2K and then leave the thymus. The central question that must be answered, therefore, is whether H-2K expression in small cortical cells is a stable marker separating potential migrants from cells bound to die within the thymus, or is it an unstable indicator of sequential maturation stages? The same question can be asked of the H-2D bright and dull cortical cells or of the TL-positive and TL-negative subcapsular and small cortical cells. In preliminary double staining experiments with H-2K and TL, we found incomplete overlap between high levels of TL and H-2K positivity; therefore there are thymic subpopulations that are $H\text{-}2K^+TL^-$ and $H\text{-}2K^-TL^+$. Thus, if the presence of high levels of TL made emigration unlikely, whereas the presence of H-2K favored emigration, then only the $H\text{-}2K^+TL^-$ population would migrate. The $H\text{-}2K^-TL^+$ cells would die *in situ*. If this hypothesis is true, then the differentiation of $H\text{-}2K^+$ subcapsular lymphoblasts to $H\text{-}2K^+TL^-$ and $H\text{-}2K^-TL^+$ subpopulations has great functional significance, and could be programmed as a result of intrathymic selection of "appropriate" *vs* "inappropriate" maturation (6, 39).

In conclusion, the question remains whether the phenotypically distinct subpopulations we have defined are representative of separate lineages, or whether they are different stages of the same lineage. Since the immediate precursors of migrants represent such a small proportion of the whole thymocyte population, this question can never be answered by phenotype analysis alone. Transfer experiments designed to test the potential of these subpopulations may give more definitive answers and are in progress in our laboratories. It is also important to reemphasize that these results were obtained with a number of monoclonal antibodies and conventional antisera on a finite number of strains. It is not correct to extrapolate these results to MHC determinants other than those described, or to mouse strains other than those studied.

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