RESTRICTION OF SPECIFICITY IN THE PRECURSORS OF BONE MARROW-ASSOCIATED LYMPHOCYTES*

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The immediate progenitors of B lymphocytes (PB cells)[1] in the adult mouse are readily distinguishable from mature B cells. PB cells can be separated from B cells by velocity sedimentation cell separation and transplanted into mice made immunologically incompetent by irradiation (1). The PB cells cannot interact with antigen and thymus-dependent lymphocytes (T cells) to give rise to antibody-producing cells, but within a few days can give rise to differentiated progeny having this functional requirement for being B cells. PB cells, in turn, appear to be derived from hemopoietic stem cells (2).

Of particular interest in previous studies was the possibility that PB cells possess surface immunoglobulins (2). Since PB cells are immunologically incompetent when injected into irradiated mice together with antigen and thymus cells (T cells), this implies that acquisition of competence requires another step in differentiation, perhaps enabling interaction with T cells or T cell products. Nevertheless, the presence of surface immunoglobulin molecules in PB cells suggests that cells at this stage of differentiation are already restricted with respect to specificity. The work described below is an attempt to determine whether PB cells are specificity restricted.

Materials and Methods

Animals.—The mice used in all experiments were 8–10-wk old F_1 hybrids between C57BL/6 × DBA/2 (BDF_1) purchased from Cumberland View Farms (Clinton, Tenn.). Males were usually used both as recipients and bone marrow donors, females as donors for thymus and spleen cells. Mice were kept three or four to a cage after irradiation and allowed free access to food and water.

Antigen.—Sheep blood (SRBC) in citrate saline was obtained weekly from Woodlyn Farms

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[1] *Abbreviations used in this paper:* B cells, precursors of antibody-producing cells; BSA, bovine serum albumin (fraction V); CFU-S, spleen colony-forming units; Fl-anti-Ig, fluorescein-labeled antimouse immunoglobulin antiserum; PB cells, precursors of B cells; PBS, phosphate-buffered saline; PFC, plaque-forming cells; RFC, rosette-forming cells; SRBC, sheep erythrocytes; T cells, thymus-dependent lymphocytes.

(Guelph, Ontario, Canada). The cells were washed three times in phosphate-buffered saline (PBS) before use.

Radiation.—Mice and single-cell suspensions were irradiated in a ^{137}Cs irradiator (3) at a dose rate of 102 rad/min. Mice received 950 rad, cell suspensions 1,000 rad. Cells were irradiated in an ice bath at a concentration of 10^8 cells/ml.

Cell Suspensions.—Bone marrow cell suspensions were prepared by flushing 1 ml of 0.2% bovine serum albumin (BSA) in PBS through the femurs. Spleen, thymus, and lymph node cell suspensions were prepared by cutting the organ into small pieces with a pair of scissors and pressing the pieces through an 80 mesh stainless steel screen. All cell suspensions were gently pipetted and washed once; the remaining cell clumps were broken up or removed by filtration through a glass capillary array filter, pore size 34 μm (Mosaic Fabrications, Sturbridge, Mass.). All suspensions were prepared in the cold.

Cell Separation.—Cells were fractionated on the basis of differences in sedimentation rate at unit gravity, a procedure which separates cells primarily on the basis of size (4). One of two techniques (4, 5), both of which give equivalent results, were used. In either case, a 1–2% stabilizing gradient was used, starting cell bands were 1–4 mm wide, sedimentation time was 4–5 h and the whole separation was performed at 4°C in a cold room.

Antiserum.—Fluorescein-labeled anti-Ig antiserum (Fl-anti-Ig) was a generous gift from Dr. E. Unanue. In the sedimentation experiments, the cells were exposed to the labeled antiserum before separation to ensure that all cells were exposed to identical labeling conditions. The suspension of cells was incubated for 3 h at 0°C with the labeled antiserum, washed three times with 0.2% BSA in PBS, and then introduced into the separation chamber. The cells were allowed to sediment for 4 h at 4°C, and fractions were collected and scored for the percent of cells showing a ring-type fluorescence. At least 200 cells were scored for each fraction.

Functional Assays.—All functional assays are based on transplantation experiments in which the quantity measured is the number of 19S antibody-producing cells as determined by the Jerne plaque-forming cell (PFC) assay (6). All the assays are designed in such a way that only B cells or their precursors limit the response obtained, i.e., the response is a linear function of the number of B cells or PB cells assayed. The conditions have been described previously (1). Briefly, B cells are defined as those cells giving rise to a detectable number of PFC within 8 days after immunization. A suspension to be tested for B cell activity is mixed with 5×10^7 thymus cells (to provide excess T cells), and the mixture is injected into irradiated recipients. Within 2 h of injection, 5×10^7 irradiated spleen cells (to provide excess accessory or A cells [7]) and 10^8 SRBC are injected intraperitoneally. 8 days later the number of PFC per spleen are determined.

By definition, PB cells are inactive in the assay for B cells. However, if an interval of several days is allowed to elapse between transplantation and immunization, PB cells differentiate into B cells that can be detected by immunization. Therefore, we have defined as PB cells any cells that give rise to functional B cells within 7 days after transplantation into an irradiated recipient. Thus, the assay for PB cells is identical with the one for B cells except that in the former 7 days elapse between transplantation and immunization while in the latter the interval is only 2 h. Under the conditions of the assay for PB cells, the PFC response is a linear function of the number of cells injected.

RESULTS

Direct Measurement of Sedimentation Profile of Immunoglobulin-Containing Cells in Bone Marrow, Spleen, and Lymph Node.—Previous characterization of the precursors of B cells sensitive to SRBC indicated that these cells sediment faster than the mature B cells specific for SRBC (1). Further work indicated

that both B lymphocytes and PB cells probably have surface immunoglobulin receptors (2). These two results can be directly verified by measuring the sedimentation distribution of cells stained with Fl-anti-Ig. Fig. 1 *a* shows, for bone marrow, the sedimentation distribution of stained cells. As expected, the

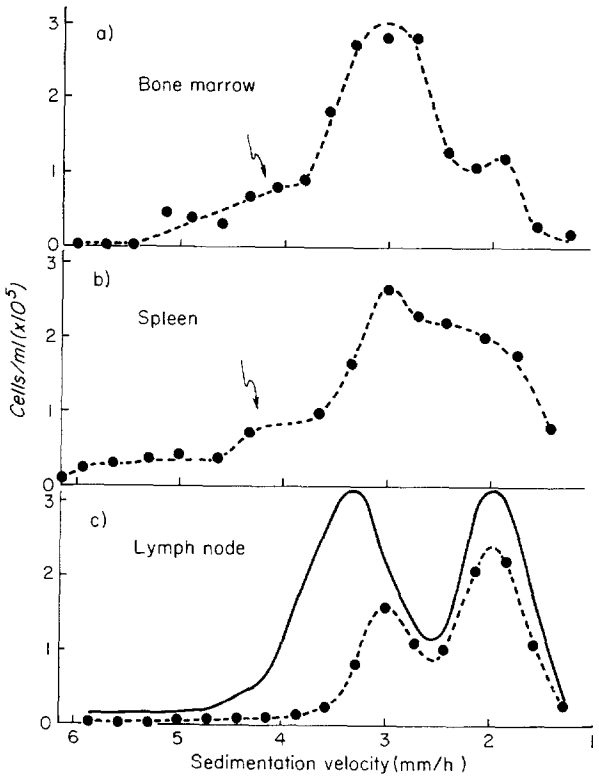

FIG. 1. Sedimentation profile of bone marrow (*a*), spleen (*b*), and lymph node (*c*) cells stained with Fl-anti-Ig. Cells at 10^7 cells/ml were incubated for 1–3 h at 0°C with Fl-anti-Ig, then washed three times in 0.2% BSA and resuspended at 5×10^6 cells/ml for fractionation. The sedimentation time varied from 4 to 12 h and individual fractions were scored for fluorescent cells (●). The nucleated cell profile (solid line) is shown only for lymph node.

majority of the activity is found in the region of small lymphocytes where, by functional assays, B cells are known to sediment (8). The peak of fluorescent cells at 2.0 mm/h is the result of nonspecific uptake by damaged cells (9) that sediment in this region of the gradient (8). In addition to the small peak in the region of slowly sedimenting cells, the main peak has a distinct shoulder (indicated by the arrow) that skews the distribution toward the rapidly sedimenting cells. If one assumes a gaussian distribution for B cells and subtracts this distribution from the measured distribution of labeled cells, the residual population (the one giving rise to the shoulder) has a modal sedimentation velocity of

about 4.8 mm/h and constitutes about 5–10% of the total cells stained with Fl-anti-Ig. One interpretation of this shoulder is that it represents PB cells that have been shown by functional assays to sediment at 5 mm/h and probably have IgM molecules on their surface (1, 2).

The shoulder could be an artifact of the separation procedure. For example, B cell doublets would sediment in this position (8). However, this explanation is unlikely since the measured frequency of doublets was too low to cause the shoulder.

In an attempt to strengthen the correlation between PB cell activity and the presence of rapidly sedimenting immunoglobulin-bearing cells, we measured for spleen and lymph node the sedimentation profiles of PB cells and B cells measured by their activities in functional assays and of Fl-anti-Ig-staining cells. For both spleen (Fig. 2) and lymph node (Fig. 3), B cells (●) sediment as a single

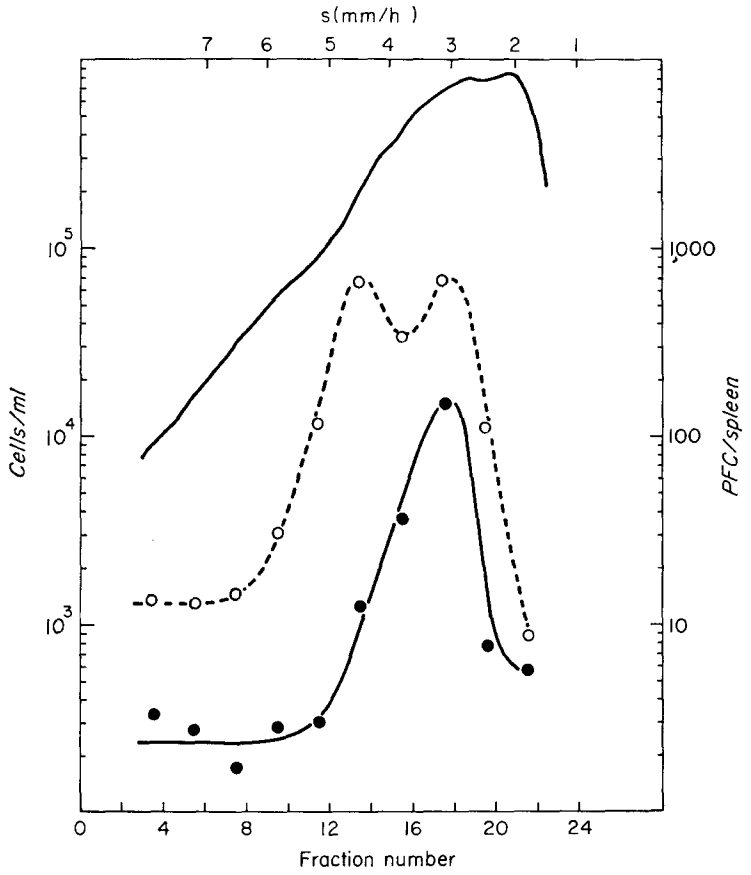

FIG. 2. Sedimentation profile of PB cells and B cells in spleen. 5×10^6 spleen cells suspended in 100 ml of 0.2% BSA were separated by velocity sedimentation for 4 h. Nucleated cells (solid line) were counted in each fraction. Pairs of adjacent fractions were tested for B cells (●) and PB cells (○) as described in the text.

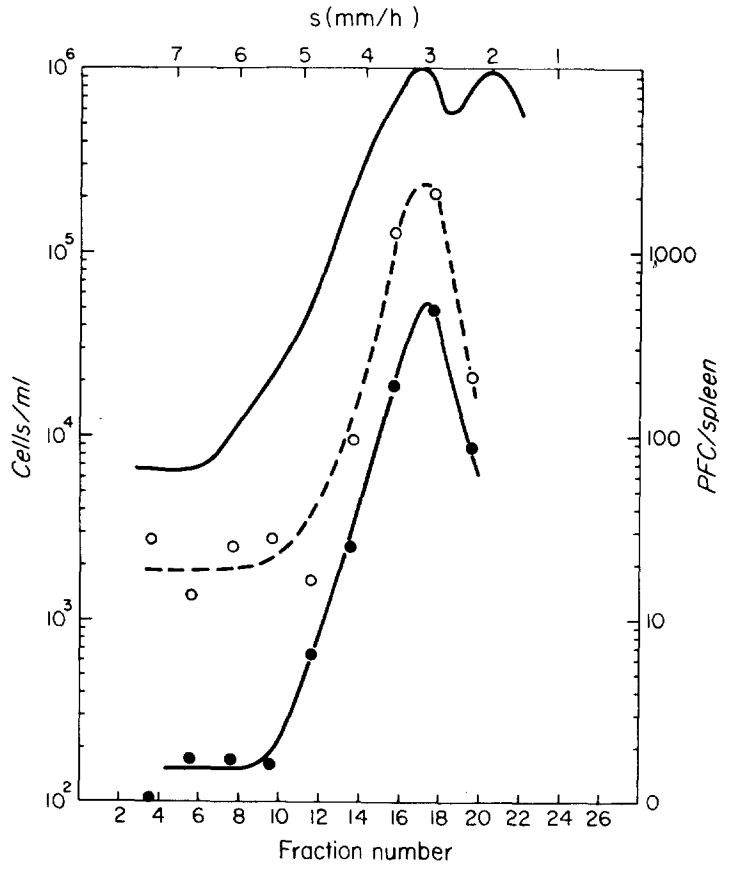

FIG. 3. Sedimentation profile of PB cells and B cells in lymph node. Sedimentation conditions were identical with those described in Fig. 2. The figure shows the distributions of nucleated cells (solid line), B cells (●), and PB cells (○).

narrow band with a sedimentation velocity of 3.0 mm/h. Under the conditions of the PB assay (○) both tissues again show the B cells initially present at 3.0 mm/h; this observation was expected since the PB cell assay also detects B cells (1, 2). The suspension of spleen cells also shows another peak of activity at 5.0 mm/h. This peak by definition represents PB cells. Lymph node, however, does not show a peak of activity in the PB cell region and, therefore, presumably contains few, if any, PB cells.

From these results, we would predict that the profile of spleen cells stained with Fl-anti-Ig should be similar to one for the bone marrow cells, whereas the distribution of stained lymph node cells should be symmetrical with no shoulder in the region of the rapidly sedimenting cells. Figs. 1 *b* and *c* verify this prediction. The sedimentation profile of labeled spleen cells (Fig. 1 *b*) has a significant

shoulder between 4.5 and 5.0 mm/h. The total number of stained cells at 4.5 mm/h is about 20% of that at 3.0 mm/h, both in bone marrow and in spleen. A similar analysis for lymph node cells gives no indication for such a population in the region of PB cells; the number of stained cells at 4.5 mm/h is less than 5% of that at 3.0 mm/h. Because the fluorescein-labeled cells sedimenting at 4.5–5.0 mm/h have the same sedimentation and tissue distribution as PB cells measured with functional assays, we have tentatively identified this population as PB cells and have used this observation to estimate the absolute frequency of both B cells and PB cells in bone marrow, spleen, and lymph node. These data (Table I) show that in both bone marrow and spleen PB cells are about 10-fold less frequent than B cells.

Frequency and Specificity of PB.—The data in the previous section indicate that in bone marrow, PB cells occur in a relatively high frequency, about 1 per 100 nucleated cells. If it is assumed that the antiserum detects the entire popu-

TABLE I

Frequency of PB and B Cells in Various Lymphoid Tissues

Tissue	no. of cells/10^3 nucleated cells	
	B cells*	PB cells*
Bone marrow	140	13
Spleen	500	32
Lymph node	250	<2

* These numbers were derived from the sedimentation distribution of Fl-anti-Ig as described in the text.

lation of PB cells, it is possible to estimate specificity restrictions among this population by looking at the frequency of cells that respond to a specific antigen. To estimate this frequency using SRBC as antigen, a standard assay for PB cells was carried out, using varying numbers of cells from bone marrow. To eliminate the background resulting from mature B cells, the initial suspension of bone marrow was separated by velocity sedimentation. All of the cells sedimenting faster than 4.5 mm/h were pooled and used as a source of PB cells. From the data shown in Figs. 1–3, this pool (the PB cell pool) should contain the majority of the PB cells and few B cells. At the completion of the assay, each group of mice was scored for responders and nonresponders. Any recipient giving a PFC response $\geq$ 200 PFC/spleen was counted as a responder. The selection of this value was somewhat arbitrary. Because of the 15 day interval between transplantation and the assay for PFC, it was not possible to look at PFC responses in mice that received only thymus cells. The mortality in such groups was too high to obtain meaningful data. However, in several experiments, it appeared that there was a clear distinction between mice that had

fewer than 200 PFC/spleen and those that had more. There were relatively few mice that gave responses between 200 and 400 PFC/spleen.

The frequency of responders as a function of the number of cells injected from the PB cell pool is shown in Fig. 4. The data fit closely to the solid line that was calculated assuming a single-hit poisson distribution with a frequency of one active cell per 4.9×10^5 cells injected (10). This estimated frequency must be corrected for two factors that alter the actual frequency. First, the measured frequency must be corrected for the enrichment resulting from the separation procedure. The PB cell pool contains 50% of the cells in the original suspension and almost all the PB cells so that the PB cell detection frequency is about 1 per

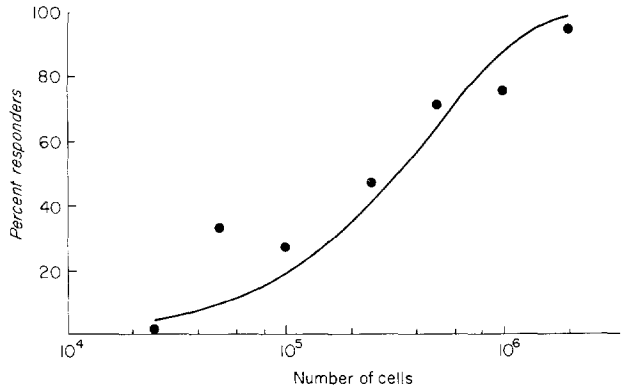

FIG. 4. Measurement of the frequency of PB cells in bone marrow by a limiting dilution assay. A suspension of cells enriched for PB cells as described in the text was assayed for PB cell activity under the standard conditions. The (●) give the percent of responding mice (response $\geq$ 200 PFC/spleen) at each dose of bone marrow cells injected. The solid line is the expected distribution assuming an activity of one functional PB cell per 4.9×10^5 bone marrow cells injected. This interpretation assumes that the active unit is a single cell.

10^6 unfractionated bone marrow cells. Second, in transplantation assays, it is necessary to correct for the fact that only a fraction of the injected cells will settle in the spleen and be subsequently detected. The standard procedure for estimating this fraction was described by Siminovitch et al. (11). Following their protocol, mice were given cells from the PB cell pool. 2 h after injection, these receipients were sacrificed and a cell was suspension made from their spleens. This suspension was then also assayed for its content of PB cells using the standard assay. By comparing the PB cell activity in this group of mice with the PB cell activity in the initial suspension, one can estimate the fraction of cells that settle in the spleen and are detected in the assay. The data in Table II indicate this to be approximately 14%; using this value to correct for the frequency of cells actually detected, we estimate that in bone marrow the frequency of PB cells giving rise to progeny that react with SRBC is 7 per 10^6 bone

marrow cells injected. These data suggest that the PB cells specific for SRBC constitute approximately 0.05% of the total PB cell population as detected by the fluorescent antiserum and imply that PB cells are restricted in specificity.

Differentiation of PB into B cells.—PB and B cells both have immunoglobulin molecules on their surface and both are apparently already restricted in their capacity to respond to specific antigens. However, they differ in two important aspects. First, B cells but not PB cells can interact with T cells to give functional PFC 8 days later, and second, they differ in size as shown by their differ-

TABLE II

Proportion of Injected Cells Trapped in the Spleen

Recipient	Source of cells	no. of cells injected	PFC/spleen*	Relative activity
Primary	PB cell pool‡	2×10^7	5,760§ (4,660–7,020)	1.00
Secondary	Spleen from primary‖ recipient, 2 h after injection of PB cell pool	Cells from ¼ spleen	211 (152–296)	0.14 ± 0.056¶

* PFC/spleen in the recipient. The values in parentheses show one standard error on each side of the mean.

‡ Cells from a pool of bone marrow sedimenting faster than 4.5 mm/h.

§ Estimated from a titration of PB cell pool. The measurement of PB activity was made of the range of 2.5×10^4 to 2×10^6 cells and showed an activity of 288 PFC/10^6 cells injected. Assuming a linear extrapolation, 2×10^7 cells would give 5,760 PFC/spleen from PB cells.

‖ Some primary recipients were sacrificed 2 h after transplantation and their spleens assayed for PB activity by injection into other irradiated recipients.

¶ The PB activity in the spleen of the primary recipient divided by the PB activity in the initial suspension is a measure of the proportion of PB trapped in the spleen of the primary recipient. $(211 \times 4) \div 5{,}760 = 0.14$. (Standard error = 0.056.)

ent sedimentation velocities and similar densities. From the measured physical properties one can estimate that B cells have a volume of 170 μm^3 and PB cells, 370 μm^3 (4). Because of these large differences in size, it is of interest to determine the physical properties of the newly produced B cells. Do B cells change in volume before they acquire the capacity to differentiate into PFC or do they become functional B cells before they decrease to the volume of mature B lymphocytes?

To answer this question we measured, at different times after transplantation of PB cells, the sedimentation velocity of cells showing B cell activity. A group of 30 mice received 2×10^7 cells from the stem cell pool of bone marrow prepared as described above. At varying times after injection, four mice were sacrificed and a cell suspension was prepared from their spleens and separated by

velocity sedimentation into two fractions: a B cell pool (2.0–4.5 mm/h) and a PB cell pool (4.5–8.0 mm/h). Estimates of B cell activity for each fraction were made using the standard assay for B cells. Fig. 5 shows the proportion of activity in each pool at various times after transplantation of the stem cell fraction. On day 7, the majority of the cells with activity in the assay for B cells are in the rapidly sedimenting pool, indicating they are much larger than mature B cells. By day 11, the activity in the two pools is about equal, and by day 20, the

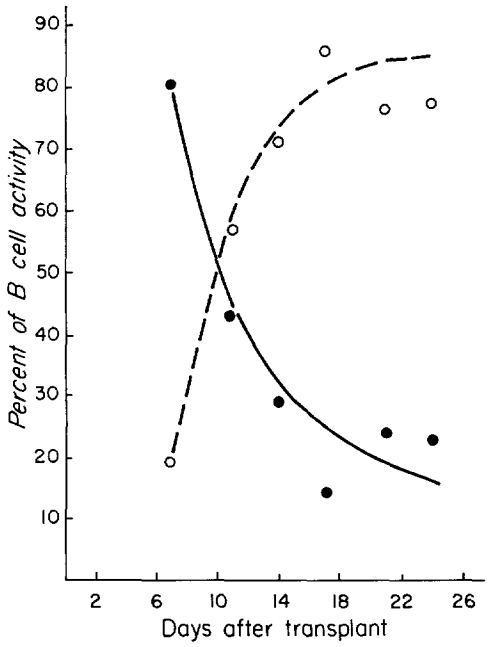

FIG. 5. Time kinetics of the transition of PB cells to B cells. A group of mice was given a large number of bone marrow cells enriched for PB cells and depleted of B cells. At increasing time after transplantation small groups were taken and their spleens were fractionated by sedimentation velocity and assayed for the proportion of the total B cell activity present in the large cells (●—●) or the small cells (○——○).

majority of the B cell activity is in the slowly sedimenting pool. Thus, while newly produced B cells have the capacity to interact with T cells and to initiate a PFC response, they are physically distinct from mature B lymphocytes.

DISCUSSION

Previous studies using a functional assay for PB cells suggested that they have immunoglobulin molecules on their surface (2). The above studies with a multispecific, fluorescein-labeled anti-immunoglobulin antiserum revealed a distinct population of stainable cells with properties similar to PB cells. Thus, there is a population of stainable cells in bone marrow and spleen that sediment at 1 g with a velocity of 4.5–5.0 mm/h. No rapidly sedimenting, staining cells were

identified in cell suspensions from lymph node. Because these properties and tissue distribution are the same as those determined by functional tests for PB cell activity, we have assumed that the antiserum binds to PB cells and that enumeration of staining cells with the sedimentation velocities in excess of 4.5 mm/h gives an estimate of the total number of PB cells.

Comparison of the total number of PB cells with the number specific for SRBC suggests that PB cells are restricted in their capacity to respond to a specific antigen. For example, PB cells in bone marrow occur with a frequency of 1,300 per 10^5 nucleated cells (Fig. 1 and Table I). This estimate, based on staining with Fl-anti-Ig, is assumed to include all of the PB cells in bone marrow. By a limiting dilution method the frequency of PB cells active in the assay with SRBC as antigen is $0.7/10^5$. Thus, the assay with a specific antigen detects only 0.05% of the total PB cells estimated to be present in bone marrow. If PB cells are restricted and each specificity is represented with the same frequency as the one for SRBC, one would predict 1,000 different PB cells representing different specificities. Because SRBC are complex antigens, this calculation probably underestimates by at least a factor of 10 the actual number of different PB cells. However, in view of this probable restriction, PB cells should not be called stem cells since this term connotes pluripotentiality (11).

It is interesting to note that B cells appear to show a similar degree of specificity restriction. Again, in bone marrow the frequency of total B cells is 1/10, while the frequency of B cells specific for SRBC is $1/10^4$ (12). As with PB cells, specific B cells represent only a small fraction, 0.1%, of the total B cell pool. Thus, we would conclude that both PB and B cells show similar restrictions in their capacity to respond to specific antigens.

The suggestion that PB cells are restricted in specificity implies that the immunoglobulin molecules on the membrane of PB cells (2) are specific receptors for the antigen to which their progeny B cells will respond. In this case, however, one would expect PB cells to form rosettes when they interact with SRBC. The data on this point are inconclusive. Studies on the sedimentation properties of rosette-forming cells (RFC) in both spleen and bone marrow give no indication of RFC among the population sedimenting at 5.0 mm/h (8). However, the density profile of RFC reveals a small population of RFC in the region now known to contain PB cells (13).

Another indirect indication of the specificity of PB cells comes from the antigen suicide experiments of Unanue (14). He incubated bone marrow cells with radioactive antigen, transplanted the population into irradiated animals, and then at a later time tested the immune response to the original antigen and to a non-cross-reacting antigen. He observed specific suppression to the antigen labeled with ^{125}I indicating that some cells in bone marrow specifically bind antigen and are destroyed by the radioactive decay of the labeled antigen. It appears that under the condition of his assays much of the immune response would be derived from the differentiation of PB cells, and we would interpret his results as evidence for the specificity of PB cells as well as B cells.

At the present time, one can only speculate about the precise physiological role for PB cells, but there are two obvious possibilities for their function. First, cells such as PB cells that occur in a relatively low frequency provide a readily available source for new B cells. Second, generation of antibody specificity may occur at the level of PB cells. For example, since they apparently have specific surface receptors but do not respond by the immediate production of antibody, they could be the stage at which specificities to self-antigens are eliminated. That is, it is possible that exposure of PB cells to antigen could induce tolerance, preventing the development of "forbidden clones" (15).

Since PB cells are the immediate progenitors of B cells, it is perhaps not surprising that the newly produced B cells have physical properties more similar to PB cells than to mature B cells. However, it is important to note that, as defined by a functional assay, intermediate B cells are different from mature B cells and should be distinguished in studies on the differentiation of B lymphocytes. We propose that these transitional B cells be designated B_1 and the mature form B_2. The precise relationship between B_1 and B_2 cells is not clear. On the basis of the kinetic data shown in Fig. 5, it appears that B_1 is a progenitor of B_2.

Table III gives a summary of some of the known properties of the cells thought to be involved in the pathway of differentiation of antibody-producing cells. For reasons described previously, it is probable that the pluripotent stem cell for the immune system is identical with the cell that forms colonies in the spleens of irradiated mice (CFU-S). Although very little is known about B_1 cells, they can be distinguished from PB and B_2 cells by two properties: their sedimentation velocity and their ability to initiate an immune response. With the accumulation of other physiological measurements, it will undoubtedly be necessary to subdivide the B lymphocyte pool into other categories. For example, Playfair and Purves (16) have shown that some B lymphocytes can initiate an immune response in the absence of added T lymphocytes. It is not clear

TABLE III

Summary of Some of the Properties of the Cells along the Differentiation Pathway to Small Lymphocytes

Cell type	Sedimentation velocity	Surface immunoglobulin	Bind antigen	Capacity to initiate PFC response	Secrete antibody
	mm/h				
CFU-S	4.0	?*	N.D.‡	No	No
PB	5.0	Yes	?	No	No
B_1	>4.5	N.D.	N.D.	Yes	No
B_2	3.0	Yes	Yes	Yes	No
PFC	4.5	No	No	No	Yes

* Equivocal results, see Discussion section.

‡ No data available.

where these lymphocytes fit into the proposed pathway of differentiation in which PB cells give rise to B_1 that in turn differentiate into B_2.

Although this paper is not concerned with the properties of T lymphocytes, the sedimentation distribution shown in Fig. 1 *c* gives information about the T lymphocytes in lymph node. Since T cells do not contain easily detectable immunoglobulins on their surface (9), the unstained lymphocytes are presumably thymus derived. The unstained lymphocytes sediment more rapidly than the stained lymphocytes, indicating that T cells have a higher sedimentation velocity than B cells. From the data in Fig. 1 *c*, one can estimate the sedimentation velocity of T cells to be 3.5 mm/h. This estimate is in contrast to our previous report of a sedimentation velocity of 3.0 mm/h for both T and B lymphocytes (8). The probable reason for this discrepancy lies in the different tissues examined in the two studies. In the previous report, the sedimentation velocity of T cells was measured directly only in thymus tissue; no measurements of T cells were made in peripheral lymphoid tissues such as spleen or lymph node. On the basis of the previous results and the results presented here, we tentatively conclude that the T cells in thymus have a slightly lower sedimentation velocity than the T cells found in peripheral lymphoid tissues such as spleen or lymph node. This conclusion is more in agreement with those of Osoba who, using a sensitive in vitro assay, found that T cells in spleen sediment at 4.0 mm/h (17).

SUMMARY

Previous work has shown that the immediate precursor of B lymphocytes (PB cell) has many properties that distinguish it from both B lymphoctes and hemopoietic stem cells. Size, density, tissue distribution, and sensitivity to cytotoxic antisera differ for each type of cell. The work described here was designed to study three aspects of the differentiation of PB cells. First, since PB cells probably have immunoglobulin surface receptors, fluorescein-labeled anti-immunoglobulin antiserum was used in an attempt to investigate directly the physical properties of PB cells. The use of this labeled antiserum revealed a population of cells with properties similar to the PB cells defined by the functional assays. Second, the differentiative potential of PB cells was studied by comparing the size of the total population of PB cells, as determined with fluorescein-labeled anti-immunoglobulin antiserum, to the size of the population of PB cells responding in a functional assay with a specific antigen. The cells responding in the functional assay represent only 0.1% of the total population of PB cells. This observation suggests that PB cells are not pluripotent stem cells of the immune system. Finally, the kinetics of the differentiation of PB cells to B lymphocytes was studied. The differentiation to mature lymphocytes involves at least one intermediate stage in which cells larger than mature B cells are active in a functional assay for B cells. These large B cells are present in irradiated mice soon after transplantation of PB cells, but by 20 days the majority of the B cells are typical small lymphocytes.

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