SEPARATION OF STAGES IN THE DEVELOPMENT OF THE "T"
CELLS INVOLVED IN CELL-MEDIATED IMMUNITY*

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Cellular immunity is thymus dependent and is mediated by thymus-derived lymphocytes (T cells)¹ (1-6). During the induction of transplantation immunity specific T cells recognizing an alloantigen are stimulated to proliferate and differentiate to form effector cells, the cytotoxic lymphocytes (CL) or killer cells. These are able to destroy target cells carrying the immunizing alloantigen, and may be assayed in vitro using the release of ⁵¹Cr from labeled targets as a measure of cytotoxic activity (7). The T cells initiating the cellular immune response (CL progenitors) may be assayed in an adoptive immune system by transfer to heavily X-irradiated allogeneic recipients (4). The number of CL progenitors responding to the alloantigens of the recipient host is measured by the amount of in vitro cytotoxic activity in the spleen cells of the recipients 4-5 days after transfer. By such assays CL progenitors are found in both mouse thymus and spleen (2, 4). Immunization of mice with tumor allografts results in the appearance of CL in the spleen, lymph nodes, and blood; but very few are detected in the thymus (8).

The aim of this study was to separate and characterize various stages in the development of this lineage of immunologically functional T cells, from the earliest detectable CL progenitors maturing in the thymus to the final population of CL in the peripheral lymphoid tissues. Cells were separated and characterized on the basis of their buoyant density by centrifugation to equilibrium in continuous gradients of albumin (9-11). In a recent study² we have demonstrated that various populations of lymphoid cells, including B and T cells of mouse spleen and two subpopulations of T cells in mouse thymus, were partially separated from each other by this technique. In addition, each individual class of lymphocyte formed a series of discrete and separable density peaks. In the present paper the density subpopulations of the T lymphocytes

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¹ Abbreviations used in this paper: CL, cytotoxic lymphocytes; T cells, thymus-derived lymphocytes.
² Shortman, K., J.-C. Cerottini, and K. T. Brunner. 1972. The separation of subpopulations of "T" and "B" lymphocytes. Manuscript in preparation.
were tested to see if they differed in immunological capacity and if they represented stages in T-cell differentiation.

**Materials and Methods**

**Mice.**—The mouse strains studied were inbred C57BL/6 (H-2^b^) and inbred CBA (H-2^k^) mice. Male mice around 12 wk of age were used in all experiments.

**Cell Suspensions.**—Cells were teased out from spleen or thymus and freed of large and small debris, as described elsewhere (12). The basic suspension medium was a N-2-hydroxyethyl)piperazine-N'-2-ethane sulfonic acid (HEPES)-buffered balanced salt solution containing 10% fetal calf serum, iso-osmotic with mouse serum (11, 12).

**Albumin Gradient Separation.**—The basic separation procedure is given in detail elsewhere (9). The only change from the original procedure was the use of albumin media iso-osmotic with mouse serum (308 milliosmolar, equivalent to 0.168 M NaCl) (11, 12). Briefly, pooled cells from three to five spleens or three to eight thymus glands were dispersed into a continuous linear gradient of bovine plasma albumin, 19-30% (w/w), pH 5.1, in a balanced salt solution. The gradient was centrifuged at 4000 g for 30 min to bring cells to their buoyant density. Fractions of known volume were collected by upward displacement from the gradient tube. The precise density of each fraction was measured. The fractions were diluted, and the cells were recovered by centrifugation and made up to a known volume. Samples of each fraction were then used for total cell counts, for assays of immunological capacity, and for estimation of the proportion of various lymphocyte subclasses.

The density distribution profiles calculated from these data represent the total numbers of cells (or the calculated total CL or CL progenitors) per fraction, divided by the density increment covered by that fraction and plotted against fraction density. All curves are normalized relative to the same peak height of 100%, regardless of absolute numbers or absolute activity.

**Total Cell Counts.**—Total nucleated cells per fraction were counted using a Coulter Model B cell counter (Coulter Electronics, Inc., Hialeah, Fla.), with the lower threshold set to eliminate red cells and cell debris. Damaged cells sedimented out of the normal gradient range and thus did not contribute to counts on individual fractions (9, 11).

**Enumeration of Lymphocyte Subclasses.**—The content of T lymphocytes in fractions from spleen gradients was obtained from a cytotoxic assay using anti-0 antiserum. The content of lymphocytes with high levels of surface H-2 antigen in fractions from thymus gradients was determined by immunofluorescent staining. Full details of both of these assays on individual gradient fractions are given in a separate paper. 2

**Immunization with Allogeneic Tumor Cells.**—P-815-X2 mastocytoma cells (13) were maintained by serial passage in ascitic form in DBA/2 (H-2^d^) mice. C57BL/6 (H-2^b^) or CBA (H-2^k^) mice were immunized by intraperitoneal injection of 3 X 10^7 viable tumor cells (7).

**Assay for Cytotoxic Lymphocytes (CL).**—The procedure was that of Brunner et al. (7), using the ^51Cr-labeled P-815-X2 mastocytoma cells (bearing the DBA/2 mouse strain alloantigens) as targets and measuring ^51Cr release over a 6-hr period. To measure the relative number of CL present in any suspension, various numbers of lymphocytes were mixed with a fixed number of target cells, and the percentage lysis was calculated as described elsewhere (14). From these data a curve relating percentage lysis to lymphocyte concentration could be constructed. The concentration of lymphocytes producing 33% lysis of 25,000 mastocyte target cells was considered to contain one unit of lytic activity (14). The relative number of CL in any preparation or gradient fraction was calculated as the number of lytic units per 10^6 lymphocytes multiplied by the total number of lymphocytes in the fraction. For each density distribution experiment, a separate, detailed, dose-response curve was constructed, with many dilutions of the unfractionated cells. This curve was assumed to apply to each gradient fraction, which was assayed at only one dilution. Errors introduced by this assumption were minimized by using concentrations of each gradient fraction that were known from preliminary
experiments to produce close to 33% lysis. All assays were done in duplicate or trilicate.

Assays for CL Progenitors.—To assay for the progenitors of CL, a fixed number of cells from each gradient fraction or test preparation was transferred intravenously into groups of four or five DBA/2 mice, previously X-irradiated with 800 rads (4). The number of viable cells transferred to each irradiated recipient was 15-20 \times 10^6 for thymus cells and 5-6 \times 10^6 for spleen cells. 5 days later the recipients were killed, and a spleen cell suspension was prepared from each. The total cell yield per spleen was determined, and samples of the spleen cell suspension were assayed for in vitro cytotoxic activity with a wide range of test cell to target cell ratios. From these data the relative cytotoxic activity per 10^6 harvested spleen cells was determined, and the total cytotoxic activity per recipient spleen was calculated. This was taken as a measure of the relative level of CL progenitors in the fixed number of cells transferred. For convenience, one unit of CL-progenitor activity was defined as the number of CL progenitors required to produce one unit of lytic activity in the spleens of the recipient animals. The total units of CL progenitors in each fraction or test sample was then the total number of cells per fraction multiplied by the units of CL progenitors per 10^6 cells transferred.

RESULTS

CL Progenitors in Thymus.—The thymus contains a small proportion of immunocompetent cells, capable of seeding in the spleen of irradiated allogeneic recipients and producing a population of cytotoxic lymphocytes (CL) (3). These CL progenitors in the thymus probably represent T cells that have just matured to the point of immunocompetence, and they should therefore be the earliest detectable elements in the differentiation sequence to be studied.

The thymus also contains a small proportion of cells that share the surface antigenic characteristics of peripheral T cells, namely high levels of H-2 antigens and relatively low levels of \(\theta\)-antigen (15). In a separate study\(^a\) we have demonstrated that this subpopulation was localized in the light-density regions after centrifugation of thymus cells to equilibrium in albumin density gradients. It was of interest to determine if the CL progenitors in thymus represented a special category of cells separable by buoyant density centrifugation and if the CL progenitors corresponded to the minor thymus population with the antigenic characteristics of peripheral T cells.

Thymus cells from 10-wk-old C57BL/6 mice were separated into five to seven density fractions by centrifugation to equilibrium in continuous gradients of bovine plasma albumin. The cells in each fraction were recovered and counted. Samples were taken for immunofluorescent assay\(^b\) (15) of the proportion of cells with high levels of surface H-2 antigens (high H-2 cells). Other samples were transferred to groups of irradiated DBA/2 mice for estimation of the relative levels of CL progenitors. The results of one such experiment are given in Fig. 1. The following are the major results: (a) The CL progenitors in the thymus showed a relatively narrow, homogeneous density distribution, around density 1.073 g/cm\(^3\), and were enriched sevenfold in this region, compared with unfractionated thymus. (b) The typical thymus small lymphocyte (density 1.080-1.090 g/cm\(^3\)) was completely inactive as a CL progenitor. (c) Although CL progenitors were in the light-density regions where the minor population of high H-2 lymphocytes concentrated, the two populations were
not completely identical. In particular, the gradient regions less dense than 1.072 g/cm\(^3\) were strongly enriched in high H-2 cells, but low in immunocompetence. (d) The single peak of CL progenitors (1.072-1.075 g/cm\(^3\)) corresponded in density to the major peak of the series of peaks of high H-2

**Fig. 1.** The density characteristics of CL progenitors in C57BL/6 mouse thymus. Thymus cells were fractionated on continuous albumin gradients, as described in Materials and Methods. The upper two curves are density distribution profiles in which the total cells and total CL-progenitor activity per fraction are divided by the density increment covered by the fraction and plotted against fraction density. Both profiles are normalized to the same peak height. The lower two curves compare the percentage "high H-2" lymphocytes in each fraction with the CL-progenitor units per 10\(^6\) cells in each fraction. In these curves the broken line gives the values for the unfractionated thymus cells.

lymphocytes found in C57BL/6 and in CBA mouse thymus in higher resolution gradients, as reported in a separate paper.\(^3\)

The results of Fig. 1 were obtained in three separate experiments. One disturbing feature of all experiments was the low over-all recovery of CL-pro-
genitor activity from the gradients (mean value 31%) compared with the un-
fractionated suspension. The mean overall recovery of nucleated cells was 83%.
The gradient procedure normally gives high recovery of biological activity (9,
10). One possible cause of low recovery would be the separation within the
gradient of two types of cells whose interaction was required to produce CL
in the recipient. A number of experiments were carried out to test for such a
synergistic effect on mixing fractions. The addition to each gradient fraction of
10% of cells from the light-density (< 1.072 g/cm³) high H-2-enriched regions
failed to produce additive effects or to change the apparent density profile of
CL progenitors. In another experiment the addition of equal parts of spleen B
cells (prepared by anti-θ treatment of C57BL/6 spleen cell suspensions) failed
to affect the results. Although these experiments were not exhaustive, no syner-
gistic effects were noted.

Comparison of CL Progenitors in Spleen and Thymus.—The CL progenitors
in the C57BL/6 mouse thymus had the density characteristics of a medium-
sized, relatively immature cell, rather than those of a typical small lympho-
cyte. It was of interest to compare this with the cells of corresponding activity in
peripheral lymphoid tissues. To this end, spleen cells from 8-wk-old C57BL/6
mice were separated by buoyant density centrifugation, and the distribution
of CL-progenitor activity was compared with that of the thymus cells. One
such comparison is given in Fig. 2, which makes the following points: (a) The
density distribution of CL progenitors in thymus and spleen was strikingly
different. The active cells in the spleen were much more dense than those in
the thymus. (b) CL progenitors in the spleen were heterogeneous in density
and were in the region of typical small lymphocytes. The active regions cor-
responded to the denser of a series of peaks of C57BL/6 spleen T cells, found
with higher resolution gradients, as reported in a separate paper² and also shown
in Fig. 3.

One difference between spleen and thymus cell experiments was the presence
of additional B cells in the spleen preparations. To test if this could affect the
CL-progenitor activity, 15 × 10⁶ viable spleen B cells (prepared by anti-θ
and rabbit complement treatment of C57BL/6 spleen cells) were added to 15
× 10⁶ thymus cells from various fractions before transfer to irradiated re-
cipients. The thymus CL-progenitor profile was unaffected.

The over-all recoveries of CL progenitors in density fractions of spleen cells
was generally low (mean 48%) compared with the total cell recovery (mean
value, 85%), just as had been observed with thymus cell fractionation.

The Effect of Antigenic Stimulation on Spleen CL Progenitors.—The results
in Fig. 2 could be interpreted in terms of continuing differentiation or matura-
tion of CL progenitors, the less mature T cells leaving the thymus to develop
into a population of typical, nondividing T-class, small lymphocytes. It was of
interest to see if these dense, mature, CL progenitors would now change their
physical characteristics on contact with antigen and become less dense, in a
manner analogous to the effects of antigen stimulation on the buoyant density of cells initiating adoptive humoral responses (16-18). Accordingly, C57BL/6 mice were immunized by intraperitoneal injection of mastocytoma cells; and the density distribution of CL progenitors in the spleen was compared at

![Diagram](image)

**Fig. 2.** A comparison of the density distribution of cells with CL-progenitor activity in the spleen and thymus of C57BL/6 mice. The curves are based on the total (not the per cell) CL-progenitor activity, and represent density distribution functions, with cells or activity per density increment plotted against density. Peak values are normalized to the same height in each case. The actual CL-progenitor activity of unfractionated thymus cells was around 20% that of spleen cells on a per cell basis.

periods after immunization by the standard assay of transfer of cells from albumin gradient fractions to irradiated DBA/2 mice. The density profiles of CL progenitors 16 hr and 63 days after immunization were compared with the normal, nonimmunized spleen population, in the hope of contrasting a recently stimulated situation with a long-term stimulated or “memory” situation. These results are summarized in Fig. 3 and are compared with a detailed density
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distribution profile of the T-cell population of unstimulated spleen. The following are the major findings:

(a) 16 hr after stimulation with alloantigens in the form of living mastocytoma cells, there was a general shift of CL progenitors toward the lighter-density regions. However, the shift was not as extensive as the shifts previously reported for cells initiating humoral responses to sheep erythrocyte antigen (16, 17). The major peak of activity remained around density 1.076–1.081 g/cm³, corresponding to one zone where T cells are concentrated on separation of normal spleen. There were a relative loss of CL-progenitor activity in the region of the most dense T-cell peak (around 1.089 g/cm³) and a relative increase in the light-density regions where some minor T-cell peaks are found in normal spleen.

(b) 63 days after stimulation, when living mastocytoma cells had been eliminated from the animals, the distribution of CL progenitors was similar to the 16-hr stimulated profile. Failure to return to the unstimulated state may have been due to persistence of antigenic stimulation. This point was not investigated further.

Density Distribution of CL in Immunized C57BL/6 Mouse Spleen.—The studies on CL progenitors in spleen indicated that these were dense cells in the unstimulated animal and that the bulk of CL progenitors remained dense even in a stimulated situation. This suggested that CL-progenitor activity was normally carried by the typical, dense, mature, small lymphocytes of the T-cell series. It was of interest to compare these physical characteristics of the CL-progenitor cells with the final effector cells, the CL. In particular, we wished to determine if CL were a clearly defined type of cell, or if they also changed their physical characteristics during maturation. Accordingly, C57BL/6 mice were immunized with living mastocytoma cells, and the density distribution of cytotoxic activity in the spleens was compared very early in the response (6 days) and very late in the response, after the mastocytoma cells had been eliminated (42 days). The effect of a second antigenic stimulus on a “mature” CL population was also investigated. The CL-activity profiles were compared with the density distribution of total T cells in a normal, nonimmunized spleen. The results are shown in Fig. 4, and may be summarized as follows: (a) Early in the response, CL were concentrated in the light-density regions of the gradient, in regions where small peaks of T cells are located in the normal spleen. (b) Even at 6 days the distribution of CL was heterogeneous, and some activity was found in the dense, small-lymphocyte region. (c) Late in the response, the distribution of the CL population had shifted to denser regions. The profile of activity now overlapped the regions where small lymphocytes concentrated, and also corresponded closely to the distribution of CL progenitors in stimulated animals (Fig. 3). (d) Restimulation of animals 36 days after the primary stimulus appeared to cause a reemergence of the immature, light-density forms of CL. (e) In the recently immunized situations, the density
distribution of the total nucleated cells of the spleen was shifted to the light-density end, compared with the normal animals. The spleens of such immunized animals were grossly enlarged. It appeared that a high proportion of the spleen

cell population was responding, specifically or nonspecifically, to the tumor allograft. (f) Recovery of CL was high (mean, 85%), in contrast to the lower recovery of CL progenitors.

Fig. 3. The effect of stimulation with a tumor allograft on the density distribution of CL progenitors in C57BL/6 spleen. The upper continuous curve gives for comparison the normal density distribution of all "T" lymphocytes in unstimulated spleen (from Shortman, Cerottini, and Brunner). Changes in density profile are given as a function of time after intraperitoneal injection of the C57BL/6 mice with $3 \times 10^7$ viable mastocytoma cells, as in the standard immunization protocol. Other details are the same as in Figs. 1 and 2.
Fig. 4. The density distribution of CL in spleens of C57BL/6 mice responding to tumor allografts. The upper curve gives the density distribution of normal, unstimulated spleen cells and of all normal T cells, for comparison (from Shortman, Cerottini, and Brunner). The spleens of immunized animals were fractionated at various times after immunization with $3 \times 10^7$ viable mastocytoma cells. The cells in each fraction were recovered, counted, and assayed for CL activity by the in vitro $^{51}$Cr release from labeled mastocyte targets. The curves represent density distribution functions based on total (not per cell) cytotoxic activities in each fraction. Peak CL values are normalized to the same height.
Density Distribution of CL in CBA Mouse Spleen.—The experiments with immunized C57BL/6 mice suggested that the density characteristics of CL developed with time, from light-density, immature cells banding in the large-medium lymphocyte-enriched zones to dense cells with the density characteristics of typical thymus-derived small lymphocytes. This recalled similar changes in antibody-forming cells in toad spleen, in which large, dividing, light-density, blast-like, antibody-forming cells early in the response increased in density and decreased in size during maturation, leading to a small-lymphocyte end product (Kraft and Shortman, 19). It was important to check if such changes in CL density with time were a general phenomenon. Since we had already observed substantial differences in the T-cell population of C57BL/6 and CBA mice, the studies on CL were repeated using the CBA strain. A comparison of CL density distribution in CBA mouse strain early and late in the response to a tumor allograft is given in Fig. 5. A comparison with the total T-cell distribution in the normal spleen was also made. The following are the major points: (a) The CL early in the response were confined to the light-density region and formed a peak around density 1.068 g/cm³, where a characteristic minor peak of T cells was observed in the normal spleen. (b) Later in the response, this “immature” peak persisted; but a series of denser peaks developed, corresponding to regions where T-cell peaks were found in the normal spleen. A small proportion of CL were in the dense, small-lymphocyte region. (c) The total nucleated cell profile of the enlarged, immunized spleens showed a marked shift to the light-density region, as with C57BL/6 mice. (d) The overall changes in CL density during the response to the allograft were similar in the two mouse strains, but the active cells always extended further into the dense regions with C57BL/6 mice. This is in general agreement with the preponderance of dense T cells in this strain, compared with CBA mice. Recovery of CL from gradients was high (mean, 108%), in agreement with the results with C57BL/6 CL.

The Characteristics of CL and CL Progenitors Developing from Dense T Cells.—The results from all the previous experiments suggested that dense, small-lymphocyte-like CL progenitors in the spleen would develop into light-density CL after antigenic stimulation. They also suggested that the most dense CL progenitors could also produce more “activated” CL progenitors of lighter density. However, a direct demonstration of this was lacking. To test this point, a very dense fraction from C57BL/6 mice was selected and transferred to heavily irradiated allogeneic (DBA/2) recipients. 5 days later, cells were obtained from the recipient spleens and separated on density gradients. Individual fractions were compared, both for CL in the in vitro test and for CL progenitors by a second transfer to another series of irradiated DBA/2 recipients. The resulting density distribution of both CL and CL progenitors is given in Fig. 6, which illustrates the following main findings: (a) Dense spleen cells, including dense CL progenitors, gave rise on transfer to a wide-density spectrum of cells, including light-density CL and light-density CL progenitors.
At this time point in the response to the continuous stimulus of the allogeneic spleen, both CL and CL progenitors included both light and dense elements. There was only a slight bias toward greater density for progenitors than effectors.

**Fig. 5.** The density distribution of CL in spleens of CBA mice responding to tumor allografts. The upper curve presents the distribution of all cells and all T lymphocytes in normal, nonimmunized spleen, for comparison (from Shortman, Cerottini, and Brunner). Other details are the same as for Fig. 4.

**DISCUSSION**

The Physical Nature of CL and CL Progenitors. One generalization that could be made from this study is that the progenitors of CL in peripheral lymphoid tissues are usually dense and are therefore likely to be typical T-class
small lymphocytes, whereas the effector cells, the CL, are usually of light to medium buoyant density and therefore likely to be “activated” large-medium cells. However, our results illustrate clearly the fallacy of trying to attribute some unique physical or morphological characteristic to these functional categories, since situations were found in which at least some CL progenitors were light in density, and a proportion of CL were usually as dense as small lymphocytes. The properties of cells with the one immunological function changed depending on the organ of origin, the time after antigenic stimulation, and the strain of mouse used. A time-based study was therefore essential to be able to associate one immunological function with a developing lineage of cells. Physical separation procedures are useful to separate and characterize cells at different stages of such a differentiation pathway and to determine the overall balance of the population between these various stages.

Density Subpopulations of T Cells.—These experiments provide some rationale for the discrete density subpopulations that density distribution analysis had revealed in lymphocyte populations (10). The studies on CL development from CL progenitors suggest that the various peaks of cells, even in the normal spleen, could represent an accumulation of lymphocytes in particular stages of development. We have not attempted to determine the immunological significance of each density subclass of T cell, but the data suggest this may be possible. For example, the CL progenitors in thymus correspond closely to one major peak of high H-2 T cells, density around 1.073 g/cm³. A second example is the earliest, least mature CL in CBA spleen, which correspond to a well-defined minor peak of T cells in normal spleen, density around 1.068 g/cm³.

Fig. 6. The density distribution of cells, CL, and CL progenitors in irradiated DBA/2 mouse spleen 5 days after transfer of a dense fraction of cells from C57BL/6 mouse spleen. CL were assayed by testing the in vitro ⁵¹Cr release from labeled mastocytoma cells of the DBA/2 allotype. CL progenitors were assayed by transfer of cells from each fraction to a second series of DBA/2 recipients.
The Differentiation of CL.—The detailed mapping of a lymphocyte differentiation pathway is difficult in whole animal studies, owing to the continual traffic of cells between tissues and the continuing balance between cell recruitment and cell death. For these reasons we did not attempt a detailed study of CL development, a study that should best be carried out in a closed, controllable, tissue-culture situation. However, the separation data, taken as a whole, indicate some of the major changes in cell buoyant density that occur during CL development and suggest ways of separating some of the principal steps in the pathway. Some aspects of this are summarized in Fig. 6. The “typical” CL progenitor in the normal mouse spleen is a very dense cell, most likely a small lymphocyte. Antigenic stimulation “activates” this cell, and the early changes are reflected by a decrease in cell density. The earliest, immature CL developing from this activated CL progenitor are extremely light in density and are probably cells of the larger lymphocyte type. As the response proceeds, the CL appear to pass through a series of maturation steps reflected by increases in cell density. The end product, mature CL, now has a density again resembling a small lymphocyte. Since these mature CL now have a buoyant density approaching that of the original CL progenitors, it is possible that after ceasing active “killer” function, they in fact revert to being CL progenitors, or “memory” cells. It is of interest that these general changes in CL resemble the stages in toad spleen antibody-forming cell maturation described by Kraft and Shortman (19).

The Differentiation of CL Progenitors.—The least mature of the immuno-competent T cells in an animal should be the CL progenitors found in the thymus. It was of interest that such cells were not typical small lymphocytes, but represented a well-defined, relatively light-density population. They corresponded in density and were probably identical to one minor thymus density subpopulation with the surface antigenic characteristics of peripheral T cells. This is presumably the population that had matured to the point of leaving the thymus to seed in peripheral tissues; it should be noted that only CL progenitors with the ability to seed in the spleen could be detected in the assay used. However, the majority of CL progenitors found in spleen were much more dense than this thymus cell. This suggests that further differentiation or maturation of CL progenitors occurs after they leave the thymus, until the mature peripheral T lymphocyte is formed. It would be of interest to know if this further maturation was dependent on some form of antigenic stimulation. A diagram of this proposed pathway of CL-progenitor development is included in Fig. 7.

The Function of the Thymus Small Lymphocyte.—In a separate study, we have demonstrated that the lymphocyte population of the thymus shares many buoyant density characteristics with spleen T cells, even though the bulk of the cells differ in their surface antigenic composition. Two separate differentiation lineages were proposed, one restricted to the thymus ("low H-2,
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high $\theta$ cells”) and one commencing in the thymus, but mainly developing in the periphery (“high H-2, low $\theta$ cells”). It was hypothesized that these share certain common “states” of differentiation, as characterized by an accumulation of lymphocytes of a particular buoyant density. The tests for immunological function suggest that the “high H-2, low $\theta$” lineage represents the series of cells involved in cell-mediated responses to alloantigens and includes both progenitor and effector cells. This poses the question of the role of the “low H-2, high $\theta$” elements, which remain in the thymus, and were without activity in our functional tests. This population represents the bulk of thymus cells, including the typical cortical thymus small lymphocyte.

Several roles could be hypothesized. One is that these cells are active in other immunological functions, such as collaboration with B cells in antibody-mediated responses. This would not explain their thymus-specific surface antigenic structure. The explanation most compatible with current concepts is that the thymus small lymphocyte is inactive because it has not fully “ma-
tured." This implies that the thymocyte first develops from large, dividing elements into small, dense, nondividing cells with a characteristic surface antigenic structure. Final maturation to the CL progenitor cell we detected in the thymus would then be a reversal of this procedure, involving a decrease in density and change in surface antigenic makeup before migration into peripheral lymphoid tissues.

A third explanation would be more compatible with the direction of maturation's being exclusively from large, light-density, dividing elements to small, dense cells. Both the "high H-2, low θ" and the "low H-2, high θ" lineages could be derived from the first steps in T-cell differentiation from hemopoietic stem cells, and in both cases further maturation could be antigen dependent. The thymus-resident population could be cells with a reactivity to self, which are "screened" by exposure to self-antigens within the thymus and directed into a sterile developmental pathway within the thymus, leading to cell death. The change in cell-surface antigenic structure may be related to this process. Cells not reactive to self would then emigrate from the thymus, free to differentiate further on contact with foreign antigens. The existence of a mitogenic response of mouse thymus cells (but not spleen cells) to self-antigens (von Bohmer and Byrd, 20) and the tolerogenic effects of antigens introduced directly into the thymus (Staples, Gery, and Waksman, 21) would lend some support to this view.

SUMMARY

Density distribution analysis in continuous gradients of albumin has been used to study the development of cytotoxic lymphocytes (CL), to separate and characterize the progenitors of CL, and to determine their relationship to subpopulations of T cells.

CL progenitors in the thymus were a homogeneous, medium-density population, distinct from the typical, dense, thymus small-lymphocyte. Activity seemed to be associated with one minor subpopulation of cells with surface antigenic properties characteristic of peripheral T cells (high levels of H-2 antigen, low levels of θ-antigen). CL progenitors in the spleen differed from those in the thymus and normally had the high buoyant density of typical small T lymphocytes. In states of antigenic stimulation, some lighter-density CL progenitors were found in the spleen.

The buoyant density of the CL population developing in the spleens of immunized animals showed progressive changes with time. Early, "immature" CL had the light-density characteristics of large, activated lymphocytes. As the response developed, the density of the CL population increased, and finally approached that of CL progenitors and of typical small lymphocytes.

The data suggest that density subpopulations of T cells represent stages in the development of immunocompetent cells. Possible differentiation pathways of T lymphocytes in the thymus and in the spleen are discussed.
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