DIFFERENTIATION OF ANTIBODY-FORMING CELLS IN TOAD SPLEEN

A Study Using Density and Sedimentation Velocity Cell Separation

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ABSTRACT

Antibody-forming cells (AFC), developing in toad spleen after stimulation with polymerized flagellin, were studied with an immune adherence assay. Differentiation was followed by several parameters: thymidine uptake to monitor dividing cells; equilibrium density centrifugation in albumin gradients to monitor cell density; microscopic measurements and sedimentation velocity separation to monitor cell size; stained preparations to follow cell morphology. Almost all AFC observed early in the response were dividing cells; the proportion of dividing AFC dropped to 4% 2 wk after stimulation. The earliest AFC detected (3 days) formed a relatively homogeneous light density population, and were purified 17-fold by equilibrium density centrifugation. As the response developed, additional denser peaks were found, so that late in the response dense AFC predominated. Dividing AFC were confined to the light density region throughout the response. Cell diameter measurements revealed that the earliest AFC were all very large cells. In a manner analogous to the density changes, smaller AFC appeared as the response developed until they finally comprised the majority of the AFC population. Dividing AFC were always relatively large, but encompassed a wide range of sizes. Sedimentation velocity separation was employed in a closer study of the immature AFC; they were purified 140-fold by this procedure. The earliest AFC consisted of several readily separable size populations in the range 9–18 μ diameter. The presence of separate peaks related by factors of two in volume suggested that the largest cells undergo a series of halving divisions before entering a division growth cycle. The results suggest an AFC differentiation sequence from a very large, light density, dividing "blast" cell to a nondividing cell with the size, density, and morphological appearance of a small lymphocyte. Stages of this sequence can be defined and selected out for investigation, using sedimentation velocity and equilibrium density centrifugation as complementary techniques.

INTRODUCTION

The development of antibody-forming cells (AFC) during an immune response is a particularly attractive model of a differentiation process, being initiated by a defined antigenic stimulus, and leading to the formation of a specific antibody as a defined end product. The advent of assays for AFC (1–4)

and the development of culture systems capable of sustaining an immune response in vitro (5–7) has made possible the detailed study of AFC maturation. Such studies would be greatly facilitated if cells at various, defined stages of the response could be separated from each other. In this way, the points at which continued stimulation by antigen (8) or feed back inhibition by antibody (9, 10), are effective could be determined, and the biochemical changes accompanying differentiation could be investigated.

In this report we investigate the feasibility of using equilibrium density gradient centrifugation in continuous albumin gradients (11) and sedimentation velocity separation at unit gravity (12, 13) to separate and define stages in AFC differentiation in terms of cell density and size. The system chosen was the development of AFC in the spleen of the toad *Bufo marinus* in response to an injection of polymerized flagellin antigen, (POL), using an immune adherence colony-forming cell assay (3) to enumerate AFC, in conjunction with an immune adherence technique which permits these cells to be examined by radioautography and phase-contrast optics (14–16). Events before the appearance of the first detectable AFC were not studied.

This work developed from earlier studies from this laboratory (17) which showed that rat AFC at the peak of the response could be separated into a series of discrete density populations. Although the validity of this finding was questioned on technical grounds (18), a reexamination of the gradient conditions has fully supported the original observation.[1] A further study of the biological basis for this situation suggested that it was in part due to the presence of cells making different classes of antibody, and in part due to the existence of different stages of AFC differentiation (19). From this study the response in the toad seemed to represent a simpler situation than in the rat; a more definite sequence of density changes with time was apparent, and in addition the response to POL was limited to IgM type antibody (16). A closer study of AFC development in the toad was therefore undertaken in the belief that experience with the simpler situation would guide later work on the differentiation of mammalian AFC.

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[1] Williams, N., N. Kraft, and K. Shortman. 1971. The separation of different cell classes from lymphoid organs. VI. The effect of osmolarity of gradient media on the density distribution of cells. *Immunology.* In press.

MATERIALS AND METHODS

Animals

Wild toads (*B. marinus*) were obtained from H. J. Honke, Paddington, Queensland, 4064, Australia. It was essential to force-feed the animals in order to obtain a normal immune response and normal spleen cell density distribution patterns (19). Before use, the animals were kept at 25°C in a dark box containing moist wood shavings and were fed weekly with one neonatal mouse. Antigenic stimulation was obtained by intraperitoneal injection of 100 μg of POL from *Salmonella adelaide* (20). The animals were then maintained at 37°C in cages containing wet wood shavings and were fed twice weekly with one neonatal mouse.

Balanced Salt Solution for Toad Cells

A pH 7.4 buffered balanced salt solution, without additional serum, was used for suspensions of toad cells. This solution was similar in composition to one described previously (11) but was diluted to be isotonic with toad serum (222 mosmolar, equivalent to 0.121 M NaCl, as determined by vapor pressure osmometer readings).

Preparation of Cell Suspensions

Toads were killed by pithing and spleens were removed into ice-cold buffered balanced salt solution. Each spleen was teased apart with stainless steel needles over a fine, stainless steel sieve. Clumps were allowed to settle out of the suspension for 15 min; the cells were then recovered by centrifugation (400 g, 7 min at 4°C).

In order to obtain blood leukocytes, blood was collected by heart puncture into heparinized tubes. Erythrocytes were separated by the technique of Hulliger and Blazkovec (21).

AFC Assay

The cytoadherence technique of Diener (3) was used. Briefly, this mixture consisted of adding motile bacteria to the washed cell suspension and plating the mixture in agar. The agar plates were then incubated at 37°C for 3.5 hr, permitting bacterial replication to occur. Cells which acquired a coating of bacteria due to the presence of specific anti-flagellar antibody on their surface gave rise to large colonies, distinguishable from the small colonies arising from single bacteria. This assay has been shown to reflect cells containing and secreting antibody (14, 15, 22, 23).

Assay for Dividing AFC

Dividing AFC were detected by in vitro thymidine uptake followed by radioautography. The cells (10⁶–

10^7) were incubated at 37°C for 2 hr in 1 ml of pre-equilibrated Eagle's medium (containing 10% fetal calf serum and adjusted to the tonicity of toad serum), in a gas phase of 10% CO_2, 8% O_2, and 82% N_2. The medium contained 1 μCi of tritiated thymidine (The Radiochemical Centre, Amersham, England; SA 5000 mCi/mmole). The cells were washed twice, then mixed with motile *S. derby* bacteria and processed for radioautography as described by Diener and Marchalonis (16). Radioautographs were normally exposed for 1 week at 4°C before development and staining with Giemsa stain. Cells with seven or more adherent bacteria were scored as AFC. Cells with 10 or more grains were scored as dividing cells. The proportion of dividing cells was not increased by longer periods of incubation or longer exposure of the radioautographs.

Diameter Measurements on AFC and Total Spleen Cells

Diameter measurements were performed on washed, viable spleen cell suspensions mixed with motile *Salmonella*, using high power phase-contrast optics and a calibrated eyepiece scale. Cells with seven or more adherent bacteria were scored as AFC. For nonspherical cells, the estimated diameter was taken as a 10% increase of the minimum diameter. In some cases, as noted, the diameter measurements were performed on the smeared and stained preparations used for the radioautographic assay of thymidine uptake.

Morphological Examination of AFC

The Giemsa-stained radioautographs for dividing AFC assay were also used in the study of the morphology of dividing and nondividing AFC.

Total Cell Counts

Total lymphoid cell counts were made with a hemocytometer, under phase-contrast optics; erythrocytes were easily distinguished and excluded from this count. Counts on density gradient fractions were performed with a model B Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.), with the low threshold set to exclude debris. In the case of density-gradient fractions, the dense erythrocytes were spun out of the gradient range and did not interfere with the lymphoid cell count. Some erythroid cells were found in the velocity sedimentation separation experiment fractions, and the Coulter Counter counts were therefore corrected after a microscopic differential count had been performed on each fraction.

Total Cell Volume Distributions

Cell volume distribution determinations were performed with a model B Coulter Counter, fitted with a 100 μ diameter and 96 μ length aperture, and a model J Size Distribution Analyzer (Coulter Electronics Inc.). It was calibrated as described by Legge and Shortman (24). The distribution of unfractionated spleen suspensions was corrected for the presence of the large toad erythrocytes, by subtracting values derived from a separate erythrocyte volume distribution determination and a percentage erythrocyte count.

Serum Antibody Titers

Serum immobilization titers were performed according to the method of Nossal (1959).

Density Distribution in Albumin Gradients

The technique of equilibrium density gradient centrifugation of cells in isotonic albumin solutions is described in detail elsewhere (11). In brief, cells were dispersed in a linear density gradient of bovine plasma albumin at pH 5.1, and spun to equilibrium (4000 g, 45 min at 4°C). Fractions (20-30) were collected by upwards displacement of the gradient. Subsequent to a density determination, each fraction was diluted with buffered balanced salt solution; the cells were then recovered by centrifugation and were resuspended in a known volume of buffered balanced salt solution.

Toad cells which were separated in gradients made from albumin solutions having the tonicity equivalent of normal saline, 0.147 M NaCl, were found in the dense regions of the gradient due to the shrinking effect of the hypertonic medium. Accordingly, all albumin solutions were made with an unbuffered balanced salt solution similar to that previously described (11) but reduced in concentration to give a final osmolairty equivalent to toad serum (222 mosmoles, equivalent to 0.121 M NaCl).

Control experiments presented elsewhere[1] demonstrated that the gradients were isosmotic with toad serum, that the density gradients did not generate osmolarity gradients, and that the separation procedure gives a valid reflection of AFC density. The density measurements on a single population of cells were reproducible to ± 0.0003 g/cm^3, about one-third of a fraction, from one experiment to another. The error in the height of a peak was $\pm 5\%$ SD for total cell counts, $\pm 15\%$ for AFC counts. Recoveries from gradients were in the range 80-100% for all viable lymphoid cells, 50-100% for AFC.

All results are given as density distribution profiles relating total cells (or AFC) per density increment to fraction density. Peak values have been adjusted to the same height of 100% regardless of the absolute numbers.

Sedimentation Velocity Separation

Sedimentation velocity separation was carried out by the general procedure described by Miller and Phillips (13). The shape of the chamber was that given by Mage et al. (25), but reduced to half diameter. The medium was "toad osmolarity" buffered balanced salt solution, containing from 6 to 24% fetal calf serum (also diluted with water to iso-osmolarity with toad serum). The shape of the stabilizing gradient was as described by Peterson and Evans (12), modified as suggested by Miller and Phillips (13). The gradient was produced by a preprogrammed Beckman model 141 (Beckman Instruments, Inc., Fullerton, Calif.) gradient pump. The gradient shape was verified by using radio-labeled albumin as a tracer in one solution. Care was taken to minimize turbulence and avoid inverse-gradients during the filling and emptying of the chamber. To reduce the cell load and avoid aggregation effects, damaged cells and erythroid cells were first removed from the starting spleen cell suspension. This was accomplished by dispersing the cells in a discontinuous bovine serum albumin density gradient containing layers of density 1.06, 1.075, and 1.08, and centrifuging for 15 min at 4000 g. The upper two layers were recovered and the bottom layer, which contained most of cellular debris and erythrocytes, was discarded. The cells, recovered from the albumin, were applied in a suspension of 5×10^6 cells/ml in 3% fetal calf serum, balanced salt solution. The length of the sedimentation run was varied as described in the text.

Calculation of Cell Volumes from Sedimentation Velocity Separation Data

It was possible to make estimates of the sizes of the cells obtained in the various fractions after a sedimentation experiment by applying the formula for the terminal velocity of spheres falling through a fluid: $V = \frac{2}{9}(d_1 - d)gr^2/\eta$ where V is the terminal velocity (calculated for each fraction from the time of sedimentation and the chamber dimensions); d_1 is cell density (measured previously by equilibrium density gradient centrifugation); d is the density of the fluid; g is the force of gravity; r is the cell radius; and η is the viscosity of the fluid.

Water and fetal calf serum have small but definite density and viscosity differences. (The viscosity of a 24% solution of fetal calf serum and balanced salt solution at 4°C was found to be 0.0194 poise, and its density was 1.0049 g/cm³). The presence of a continuously changing gradient presents difficulties in assigning a value for| η and for d. In addition, V is usually calculated by taking into account the time the cells have spent in the cylindrical portion of the chamber and ignoring the passage through the tapered section. This assumption appears quite valid

if the period of sedimentation is a matter of hours; however, in our experiments the presence of very large cells necessitated the use of short sedimentation periods, and the period in the tapered section became significant. The effect of these factors was assessed by using a computer program written by Mr. J. Pye for the University of Melbourne IBM 7044 computer. All volume estimations presented were precisely computed. It was found that good approximations to the precisely computed cell volumes were obtained if mean values for the fluid density and viscosity (1.0024 g/cm³ and 0.0176 poise, respectively) were used and if half the time the cells spent in the tapered sections was added to the time they had spent in the chamber.

RESULTS

General Kinetics of the Response

The kinetics of the humoral response of toads to a single intraperitoneal injection of 100 µg POL was determined to facilitate comparison with higher animals, and to determine when spleens should be harvested to provide AFC in early, peak, or late stages of the response, for cell separation experiments. The animals were maintained at 37°C. Forced feeding was employed to maintain the animals in a good nutritional state, a factor which has been found essential to obtain a normal response (19). Under these conditions, the over-all kinetics of the toad response was similar to that seen in mice and rats (3, 4), as shown in Fig. 1.

The eariest time at which AFC could be detected in spleen or blood was 3 days after stimulation. Two peaks of AFC numbers were seen in the spleen, one at day 5 and another at day 7. These were seen in three separate kinetics experiments as well as in the pooled data of Fig. 1; both the levels of AFC in blood and the serum show suggestions of similar peaks. Similar fluctuations of the immune response to this and other antigens have been observed previously in other species (3, 4, 26). After day 7, there was a gradual decline in AFC numbers. It is of interest that the total number of AFC found in the circulation was in fact higher than that in the spleen.

Serum Antibody

The development of serum antibody against POL was also determined, and the results are included in Fig. 1. The levels followed the rise in AFC numbers, as expected. The class of antibody found in pooled sera 5 and 14 days after stimulation was investigated by Sephadex gel (Pharmacia
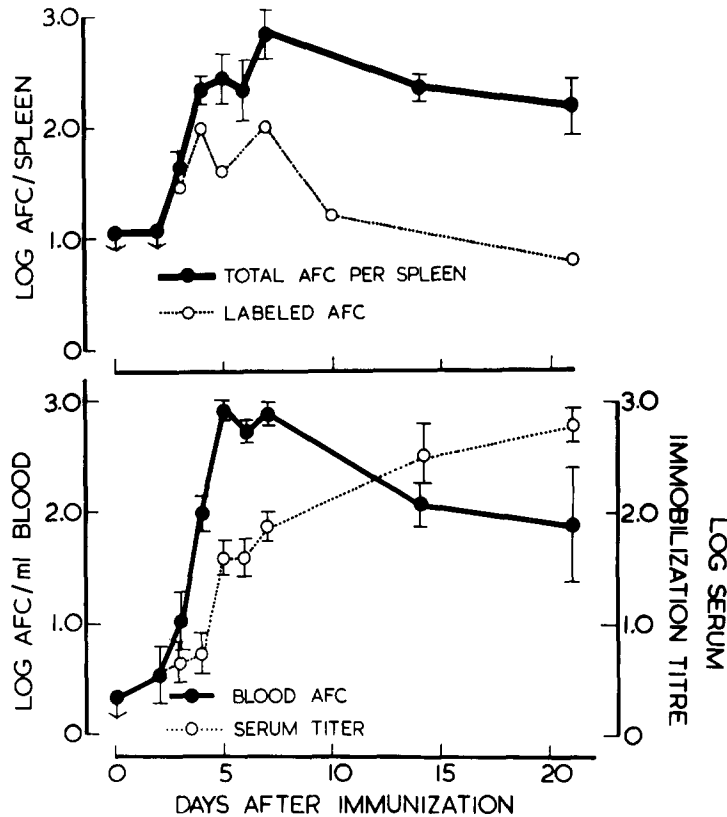
FIGURE 1 The kinetics of the immune response of toads to POL antigen. 100μg POL antigen was given intraperitoneally at day 0. All points are geometric means; error bars represent SEM. The spleen total AFC and the serum titrations are pooled data from three separate kinetic experiments; each point represents a total of 12 animals. Blood AFC data were derived from a single experiment; each point represents 4 animals. Labeled AFC data represent the estimate of the total number of dividing AFC per spleen. The values were calculated from the total AFC curve, together with separate determinations (2–4 experiments) of the proportion of AFC taking up tritiated thymidine in vitro.

Fine Chemicals Inc., Uppsala, Sweden) filtration and mercaptoethanol sensitivity. The results, given in detail elsewhere (27), showed that 97% of the serum antibody was IgM type, in agreement with the conclusions of Diener and Marchalonis (16).

Dividing Cells

The numbers of dividing AFC in the spleen at various times after stimulation was determined by in vitro thymidine uptake followed by radioautography, and these results are also included in Fig. 1. Virtually all the AFC found early in the response were dividing cells. There were two waves of cell division, around days 4 and 7. After day 7 the number of dividing AFC declined rapidly.

Separate experiments established that the non-dividing AFC seen later in the response were

nonetheless products of earlier cell division. Toads were subjected to intraperitoneal injections of 10 μCi tritiated thymidine every 8 hr after POL stimulation. Under these conditions of "continuous" labeling, 37 out of 39 AFC found on radioautographs at day 5 had incorporated tritiated thymidine and were therefore the product of cell division occurring during the labeling interval. Only 15% of AFC were found to be actually dividing cells at this same time point, as determined by in vitro thymidine uptake studies.

Size and Density Studies on Toad Spleen Lymphoid Cells

Before attempting separation of differentiating AFC on the basis of cell density or size, some background information was sought on the total toad

spleen lymphoid cell population. In particular, the relationship between size and density was examined, to assess the relative value of the two parameters. This was done by separating spleen cells according to density, by equilibrium density gradient centrifugation in albumin solutions, and then performing electronic volume distribution studies on the isolated fractions. This investigation is summarized in Figs. 2 and 3. The main points to be made from these results are as follows:

(a) Toad spleen cells showed a spread of buoyant densities, the main small lymphocyte peak being found in the region 1.071–1.078 g/cm. (b) Very large cells were confined to the lighter regions of the gradient and the smaller "small lymphocytes"

were only found in the dense regions. However, several distinct density types or peaks of medium-sized cells were also obtained. (c) Cells in the light density regions (even from narrow density "cuts") contained a wide range of cell sizes, although the smallest elements were absent. Denser cells showed sharper size distribution profiles and represented small lymphocytes with a minor but distinct population of medium-sized cells. (d) The results were simpler than those obtained with rat or mouse lymphoid cells, but showed the same general characteristics (28). Size and density appeared to be related parameters, but one did not necessarily predetermine the other.
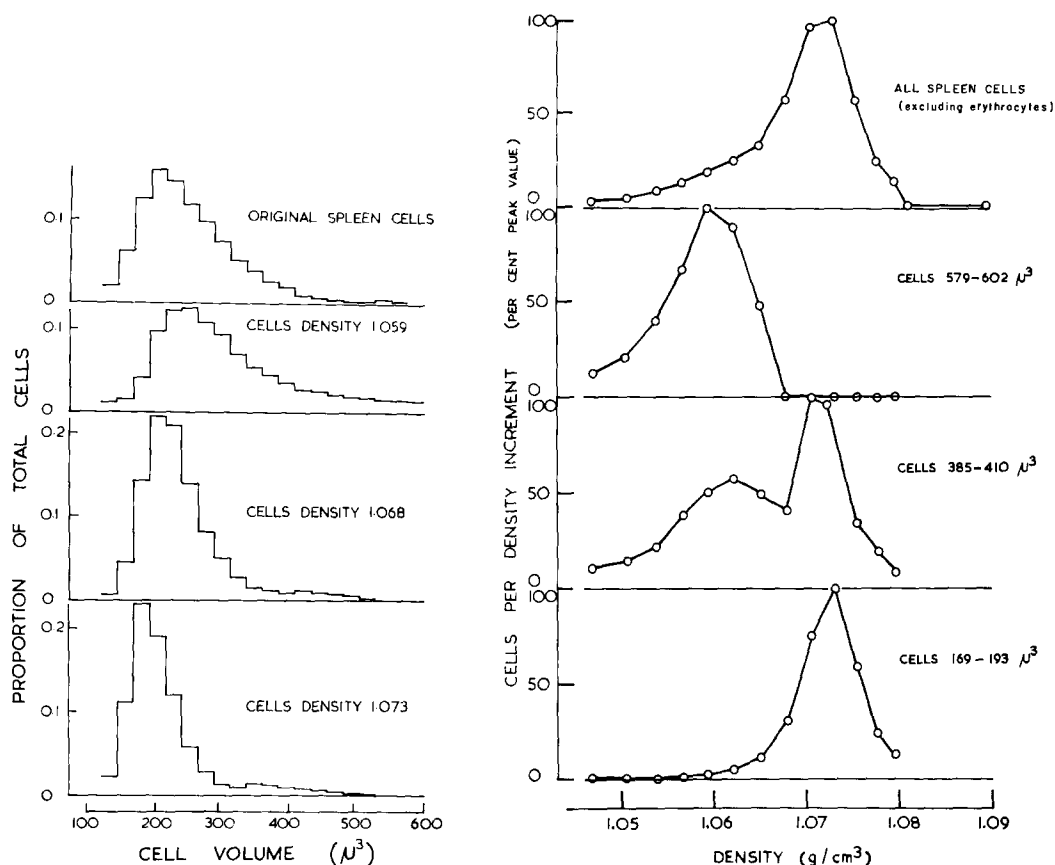
FIGURES 2 and 3 The relationship between the density and the volume of toad spleen lymphoid cells. Cells from 10 normal toad spleens (10^7 cells) were separated on albumin density gradients. An electronic cell counter was then used to obtain the volume distribution of cells in each fraction, as well as in a sample of the original material. The density distribution of a given size cell ws then calculated from the total lymphoid cell distribution and the proportion of cells of that size in each fraction. All density profiles were normalized to a peak of 100% regardless of absolute numbers. Toad spleen erythrocytes were more dense than 1.09 g/cm^3, and were spun out of the density gradient.

Density Distribution of AFC during the Immune Response

Since toad spleen lymphoid cells could be effectively separated in albumin gradients, provided these were iso-osmotic with toad serum, experiments were performed to study in more detail the changes in AFC density that were noted in a preliminary study (19). A complete series of AFC density distribution profiles, covering all stages of the response, is shown in Fig. 4. For simplicity, only AFC distributions are shown; total nucleated cell distributions showed little change, and a typical example is given in Fig. 5. The following conclusions may be drawn:

(*a*) The earliest detected AFC were exclusively light density cells, confined to a narrow region of the gradient, around density 1.06 g/cm³. (*b*) A series of denser peaks of AFC appeared with time, and gradually assumed major importance. (*c*) Very late in the response the cells were predominantly dense, around density 1.07 g/cm³, and approached the density of the major small lymphocyte peak. (*d*) AFC were purified up to 17-fold (average value 10-fold) at 3 days, and fourfold at 5 days, compared to unfractionated spleen.

The data of Fig. 4 suggested that the light density AFC seen early in the response could still be found, but in much smaller proportion, later in the response. In many cases the profiles showed evidence of definite peaks, suggesting the existence of definite stages in the progressive density increase. However, the relatively wide density range used in these experiments reduced the resolution obtained (11). In order to examine these questions more closely, a series of narrow range gradients of improved resolution was used. Some results of these experiments are shown in Fig. 5. These confirmed the following points:

(*a*) At intermediate times during the response, the AFC were heterogeneous in density, and several readily separable density peaks could be isolated, suggesting a discontinuous density increase during maturation. (*b*) Separate peaks characteristic of the early stages of the response persisted at late stages, although their relative importance diminished.

Blood AFC Density Distribution

In view of the quantitative importance of AFC in toad blood (see Fig. 1), and of earlier work suggesting differences between tissue-fixed and cir-

culating AFC in the rat (17), the density profiles of toad spleen and blood AFC were compared. Some results at 5 days after stimulation are given in Fig. 6. The density distribution of AFC in the blood was similar to that in the spleen, except for a higher proportion of dense elements. This suggests that
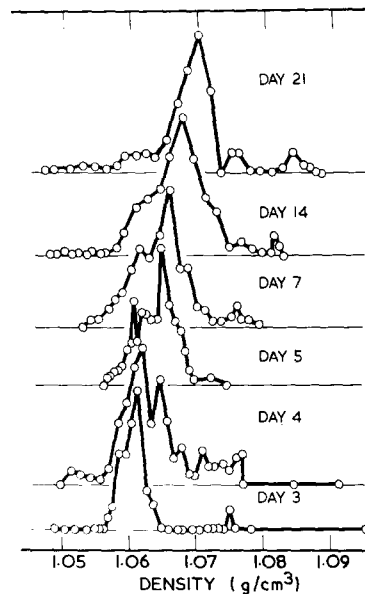

FIGURE 4 Changes in toad spleen AFC density distribution during the course of the response to POL. Cells from six spleens, comprising approximately 6 × 10⁶ lymphoid cells, were used for each experiment. Each point was repeated 2-6 times, with little variation in the basic pattern. Typical profiles are presented. All curves are normalized to the same peak height, regardless of absolute AFC numbers in fractions. The actual AFC concentration in the spleen at each point can be obtained from Fig. 1.
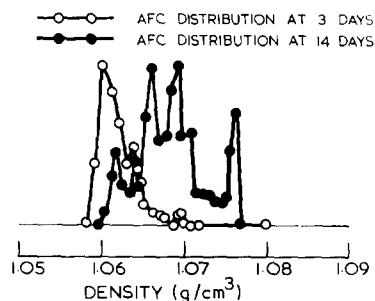

FIGURE 5 Higher resolution comparison of toad spleen AFC density distribution profiles 3 and 14 days post-POL stimulation. Conditions are as in Fig. 4, except that the analysis involved more points in gradients encompassing a narrower density range.

blood AFC were more mature than those in the spleen. Occasional dividing AFC were, however, found in toad blood.

Density Distribution of Dividing AFC

Since the results of Fig. 3 suggested a definite correlation between AFC density and the degree of maturation, experiments were undertaken to see if certain density peaks represented dividing elements and others nondividing product cells. Fractions from spleen density gradients at 3, 5, and 7 days after antigen stimulation were incubated with tritiated thymidine, and the proportion of dividing AFC was determined by the radioautographic technique. From this proportion, and the total AFC count per fraction, the density distribution of all dividing AFC could be calculated. This is compared to the total AFC density distribution in Fig. 7. The following main points should be noted:

(a) Most of the AFC in the narrow density region around 1.06 g/cm³ at day 3 were dividing cells, as would be predicted from the data of Fig. 1 and Fig. 4. (b) As the response developed, and the bulk of AFC increased in density, more dense dividing cells were found. However, dividing cells of density greater than 1.066 g/cm³ were never found. (c) As the proportion of dividing cells in the total population of AFC declined, the proportion of light density AFC in division also declined. Hence, light

FIGURE 7 The density distribution of dividing AFC at various stages of the response to POL. Dividing cells were assayed by tritiated thymidine uptake in vitro, after fractionation by density. The total AFC curves are normalized to the same peak height, as in Fig. 3. The calculated, dividing AFC density profiles are normalized, so the shaded area, relative to the total AFC area, represents the proportion of dividing cells. Absolute levels of dividing AFC are given in Fig. 1.

density peaks persisting late in the response differed in this respect from those found at 3 days. A low cell density clearly did not predetermine continuous cell division. (d) The data of Figs. 1, 4, and 7 demonstrate that progressive changes in AFC density continued after cell division ceased.

Size Distribution of AFC

The second physical parameter used to follow AFC differentiation was cell size. The studies on the total lymphoid population in Figs. 2 and 3 suggested that the early, light density AFC were likely to be large and that the late dense AFC were likely to be small. Direct diameter measurements on AFC were used to form an approximate size distribution at various stages of the response. The results are shown in Fig. 8, from which the following points can be made:

(a) AFC found earlier in the response were very much larger (typically 10–14 μ diameter) than the typical spleen small lymphocyte (6–8 μ diameter) (b) The size distribution of the early AFC was very broad, in contrast to their narrow density distribu-
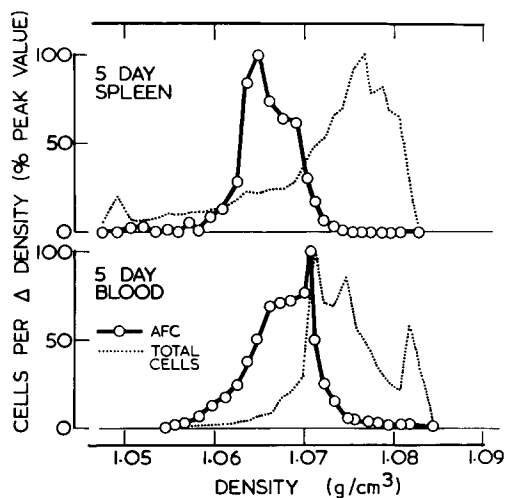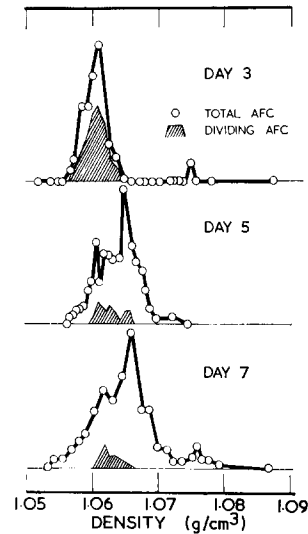

FIGURE 6 Comparison of toad spleen and blood AFC density distribution profiles. Cells were obtained 5 days after stimulation with POL. Six animals were used for each experiment. Approximately 3000 spleen AFC or 5000 blood AFC were applied to the gradients.
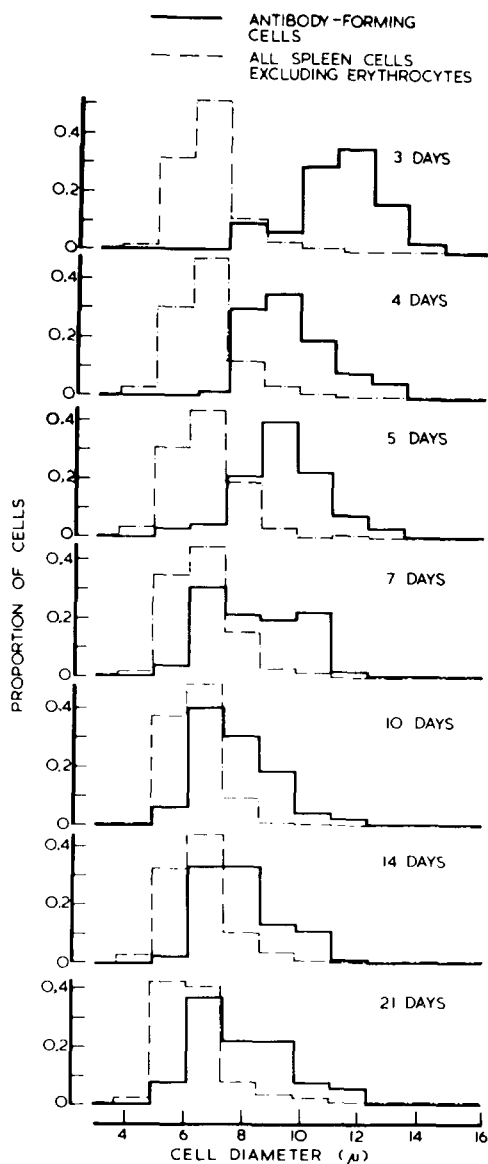
FIGURE 8 Changes in the size-range distribution of AFC during the response to POL. Viable cell suspensions were mixed with motile *Salmonella*, and AFC with adherent bacteria were measured under phase-contrast optics. Each curve represents measurements on 50–70 cells.

wave of light density cells. (*d*) Many of the AFC late in the response were of the same size as spleen small lymphocytes (6–8 μ diameter) (*e*) The range of volumes, calculated from these diameter measurements, from the largest early AFC to the smaller late AFC was 16- to 17-fold.

Size Distribution of Dividing AFC

Since the results of Fig. 7 suggested a correlation between AFC size and the degree of maturation, similar to the correlation observed between AFC density and the degree of maturation, experiments were undertaken to determine which of the size categories of Fig. 7 represented dividing cells. Spleen cells from animals at various stages of the immune response were incubated with tritiated thymidine in vitro, and the dividing AFC were located by the radioautographic procedure. The size distribution of dividing AFC, as measured by cell diameter on the smeared preparations, is com-
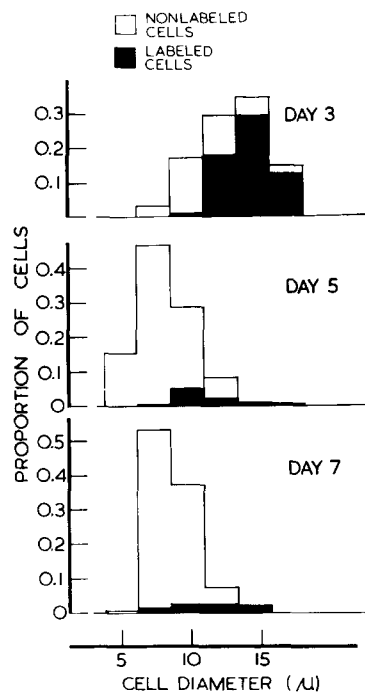

FIGURE 9 Comparison of the size range distribution of dividing and nondividing AFC at various stages of the response to POL. Spleen cells were incubated with tritiated thymidine in vitro; the cells were recovered, mixed with motile *Salmonella*, and then smeared for radioautographic study. AFC and dividing AFC diameter measurements were performed on the smeared, stained radioautographic preparations.

tion (Fig. 3). (*c*) As the response developed, the AFC population showed a continuous shift towards a smaller size, roughly paralleling the density shift shown in Fig. 3. There was evidence of a slight resurgence of large cells at day 7, corresponding to the second peak in the kinetic curve and the second

pared to that of all AFC found on the same smears in Fig. 9. These data demonstrate the following points:

(a) Dividing AFC found early in the response were all large cells, as predicted, but embraced a wide range of sizes. This is in contrast to their narrow density distribution at this stage. (b) As the response developed, and the mean size of all AFC decreased, there was marked reduction in the mean size of dividing AFC. (c) Only a very minor proportion of the smaller (6–8 μ) AFC showed uptake of tritiated thymidine.

Two-Dimensional Size:Density Characterization of AFC Population

Much of the data obtained by these analyses on AFC suggested a relationship between cell size and cell density similar to that described for the total lymphoid population of toad spleen; in general, large cells were light and small cells dense, but these parameters were not always directly related. To examine more closely the relationship between size and density, AFC at different stages of the response were first separated on an albumin density gradient, then the size distribution of AFC in each fraction was determined by diameter measurements or cell suspensions under phase-contrast optics. These experiments provided data on AFC which were then comparable with the data on total lymphoid cells in Figs. 2 and 3. The data could also be presented more completely in the form of a "map" showing simultaneously the size distribution and the density distribution of the AFC population at any given stage of the response. Such a two-dimensional analysis of AFC early in the response is given in Fig. 10, where the number of "dots" in any segment represents the relative number of AFC of a given size and given density. This produced a more detailed and more meaningful picture of the population than any one parameter considered alone. It also confirmed the following four main points:

(a) Large AFC cells tended to be light cells, small AFC dense cells. (b) All AFC early in the response were simultaneously large and lighter than the typical toad spleen lymphocyte. (c) Cells of a given size (e.g., 12 μ diameter) could exist in several different density categories. (d) Cells of a given density (e.g., 1.066 g/cm^3) could be of more than one size category.

Sedimentation Velocity Separation of AFC

In all the data presented up to this stage, cells were analyzed by both size and density, but only separated on the basis of density. Sedimentation velocity separation at unit gravity (12, 13) represents a separation procedure that predominantly reflects cell size; the effects of cell density on sedimentation velocity are quantitatively less important. Studies using this technique were initiated for two reasons. Firstly, the size range data of Fig. 4 suggested that a high degree of purification of AFC
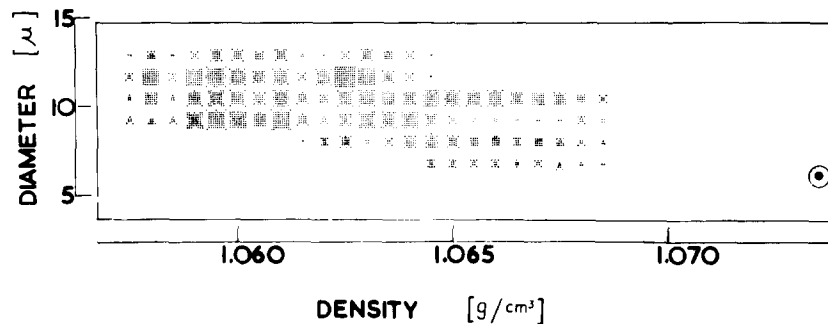

FIGURE 10 The relationship between the diameter and the density of toad spleen AFC 3 days after POL stimulation. The number of dots in each segment represents the relative number of AFC of a given size and a given density, relative to a peak of 100. ⊙ indicates the position of the diameter/density peak for the total lymphoid cell population, and is thus a "typical" small lymphocyte. The map was constructed from two sets of data. A density distribution profile of AFC was first calculated as the average of five separate distribution profiles. The relative number of cells of a given density interval was then subdivided on the basis of a second set of data giving the diameter distribution for each interval. This was obtained by separating a pool of six 3-day primed spleens into density fractions on albumin gradients, then performing AFC diameter distributions on 20–40 viable AFC from each individual fraction.

would be obtained. Secondly, the size distribution data were relatively unprecise and of low resolution, being based on microscopic diameter measurements, and the separation technique allowed the calculation of more precise size distribution profiles. These studies were restricted to the earliest stages of the response (3–3.5 days poststimulation) when greatest purification was likely and the most marked size changes were expected. The relatively narrow density distribution of AFC at this early stage meant that any separation between AFC was almost exclusively on the basis of cell size. The settling times used varied from 70 to 120 min. The largest cells were only recoverable when the shorter times were used; the longer runs were used to determine more accurately the sedimentation velocity of the smaller AFC peaks. The separations obtained are shown in Fig. 11, and the volume distributions calculated from these data are shown in Fig. 12. The following are the main findings:

(a) AFC could be purified 140-fold over other spleen lymphoid cells. The chief contaminant in the AFC region was the large, dense red cell, easily separable in a subsequent density step. (b) AFC were heterogeneous in size, and at this early stage of the response gave a number of very distinct peaks rather than a continuous distribution. (c)

Some of the peaks differed from each other by factors of two in volume (e.g., 700, 1400, 2750 μ^3; 1500, 2950 μ^3). Microscopic examination of AFC in the faster sedimenting peaks confirmed that these were large cells, not just doublets or aggregates of AFC.

Morphological Characterization of AFC

Although part of the aim of this study was to substitute precise characterization by measurable parameters for a subjective assessment of cell morphology, the appearance of the AFC in smeared and stained preparations was nevertheless recorded. The general appearance of these morphological types was similar to that described by Diener and Marchalonis (16), although our results differ in the sequence of appearance of the various types. The earliest AFC, large in size and light in density, generally had the staining characteristics of a typical "blast"-type cell (11–17 μ diameter, a marked degree of basophilia and a nucleus/cytoplasm ratio of < 0.5). Some cells with the general appearance of immature plasma cells were observed at days 3 and 4, but at no stage in this series did the AFC have the appearance of a typical mature plasma cell, although these have been
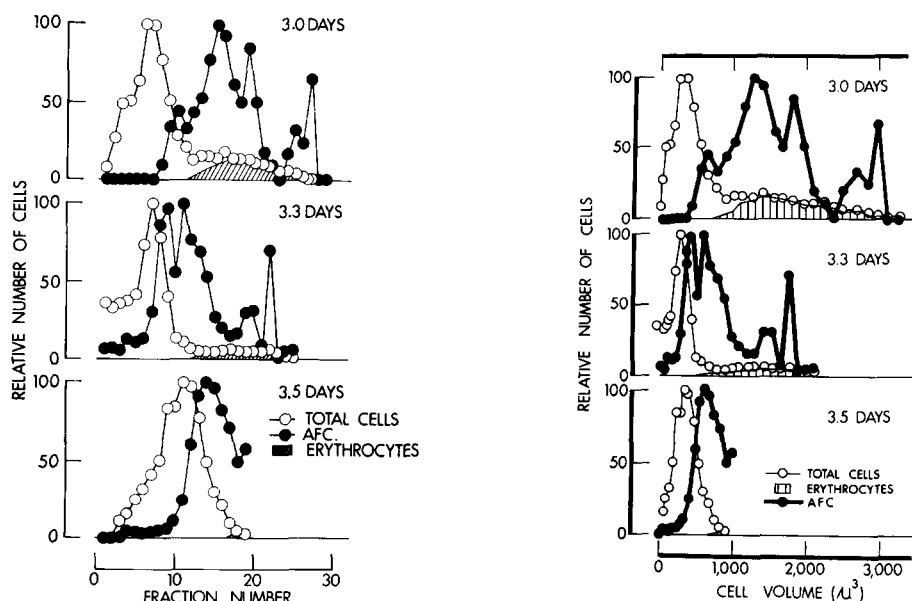
Figures 11 and 12   Sedimentation velocity separation of toad spleen AFC obtained 3–3.5 days after stimulation. The sedimentation times for the three experiments were 70, 90, and 120 min, respectively, for the 3.0, 3.3, and 3.5 day cells. The data are presented as the relative numbers of AFC plotted against fraction number, or against the calculated cell volume that each fraction represents.

observed in the toad (29). Rather, the bulk of the active cells had the general appearance of a relatively basophilic medium lymphocyte. Late in the response, many of the small, dense AFC were not distinguishable morphologically from small lymphocytes.

DISCUSSION

AFC Development in the Toad as a Model of Cell Differentiation

The toad, *B. marinus*, was selected for this project as a particularly favorable situation to study AFC development and maturation. There was a clear-cut progression of both density changes and size changes in the AFC population as a function of time after antigenic stimulation. A much more complex situation, lacking this striking progression, was noted when AFC development was followed by density in the rat (17, 4) or by size in the mouse (30). In part, this has been ascribed to the restriction of the response to a single antibody class in the toad, and to a generally simpler immune apparatus in the phylogenetically more primitive animal (4). The question of the relative degree of synchrony of the response in the different species may also be important. In the present study some "immature" forms were found throughout the response, suggesting some continual recruitment; in addition, a second major wave or cycle of cell division at day 7 somewhat complicated the situation. It is therefore easy to imagine that the total AFC population in some situations rapidly attains a "steady state", with continual recruitment of immature AFC and loss of mature elements. This would mask the sequential changes in density or size as individual cells matured. If this were true, synchronous populations of AFC could also be obtained from higher animals, by selecting out discrete stages of the differentiation process with cell separation techniques.

Some limitations of the chosen model system should also be noted, since these have prevented the construction of a detailed pathway of the differentiation sequence, and limit the value of further studies with this animal. The main limitations were:

(*a*) Events before the detection of the first AFC around 3 days could not be followed. (*b*) AFC development in the spleen was not a closed system, as the high AFC levels in the blood emphasized. (*c*) Precursor-product relationships between "im-

mature" and "mature" AFC could only be inferred, not proven. Thus it remained possible that certain AFC considered to be the product of the blast-like "immature" AFC had in fact an independent origin from a non-AFC precursor. (*d*) It was assumed, on the basis of extensive tests by others (14, 15, 22, 23), that the immune-adherence of motile *Salmonella* reflected antibody synthesis and secretion by the cell. It still remained possible that some of the isolated AFC population, in particular the small-lymphocyte "end product" cell, had in fact ceased active antibody production.

Most of these limitations could be overcome in studies of AFC development in tissue culture. Despite the limitations, the study provided the following guidelines for future work.

Following Differentiation by Cell Size

The use of cell size measurements to monitor AFC development, and especially the use of the largely size-based sedimentation velocity separation procedure, offered many advantages. The most extensive purification of immature AFC was achieved on this basis, since these cells differed much more in size than in density from the bulk of the spleen lymphocytes. Size could produce a high degree of selection between dividing and nondividing AFC. There appeared to be a continual reduction in AFC size even after division ceased; this might reflect continuing loss of cytoplasmic material in the process of antibody secretion (31). The greatest value of the size-based analysis, however, was its sensitivity to the halving of cell volume at division, a point at which cell density would not be expected to change markedly. This was reflected in the analyses early in the response, where the immature, dividing cells were relatively homogeneous by density, but included a wide range of sizes.

The AFC found in toad spleen represented a 16-fold range of cell volume as determined by microscope diameter measurements, the volume-span of four successive halving divisions. Sedimentation velocity analysis of dividing AFC early in the response revealed different cells spanning a volume range from 3000 to 400 μ^3, and demonstrated a number of sharp peaks, some related to others by factors of two. This suggests that part of the differentiation pathway of AFC in the toad involves a series of halving divisions without intermediate growth, a cell of volume 16 *a* dividing to give cells of 8 *a*, 4 *a*, etc. It is probable that cycles

of division with intermediate growth are also involved.

In a study of AFC to sheep erythrocytes in mouse spleen at the peak of the response, Phillips and Miller (32) obtained by sedimentation velocity analysis a single, broad peak of AFC spanning the velocity sedimentation range 3–7 mm/hr. They proposed a simple cycle of division and growth, cells of volume 2 a dividing to give volume a, then growing back to volume 2 a. (Although cells of identical density sedimenting at 3 and at 7 mm/hr would differ in volume by a factor of 3.5). While this probably represents the behavior of the bulk of the AFC at this point of the response, it would now be interesting to carry out high resolution analyses at earlier times, to see if larger AFC and sequential halving divisions can be detected in a mammalian spleen. In this context it should be noted that other workers have found AFC in mammals embracing a wider volume range than the simple model of Phillips and Miller above. For example, early in the response of mice to POL antigen, Russell and Diener (30) found AFC from 8 to 16 μ in diameter, an eightfold volume span, although the size range was narrow later in the response.

The sequential changes in the size of the AFC population in the toad described in this study are in direct contradiction to one aspect of the report of Diener and Marchalonis (16) who described many small cells early in the response, and predominantly large cells by 10 days. There are several possible explanations for this radical difference. It could be that the early cells of Diener and Marchalonis represented a still earlier step of the differentiation pathway, preceding our large blast cells; if so, it is surprising that we failed to detect them even by separation procedures. A second explanation derives from the two waves of proliferation seen in our kinetics curve. Diener and Marchalonis had relatively few points early in the response and they may fortuitously have taken their first sample late in the first wave, their later samples early in the second, thus reversing the maturation sequence. The most probable explanation derives from our previous demonstration (4) that the nutritional state of the toads has a radical effect on the kinetics of the response, the density distribution of AFC, and the total lymphocyte population. In our study the animals were force-fed to maintain a normal spleen lymphoid population; it is possible that after 10 days at 37°C the toads used by Diener and Marchalonis had selectively lost most of their spleen small lymphocytes, leaving a high proportion of large cells and large AFC dominating the picture.

Following Differentiation by Cell Density

The density of a cell must reflect its average chemical composition, and is thus a fundamentally different parameter than the total cell volume. Although the size decrease and the density increase of the total AFC population followed approximately the same over-all timetable, this study also demonstrated that the two parameters were not obligatorily linked, each reflecting a different aspect of the maturation process. Processes associated with cell division may have caused some of the density changes observed, but such effects are likely to be relatively small. For example, doubling the DNA content of a medium-sized cell would change its density by only 0.004 g/cm^3. The general density increase of AFC continued well after division ceased, and in part must have reflected large-scale changes in the cell cytoplasm, such as the progressive accumulation of endoplasmic reticulum, of polysomes, and of immunoglobulin. The cell nucleus would also be expected to become more dense, as it decreased in size, while maintaining a constant DNA and associated protein content. Finally, the process of secretion of immunoglobulin could have effects on cell buoyant density.

It is of particular interest that definite density peaks of AFC were isolated. This suggests that the differentiation process was discontinuous, and that particular steps in the process could be defined. It would therefore be of interest to characterize these stages in biochemical terms, and to study the factors controlling transfer from one stage to another.

Immunological Implications

The sequential changes we have described raise a number of further questions about the AFC response. The origin of the large, light density, immature AFC is of particular interest. Did they derive from preexistent dividing blast cells or from nondividing small lymphocytes? The persistence of some of these "immature" cells throughout the response calls for some explanation. It would be possible to account for these by continual low-level recruitment from the stem-cell pool, since Diener and Nossal (33) have demonstrated the persistence of injected POL in the toad for

several weeks. An alternative explanation is that they were arrested from reaching maturity by the absence of a sufficient antigenic stimulus; support for this view is the observation that all light density cells were dividing early in the response, but that the majority of cells of the same density were nondividing at later stages. This raises the question of whether antigen is required to drive AFC throughout all stages of differentiation, or whether it is only needed at the initial steps. The nature of the end-product cells of this sequence is also of interest; by density, by size, and by morphological appearance, they resembled small or small-medium lymphocytes, and they persisted for a considerable time in both the spleen and the blood. Did they represent a form of memory, capable of antibody production or proliferation or later stimulation? What proportion of the normal "lymphocyte" population of the animal represents the end product of other AFC maturation sequences?

Many of these questions are being explored, using tissue culture studies to overcome the limitation of this in vivo model.

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