T CELL ONTOGENY

Organ Location of Maturing Populations as Defined by Surface Antigen Markers Is Similar in Neonates and Adults*

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Cytotoxic depletion studies with anti-Lyt-1, anti-Lyt-2, and anti-Lyt-3 antisera demonstrated that functional mouse T cell subpopulations differ in their expression of these surface antigens. Helper T cells were sensitive to depletion only with anti-Lyt-1, whereas suppressor and cytotoxic T cells were depleted only with anti-Lyt-2 or anti-Lyt-3 (1–3). Thus, helper T cells were classified as Lyt-$1^+2^-3^-$ and suppressor and cytotoxic T cells as Lyt-$1^-2^+3^+$. Supposedly immature T cells, in contrast, were sensitive to depletion by all three antisera and were therefore classified as Lyt-$1^+2^+3^+$. These latter cells constituted ~50% of T cells in the adult spleen, but almost 100% in spleens from 7-d-old mice (1).

With the advent of monoclonal antibodies directed against each of these three Lyt antigens, it became possible to take full advantage of the analytic capabilities of the fluorescence-activated cell sorter (FACS)[1] to examine the quantitative expression of these antigens on lymphocyte subpopulations by immunofluorescence. These studies, conducted with adult mice, confirmed that Lyt-1 is present on all thymocytes and, surprisingly, showed that Lyt-1 is also found on essentially all peripheral T cells, albeit to a lesser extent on Lyt-2^+ than on Lyt-2^- cells (4, 5). This corroborated earlier reports demonstrating that cytotoxic T cells can be depleted under optimal conditions with either conventional or monoclonal anti-Lyt-1 antibodies (6, 7). It therefore established Lyt-1 as a quantitative rather than a qualitative marker for distinguishing functional and maturational T cell subpopulations.

Lyt-2, in contrast, was shown to be present on fewer peripheral T cells than originally reported but, as before, to be present on most thymocytes (4, 5). Thus it remains as a qualitative marker for a T cell subpopulation in the periphery and, because of its higher frequency in the thymus, as a marker for distinguishing immature from mature T cell populations during ontogeny. Lyt-3, which is located on the same

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[1] *Abbreviation used in this paper*: FACS, fluorescence-activated cell sorter.

surface macromolecule as Lyt-2 (8), constitutes a second determinant marking the same subpopulations.

In this paper, we use the Lyt-1, Lyt-2, Thy-1, and ThB markers to examine the development of T cell subpopulations in thymus and spleen during the neonatal period. (ThB is present on approximately one-half the adult thymocyte population, as well as on B cells, but is not detectable on peripheral T cells [9, 10]). The data obtained do not support the view that the splenic T cell population of nursling mice is less mature than in adults. Rather, they suggest that the sites for T cell maturation are established on the adult pattern during the first 2 wk of life.

Materials and Methods

Mice. BALB/cNHz and SJL/JHz mice were bred at Stanford University School of Medicine, Stanford, Calif. All mice received food and water without restrictions. Only female animals were used for these studies because aggressive behavior among adult male animals was associated with substantial alterations in T cell numbers and distribution.

Antibodies

MONOCLONAL ANTIBODIES. The preparation and characterization of the monoclonal rat antibodies against Thy-1 (59-AD2.2, 53-2.1, and 30-H12), Lyt-1 (53-7.3), Lyt-2 (53-6.7), Lyt-3 (53-5.8), and ThB (53-9.2 and 49 h4) has been described previously (4, 8, 10). In earlier experiments, these were used as indirect staining reagents; however, all density-distribution curves (FACS profiles) shown were obtained by direct staining with fluorescein-conjugated antibodies.

OTHER ANTIBODIES. A non-protein A-binding fraction (primarily IgG_1 antibodies) of hyperimmune SJL/J anti-rat IgG was conjugated with fluorescein isothiocyanate; this was used as a second-step reagent to demonstrate binding of the rat antibodies to the T cells. A fluorescein-labeled goat anti-mouse Ig (5-178; Nordic Immunological Laboratories, Tilburg, The Netherlands) was used to enumerate surface Ig-bearing B cells.

Cell Suspensions. Spleen or thymus from at least three animals were pooled for each age group in each experiment. The cells were suspended by gently rubbing the spleens between slides with frosted ends. The medium consisted of RPMI-1640 (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 5% heat-inactivated and filtered fetal calf serum, 10 mM Hepes, and 0.1% NaN_3. Cells were washed twice by centrifugation and their number and percentage of viability were determined by staining an aliquot with ethidium bromide and acridine orange (1 μg/ml of each) and counting with a fluorescence microscope. The cells were kept at 4°C throughout the experiments.

Staining. 10^6 live cells in 25–50 μl of medium were incubated for 30 min at 4°C in 96-well flexible microtiter plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.) with the various monoclonal antibodies, either unlabeled or fluorescein-conjugated. For indirect stains, the cells were then washed twice with 150 μl of medium, 2 μl of fluorescein-labeled SJL/J anti-rat IgG was added in a total vol of 50 μl, and incubated for 30 min at 4°C. Finally, the stained cells were washed and resuspended in 1 ml of medium for FACS analysis.

FACS Analysis. Scatter and fluorescence profiles of the various samples were obtained with a modified fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Mountain View, Calif.) equipped with a logarithmic amplifier (11). Standard FACS analytical procedures were used (12, 13): low-angle light scatter gates were used to exclude fluorescence signals from cells or other objects falling outside the range characteristic for viable cells. The percentage of cells falling within a given fluorescence range was determined by integration from profiles based on 10,000 viable cells. Integration thresholds were established from profiles with clearly defined minima between positive and negative populations. These thresholds (different for each of the three antibodies) were then used for all curves obtained with the respective antibodies.

Results

Adult Lyt-1 and Lyt-2 Frequencies in Splenic T Cell Populations Are Established within the First 2 wk of Life. The overall frequency of Thy-1$^+$ cells in spleen increased rapidly in animals between 1 and 3 wk of age and somewhat more slowly thereafter (Fig. 1). It reached its adult range ~6 wk after birth and then stayed relatively constant for at least the next 18 mo (unpublished data). Lyt-1$^+$ cells and Lyt-2$^+$ cells increased concordantly with Thy-1$^+$ cells so that the frequencies of these cells relative to the Thy-1$^+$ frequency remained relatively constant from the earliest times when such frequencies were accurately measurable (~10 d of age).

The frequency of splenic Lyt-1$^+$ cells in both nurslings and adults was routinely somewhat higher than the frequency of Thy-1$^+$ cells (Fig. 1). The ratio of Lyt-1$^+$/Thy-1$^+$ cells may have decreased slightly with age but was still greater than one in adults. This is consistent with recent observations (4) demonstrating the existence in adult spleen of a small Lyt-1$^+$ population devoid of detectable Thy-1. Because the T cell origin of this "Lyt-1 only" population has not been established, we use the Thy-1$^+$ frequency as a reference value for T cell frequency in the various spleen cell populations discussed below.

Lyt-2$^+$ cell frequencies contrasted sharply with Lyt-1$^+$ frequencies in that they were always substantially lower than Thy-1$^+$ cell frequencies. Calculation of the ratios of Lyt-2$^+$:Thy-1$^+$ cells shows that the proportion of T cells carrying Lyt-2 was also essentially constant throughout life regardless of the overall increase in splenic T cell frequency that occurs with age (Fig. 2). Thus, although there were many fewer T cells in 2-wk-old animals than in adults, the Lyt-2$^+$ cells represented about one-third of the total T cell population in both cases. There was therefore no evidence for a relatively large neonatal population of splenic Lyt-1$^+$2$^+$3$^+$ (immature) T cells that reduced in size as animals matured.

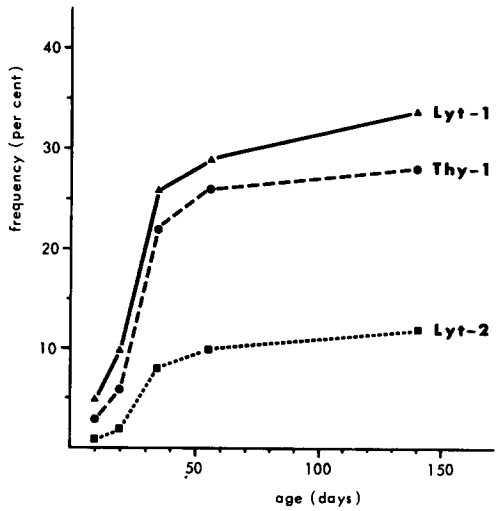

FIG. 1. Frequencies of Lyt-1$^+$ cells, Lyt-2$^+$ cells, and Thy-1$^+$ cells increase with age. Cell frequencies in BALB/c spleen were determined by integration of cells under the positive peak in FACS profiles similar to those shown in Figs. 3–5 but obtained using an indirect staining protocol; frequencies were corrected by subtraction of the small percentage of positive cells in control preparations exposed only to the fluoresceinated second-step (mouse anti-rat Ig) reagent.

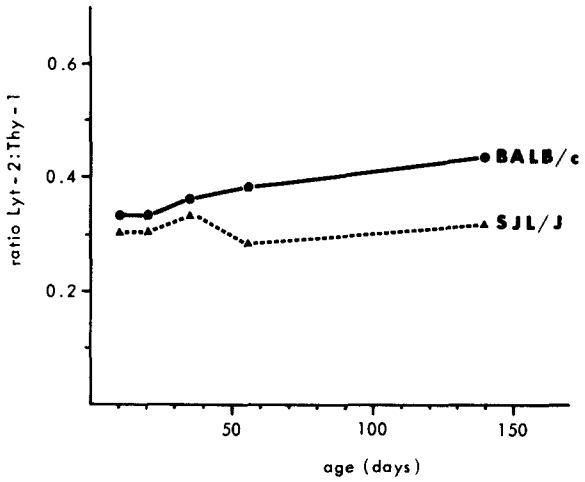

FIG. 2. Ratio of Lyt-2^+:Thy-1^+ cells remains essentially constant as animals mature. Ratios shown for BALB/c were calculated from data in Fig. 1.

Fig. 1 shows T cell frequencies for animals 10 d of age and older. Before this time, the frequency of Thy-1^+ cells in spleen was roughly 1%, even in neonatal animals (<24 h of age). We also detected a small number of Lyt-1^+ cells in these animals but did not clearly establish the existence in them of an Lyt-2^+ population, because the frequency of these cells was substantially lower than Thy-1^+ or Lyt-1^+ cells.

Surface Antigen-Density Profiles Indicate Comparable Splenic T Cell Maturity in Nurslings and Adult Animals. The distributions (FACS profiles) of cells according to the amounts of Lyt-1, Lyt-2, Lyt-3, and Thy-1 displayed indicated again that splenic T cell populations in 2-wk-old and adult animals are similar (Fig. 3). The younger animals had relatively fewer splenic T cells and consequently showed larger negative peaks in the FACS profiles. Nevertheless, the shapes of the profiles for positive cells were remarkably like the shapes of profiles for adult spleen cells stained with the same reagents. In fact, taking into account the smaller number of T cells in the younger animals, there appeared to be no significant differences between adult and nursling profiles.

In addition to demonstrating this similarity, comparison of the Thy-1 and Lyt-1 profiles shown in Fig. 3 clearly distinguished the splenic T cell population in nurslings (and adults) from the cortical thymocyte population whose analogue might be expected to be present in nursling spleen if this spleen contained mainly immature T cells. Comparison of these profiles with the profiles for thymocytes (Figs. 4 and 5) demonstrated, in agreement with previous reports (4, 14, 15), that splenic T cells carry substantially less Thy-1 and more Lyt-1 than the predominant (cortical) population in thymus. Thus both the Lyt-2 frequency data discussed earlier and the quantitative immunofluorescence data presented in this section indicated that splenic T cell populations in nurslings are more comparable to adult peripheral T cells than to the immature T cells in thymus.

A Subpopulation with the Thy-1 and Lyt-1 Antigen Density Profiles of Mature T Cells Appears in the Thymus within 24 h of Birth. The adult thymus contains a medullary subpopulation of cells, resistant to depletion by hydrocortisone, which is believed to

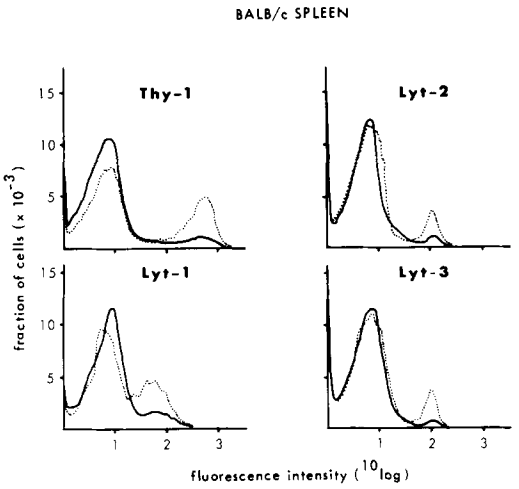

FIG. 3. Determinant density distributions for splenic T cells are similar in nurslings and adults. The FACS profiles in the Figures show cell frequencies as a function of the $\log_{10}$ of the intensity of cell-associated fluorescence obtained after staining with the indicated reagents directly conjugated with fluorescein. The logarithmic amplifier used to obtain these profiles, which is accurate over roughly a four-decade range, allows a display of frequencies for all cells in the population, including those which show no detectable staining with the reagents used. Signals from these latter cells fall within the intensity range for autofluorescence signals observable from cells that have not been exposed to any fluorescent staining reagents. Thus cells that do not stain with a given staining reagent, i.e., do not carry the determinant detected, appear as a peak on the left of the profile extending approximately to about 1.4 ($\log_{10}$) fluorescence intensity units. (···) 56 d; (—) 14 d.

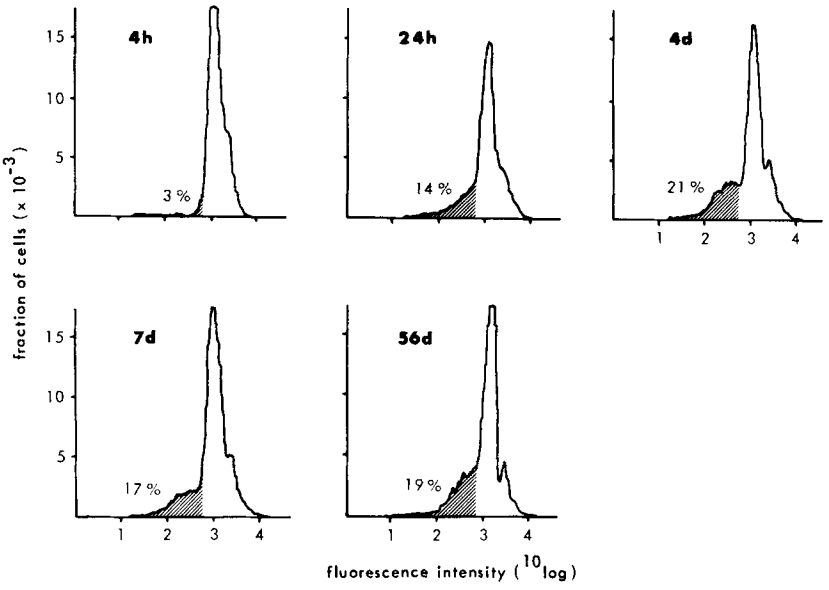

FIG. 4. Neonatal thymus rapidly acquires a dull Thy-1^+ population similar in size to the dull Thy-1^+ population in adults. (For analysis details, see legend to Fig. 3.)

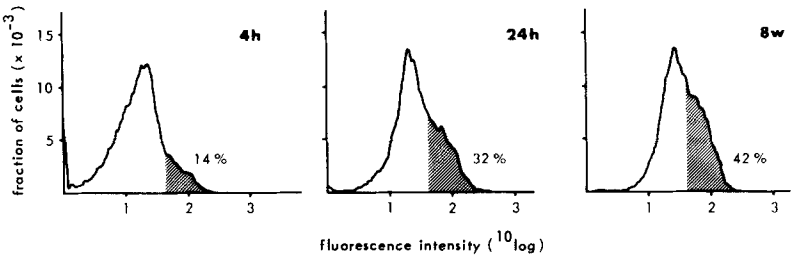

FIG. 5. Neonatal thymus rapidly acquires a bright Lyt-1$^+$ population similar in size to the bright Lyt-1$^+$ medullary population in adults. (For analysis, see legend to Fig. 3.)

be relatively mature and to include cells that will emigrate and contribute to the peripheral T cell pool (16). On average, this population has less surface Thy-1 and more surface Lyt-1 than cortical thymocytes (17), so that its presence in suspensions of intact thymus can be recognized by shoulders on FACS profiles of thymocytes stained with these reagents. These shoulders, which were particularly well defined with the aid of logarithmic amplification, appeared on the high antigen-density side of the profile for thymocytes stained with anti-Lyt-1 (Fig. 4) and the low antigen-density side for cells stained with anti-Thy-1 (Fig. 5). The comparatively high frequency of bright Lyt-1$^+$ cells suggested that they were present in a proportion of cortical as well as the medullary cells; this Lyt-1-bright subpopulation evidently included some cells that retained a high density of Thy-1.

The emergence of a thymocyte population whose antigen-density characteristics were similar to the adult medullary population occurred rapidly in the neonatal animal. Small but discernible shoulders were visible on FACS profiles of thymocytes from newborn animals (<4 h old). In 1-d-old animals, these shoulders had enlarged markedly and in 4-d-old animals, they had grown to almost the same size as the shoulders on profiles of adult thymocytes (Figs. 4 and 5). Thus, at a very early age the thymus had developed a subpopulation, proportionally the same size as in adults, of relatively mature cells. These were thus available to serve as the source of the emerging peripheral T cell population.

Changes in the thymocyte population, however, continued well beyond 4 d of age when, as indicated above, the adult Lyt-1 and Thy-1 density distributions had become clearly established. The ThB antigen, which is present on ~50% of the adult thymocyte population (9, 10), made its appearance in the thymus only gradually after birth. Less than 10% of BALB/c thymus cells carried detectable ThB at 4 h of age. This proportion increased only slowly, so that at 7 d after birth it was still only 16%, considerably below the adult frequency which was attained by 8 wk of age.

It has previously been suggested that ThB might mark a common T and B cell precursor population because, in adults, this antigen is present on virtually all splenic B cells and a large fraction of immature (cortical) thymocytes, but is found infrequently on mature (medullary) thymocytes and is undetectable on peripheral T cells (9, 10). Our demonstration that ThB$^+$ cells appear relatively gradually in the thymus argues against ThB as a differentiation marker for a prelymphocyte population. However, because even in 4-h-old animals a few ThB$^+$ cells were present; an alternative interpretation is that cortical thymocytes lose the antigen more rapidly as they mature in the neonate than in the adult.

Discussion

The introduction and use of the Lyt antigens as markers for T cell subpopulations (1–3) constituted one of the major advances in cellular immunology. Even though more sensitive detection systems have now shown that Lyt-1 is on more adult T cells and Lyt-2 is on fewer than initially believed (4, 5), the basic utility of the Lyt antigens as functional subpopulation markers still remains unchallenged. In fact, the newer methods using monoclonal antibodies and FACS analysis mainly serve to broaden the basis for subpopulation definition with anti-Lyt reagents: they allow both the unequivocal identification of determinants and the use of quantitative as well as qualitative criteria in relating antigen expression to cell subpopulations.

The use of these methods here to locate immature and mature T cell populations during ontogeny demonstrates that the pattern of neonatal T cell development differs in some respects from that defined previously by cytotoxic depletion studies with conventional anti-Lyt antisera (1); however, as in the studies on adults mentioned above, the differences we observed do not detract from the basic principles revealed by the earlier studies, i.e., that changes in the expression of the Lyt markers distinguish immature T cell populations from their more mature progeny.

These changes, defined in quantitative terms, provide the grounds for identification of mature and immature populations in spleen and thymus and thus for the conclusion that the maturity of T cells in the spleen at 10–14 d of age is similar to that of adult splenic T cells. This finding does not agree with previous reports that >80% of T cells in spleens of similar age were killed by cytotoxic treatment with either anti-Lyt-1 or anti-Lyt-2 and were therefore considered as a predominantly immature population (1). The reasons for this disparity are unclear but may involve a greater fragility of neonatal spleen cells or the presence of contaminating antibodies in the conventional anti-Lyt antisera. In this regard, it should be noted that Cantor and Boyse (1) found 30% of nucleated spleen cells from 1- to 2-wk-old mice to carry Lyt-1, -2, and -3 antigens—a frequency similar to that in adults and far higher than we have found using anti-Lyt or anti-Thy-1 monoclonal antibodies.

Whether splenic T cell populations should be considered as mature in nurslings as in adults depends on the criteria established for maturity. We have used the frequencies of Lyt antigen-defined subpopulations and the antigen-density distributions. By these limited criteria, the splenic T cells in 2-wk-old animals, although quite small in number, clearly constitute a mature population, as they show the Thy-1 and Lyt-1 surface densities and the Lyt-2 frequency characteristic of adult splenic T cell populations.

These findings, which suggest that nursling T cells mature before arriving in the spleen, are consistent with our evidence for the emergence of a relatively mature thymocyte subpopulation within 4 d of birth, a period when thymic helper T cell function also increases rapidly (18). This thymocyte subpopulation appears similar to the adult medullary (cortisone-resistant) thymocyte population which displays the antigen-density characteristics of peripheral T cells (lower Thy-1 and higher Lyt-1) (17). The composition of this population in the adult and its early appearance in the neonate make it a probable source of peripheral T cells.

Combining data presented here with those obtained in other studies, we arrived at the following tentative outline for the major events in the ontogeny of T cell populations: an immature population of T cells is present in the thymus at birth.

Within 24 h, this population begins to generate a more mature thymocyte subpopulation which reaches its adult proportion by ~4 d of age. Peripheral T cells, which may be derived from the more mature thymic subpopulation, begin to accumulate in the spleen by 1 day of age but remain at very low frequencies (1% of splenocytes) during the first week of life before beginning to expand relative to other cell types in the spleen. By 2 wk of age or earlier, however, the splenic T cell population mirrors the antigen-density distributions and relative frequencies observed in the adult. Thus despite large increases in the size of the spleen and thymus during development, and despite the increase in the frequency of T cells in the spleen, the overall pattern of subpopulation representation in spleen and thymus (as measured by Thy-1, Lyt-1, and Lyt-2 expression), is established in the first 2 wk of life and remains constant thereafter.

It is interesting to note that the period during which the neonate is most sensitive to tolerance induction coincides with the time at which relatively few mature T cells are present in the thymus and considerably precedes the development of substantial numbers of T cells in the periphery. With regard to this last point, we find proportionally far fewer splenic T cells during the first 2 wk of life than have been described by Cantor and Boyse (1) and Thomas et al. (19). Thomas et al. (19) claimed that neonatal splenic T cells lacked some Thy-1-related determinants that were present on most adult T cells. They predicted that some monoclonal anti-Thy-1 reagents might fail to recognize all neonatal and a considerable proportion of adult T cells. However, all the three monoclonal anti-Thy-1 antibodies (two anti-Thy-1.2 and one anti-Thy-1 framework) used in our studies stain identical-sized cell populations, which constitute up to 99% of non-B cells in spleen (4, 5). Thus we conclude that these antibodies can stain all cells bearing Thy-1 determinants and that the low frequency of stained cells in nursling spleen reflects a genuine paucity of T cells; this is confirmed also by the low frequency of Lyt-1^+ cells. The frequency of T cells in lymph nodes during the neonatal period is doubtless much higher than in the spleen because of the absence of concomitant erythropoiesis. Near-adult frequencies were described by Raff and Owen (20) in the mesenteric lymph node, an observation we have confirmed using monoclonal antibodies (data not shown).

Because our studies do not support the belief that T cells in nursling spleens include any large proportion of putatively immature (Lyt-$1^+2^+3^+$) cells, they point to a relatively simplified developmental pattern in which the major T cell maturational events occur similarly in neonates and adults. They do not, however, necessarily imply that nurslings and adults are identical with respect to their thymocyte and peripheral T lymphocyte populations; for example, our evidence that the frequency of ThB^+ thymocytes changes substantially during the early weeks of life suggests that they are not. Thus, more refined criteria for the differentiation and maturation of thymocyte and T lymphocyte subpopulations may reveal other changes in these populations as animals develop.

Summary

Earlier studies have suggested that splenic T cell populations in nursling mice (<18 d of age) have Lyt cell surface antigens that identify them as less mature than their adult counterparts. Studies presented here, however, demonstrate that the expression of the Thy-1, Lyt-1, Lyt-2, and Lyt-3 T cell antigens is virtually identical in 14-d-old

and adult T cell populations even though at 14 d, T cells constitute <10% of the total spleen cell population. Because the expression of these antigens on the immature (cortical) thymocyte population differs substantially from their expression on peripheral T cells, the maturity of splenic T cells as judged by these criteria is similar in nurslings and adults.

Very few cells in the neonatal thymus 4 h after birth correspond, in terms of antigen expression, to the more mature (medullary) thymocyte population of adults, but such cells develop rapidly during the first few days of life. They are present, therefore, sufficiently early to serve as the immediate source of peripheral T cells, as they apparently do in the adult. This then suggests that the locations for the major T cell maturational events are established within the first 2 wk of life of the mouse and maintained as such thereafter.

The use of monoclonal antibodies and quantitative immunofluorescence analysis in our studies probably explains the differences between our findings and those reported previously, which relied on cytotoxic depletion by alloantisera and complement to estimate the frequencies of cells carrying the Lyt differentiation antigens in nurslings.

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