ANTIGEN-INITIATED B-LYMPHOCYTE DIFFERENTIATION

VIII. Sedimentation Velocity and Buoyant Density Characterization of Virgin Antibody-Forming Cell Progenitors in the Adoptive Immune Response of Unprimed CBA Mice to 4-Hydroxy-3-Iodo-5-Nitrophenylacetic Acid-Polymerized Bacterial Flagellin Antigen*,‡

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Cells of the immune system are heterogeneous by function, origin, and physical properties and there is increasing interest in isolation and characterization of the various lymphocyte subsets. Previous studies in this laboratory have made use of a battery of physical separation methods (cell electrophoresis, adherence column fractionation, sedimentation velocity separation, and buoyant density separation) to characterize the bone marrow-derived, bursal-equivalent (B) lymphocyte population of the adult CBA mouse, and have shown this population to be heterogeneous by all physical criteria investigated (1). These physically distinct B-lymphocyte subpopulations are being investigated in the context of the B-cell pathway of development, and their place in this sequence.

The newborn, functional B lymphocyte bearing a full complement of immunoglobulin receptors develops from a multipotent hemopoietic stem cell (2-6), probably via some intermediate "pre-B"-cell stage. Lafleur et al. have studied this maturation sequence using cell separation methodology (7-9), while Osmond and Nossal have investigated the phase of B-cell development in a kinetic study of marrow B-lymphocyte production (10). Both approaches suggested that the functional antigen-inexperienced "virgin" B cell is a typical small lymphocyte. Earlier studies from this laboratory, however, seemed to indicate that typical small lymphocytes of the adult CBA mouse were largely memory cells, the virgin B cells constituting a minor, atypical subset of B cells in terms of surface properties (11, 12). In contrast to the finding of Lafleur et al. of slowly sedimenting functional B cells (7-9), Strober has recently reported unprimed antibody-forming cell (AFC)[1] progenitor activity among rapidly sedimenting

cells (13). The nature of the virgin AFC progenitor B-lymphocyte population is therefore not clearly delineated and requires closer study.

In this communication the techniques of sedimentation velocity and buoyant density separation were used in a complementary fashion in the physical and functional characterization of virgin B cells. Both techniques were needed to give an estimate of cell size (14). These methods were used in conjunction with an adoptive immune assay of IgM AFC progenitors reactive to the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) conjugated to the carrier polymerized bacterial flagellin (POL) (11, 15–17). Previous studies have indicated that the CBA mouse is in a relatively unprimed state with respect to this hapten (11, 15, 16). The assay allows a direct measurement of AFC progenitor function, under conditions where AFC production bears a direct, linear, arithmetic relationship to the number of progenitors transferred (17). A combination of cell separation methods and studies of B cells from mice of various ages and different immunological states has suggested the existence of three major physically and functionally distinct states of AFC progenitors. This paper describes two states of unprimed IgM AFC progenitors which can be physically separated and characterized: (a) A small newborn B cell found in neonatal mice and (b) a range of larger, apparently nonspecifically activated B cells found in adult mice. A further class, the memory B lymphocyte, will be the subject of a forthcoming publication.[2]

Materials and Methods

Mice. Inbred CBA/CaH/Wehi mice, either conventionally reared, specific pathogen free (SPF), or germfree were used at 6–7 wk of age (adult) or 7 days after birth (neonatal). Congenitally athymic nude inbred CBA mice were used when 5- to 8-wk old. The nude CBA mice and the germfree CBA mice were kindly provided by Dr. M. Holmes, Walter and Eliza Hall Institute of Medical Research. All mice were bred and maintained at The Walter and Eliza Hall Institute of Medical Research.

Antigen. POL conjugated with NIP (NIP-POL) was prepared as described previously (15).

Preparation of Cells. The basic cell suspension medium was a pH 7.2 balanced salt solution (BSS) buffered with HEPES, (Calbiochem, San Diego, Calif.) and isosmotic with mouse serum (308 mosM, equivalent to 0.168 M NaCl) (18, 19) and containing 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia). Cells were maintained and prepared at 0–7 °C. Spleens were harvested from 6 to 8 adult mice, or 10 to 25 neonatal mice, and bone marrow was collected from the femurs and tibias of 20 to 25 adult mice. Cells were prepared as described previously (18).

Sedimentation Velocity Separation. Cells were separated on the basis of their sedimentation velocity at unit gravity essentially as described by Miller and Phillips (14, 20). The 14-cm wide chamber was a streamlined version of the chamber of Peterson and Evans (21), having an additional conical exit at the top without a baffle.

Cells freed of erythrocytes and damaged cells (18, 22) were suspended in 4% FCS in BSS, rapidly passed through a 25 gauge needle to eliminate doublets, and adjusted to a concentration of 10×10^6 cells/ml. These cells were introduced into the chamber through the lower baffled entry port under a 12 ml layer of saline (to reduce surface effects when loading the chamber). A stabilizing buffered step gradient ranging from 7.5 to 25% FCS in BSS was formed and introduced under the cell band

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with a Beckman 141 programmable gradient pump (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The cells were allowed to sediment for 3 h at 6°C and 12-ml fractions were collected by upward displacement of the gradient with 20% sucrose.

Precise sedimentation velocity values of the separated cells were calculated from the fraction number and volume. A computer program, kindly provided by Mr. J. Pye, Walter and Eliza Hall Institute of Medical Research, was used for these calculations. It is important to note that the sedimentation velocity (S) values cited are 10% higher than those obtained in separations performed with "human osmolarity" media at 4°C. A small part of this difference was due to alterations in cell density with changes in osmolarity. The main difference was caused by the decreased viscosity of the sedimentation medium at 6°C.

With the exception of "specific activity" curves, all sedimentation velocity results are presented as a plot of the proportion of the total activity or total cell number vs. sedimentation velocity value, resulting in equivalent areas being occupied by all profiles. Specific activity values were determined by dividing the AFC produced by the progenitors in an individual fraction by the number of cells in the fraction and multiplying by 10^6.

Buoyant Density Separation.   Cells (with damaged cells removed) (18, 22) were separated on the basis of their buoyant density into 10-25 fractions by centrifugation to equilibrium (4,000 g, 30 min, 4 °C) in a continuous linear gradient of bovine serum albumin, pH 5.1, isosmotic with mouse serum, using techniques previously described (19, 23-25). The fractions were collected by upward displacement through a conical exit. Precise density measurements were made on cell fractions with microdroplet samples using a nonaqueous, bromobenzene-petroleum spirit gradient in a calibrated burette. All distribution profiles are expressed as cells per density increment (rather than cells per fraction) plotted against fraction density, in order to correct for small deviations from nonlinearity in the gradient and provide a true density distribution profile. All density profiles are normalized to a peak value of 100.

Cell Counts.   Total cell counts were obtained with a Model B Coulter Cell Counter (Coulter Electronics, Inc., Hialeah, Fla.), using a lower threshold set to exclude erythrocytes and debris. Counts on cell fractions, as used in the total cell distribution profiles, represent only viable nucleated cells because of the coulter gate settings, and since separation procedures were preceded by damaged cell and erythrocyte removal.

Assay of AFC Progenitors.   AFC progenitors specific for the NIP hapten were determined using an adoptive immune assay in which thymic-derived (T) cells and accessory cells were not limiting, as described in detail elsewhere (16, 17). All the cells from individual fractions (or pools of fractions) were mixed with 40×10^6 spleen cells from 850 rad X-irradiated mice (filler cells) and injected intravenously into four lethally X-irradiated (800-850 rad) syngeneic recipients. The X-irradiated spleen filler cells were used in order to obtain a linear dose-response relationship between cells injected (i.e., AFC progenitors) and anti-NIP AFC produced (17). A typical experiment involved the injection of 1-8 × 10 ^{6} cells per recipient together with 10 × 10 ^{6} viable irradiated filler cells. Antigen (25 µg of NIP-POL) was injected intraperitoneally immediately after the cells. The number of NIP-specific AFC per recipient spleen was determined 7 days later. All experiments included a negative control of X-irradiated recipients which received only filler cells and antigen and a positive control consisting of the unseparated original cell suspension with filler cells and antigen.

Pilarski and Cunningham (26) have recently pointed out the distinct possibility of host contribution to the AFC produced in adoptive immune assays. This has been checked by Stocker et al. (27) in a system similar to that used here, and no host reconstitution was detected. This point has also been tested by us in the NIP-POL adoptive immune assay using doses of unseparated cells characteristic of the experiments reported here. Using F_1 recipients and antihistocompatibility antiserum to determine the origin of AFC, no host contribution to the AFC response to spleen cells could be detected. With bone marrow, however, this host-derived component was small but significantly greater than zero.

AFC Assays.   Direct NIP-specific AFC were assayed by a modification (15, 28) of the procedure of Cunningham and Szenberg (29) using NIP coupled to sheep erythrocytes (SRBC) and subtracting the background anti-SRBC AFC. NIP-coupled SRBC were prepared using NIP-conjugated to the Fab' fragment of rabbit anti-SRBC antibody (30). All assays were performed in duplicate.

Anti-θ Serum Treatment.   Spleen cell suspensions were treated with high titer anti-θ antise-

rum (AKR/J anti-CBA/H thymocyte serum), directed against θ-C3H (Thy 1.2) plus absorbed rabbit complement (C) as described previously (31).

Results

Sedimentation Velocity Characteristics of Splenic IgM AFC Progenitors of Adult CBA Mice

CONVENTIONAL SPLEEN. In a previous study from this institute, the sedimentation velocity and buoyant density distribution of B lymphocytes in conventional adult CBA mouse spleen was investigated in detail, using a high level of accessible surface immunoglobulin (Ig) as measured by autoradiography as a criterion for the identification of B cells (1). Extensive physical heterogeneity in the B-lymphocyte population was found by both separation methods and a summary of the sedimentation results is included in Fig. 1 for comparative purposes. In the work presented here, the ability of these physically distinct
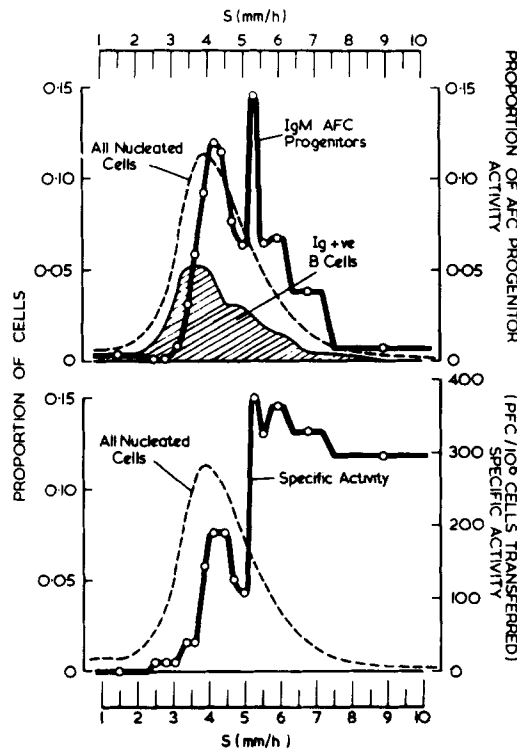

FIG. 1. Sedimentation velocity characteristics of adult conventional CBA mouse spleen cells. 240 × 10⁶ viable spleen cells from six to seven adult conventional mice were separated by sedimentation velocity as described in the Materials and Methods. The fractions were used in the adoptive immune IgM AFC progenitor assay with the antigen NIP-POL. The results are the mean of four separate experiments with the exception of the specific activity curve which is from a typical experiment. This distribution of Ig-positive B cells was reproduced from results described elsewhere (1). The upper graph presents the data in the form of a distribution profile of the total nucleated cells, the total Ig-positive cells, and the total IgM AFC progenitor activity per fraction. In the lower graph, the specific activity has been calculated by division as the AFC/10⁶ cells.

B-lymphocyte subsets from unprimed mice to function as IgM AFC progenitors in an adoptive immune response to NIP-POL was assessed. There was a marked discrepancy between the AFC progenitor activity profile and the distribution of Ig-positive B lymphocytes. The AFC progenitor activity was displaced toward the faster sedimenting region, exhibiting two distinct peaks (4.2 and 5.3 mm/h) and a definite skewing of the activity into the fast sedimenting region (above 5.5 mm/h). A recovery of 85% of cells and 93% of progenitor activity was achieved. Enrichment was obtained in the fast sedimenting region (up to 22-fold enrichment with an average peak value of 6-fold) as seen when the AFC progenitor activity is plotted on the basis of specific activity. There was little or no progenitor activity in the region of the typical B cell (3–4 mm/h). This is in contrast to previous findings by Lafleur, Miller, and Phillips, using the adoptive response to SRBC, of AFC progenitor activity only in this region of typical B cells (3–4 mm/h) (7–9, 32).

GERMFREE AND SPF SPLEEN.   It was found in a previous study (1) that antigen stimulation caused a marked physical alteration in the total B-cell population of adult CBA mice, in an apparently nonspecific manner. Since this type of effect could be the cause of the extensive heterogeneity observed in conventional CBA mouse splenic B cells, studies were undertaken using adult SPF and germfree CBA mice where the level of stimulation from infection should be reduced.

The AFC progenitor profile of adult germfree CBA spleen cells was basically similar to conventional adult spleen. The activity profile deviated from the total nucleated cell distribution, with enrichment of activity in the faster sedimenting region (Fig. 2). Two peaks of activity were found, at 4.2 and 5.0 mm/h. In contrast to conventional spleen, there was a slight shoulder (3–3.5 mm/h) on the AFC progenitor activity curve in the region of typical slowly sedimenting B cells. Similar results were obtained with SPF spleen cells (not shown).

ATHYMIC NUDE SPLEEN CELLS AND ANTI-θ-TREATED SPLEEN CELLS.   As a further check on the finding that IgM AFC progenitor activity of spleen cells from unprimed adult CBA mice was associated with faster sedimenting cells, the possible effects of T cells were considered (33, 34). Although the adoptive assay was independent of T-cell function, it seemed possible that T cells might act either in the adoptive hosts to suppress responses of B cells sedimenting in certain regions, or in the cell donors by influencing the physiological state of the B cells. In order to test the effect of T cells in the adoptive assay, spleen cells from conventional adult CBA mice were treated with anti-θ antiserum and C (31) after sedimentation velocity separation. No significant alteration in the AFC progenitor profile was obtained (not shown). As a related test, spleen cells from adult athymic nude CBA mice, lacking functional T cells, were subjected to sedimentation velocity separation and functional assay. Similar to conventional mice, the AFC progenitor activity was displaced toward the faster sedimenting zone, and several activity peaks were evident. However, as was seen to a small extent with SPF and germfree mouse spleens, there was a significant increase of AFC progenitor activity in the region of slowly sedimenting, small B lymphocytes (3–4 mm/h) in comparison to conventional spleen.
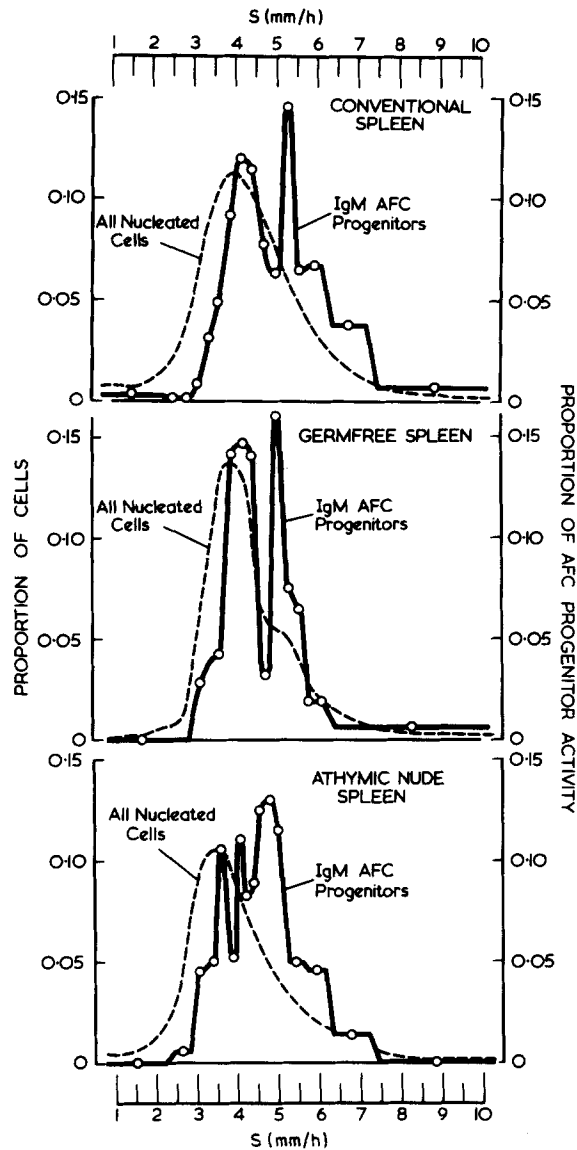
FIG. 2. Sedimentation velocity characteristics of IgM AFC progenitors in adult germfree and athymic nude CBA mouse spleens. The profiles are the mean of two separate experiments, and are presented with the results obtained with conventional spleen for purposes of comparison. See Fig. 1 for details.

Buoyant Density Characteristics of Splenic IgM AFC Progenitors of Adult CBA Mice

CONVENTIONAL SPLEEN. Previous studies have shown that both the total nucleated cell population and the B-cell population of unprimed adult CBA mouse spleen are heterogeneous by buoyant density criteria (1). When the physically separated cells were transferred in the adoptive assay for B cells

responsive to NIP-POL, the IgM AFC progenitors were heterogeneous, with some activity associated with all B-cell peaks (Fig. 3). However, the activity profile was displaced from the B-lymphocyte distribution, with lighter density B cells displaying more activity than the typical mid-density or higher density B cells. From six separate experiments, a mean of 82% of the input cells, and 65% of the AFC progenitor activity was recovered. Taking into account the two peaks of activity by sedimentation velocity separation and the average density of the progenitors, the diameter of the B cells of unprimed conventional adult spleen was 6.9 and 7.8 μm (in suspension) and the vol was 175 and 245 μl^3. Due to the skewing of the profile into the faster sedimenting zone, significant numbers of active B cells were found greater than 8.8 μm in diameter and 360 μl^3 in vol.

GERMFREE SPLEEN. In order to check the possible effects of infection on the density distribution of AFC progenitors, adult germfree spleen cells were next investigated (Fig. 4). The total nucleated cell profile and the AFC progenitor density distribution were heterogeneous and resembled those for conventional spleen cells. However, the distribution of germfree spleen cells was sharper, being dominated by medium density cells ($\sim$1.078 g/cm^3). The major peak of AFC progenitor activity was found in the region of these more typical cells, a result which seems to parallel the appearance of some activity in B cells of low sedimentation velocity in the adult germfree spleen.

ATHYMIC NUDE SPLEEN. To investigate the possible effects of T cells on the physical characteristics of B lymphocytes, athymic nude CBA spleen cells were studied (Fig. 4). The nucleated cells were heterogeneous by density as was the AFC progenitor activity. The major activity peak corresponded to a cell peak, and was found in a more dense region of the gradient than with conventional or germfree spleens (1.081–1.084 g/cm^3). Although there was overlap, proportionally less activity appeared in the lower density regions. If the significant level of activity exhibited by slowly sedimenting nude spleen cells corresponds to the dense cell peak, the cell would be a small lymphocyte. The data on nude spleen cells thus suggest that small lymphocytes can express virgin B-cell AFC progenitor activity.

Sedimentation Velocity Characteristics of Bone Marrow IgM AFC Progenitors of Adult CBA Mice. Although B cells are probably generated in both the spleen and the bone marrow, the marrow seems to be the major site of B-lymphocyte production (35, 36). The bone marrow was studied in order to search for a population of functional B lymphocytes closer to their site of production and state of origin. Marrow (femur and tibia) from 6-wk-old conventional CBA mice was separated on the basis of sedimentation velocity. As seen in Fig. 5, the total nucleated cell profile was broader and more obviously heterogeneous than spleen. Assay of the separated fractions for IgM AFC progenitors resulted in an activity profile that was no less complex than adult conventional spleen, and was, in fact, similar to it in many respects. Two major peaks of AFC progenitor activity were evident, the 4.2 mm/h peak corresponding directly to one in the adult spleen, and the second peak (5.7–6.1 mm/h) occupying a faster sedimenting position than in spleen. A skewing of the activity was evident toward the faster sedimenting region (>6.5 mm/h). Similar to athymic nude spleen, but unlike conventional spleen, there was an additional activity peak in the slowly sedimenting small lymphocyte region (3–3.5 mm/h).
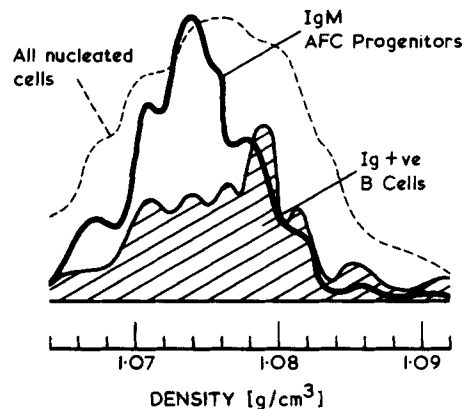
Fig. 3.   Buoyant density characteristics of IgM AFC progenitors from adult conventional CBA mouse spleen. Spleen cells were centrifuged to equilibrium in isosmotic albumin density gradients, as described in the Materials and Methods. The fractions were used in the adoptive immune assay for IgM AFC progenitors responding to the NIP-POL antigen. The distribution of Ig-positive B cells is reproduced from results described elsewhere (1). The results are the mean of six separate experiments, each on a pool of six to eight spleens. The data are presented as total progenitor activity per density increment, with the values normalized to a maximum of 100%.

Buoyant Density Characteristics of Bone Marrow IgM AFC Progenitors of Adult CBA Mice.   Bone marrow was also heterogeneous when examined by equilibrium density centrifugation, with cells being found throughout the density range. As shown in Fig. 6, bone marrow (femur and tibia) from conventional mice exhibited two main peaks of AFC progenitor activity, the larger corresponding to the main activity peak of adult spleen (~1.072 g/cm). The denser AFC progenitor peak of marrow was not present in spleen, and could correspond to the slowly sedimenting progenitor activity peak of marrow. If such were the case, this cell would be a small lymphocyte.

Sedimentation Velocity Characteristics of Neonatal CBA Mouse Spleen IgM AFC Progenitors.   An unexpected, and yet consistent characteristic of the IgM AFC progenitor activity of unprimed mice was the relative lack of activity in the region of typical small B cells, and the association of activity with larger, faster sedimenting, less dense B lymphocytes. This was true of both bone marrow and spleen cells, and of conventional, SPF, germfree, and athymic nude CBA mice. However there were cases where a proportion of the progenitor activity was found among slowly sedimenting small B lymphocytes, and these were situations where there was probably some effective reduction in the level of overt environmental exposure. It seemed possible that the small B lymphocyte was actually the original virgin AFC progenitor, the atypical active forms arising from this cell, perhaps by environmental stimulation. Accordingly, a more favorable biological situation was sought, where more newborn and unstimulated AFC progenitors might predominate. Neonatal CBA mice were studied, and conventional, SPF, and germfree mice were compared.

The total nucleated cell profile of neonatal spleen cells was broader than for adult spleen cells (Fig. 7). The IgM AFC progenitor activity of neonatal conventional spleen cells was similar to adult conventional spleen cells with activity
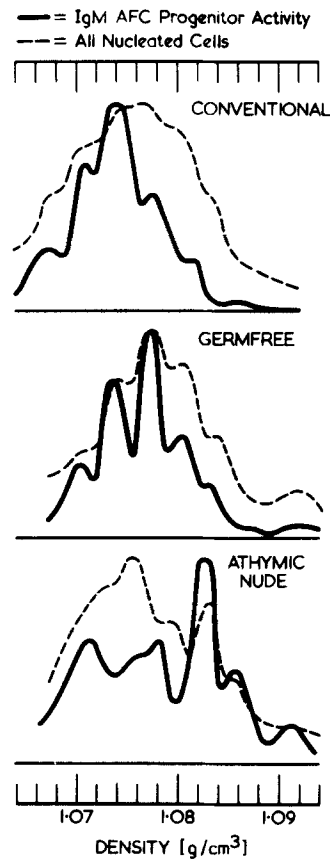
Fig. 4.   Buoyant density characteristics of IgM AFC progenitors from adult germfree and athymic nude CBA mouse spleen. The results are the mean of two separate experiments each, and are presented with the results obtained with conventional spleen for purposes of comparison. See Fig. 3 for details.

among more rapidly sedimenting cells. However there was an additional minor activity peak in the slowly sedimenting region coincident with the total cell peak. The AFC progenitor profile of neonatal SPF spleen cells was similar, but now a significant proportion of the progenitor function appeared in the small B-cell activity peak. Neonatal germfree spleen cells gave a predominant peak of AFC progenitor activity among slowly sedimenting small B lymphocytes (3.5 mm/h), with less activity being found in the region of faster sedimenting cells. This last result is in strong contrast with conventional adult CBA spleen cells (Fig. 1) where no peak and very little AFC progenitor activity was found at 3.5 mm/h.

Buoyant Density Characteristics of Neonatal Germfree Mouse Spleen IgM AFC Progenitors.   To further characterize this virgin AFC progenitor from neonatal germfree spleen, and to allow an estimate of size, the spleen cells were next separated on the basis of buoyant density. As shown in Fig. 8, the total nucleated cell density distribution of neonatal germfree spleen was heteroge-
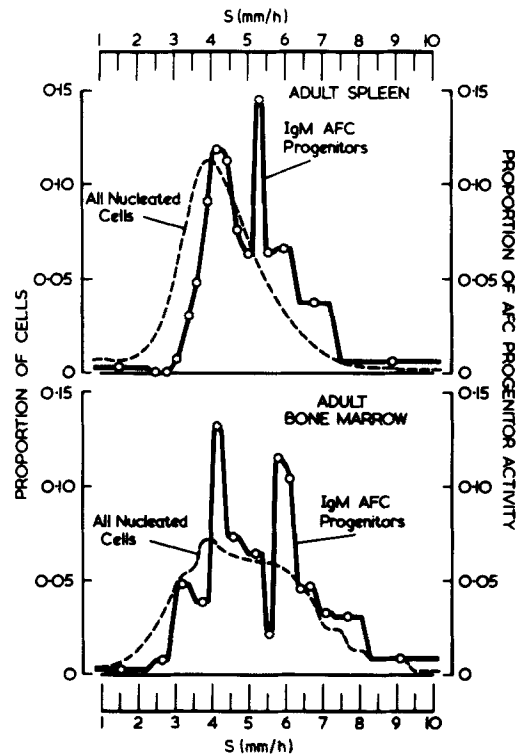
FIG. 5.   Sedimentation velocity characteristics of IgM AFC progenitors from adult conventional CBA bone marrow. The profile is the mean of three separate experiments, and is presented with the results obtained with conventional spleen for purposes of comparison. See Fig. 1 for details.

neous. The AFC progenitor activity was displaced toward the denser cells compared to the adult conventional spleen. The lighter of the two progenitor peaks was considerably denser than the vast majority of activity in adult conventional spleen, while the denser of the two major cell peaks ($\sim$1.084 g/cm^3) was most prominent. Combined with the sedimentation velocity results, the average buoyant density data were used to calculate the size of the virgin AFC progenitors. The diameter was 5.9 μm and the volume 110 μl^3, consistent with classification as a small lymphocyte.

Effect of Age Upon the Sedimentation Velocity Characteristics of IgM AFC Progenitors of Germfree Mouse Spleen.   Slow sedimenting, dense IgM AFC progenitors were predominant in neonatal germfree mouse spleen. However, the active B cells of adult germfree spleen were similar in physical characteristics to those of adult conventional spleen, being light and fast sedimenting. To investigate this difference, the spleens of germfree mice of various ages were separated on the basis of sedimentation velocity and were assayed for IgM AFC progenitor activity (Fig. 9). Although some AFC progenitor activity in 12-day-old germfree spleen was found in the slowly sedimenting B-cell region, most of the functional B lymphocytes were faster sedimenting. Most AFC progenitor
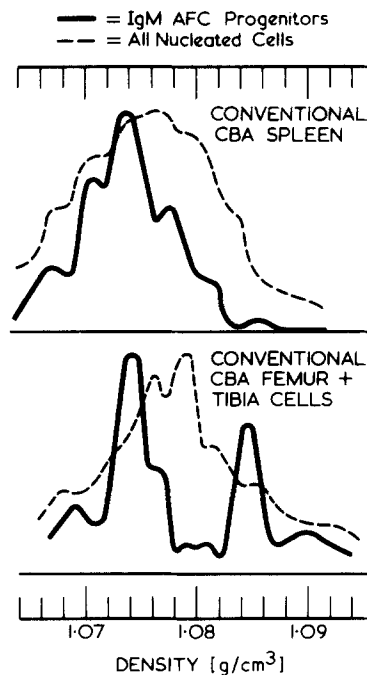
Fig. 6. Buoyant density characteristics of IgM AFC progenitors from adult conventional CBA mouse bone marrow. The results are the mean of three separate experiments, and are presented with the results obtained with conventional spleen for purposes of comparison. See Fig. 3 for details.

activity in 16-day-old germfree spleen was as rapidly sedimenting as in the adult spleen. The physically atypical AFC progenitors characteristic of the adult mouse appear to arise early in maturation.

Discussion

These studies are part of an investigation of the B-lymphocyte pathway of development. In this paper, cell separation techniques were used to characterize the functional, newborn B cell on the basis of physical properties. This approach should complement work on surface immunoglobulin, differentiation antigens, and receptors, as well as providing a means of isolating the cells for closer investigation. The assay procedure measured the ability of physically separated B-cell subsets from unprimed CBA mice to mount an adoptive IgM AFC response to the antigen NIP-POL. The assay system has the following important advantages: (a) it enables a direct assessment of B-lymphocyte function, (b) the number of AFC produced bears a simple linear relationship to the number of progenitors present, and (c) it has characteristics which strongly suggest that the donor CBA mice are in an unprimed state with respect to the NIP determinant (15–17).

The Ig-bearing B-cell population of the adult CBA mouse is heterogeneous by both density and sedimentation velocity criteria (1). The AFC progenitor assay indicates that all these cells are not equally active in initiating an IgM AFC response to NIP-POL. These unprimed AFC progenitors are quite heteroge-
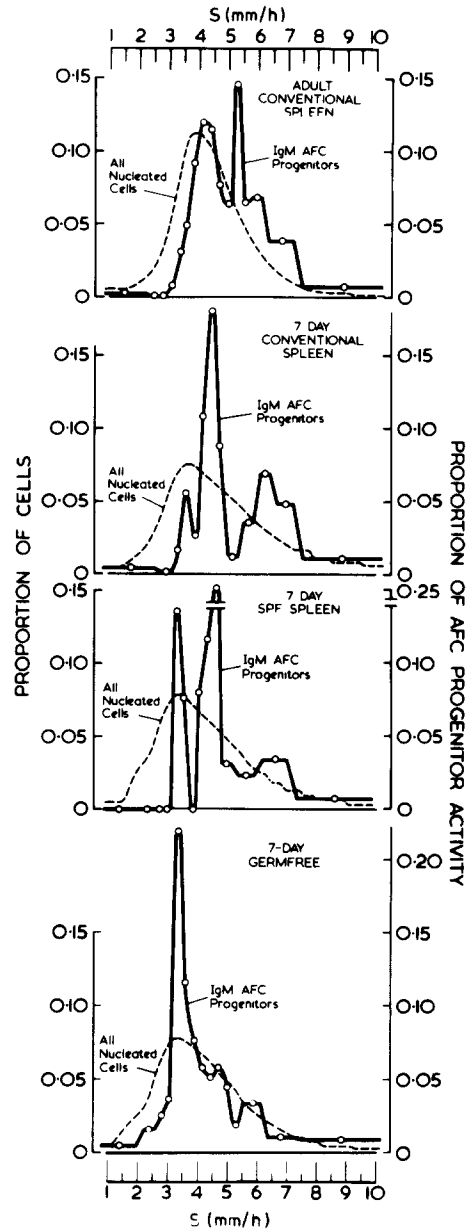
Fig. 7. Sedimentation velocity characteristics of IgM AFC progenitors from neonatal CBA spleen. The results with conventional and SPF spleen are from one experiment each, and the result with germfree spleen is the mean of three separate experiments. Each experiment involved a pool of 10-15 neonatal spleens. For purposes of comparison, the results obtained with conventional adult spleen are also reproduced. See Fig. 1 for details.

neous and are, in fact, atypical, being faster sedimenting and generally less dense than the typical small B cell. Based on an average density value, the sedimentation velocity peaks of active cells suggest that most functional un-primed B cells range from 175 to 245 μm^3, or 6.9 to 7.8 μm diameter in
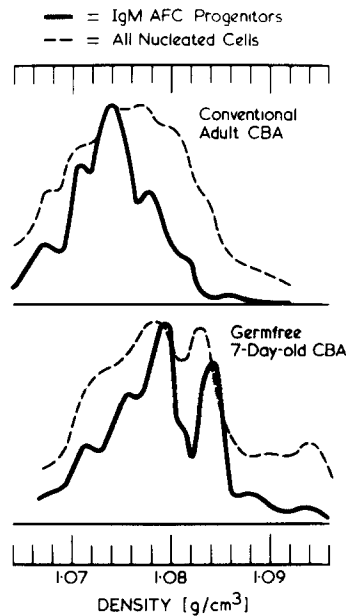
FIG. 8.   Buoyant density characteristics of IgM AFC progenitors from neonatal germfree
CBA mouse spleen. The results are the mean of three separate experiments, each on a pool
of 15–25 spleens, and are presented with the results obtained with conventional adult
spleen for purposes of comparison. See Fig. 3 for details.

suspension and are therefore predominantly medium sized cells (although significant activity is found among cells greater than 8.8 μm in diameter and 360 μl^3 in vol). This result agrees with previous cell electrophoresis and adherence column studies in this laboratory (11, 12) which characterized most of the unprimed AFC progenitors as atypically fast migrating and exceptionally adherent B cells. The earlier density separation studies of Haskill (37) and Haskill and Marbrook (38) are in general agreement with these conclusions. They are also consistent with recent findings of Strober (13) on the sedimentation characteristics of unprimed AFC progenitors in the rat. The results are in apparent disagreement with the sedimentation velocity studies of Lafleur, Miller, and Phillips (7–9, 32), and Diener et al. (39), both of whom found the AFC progenitors to be slowly sedimenting cells.

The physical characteristics of the unprimed AFC progenitor population in conventional adult mouse spleen are, in fact, those expected of activated B-cell forms. These characteristics are shared with larger, dividing B cells and with many AFC (1). This similarity suggested that the AFC progenitors assayed might already be in a primed or activated state. This possibility prompted a range of control experiments in adult mice, where attempts were made to eliminate certain stimulatory influences. These included the use of germfree mice (where the possibility of infections was removed), nude mice (where T-cell stimulatory effects were eliminated or reduced), and studies on bone marrow cells (which might represent a source closer to the origin of B lymphocytes). The dominant result was that, in all situations, the IgM AFC progenitor population retained its atypical and heterogeneous characteristics. However there was a
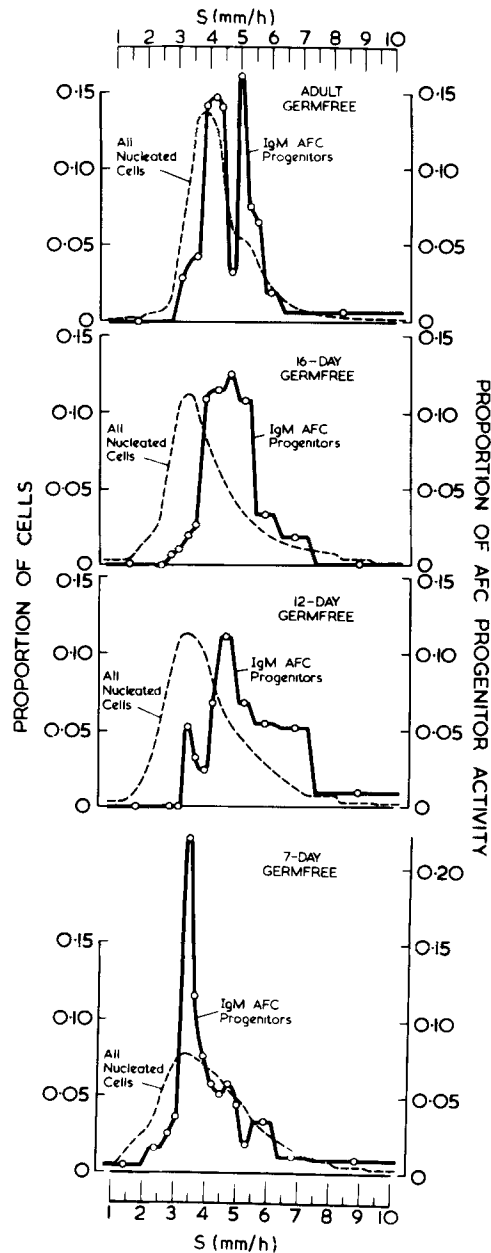
Fig. 9.   Sedimentation velocity characteristics of IgM AFC progenitors among spleen cells from germfree CBA mice of various ages. The results with 12- and 16-day-old mice are from one experiment only while the results obtained with adult and 7-day-old germfree spleen are the means of three experiments. Each experiment involved a pool of 10-15 neonatal spleens.

suggestion of some activity appearing in slowly sedimenting, medium to high density, small B cells.

There are a number of possible explanations for the lack of unprimed AFC progenitor activity among smaller B lymphocytes. Despite optimization of ex-

perimental conditions, the adoptive immune assay system may not allow this type of cell to express activity. While this possibility is currently under investigation, the fact that certain alterations of the immunological status of the cell donor enables the production of positive responses by cells in this region argues against this view. Another explanation is that a T-cell-independent antigen is used, whereas Lafleur et al. (7–9) and Miller and Phillips (32) obtained activity from smaller B cells using a T-dependent antigen. The existence of two classes of B lymphocytes, T dependent and T independent, has been suggested by Gershon (34) and Playfair and Purves (40), and evidence for this point has been advanced by Gorczynski and Feldmann (41). However, the data of the latter group is expressed only in terms of relative enrichment (or activity per cell) and, when calculated on the basis of total progenitor distribution as we have done, does not suggest sufficiently marked separation of the proposed B-cell subgroups to explain our results. This aspect is currently being investigated with a T-dependent form of the NIP hapten.[3] Our preferred explanation is that most of the inactive, small B cells in our CBA mice are in fact IgM and IgG progenitor memory cells, formed by prior contact with a range of antigenic stimuli to which the synthetic NIP determinant shows little cross-reactivity. In favor of this suggestion is our evidence from electrophoretic and column fractionation experiments (11, 12), and from density and sedimentation separation studies, that NIP memory B cells in hapten-primed animals are smaller, more "typical" B lymphocytes. It is possible that the AFC progenitors found in other studies among small B lymphocytes in animals not experimentally primed were in fact memory cells formed by encounter with antigens cross-reacting with SRBC.

If the majority of IgM AFC progenitors in the unprimed adult animal are atypical larger B-cell forms, resembling activated B cells, the question arises whether this is indeed the basic state of antigen-inexperienced B cell, or whether the newborn B lymphocyte differs in physical properties. The studies of Osmond and Nossal (10) on the generation of Ig-bearing cells in bone marrow would suggest a typical small lymphocyte as the first functional B cell. A hint that the initial functional B lymphocyte in our system could be a small, dense B lymphocyte came from the appearance of some activity in cells of this type in bone marrow, and in spleens of germfree and athymic nude mice. This prompted the investigation of neonatal animals as a possible B-cell source that would be in a less environmentally stimulated state. In marked contrast to the situation in adult animals, the vast majority of the IgM AFC progenitors responsive to NIP-POL in 7-day-old germfree CBA mice were slowly sedimenting, dense cells, presumably typical small virgin B lymphocytes. It appeared that this simple situation changed rapidly towards the larger, lighter, more heterogeneous AFC progenitors as either the germfree mice themselves aged, or when age-matched mice had been exposed to increasing levels of exposure to the bacterial environment. Based upon this data, our hypothesis is that the small newborn virgin B cell is in fact hypersensitive to nonspecific effects of environmental stimuli, perhaps indirectly mediated by the responses of other cells to particular anti-

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[3] Fidler, J. M., M. C. Howard, and K. Shortman. Manuscript in preparation.

gens. Thus the specific antigen-inexperienced cell in unprimed mice is normally in a partially stimulated state, in contrast, for example, to a memory B cell that generally requires contact with specific antigen to become even partially activated. Experimental testing of this hypothesis will be the subject of a later study.[4]

We have directed attention in this series of papers to developmental events occurring after the birth of the first functional B cell (i.e., the antigen-dependent differentiation stages). However, the results may have some bearing on studies designed to elucidate earlier steps in the B-cell pathways of development, where larger, dividing immature B or pre-B cells have been described (7–9, 10, 13, 32, 42, 43). Strober reported a large, dividing Ig-bearing AFC progenitor cell in rat spleen, which appears to produce a population of small mature B cells (13, 42). Melchers et al. have described a large Ig-synthesizing cell in fetal liver, as well as adult spleen and bone marrow, and considered this to be a transitional stage of development before an Ig-positive mature virgin B cell (43). Lafleur, Miller, and Phillips have found a pre-B cell to be a large, Ig-bearing cell, found in spleen and marrow (7–9, 32). The B-cell precursor of Osmond and Nossal (10) is a larger, dividing but Ig-negative cell found in marrow. It is conceivable that some or all of these supposedly immature forms correspond to our large "activated" but functional B cell which we detect as the form of the antigen-inexperienced AFC progenitor in unprimed adult CBA mice. Classification of this cell as a pre-B cell or as a cell derived from the newborn virgin B lymphocytes may depend on whether particular assay conditions allow it to develop into AFC. Under the optimized conditions used here, cells with these general physical properties appear to be active as AFC progenitors, and therefore develop from, rather than precede, the newborn functional virgin B lymphocytes.

Summary

The characteristics of antibody-forming cell (AFC) progenitors lacking previous contact with specific antigen (virgin AFC progenitors) has been studied using sedimentation velocity and buoyant density separation for the investigation of physically distinct B-cell subpopulations. Functional characterization of isolated subsets was made using a quantitative adoptive immune assay for the IgM AFC progenitors responding to the antigen 4-hydroxy-3-iodo-5-nitrophenyl-acetic acid conjugated polymerized bacterial flagellin. Extensive heterogeneity is present among B lymphocytes, only some subpopulations of which exhibit AFC progenitor function. In the spleen of adult conventional CBA mice, atypically fast sedimenting cells of low buoyant density are active, while typical small B lymphocytes do not appear to be progenitors of IgM AFC. Spleens of adult specific pathogen-free (SPF), germfree, and athymic nude mice give similar results, although a minor population of typical slowly sedimenting dense

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cells are active in the latter two sources. Adult conventional bone marrow cells are as physically and functionally heterogeneous as splenic B cells, and although a significant proportion of AFC progenitor activity is found among dense, slowly sedimenting cells, most of the activity is among low density, faster sedimenting cells. In contrast to this situation in adult animals, where most of the unprimed AFC progenitors are large, atypical B cells, the spleens of neonatal mice provide a site where virgin AFC progenitors with the physical properties of typical small B lymphocytes are found. While being present in conventional and SPF neonatal spleens, these virgin cells are predominant in 7-day-old germfree mouse spleen.

These findings suggest that the newborn virgin B cell is a typical small lymphocyte. However, few cells of this type are found in the adult animal. The unprimed AFC-progenitor population in the adult consists of large, fast sedimenting, low buoyant density, adherent cells, the physical properties of which are characteristic of activated B lymphocytes. It is suggested that these atypical cells are derived from the small newborn virgin B cell by the nonspecific effects of environmental antigenic stimuli.

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