A QUANTITATIVE ASSAY FOR THE PROGENITORS OF BONE MARROW-ASSOCIATED LYMPHOCYTES

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Three cell types are required for initiation of antibody synthesis after immunization of mice with sheep erythrocytes (SRBC):[1] a bone marrow-associated cell (the B cell), a thymus-associated cell (the T cell), and an accessory cell (the A cell) (1–3). The existence of suitable assays for each of these cells has led to a rapid accumulation of information concerning their properties. However, because of a lack of adequate assays, there are few data concerning their progenitors, the stem cells of the immune system.

Studies on the stem cells of the immune system have generally involved measurement of the restoration of the immune response in irradiated mice given syngeneic bone marrow cells (4). Restoration, as measured by the ability of the recipients to respond to a test antigen, starts about 2 wk after transplantation and reaches near-normal levels 1–2 months later. These data have led many investigators (5–8) to suggest that the bone marrow of adult mice contains pluripotent stem cells capable of reconstituting the immune system with all of the functional cells described above. However, interpretation of the kinetics of the recovery of the immune response after restoration by bone marrow is complicated by not knowing which of the three required cell types is limiting the response at a particular time. For example, in the early recovery phase, the immune response is limited primarily by the slow recovery of T cells (9). To overcome the uncertainties associated with the kinetic experiments, it is necessary to develop precise, quantitative assays for the progenitor of interest. Because the specificity of the humoral immune response appears to reside in the B cell (10), we have restricted our attention to studying the progenitors of this cell.

Bone marrow from adult mice is known to be a good source of stem cells. However, when bone marrow is used to provide precursors of B cells (PB), an additional problem arises in that bone marrow also contains mature B cells (10). To avoid this difficulty, we have used a cell separation technique to obtain a population enriched for PB and depleted in B cells. Using such a population, it was possible to devise a rapid, quantitative assay for this stem cell and to show that its physical properties and tissue distribution are different from those of mature B cells.

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[1] *Abbreviations used in this paper*: A cell, accessory cell; B cell, bone marrow-associated cell; BSA, bovine serum albumin; CFU-S, hemopoietic stem cells; PB, precursors of B cells; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes; T cell, thymus-associated cell.

Materials and Methods

Mice.—The mice used throughout were F_1 hybrids between either C3H/HeJOci and C57BL/6JOci ($C3B6F_1$) or C57BL/6Cum and DBA/2Cum ($B6D2F_1$). In one experiment C57BL/6JOci mice were used. Mice were used without regard to sex. They were housed three or four to a cage and allowed free access to food and water.

Antigens.—Sheep and horse erythrocytes were used as antigen. Fresh blood was obtained weekly from Woodland Farms (Guelph, Ontario, Canada) and kept in citrate saline. The red cells were washed three times in phosphate-buffered saline (PBS) before use. Freshly washed erythrocytes were used for all immunizations and plaque assays.

Cell Suspensions.—Cells were taken from normal 6–8-wk-old donors. Bone marrow cells were prepared by gently flushing the femurs with PBS containing 1% bovine serum albumin (BSA); the resulting marrow plug was made into a single cell suspension by gentle aspiration with a pipette. Thymus tissue was taken from exsanguinated donors. Suspensions of both spleen and thymus cells were prepared by chopping the tissue into fine pieces with scissors and rubbing the pieces through a fine wire mesh screen. All cell suspensions were filtered using a capillary array filter with a pore size of 37 μ (Mosaic Fabrications, Sturbridge, Mass.).

Cell Separation.—Bone marrow cells were fractionated by velocity sedimentation as described previously (11). A Lucite sedimentation chamber 24 cm in diameter was used. Cell loads were 5×10^8–2×10^9 cells in 50–200 ml of 0.2% BSA in PBS; a buffered step gradient (0.35–2% BSA in PBS) was used. Cells were sedimented for 4–5½ hr at 4°C.

Bone marrow fractionation on the basis of density was carried out as described previously (12). The cells were spun for 45 min at 6000 *g* in a 10–19% Ficoll gradient at pH 5.5.

Irradiation.—Recipient mice were exposed to 900 rads whole body irradiation from a ^{137}Cs irradiator at a dose rate of 96 rads/min. Single cell suspensions were exposed to doses of 950 rads. Cell suspensions were diluted to give 10^8 cells/ml and kept in an ice bath throughout irradiation.

Plaque Assay.—Antibody-producing cells were enumerated by the plaque-forming cell (PFC) assay, described by Jerne and Nordin (13). Only direct or 19S PFC were measured.

Measurement of B Cells.—The experiments to be described below depend on the availability of a precise, quantitative method for estimating the number of B cells in irradiated animals. The most reliable functional test for B cells is to immunize the animals and enumerate PFC at some later time. As long as the number of B cells (rather than T or A cells) is limiting the PFC response, the enumeration of PFC provides an indirect estimate of the number of B cells present at the time of immunization. Therefore, it was necessary to ensure in the experiments to follow that the immune response was being limited by B cells and not one of the other cell types. To fulfil this requirement, extra A cells and T cells were injected into mice before their immunization for the measurement of B cell activity. Irradiated spleen cells were used as a source of A cells and thymus cells as a source of T cells. The mice received 5×10^7 cells of each type. Preliminary experiments indicated that these doses of cells, which are the maximum that were tolerated by the recipient mice, are close to saturating levels and are sufficient to ensure that the subsequent PFC response is a relative measure of the B cells present at the time of immunization.

Another problem when using the PFC response as a measure of B cells is choosing the time for measurement of PFC. The time of the peak response can vary according to the experimental conditions. For example, the PFC response in normal mice given optimal doses of antigen is 4 days after immunization (14), but in irradiated mice given normal spleen cells and antigen, the peak response is obtained 8 days after immunization (15). Under conditions where the immune response is undergoing rapid regeneration, so that the pool of B cells is continuously changing, the PFC kinetics after immunization are very complicated, and in some preliminary experiments it was difficult to define the precise time of the peak PFC response. For these reasons we have chosen to assay at a specific time, 8 days, after immuni-

zation in all experiments. The reason for choosing this time is that in standard experiments with B and T cell synergism (1), the peak response is generally obtained 8 days after immunization. Therefore, we have defined the PFCs obtained 8 days after immunization as being an indirect measure of the number of B cells present at the time of immunization. As shown below, these experimental conditions for the estimation of B cell activity show a linear relationship between the PFCs obtained and the number of B cells or stem cells transplanted. This linear relationship suggests that the experimental conditions are sufficient for making relative comparisons between different groups in the same experiment.

RESULTS

Kinetics of Regeneration of B Cells.—One of the problems in studying the progenitors of B cells is to obtain a source of progenitors that is not also contaminated with mature B cells. In the mouse, the bone marrow and spleen are known to be good sources of stem cells, but each tissue is also rich in B cells (10). Previous data, however, indicated that mature lymphoid cells could be separated from stem cells by velocity sedimentation; stem cells sediment more rapidly than B cells. In addition, since B cell activity falls under the peak of small lymphocytes which sediment at 3 mm/hr (16), this peak provides a good morphological marker for the location of B cells on the gradient. In the experiments that follow, suspensions of cells were separated by velocity sedimentation. Those fractions containing cells sedimenting faster than 4.5 mm/hr were pooled to give the "stem cell pool," and the fractions with cells sedimenting slower than 4.5 mm/hr were pooled to give the "B cell pool." The data that follow will show that this division is justified. Fig. 1 shows the kinetics of regeneration of the immune response for groups of lethally irradiated mice given cells from the bone marrow stem cell pool either with (○) or without (□) exogenous T and A cells as described above. The rapid recovery observed when the system is saturated with T and A cells indicates that B cells regenerate more rapidly than one of the other components; a significant increase in activity is seen as early as 3 days after transplantation, compared with about 12 days before detectable activity is seen when T and A cells are not provided. For comparison, the figure also shows for a different experiment the slow recovery obtained with small numbers of unfractionated bone marrow, when no T or A cells are given. As suggested by Gregory and Lajtha (9), the prolonged delay probably results from the slow rate of development of T cells since A cells also appear to recover more rapidly than T cells (17).

Table I outlines in more detail the properties of the stem cell pool and the B cell pool. Groups of lethally irradiated mice were transplanted with cells from each pool and assayed for their content of B cells on either day 0 or day 7 posttransplantation. On the basis of other separation data (16) it is reasonable to assume that the day 0 immunization detects only those B cells present in the original inoculum. Thus, comparison of the response on day 7 with the response on day 0 should give a measure of new B cells formed by regeneration.

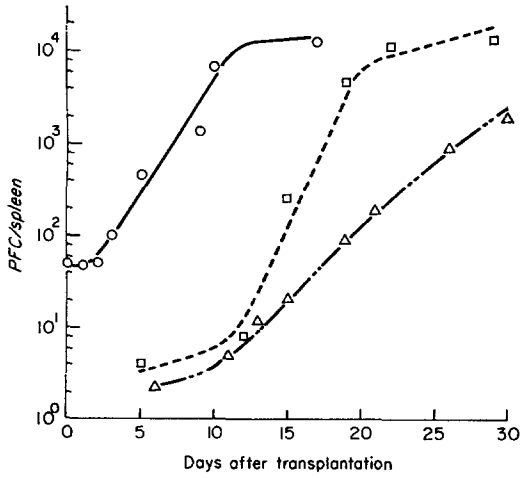

FIG. 1. Regeneration of the immune system of three groups of lethally irradiated mice transplanted with either 3×10^5 unfractionated bone marrow cells (△), or 7×10^5 cells from the stem cell pool of fractionated bone marrow with (○) or without (□) addition of exogenous A and T cells. Each value shown is the geometric mean of the PFC per spleen in a group of mice immunized at the time indicated on the abscissa.

TABLE I

Increase in B Cell Activity from Fractionated and Unfractionated Bone Marrow Cells

Cells injected	PFC/spleen*		
	Immunized day 0 (B cell)	Immunized day 7 (PB + B cells)	Index of proliferation‡
(*a*) Unfractionated bone marrow	230 (160–330)	1300 (960–1700)	5.6
(*b*) Stem cell pool§	90 (60–130)	1800 (1300–2600)	20.0
(*c*) B cell pool‖	310 (190–520)	1000 (700–1500)	3.3

* On day 0, each recipient mouse was given an irradiation dose of 900 rads followed by 2×10^6 cells from the marrow suspension being used mixed with 5×10^7 thymus cells, all given intravenously. On either day 0 or day 7 mice were given 4×10^8 SRBC and 5×10^7 irradiated (950 rads) spleen cells intraperitoneally. They were assayed after an additional 8 days for PFC. The data given are geometric means of measurements made on individual spleens; the standard error of the mean is given in parentheses.

‡ Ratio of PFC/spleen for group immunized on day 7 to PFC/spleen for group immunized on day 0.

§ Pool of bone marrow cells sedimenting faster than 4.5 mm/hr.

‖ Pool of bone marrow cells sedimenting slower than 4.5 mm/hr.

If PB are the primary source of new B cells, the major increase in B cells should occur in mice given the stem cell pool. This is in fact observed (Table I).

With this experimental procedure there is always a two to fivefold increase in activity in the B cell pool. Various factors could account for this increased

activity in the B cell pool: (*a*) the separation between PB and B cells is probably not complete and a small contamination of the B cell pool could occur (see Fig. 3); (*b*) the fraction of B cells trapped in the spleen might increase with time so that more activity is observed when mice are immunized on day 7 compared with day 0; (*c*) the 7 day interval between transplantation and immunization might allow time for more interaction between B and T cells and, therefore, a more efficient detection of the B cells initially injected; (*d*) B cells may have some self-renewal capacity. In spite of the increased activity

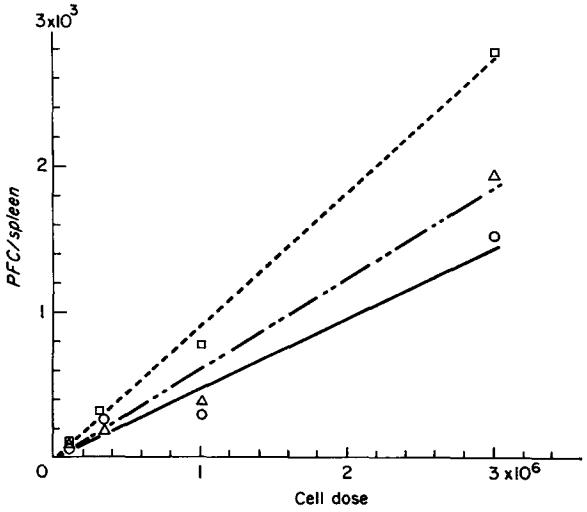

FIG. 2. Linearity of the PB assay. Titration of the B cell (○) and stem cell (□) pools and unfractionated bone marrow (△) in the presence of 5×10^7 thymus cells. Antigen (4×10^8 SRBC) and 5×10^7 irradiated spleen cells were injected intraperitoneally 7 days after transplantation and the number of PFC per spleen was measured 8 days later. The data in the figure are the geometric means for each group of mice. In a log-log regression analysis, the slopes of all three curves are not significantly different from 1.0 and do not exceed 1.5 at the 95% confidence level.

in the B cell pool, it is important to note that the majority of the increase in activity on day 7 comes from the stem cell pool.

Quantitative Assay for PB.—The above experiments indicated that B cells are formed soon after transplantation of bone marrow and suggested a procedure for obtaining a quantitative estimate of PB activity. Fig. 2 gives the results of an experiment designed to test this possibility. Irradiated recipients were given varying numbers of fractionated or intact bone marrow cells and assayed on day 7 for their content of B cells. Exogenous A and T cells were provided in the standard manner, T cells on day 0 and A cells on day 7. The response from all cell populations is linear over a wide range of cell doses. The stem cell pool is more active on a per cell basis than either unfractionated bone marrow or the B cell pool, establishing that the B cells detected in mice in-

jected with cells from this pool must have arisen primarily by differentiation from PB. The linear dose-response curve of the stem cell pool (Fig. 2) and the large increase in B cells observed in mice given cells from the stem cell pool indicate that the experimental conditions provide a quantitative assay for PB.

In the experiments that follow we use the following operational definitions for PB and B cell activities: B cell activity is proportional to the PFC obtained when irradiated mice are transplanted with B cells, immunized on day 0, and assayed for PFC on day 8; PB activity is proportional to the PFC obtained when mice are immunized 7 days after injection of cells containing PB (but no B) cells and PFC measured 8 days later. For both assays, thymus cells are injected on day 0 and irradiated spleen cells at the time of immunization (day 0 or day 7) to ensure saturation for A and T cells. By doing both assays on the same cell suspension, it is possible to estimate the relative contribution of PB and B cells to the PFC activities measured. Thus, the increase in PFC between day 0 (background B cell activity) and day 7 is used as a measure of PB activity.

Physical Characterization of PB.—

Sedimentation distribution: The separation data presented in Table I suggest that PB and B cells are physically distinct, but these crude separations do not provide accurate data on the sedimentation properties of these cells. To determine the precise sedimentation profile for PB, bone marrow cells were fractionated, mixed with thymus cells, and injected into groups of irradiated mice. One half of each group was given SRBC and irradiated spleen cells on day 0; the other half was similarly treated on day 7. This procedure measures B cell and PB activity, respectively. For both groups the PFC assay was 8 days after immunization. Fig. 3 shows the distribution of nucleated cells (solid line), B cell activity (●), and PB activity (○) found in the pooled fractions. The region of B cell activity (peak at 3 mm/hr) in the original marrow sample also gives rise to PFC under the conditions of the PB assay. However, the majority of the increase in activity between day 0 and day 7 is in the stem cell region (peak at 5 mm/hr). Cells sedimenting faster than 4.5 mm/hr do not have appreciable B cell activity initially, i.e., they do not synergize with thymus and antigen to give a PFC response. However, these cells differentiate into B cells, because synergism with T cells is observed if 7 days are allowed for differentiation. Thus, by definition, the cells sedimenting at 5 mm/hr are PB, the progenitors of B cells.

Results similar to those of Fig. 3 were obtained using horse erythrocytes instead of sheep erythrocytes as the immunizing antigen, ruling out the possibility that the effect is due to some odd property of sheep erythrocytes when used as antigen (for example, see reference 18). Similar sedimentation profiles have also been obtained using two other strains of mice, C57BL/6 and B6D2F_1.

Density distribution: To measure the density profile of PB, 2×10^8 bone marrow cells were mixed in a 30 ml Ficoll gradient and centrifuged to their

equilibrium density. Fractions of 1 ml were collected. Each was diluted to 6 ml, mixed with 6 ml of thymus cells at a concentration of 2×10^8 cells/ml, and injected into a group of 20 mice such that each mouse received $1/24$ of the

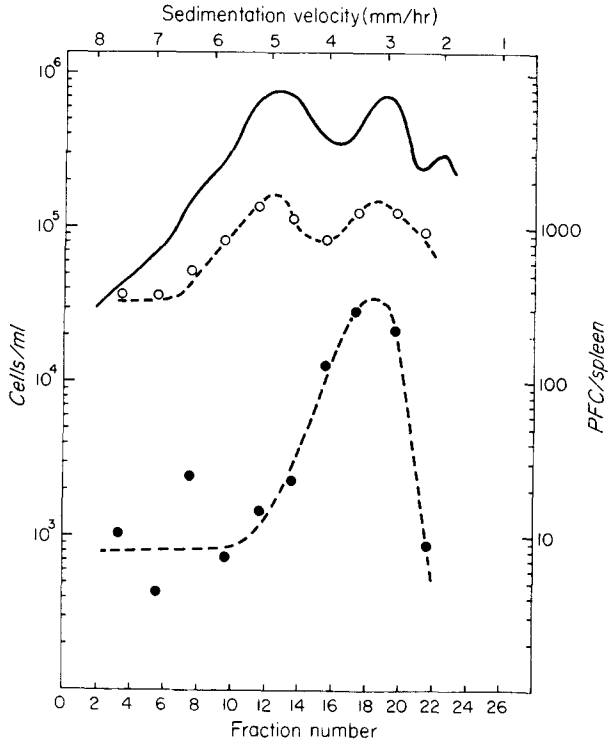

FIG. 3. Sedimentation profile of PB. A bone marrow suspension (5×10^8 cells total) from $C3B6F_1$ mice was sedimented for 4 hr. 50-ml fractions were collected, counted, and concentrated by centrifugation. The solid line indicates the nucleated cell distribution as measured in the original fractions. The predominant cell classes in the peaks at s values of 3 and 5 mm/hr are lymphocytes and granulocytes, respectively. Fractions were pooled in pairs and thymus cells added. The resultant suspensions were injected into lethally irradiated (900 rads) mice such that each mouse received 5×10^7 thymus cells and 1/24 of the bone marrow cells in the pool. The rationale for injecting a constant proportion rather than a constant number of cells from each fraction has been discussed previously (16). Half of each group of mice was immediately given 4×10^8 SRBC and 5×10^7 irradiated spleen cells to measure B cells (●). 7 days later the remaining mice were similarly treated to measure B cells and PB (○). For each group the PFC per spleen were measured 8 days after immunization. Because of the linearity of the assay, the geometric mean of the response in each group is a measure of the activity in that fraction.

cells in the fraction. Half of each group was assayed for B cells, the other half for PB following the standard protocol. The results of this experiment are shown in Fig. 4 with the results of another similar experiment where only PB

activity was measured. The PFC response in the B cell assay shows a peak at a density of 1.065 g/cm^3, which is consistent with a previous report from this laboratory (10). The results of the PB assays are somewhat variable but nevertheless show that PB and B cells are physically different. The density profile for PB is skewed to lower density compared with the B cell profile, and

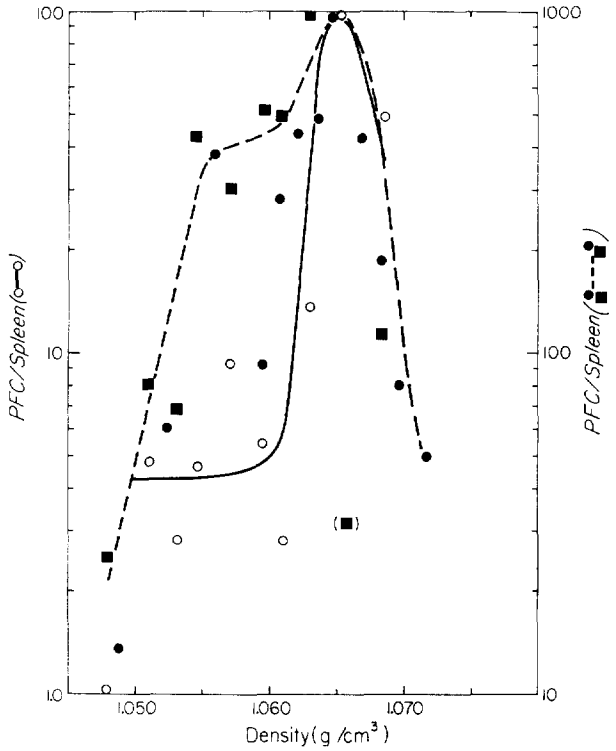

FIG. 4. Density profie of PB. The details of this experiment are given in the text. B cell (○) and PB assays (■: and●) were performed as described in Fig. 3 and the maximum response normalized to 1000 PFC/spleen for PB and to 100 PFC/spleen for B cells to compare two independent experiments. The point in parentheses at 1.065 (g/cm^3) is abnormally low and probably represents an experimental error.

indicates that PB have a lower average density than B cells. However, the relatively large increase in activity under the B cell cell peak also suggests considerable overlap in the density profiles of PB and B cells. It is of interest to note that hemopoietic stem cells are also found in the region of high PB activity (19).

Tissue Distribution of PB.—The data in the previous sections indicate that PB and B cells are physically distinct types of cells. However, the precise relationship between them remains unclear. If they are extremely closely re-

lated, one might expect them to occur together in various tissues. The data in Table II show the relative activities of B cells and PB in spleen, bone marrow, and lymph node. In these experiments, cell suspensions were made from each tissue, separated by velocity sedimentation, and the total activity in the B and PB regions assayed as described above. As expected, B cells were found in high proportions in all three tissues. The increase in PFC in the B cell region (<4.5 mm/hr) between day 0 and day 7 was five- to sixfold for all tissues; similar increases in this region were observed in other experiments (Table I, Fig. 3). In contrast, only the rapidly sedimenting cells (>4.5 mm/hr) from bone marrow and spleen showed large increases in PFC, 58- and 42-fold,

TABLE II
Tissue Distribution of B Cells and PB

Tissue	PFC/spleen*			
	Immunized on day 0		Immunized on day 7	
	<4.5 mm/hr	>4.5 mm/hr	<4.5 mm/hr	>4.5 mm/hr
Bone marrow	620 (420–920)	67 (44–105)	3700 (2800–5400)	3900 (2600–5800)
Spleen	200 (70–600)	20 (14–28)	1150 (870–1500)	840 (540–1100)
Lymph node	680 (420–1100)	35 (15–80)	3700 (3000–4600)	160 (100–260)

* In these experiments, cells from the various tissues were separated and the various fractions assayed as described for Fig. 3. The values in the table are the integral of the PFC/spleen measured for individual fractions sedimenting >4.5 mm/hr (PB) or <4.5 mm/hr (B cells). Mice were immunized on day 0 or day 7 to estimate B cell and PB activity, respectively. The values in parentheses give the standard error.

respectively, in the PB region. The activity from large cells in lymph node increased only fivefold between day 0 and day 7, a value characteristic of a small B cell contaminant in the pool rather than PB.

DISCUSSION

The early appearance of B cell activity after the transplantation of a stem cell pool confirms the results of Gregory and Lajtha (9) obtained using unfractionated bone marrow: the regeneration of B cells occurs soon after transplantation, indicating that the delayed recovery of immunological competence in mice given only bone marrow is most likely due to a slow recovery of functional T cells. It is important to note that in our experiments the stem cells injected had been depleted of mature B cells and that regeneration still occurred rapidly. The rapid appearance of specific B cells after transplantation of PB may be useful in studies of the generation of antibody specificity.

In the experiments presented above, it was assumed that the only function of the added thymus cells was to provide the T cells required in the initiation

of an immune response to erythrocyte antigens. However, in view of reports that the thymus can secrete a hormone as well as provide T cells (20, 21), it is possible that the thymus has a regulatory influence on the differentiation of PB to B cells. Two pieces of data make such a possibility unlikely. First, in adult thymectomized, irradiated, bone marrow–reconstituted mice there is apparently normal differentiation of B cells (22). Second, experiments in our laboratory indicate that similar results are obtained when thymus cells are injected either on day 0 or day 7 in the PB assay.[2] Although the irradiated recipients in these experiments have a thymus, the results suggest that the thymus cells injected for the assay have little effect on the PB to B cell transition.

Although no cytogenetic markers were used to confirm that the newly produced B cells were derived from the transfused stem cells and not from the host, there are several arguments in favor of the generation of B cells from the bone marrow stem cell pool. First, both the sedimentation and density profiles show that PB activity is confined to only a small proportion of the total cells in the suspension. If B cells were being generated from surviving host cells, it is unlikely that only a small, select population of cells would have such a nonspecific stimulatory property. Second, the PB pool has been shown to be sensitive to ionizing radiation with a D_0 of 85 rads.[2] This observation suggests that the transplanted PB must proliferate to generate B cells. Third, it is unlikely that a linear dose-response curve for PB would have been possible if B cells were being generated through some sort of experimental artifact. Fourth, B cells and PB cells appear to be differentially distributed. Thus, bone marrow, spleen, and lymph node all contain appreciable numbers of B cells but only the first two contain appreciable numbers of PB cells. These arguments provide compelling reasons for believing that the procedure as described provides a quantitative assay for the progenitors of B-type lymphocytes.

Although PB are clearly distinct from B cells, it is not clear whether or not PB have the properties of stem cells: namely, capacity for extensive proliferation, differentiation, and self-renewal as well as response to regulatory mechanisms (23). However, the availability of a quantitative assay for PB makes it possible to investigate the properties of PB. In this regard, it will also be of interest to determine the relationship between PB and the hemopoietic stem cell (CFU-S) that forms colonies in the spleens of irradiated mice (23). It has been reported by other investigators (5, 7, 8) that these two stem cells are identical. However, comparison of the sedimentation profile for PB (Fig. 4) with the sedimentation profile of spleen colony-forming cells as determined by Worton et al. (24) suggests that the two stem cells are not completely identical. Our data do not exclude the possibility that the two stem cells are closely related in a manner similar to that observed for CFU-S and cells that

[2] Lafleur, L. Unpublished data.

form colonies in tissue culture (23). Recent experiments have confirmed that CFU-S and PB are different and are probably related as parent to progeny (25).

SUMMARY

A cell transfer assay system was developed to study the precursors of bone marrow–associated (B) lymphocytes in the adult mouse. The rationale of the assay is to inject into irradiated mice a cell suspension depleted of B lymphocytes, to wait a period of time to let precursor cells differentiate to B lymphocytes, then to correlate the number of B cells present in the recipient mice with the number of precursor cells injected. The assay as described was shown to be linear in the range of 10^5–3×10^6 fractionated bone marrow cells. Kinetic studies indicated that precursor cells start producing detectable numbers of B cells within 3 days after transplantation; B cell activity then increases with a doubling time of 24 hr. Physical characterization of that precursor cell has shown that it is lighter and sediments faster than small lymphocytes. Precursor cells were found in bone marrow and spleen but could not be detected in peripheral lymph nodes. Results of physical analysis also indicate that the precursors of B lymphocytes described here may not be pluripotent stem cells for the immune system.

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