# PROGENITORS FOR Ly-1 B CELLS ARE DISTINCT FROM PROGENITORS FOR OTHER B CELLS

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Within the last several years, murine B cells have been classified into subsets that differ with respect to surface marker phenotype, tissue distribution, and/or functional responsiveness to a variety of T-dependent and T-independent antigens (1–8). In general, these subsets have been considered representative of different developmental stages of a single B cell lineage; however, arguments have also been advanced for separate lineages (5, 7, 8). This report demonstrates that one such subset, Ly-1 B, has the properties of a distinct lineage in that it fails to develop from the bone marrow–based progenitors that give rise to other B cells in irradiated recipients.

Ly-1 B cells carry the Ly-1 surface antigen in conjunction with IgM, IgD, Ia, and other typical B cell surface markers (9). They are distinguishable from the predominant B cell populations in spleen and lymph nodes by a variety of characteristics, e.g, low levels of surface IgD, high levels of surface IgM, presence in spleen but not lymph node, predominance in peritoneal populations, increased frequency in NZB-related mice, and decreased frequency in SJL mice (9, 10). In addition, they are functionally distinct from other B cells in that they rarely participate in IgM antibody responses to exogenous (laboratory) antigens and frequently contribute to IgM autoantibody production in normal and (NZB-related) autoimmune mice (11).

Ly-1 is not simply a B cell "activation" marker, since it is not detectable on mitogen-stimulated B cell "blasts" activated in vitro or on antigen-stimulated germinal center cells and antibody-producing cells activated in vivo (11). Furthermore, it is not a maturation marker, since it is present both on immature and mature cell types, e.g., on pre-B tumor cells and on normal B cells in neonates, as well as on antibody-secreting tumor lines and autoantibody-secreting splenic and peritoneal cells in adult animals. This evidence, which indicates that the Ly-1-bearing B cell subset contains cells at different stages of maturation and activation, suggests that this subset represents a distinct B cell lineage.

Data from cell transfer studies presented here support this hypothesis. In these precursor/progeny experiments, we compare cell populations from various donor organs for their ability to reconstitute particular B cell subsets when transferred to lethally irradiated allotype-congenic recipients. On the whole, our

results are consistent with previous evidence (12–16) demonstrating that progenitors present in newborn liver and in spleen and bone marrow from mice of all ages reconstitute B cells in these recipients. Surprisingly, however, we find that adult bone marrow and spleen specifically lack the ability to reconstitute Ly-1 B cells. Since our studies also demonstrate that Ly-1 B cells are readily reconstituted by progenitors from other sources (e.g., newborn liver, adult peritoneum), we interpret our findings as favoring the existence of separate progenitors (and hence separate lineages) for Ly-1 B and for other B cells.

## Materials and Methods

*Mice.* BALB/cN, BAB/14 mice were raised in our breeding colony. CBA mice congenic for Ig allotype (CBA-Igh$^b$) were established by Dr. H. S. Micklem (Immunology Unit, University of Edinburgh, Edinburgh, Scotland) and were his generous gift. CBA/HN mice were also obtained from Dr. Micklem and bred in our animal facility. 3-mo-old female or male mice were used in most experiments as adult donors except in certain experiments described below. 4–6-mo-old mice were used as irradiated recipients.

*Preparation of Cells.* Femur and tibia were used as sources of bone marrow cells. Peritoneal washout cells (PerC)[1] were obtained by injecting 8–10 ml of chilled medium (RPMI 1640; Irvine Scientific, Santa Ana, CA) containing 3% newborn calf serum (NCS) into the peritoneal cavity of mice. 90% of the injected volume was usually recovered by this procedure. Cells were passed through nylon mesh and washed twice with 3% NCS-RPMI.

*Radiation and Transfer.* Mice were irradiated with 600 rad by x-ray 1 d before transfer. Cells were resuspended in serum-free medium, and $10^7$ cells were injected intravenously into the irradiated mice.

*Antibodies and Reagents.* Anti-Igh-6b (IgM$^b$, AF6-78.25) and anti-Igh-5b(IgD$^b$, AF6-122.2) were made by Dr. A. Stall (Becton, Dickinson & Co., Mountain View, CA) as described elsewhere (17). Anti-Igh-6b, anti-Igh5b, and anti-Igh-5a (10-4.2) (18) were purified from mice ascites. Rat monoclonal antibodies anti-IgM (331.12) (19), anti-Ly-1 (53-7.3) (20), and anti-Lyt-2 (53-6.7) (20) were purified from supernatant obtained by culture in serum-free medium (HB101; Hana Media, Inc., Berkeley, CA) (21). Avidin was purchased from Vector Laboratories, Inc. (Burlingame, CA). The methods for Texas Red (Molecular Probes, Inc., Junction City, OR) conjugation to avidin and for biotination or fluoresceination of antibodies have been described previously (9).

Anti-Ly-1 and anti-Lyt-2 antibodies were used as F(ab')$_2$ reagents to avoid labeling Fc-binding cells. Both antibodies are rat IgG2a, whose chemical characteristics are similar to mouse IgG1 (crossreactive with anti–mouse IgG1). After digestion of antibody with 5% pepsin in 0.1 M sodium acetate buffer (pH 4.5) at 37°C for 12 h, the mixture was fractionated on an AcA 44 (LKB Instruments, Inc., Gaithersburg, MD) column (22). The fractions that do not bind anti–mouse IgG1 but still show specific cell-binding activity were pooled and used for biotination.

*Staining Procedure and Data Analysis.* Erythrocytes in spleen cells were depleted by treatment with 0.165 M NH$_4$Cl. PerC were prepared as described above. The two-color staining procedure and data analysis by modified fluorescence-activated cell sorter (FACS II; Becton-Dickinson Immunocytometry Systems, Inc., Mountain View, CA) and VAX computer have been described previously (9). An extra channel was added to detect scattered light from cells at large angles (obtuse scatter), which was particularly useful for peritoneal cell analysis. Since cells that scatter a large amount of light to the obtuse scatter photodetector are mostly granulocytes, monocytes, polymorphonuclear cells, or other nonlymphoid cells (23), these cells (15–40% in PerC from the irradiated reconstituted mice) were gated out in the analysis of the lymphocyte population.

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[1] *Abbreviations used in this paper:* BrMRBC, bromelain-treated mouse red blood cell; FACS, fluorescence-activated cell sorter; NCS, newborn calf serum; PerC, peritoneal "wash-out" cell; PFC, plaque-forming cell.

*Inhibition of Anti-BrMRBC PFC.* B10 mice were used as the source of mouse red blood cells (MRBC). The bromelain (Br) treatment of MRBC and the plaque-forming cell (PFC) procedure were described previously (11). 30 $\mu$g of anti-Igh-6b antibody (IgG1 anti-IgM) was used to inhibit the generation of anti-BrMRBC PFC from $2 \times 10^7$ spleen cells (24).

## Results

*Bone Marrow Cells from Adult Animals Contain Progenitors That Reconstitute Most of the B Cells in Spleen.*    Virtually all B cells in lethally irradiated mice reconstituted with allotype-congenic. bone marrow cells are derived from the donor inoculum. These B cells carry donor (rather than recipient) IgD and IgM allotypes, and are therefore specifically recognizable in FACS analyses with monoclonal antibody reagents that detect surface Ig allotypes. Thus, for example, B cells in 600 rad–irradiated BALB/c (Igh$^a$) mice reconstituted with BAB/14 (Igh$^b$) bone marrow are marked with the Igh-5b (IgD) and Igh-6b (IgM) allotypes, which are expressed on the BAB/14 (Igh$^b$) donor B cells (see Fig. 1).

The frequencies of donor-derived B cells in reconstituted recipients rise to nearly normal levels by 1 mo after transfer and stabilize at somewhat higher (effectively normal) frequencies shortly thereafter. Surface IgM a93 IgD expression patterns shift during this reconstitution period. Initially, the IgM/IgD FACS contour plots resemble plots obtained with cells from very young animals. However, by 1 mo after transfer, the reconstituted B cells have established overall patterns of surface IgD and IgM expression that are almost indistinguishable from the patterns displayed by B cells in the same locations in normal adult (nonirradiated) animals; e.g., compare IgM/IgD FACS contour plots for spleen cells in Figs. 1 and 2. Thus, adult bone marrow transfers are clearly capable of restoring most of the B cells normally found in spleen and lymph nodes.

Bone marrow transfers do not, however, restore all B cells. As we will show, 2 mo after transfer, animals reconstituted with bone marrow still lack significant numbers of donor-derived Ly-1 B cells. The FACS analysis of splenic and lymph node B cells discussed above largely failed to reveal this deficit because lymph nodes almost never contain detectable numbers of Ly-1 B cells and spleens
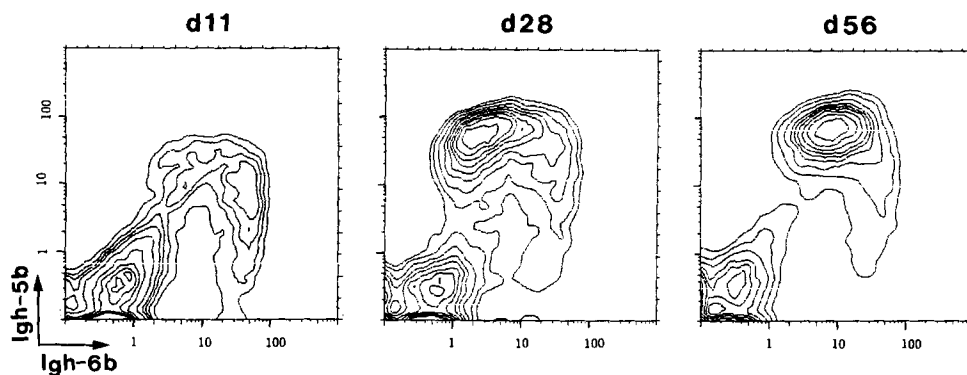


FIGURE 1.    Bone marrow transfers reconstitute most splenic B cells. BAB/14 (Igh$^b$) bone marrow cells from 12-wk-old (adult) animals were transferred to 600-rad irradiated BALB/c (Igh$^a$) mice. Mice were killed 11, 28 (1 mo), and 56 d (2 mo) after transfer, and were analyzed for donor-derived B cells by simultaneous staining with fluoresceinated anti-Igh6$^b$ and biotinated anti-Igh5$^b$ antibodies that detect donor allotypic determinants.

usually contain so few Ly-1 B that frequency reductions are difficult to document, particularly in animals that have a reasonable number of B cells with the same bright IgM, dull IgD phenotype as Ly-1 B.

On the other hand, the Ly-1 B deficit in irradiated recipients of adult bone marrow is readily demonstrated by functional studies that detect autoantibody production by splenic Ly-1 B cells (see below). Furthermore, it is clearly visible in FACS analysis of recipient PerC populations since, as we show next, Ly-1 B frequencies are normally quite high in these populations.

*Ly-1 B Represent a Major Proportion of Peritoneal B Cells in Normal Animals.*    One-third to one-half of the IgM-bearing PerC are typical Ly-1 B cells that express relatively large amounts of IgM, small amounts of IgD, and small amounts of Ly-1. Thus, PerC Ly-1 B are recognizable either as a clearly delineated population of dull IgD, bright IgM cells in IgM/IgD FACS plots, or as a relatively well-delineated population of dull Ly-1, bright IgM cells in Ly-1/IgM plots (Fig. 2). Data from three-color immunofluorescence studies (not shown) confirm that these individual analytic populations reflect different properties of the same (Ly-1 B) cells.

The other B cells in the peritoneum are, on the whole, similar in their IgM/IgD expression to the predominant B cells in lymph node and spleen, and do not carry Ly-1. Thus, they are well resolved in the IgM/IgD contour plot as a bright IgD, dull IgM population, and reside in the Ly-1⁻, dull IgM region of the Ly-1/IgM contour plot (see Fig. 2). Given the lack of anatomical organization of the apparently free-living lymphocytes in the peritoneum, the relative frequencies of these two PerC populations are surprisingly constant in most normal mouse strains.

*Bone Marrow and Spleen Cells from Adults Selectively Reconstitute Ly-1⁻ B Cells.*    In addition to reconstituting the major B cell populations in spleen and lymph nodes, adult bone marrow transfers selectively reconstitute the peritoneal B cells that display the predominant lymph node or splenic B cell phenotype (Ly-1⁻, bright IgD, dull IgM). As Fig. 3 shows, there are essentially no donor-type Ly-1 B in PerC harvested from marrow-reconstituted recipients 2 mo after transfer. Nevertheless, Ly-1⁻ PerC B cells are clearly reconstituted to normal levels by this time.

The B cell progenitors in spleen are very similar to the progenitors in bone marrow in that they reconstitute most B cells in irradiated recipients, but do not reconstitute Ly-1 B (see Table I). Thus, even though Ly-1 B make up 1% of the cells in spleen, animals that received $10^7$ allotype congenic spleen cells do not have detectable numbers of Ly-1 B in the peritoneal cavity 1 mo after transfer.

*Peritoneal Cells from Adults Selectively Reconstitute Ly-1 B.*    In contrast to bone marrow, transfers of PerC from normal adult (allotype congenic) donors fully reconstituted recipient PerC Ly-1 B populations. 11 d after transfer, recipients showed low but detectable numbers of peritoneal Ly-1 B marked with the donor allotype (data not shown). Over the next 2 wk, the frequency and absolute number of these donor-derived cells increased >10-fold. Thus, 1 mo after transfer, PerC recipients had roughly normal numbers of donor-type Ly-1 B in peritonea (Fig. 3).

These PerC transfers did not reconstitute the PerC B cells reconstituted by
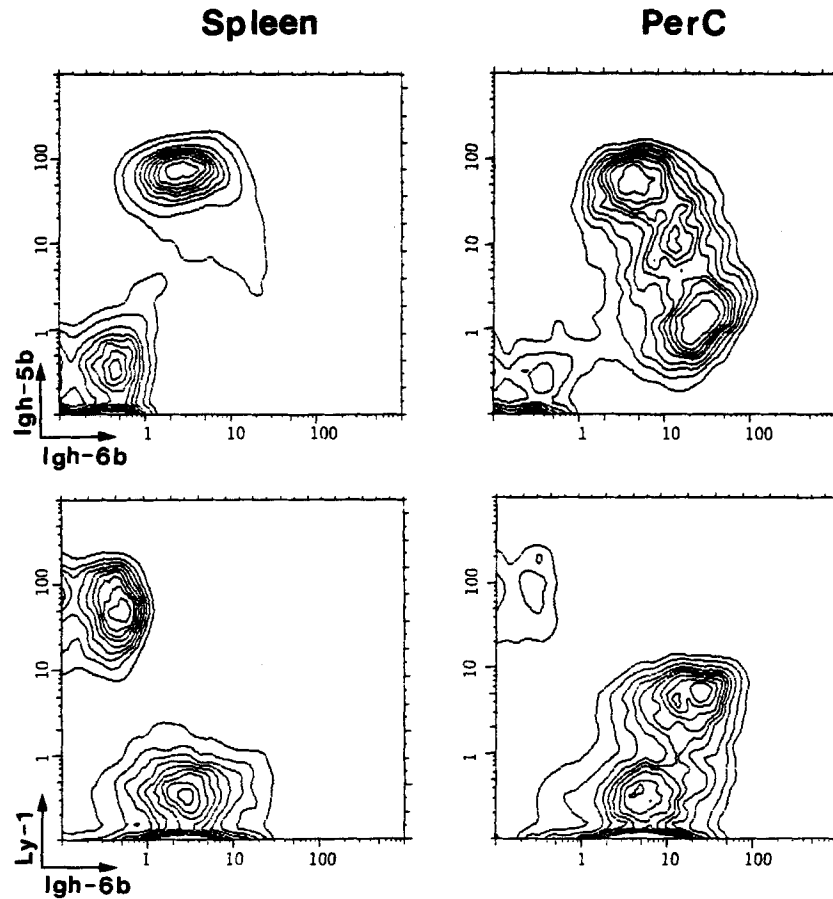
FIGURE 2. Ly-1 B frequencies are higher in peritoneum than in spleen. Spleen and peritoneal cells (PerC) from 3-mo-old BAB/14 mice were simultaneously stained with fluoresceinated anti-Igh6b and biotinylated anti-Igh5b (*top*) or fluoresceinated anti-Igh6b and biotinylated anti-Ly-1 (*bottom*). PerC IgM/IgD analysis revealed a relatively large population of cells that have high levels of surface IgM (Igh-6b) and low levels of IgD (Igh-5b). IgM/Ly-1 analysis showed that most of these cells express low levels of Ly-1. Ly-1 B cell frequency in spleen (1%) is often too low to visualize on this type of contour plot.

bone marrow transfers; however, they did reconstitute a minor PerC B cell population that has the same IgM/IgD phenotype as Ly-1 B (dull IgD, bright IgM) while lacking Ly-1. PerC-reconstituted recipients therefore have at least two kinds of B cells (Ly-1 B and this second population), even though they lack the Ly-1⁻ bright IgD, dull IgM B cells that predominate in lymph node and spleen.

*Liver, Spleen, and Bone Marrow Cells from Young Mice Reconstitute All B Cells.* The physical segregation of cells that reconstitute Ly-1 B from cells that reconstitute other B cell populations occurs relatively late in postnatal life (Table I). Liver and spleen from neonatal animals (1–7 d old) still contain both kinds of progenitor cells. Similarly, bone marrow taken from animals as much as 3 wk old can reconstitute a sizable proportion of Ly-1 B cells in addition to the typical
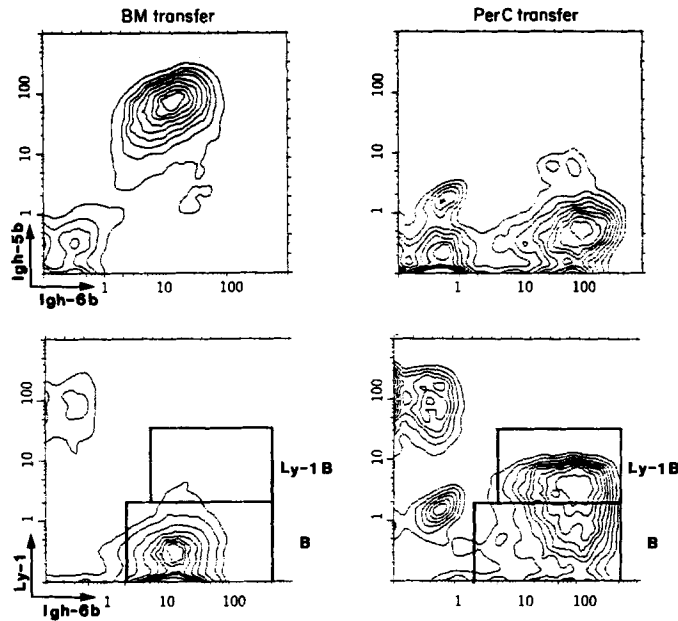
FIGURE 3. Adult PerC and adult bone marrow reconstitute different B cell populations in irradiated recipients. $10^7$ bone marrow cells (BM) or $10^7$ PerC from 12-wk-old adult BAB/14 (Igh$^b$) mice were transferred to 600-rad irradiated BALB/c (Igh$^a$) mice. 1 mo after transfer, PerC were analyzed for donor-derived (Igh$^b$) B cells. The boxes in the figure show the analytic gates used to determine the frequencies of Ly-1 B and Ly-1$^-$ B. Ly-1 B frequencies were corrected for background staining by subtracting the frequency of anti-Lyt-2–stained cells that fell within the Ly-1 B gates (usually 1–2% of total cells). In the experiments shown, the bone marrow–reconstituted animals had ~1% Ly-1$^-$ B and 40% Ly-1$^-$ B, while the PerC-reconstituted animals had 20% Ly-1 B and 30% Ly-1$^-$ B, nearly all of which showed a surface phenotype (high IgM, low IgD) that is rare among normal splenic or peritoneal B cells.

B cells reconstituted by adult bone marrow. We have not determined exactly when bone marrow completely loses the ability to reconstitute sizable numbers of Ly-1 B; however, this capability tends to be reduced by 4 wk and is clearly gone by 12 wk of age.

*Joint Transfers of PerC and Bone Marrow Cells from Adults Reconstitute All B Cells.* Transferring bone marrow cells from Igh$^a$ donors and PerC from congenic Igh$^b$ donors creates chimeric animals that have all of the FACS-detectable B cell populations present in normal (nonirradiated) mice. The Ly-1 B cells in these animals express Igh$^b$ allotypes, and thus derive solely from the transferred PerC. The Ly-1$^-$ B cells, in contrast, express Igh$^a$ allotypes, and thus derive mostly from the transferred bone marrow cells (see the FACS plots for recipient PerC in Fig. 4). Thus, neither of the progenitor sources blocks the development of progenitors supplied by the other, and neither source induces the other to give rise to a broader spectrum of B cell subpopulations than it gives rise to when transferred individually.

Joint transfers of bone marrow and allotype-congenic neonatal liver yield similar results in that Ly-1 B reconstitution proceeds normally, and the reconstituted Ly-1 B cells derive solely from the neonatal liver donor. Since bone marrow

TABLE I

*Ly-1 B Are Reconstituted by Newborn Liver, Newborn Spleen, Weanling Bone Marrow, and Adult Peritoneal Cells*

| Cells injected* | | | Donor-derived B cells present in recipient peritoneum‡ | | |
|---|---|---|---|---|---|
| Source | Donor age | Number (×10⁶) | All IgM⁺ | LY-1 B | Mice tested |
| | | | % | % | |
| Liver | 1–7 d | 10 | 43 ± 6 | 8 ± 2 | 4 |
| Spleen | 1–7 d | 10 | 49 ± 7 | 7 ± 1 | 5 |
| Bone marrow | 3 wk | 10 | 43 ± 13 | 6 ± 2 | 5 |
| Peritoneum | 12 wk | 10 | 50 ± 5 | 20 ± 4 | 5 |
| | | 4 | 26, 28 | 9, 12 | 2 |
| | | 1 | 16 ± 4 | 5 ± 2 | 3 |
| Spleen | 12 wk | 10 | 17 ± 7 | 1 ± 1 | 3 |
| Bone marrow | 12 wk | 10 | 39 ± 21 | 1 ± 1 | 8 |

\* Cells from BAB/14 (Igh$^b$) donor were injected intravenously to 600 rad–irradiated BALB/c (Igh$^a$) recipients.
‡ Frequencies were determined by FACS analysis of recipient PerC 1 mo after transfer. Donor-derived B cells were identified by staining with monoclonal antibodies to donor IgM and IgD allotypes. No recipient-derived B cells were detected (<1%) in any group of mice.

and neonatal liver both contain progenitors for Ly-1⁻ B cells, about half of the Ly-1⁻ B cells in these recipients express the allotype of the bone marrow donor, while the remainder express the allotype of the neonatal liver donor; all Ly-1 B cells, in contrast, express the allotype of the neonatal liver donor (data not shown).

These findings demonstrate that conditions in irradiated recipients of bone marrow allow Ly-1 B reconstitution to proceed normally, regardless of whether the Ly-1 B progenitors are drawn from mature or immature animals, or whether they come from locations that potentially differ with respect to the average maturity of the B cells present. Thus, the failure to reconstitute Ly-1 B with bone marrow cells is the result of a functional deficit of Ly-1 B progenitors in bone marrow from normal mice.

*Ly-1 B Are More Radioresistant Than Other B Cells.* In keeping with previous evidence (13, 25), our studies show that most splenic and lymph node B cells are highly sensitive to radiation. Ly-1 B, however, tend to persist, and thus make up a relatively large percentage of the surviving splenic B cells 7 d after irradiation (600 rad in BALB/c); e.g., host-type Ly-1 B made up 20–30% of the B cells in a spleen that had decreased ∼300-fold in cellularity at this time (data not shown).

Analysis of peritoneal cells in the irradiated animals demonstrates this relative radioresistance of Ly-1 B even more clearly. 7 d after irradiation, all of the surviving peritoneal B cells showed the bright IgM, dull IgD phenotype of the Ly-1 B population, and at least half express Ly-1. Similarly, host-type Ly-1 B were usually detectable in PerC harvested from irradiated animals reconstituted with allotype-congenic bone marrow cells, particularly when assayed within 2 wk of transfer (data not shown).
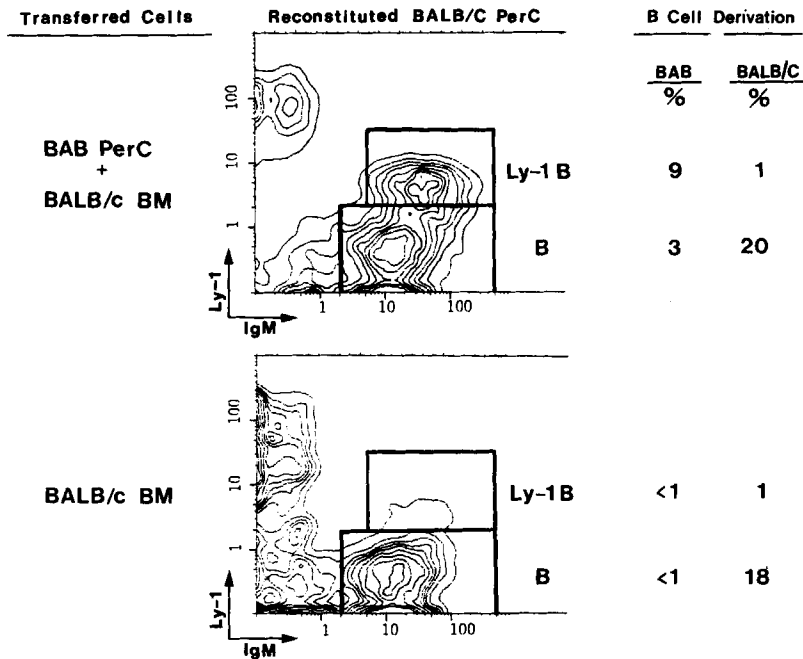
FIGURE 4. Combined transfers of bone marrow (BM) and PerC fully reconstitute PerC B cells. $5 \times 10^6$ adult bone marrow from BALB/c (Igh$^a$) donors were transferred with or without $5 \times 10^6$ PerC from BAB/14 (Igh$^b$) donors to 600-rad irradiated BALB/c recipients. Data show the IgM/Ly-1 distribution of all PerC in recipients. The frequency of PerC (BAB/14)–derived B cells was determined by staining with anti-Igh-6b and anti-Ly-1. The frequency of bone marrow–derived (BALB/c) B cells was determined by subtracting the frequency of Igh-6b-bearing B cells from the total IgM$^+$ B cell frequency.

*Reconstitution of Splenic Ly-1 B Parallels the Reconstitution of PerC Ly-1 B.* Complementary data from FACS and functional studies show that splenic Ly-1 B are reconstituted by PerC and tend to survive irradiation much as PerC Ly-1 B do (see Fig. 5 and Table II). 1 mo after transfer, FACS analysis clearly shows donor-derived Ly-1 B in spleens from irradiated Igh$^j$ CBA/N mice reconstituted with PerC from allotype-congenic CBA-Igh$^b$ mice (Fig. 5). Similar analysis revealed Ly-1 B populations at lower frequencies in spleens from BALB and CBA mice reconstituted with allotype-congenic PerC (data not shown). Thus, cells recovered from the donor peritoneum are capable of reconstituting splenic Ly-1 B, in addition to reconstituting the peritoneal Ly-1 B populations discussed earlier.

Data from functional studies independently confirm this conclusion. Virtually all of the spleen cells that produce IgM autoantibodies to BrMRBC (anti-BrMRBC) are Ly-1 B cells (11). The activity of these cells is detectable in a hemolytic PFC assay modified to allow determination of the IgM allotype produced by individual PFC, i.e., by inclusion of monoclonal anti–IgM allotype antibodies that specifically block PFC formation (Table II). This assay therefore provides a highly sensitive probe for detecting the presence of Ly-1 B in transfer recipients, and a direct method for determining the origin (allotype) of the PFC detected.
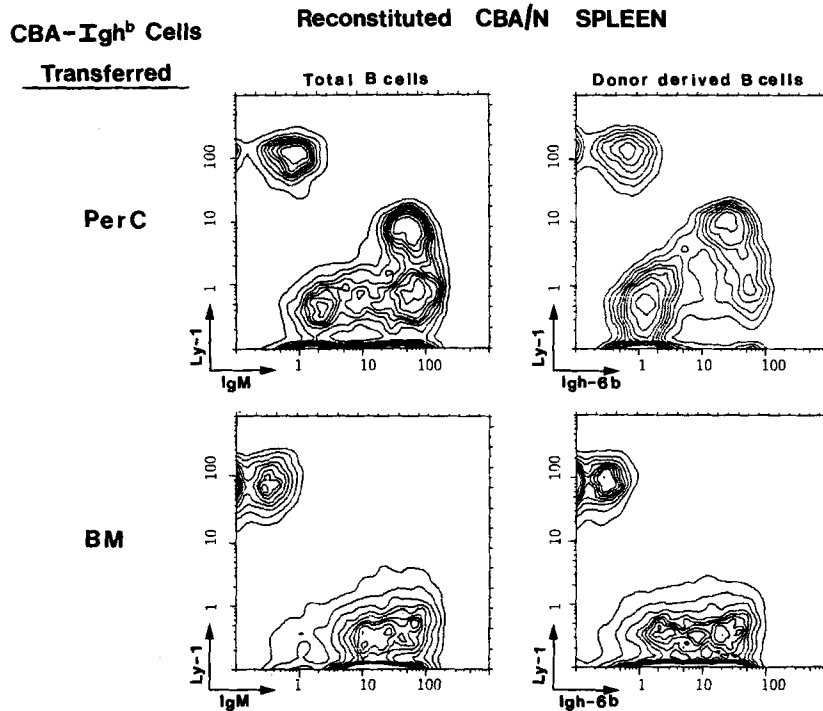


Ly-1 B PROGENITORS



FIGURE 5.   Adult PerC reconstitute Ly-1 B in CBA/N spleen. $10^7$ adult PerC or bone marrow (*BM*) cells from CBA-Igh donors were transferred to irradiated CBA/N mice. 1 mo after transfer, PerC-reconstituted spleen showed 20% donor-derived B cells (10% Ly-1 B and 10% Ly-1⁻ B), and 30% host-type B cells (<1% Ly-1 B and 30% Ly-1⁻ B).

TABLE II

*Anti-BrMRBC PFC (Ly-1 B) Are Reconstituted by PerC But Not by Bone Marrow*

| Strain | Irradiated | Allotype congenic cells transferred* | | Anti-BrMRBC PFC per $10^6$ spleen‡ | |
| --- | --- | --- | --- | --- | --- |
| | | Source | Strain | Total | Donor-derived |
| BALB/c | Yes | Bone marrow | BAB/14 | 30 | <3 |
| BALB/c | Yes | PerC | BAB/14 | 300 | 280 |
| CBA/N | Yes | PerC | CBA-Igh^b | 150 | 150 |

* $10^7$ cells were transferred intravenously to 600 rad–irradiated recipients. BALB/c recipients are Igh^a; CBA/N recipients are Igh^j. Donors are all Igh^b.
‡ Spleens from reconstituted mice were analyzed 1 mo after transfer. Three mice per group were tested; average values are shown. Spleens from normal BALB/c and BAB/14 controls yielded 15 PFC/$10^6$ cells in this assay; CBA/N had no detectable PFC.
§ Donor-derived (Igh^b) PFC were identified by inhibition with monoclonal anti Igh-6b antibodies. This antibody inhibits all BAB/14 PFC and does not inhibit any BALB/c or CBA PFC.

The ability to determine PFC allotype is particularly crucial for these studies, since radioresistant (host derived) anti-BrMRBC PFC are found in spleens in all recipients that show anti-BrMRBC PFC activity before irradiation, i.e., in essentially all mice except CBA/N. Donor-derived PFC, in contrast, are only detectable in spleens from PerC recipients and are clearly lacking in recipients of adult

bone marrow (Table II). Thus, these highly sensitive functional studies confirm conclusions from FACS studies demonstrating that PerC transfers reconstitute Ly-1 B, whereas adult bone marrow transfers do not.

*Persistence of Transferred Ly-1 B Cells Does Not Account for Ly-1 B Reconstitution.* Since host-derived Ly-1 B tend to persist for relatively long periods after irradiation, there is reason to suspect that at least some of the donor-derived Ly-1 B found in reconstituted recipients represent transferred Ly-1 B cells that merely persist in the new host. The potential persistence of such cells cannot account for Ly-1 B reconstitution in recipients of neonatal liver or spleen, since too few Ig-bearing cells (including Ly-1 B) are present in the donor organs to account for the number of Ly-1 B present when reconstitution is complete. Therefore, Ly-1 B reconstitution from neonatal sources must involve the expansion and differentiation of immature progenitor cells that probably do not express surface Ig until sometime after transfer.

Ly-1 B reconstitution from adult PerC, in contrast, could potentially be explained by the persistence of fully mature Ly-1 B in the inoculum, since the estimated number of donor-derived Ly-1 B in fully reconstituted PerC recipients (1–2 mo after transfer) is, at most, several-fold higher than the number of Ly-1 B injected initially. However, if this hypothesis were correct, virtually all of the injected Ly-1 B cells would have to survive substantially longer than mature cells usually survive in adoptive recipients. Furthermore, virtually all of the injected Ly-1 B would have to migrate initially to inaccessible locations and later find their way to the peritoneum and the spleen, since very few donor-derived Ly-1 B are detectable in PerC recipients until several weeks after transfer.

The failure to reconstitute Ly-1 B cell populations in recipients of spleen cells from adults also argues against a simple persistence hypothesis. If we assume that splenic Ly-1 B are equivalent to PerC Ly-1 B vis-á-vis persistence, then transfers of $10^7$ spleen cells should reconstitute Ly-1 B about as well as transfers of 1–2 × $10^6$ PerC (since PerC Ly-1 B frequencies are ~10-fold higher than splenic Ly-1 B frequencies). 2 × $10^6$ transferred PerC, however, reconstituted substantial numbers of Ly-1 B, whereas spleen cells did not detectably reconstitute Ly-1 B in recipients (Table I). The persistence of Ly-1 B in recipients, therefore, does not appear sufficient to account for the Ly-1 B reconstitution by PerC.

Curiously, preliminary evidence from FACS sorting and transfer experiments suggests that Ly-1 B are derived from Ig-bearing cells in PerC (K. Hayakawa, R. R. Hardy, and L. A. Herzenberg, unpublished data). Thus, since the evidence discussed above indicates that Ly-1 B reconstitution is not due to the persistence of mature transferred cells, the reconstituted Ly-1 B population apparently derives from a small number of relatively mature (Ig-bearing) progenitors that still retain the potential for long-term self-renewal.

## Discussion

The simplest and most straightforward way to interpret the cell transfer data presented here is to define two independent B cell lineages whose progenitors inhabit different sites in adult animals (Fig. 6). The first lineage culminates in the predominant B cell populations in spleen and lymph nodes, and derives from classical B cell progenitors that are present in neonatal and adult spleen and
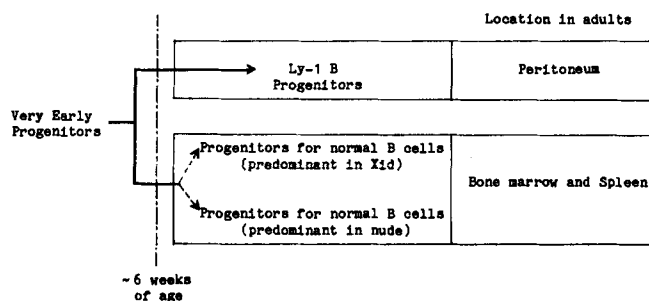
FIGURE 6.   Progenitors for Ly-1 B diverge before animals reach adulthood. This figure presents a tentative interpretation of the current B cell lineage data. The decision to draw two independent lineages (Ly-1 B and other B) is based on data presented here, indicating that these lineages exist, and that their progenitors have already diverged and are present in different locations (peritoneum vs. bone marrow) in animals >6 wk of age. The decision to include a tentative split in the "other" B cell lineage is based on previously published evidence (7, 8).

bone marrow, but are missing from adult PerC suspensions. The second lineage, in contrast, derives from previously unrecognized B cell progenitors that are present in adult PerC suspensions and neonatal hematopoietic organs, but are missing from adult spleen and bone marrow. This novel lineage culminates in the Ly-1-bearing B cell (Ly-1 B) populations in spleen (9) and peritoneum (10) that produce many of the commonly studied IgM autoantibodies found in normal and autoimmune mice (11).

This construct is based on analytical (FACS) and functional studies of the progeny of individually and jointly transferred cell populations. The individual transfers showed that (a) adult PerC selectively reconstitute Ly-1 B in irradiated recipients; (b) adult bone marrow selectively reconstitutes other B cells; and (c) spleen, liver, and bone marrow from very young animals reconstitute both the Ly-1 B population and the other (Ly-1⁻) B cell populations. In addition, joint transfer studies (adult bone marrow with either neonatal liver or adult PerC) showed that each progenitor source reconstitutes all of the B cells that it reconstitutes when transferred alone.

These findings largely rule out the presence of cryptic Ly-1 B progenitors in bone marrow: first, bone marrow does not block Ly-1 B development when appropriate progenitors are present (from PerC or neonatal liver donors); second, although both PerC and neonatal liver give rise to Ly-1 B, neither of these sources supports the development of Ly-1 B from cotransferred, allotypically distinguished bone marrow cells. Similarly, these findings (not surprisingly) rule out the presence of bone marrow–type B cell progenitors in PerC. Thus they provide a strong base for concluding that Ly-1 B do not belong to the same lineage as the predominant splenic and lymph node B cell populations (whose progenitors are clearly detectable in bone marrow in adults).

Previous suggestions (7) of lineage differences among B cells do not pertain to this Ly-1 B lineage distinction. In essence, these earlier studies focused on genetically controlled differences in the predominant B cell populations. The evidence they generated can now be interpreted as indicating that bone marrow–derived (Ly-1⁻) B cells are further subdivided into two lineages, one that

predominates in nude mice, and the other in X-linked immunodefective (*xid*) animals (7). We show these putative lineages in the summary diagram in Fig. 6; however, we consider their existence tentative, since evidence from progenitor studies such as those presented here is still lacking.

The developmental stage at which the Ly-1 B lineage diverges from other B cell lineage(s) has yet to be determined. Although these lineages are clearly derived from different progenitors in adults, they quite possibly may be derived from a common progenitor in immature animals (since progenitors for both lineages are found in spleen, liver, and bone marrow from neonatal and weanling animals). Alternatively, they may be derived from progenitors that are already distinct in immature animals but do not migrate to unique sites until relatively late in development. In either case, these lineages must ultimately be traceable to a single progenitor, since (by definition) all lineages bifurcate at some point during fetal or neonatal development.

If Ly-1 B progenitors are already distinct from other B cell progenitors in fetal and neonatal organs, then, ironically, current concepts of pre-B cells may be based largely on the behavior of cells drawn from the Ly-1 B lineage. Much of our understanding of B cell development derives from studies with pre-B and B cell lines, and tumors such as 70Z (26), $BCL_1$ (27), CH1 (28), and NFS-5 (29), all of which carry Ly-1 surface molecules (R. Hardy, K. Hayakawa, and L. A. Herzenberg, unpublished observations). Furthermore, much of what is known about the properties of normal (nonmalignant) pre-B cells derives from studies of cell populations from immature (fetal and neonatal) animals, and thus may mainly reflect the properties of Ly-1 B progenitors (since Ly-1 B cells represent such a large proportion of neonatal B cells) (9). Although spleen, liver, and bone marrow from young animals contain progenitors that reconstitute all B cells, studies of pre-B cells derived from adult bone marrow may be required to unambiguously characterize the progenitors of the majority of the B cells found in adults.

These considerations may be key to understanding recently reported differences in the properties of pre-B cells derived from different sources (30–33). The IgH gene rearrangements that occur in virally induced (Abelson) pre-B tumors derived from adult bone marrow differ, for example, from the IgH rearrangements that occur in similarly induced pre-B tumors from neonatal animals (33). These differences may be due to differences in the properties of immature vs. mature B cell progenitors; however, they may equally well be ascribable to basic biological differences that distinguish Ly-1 B progenitors from the progenitors of other B cells.

Similarly, evidence demonstrating that hybridoma fusions with fetal and neonatal B cells tend to yield more self-reactive antibodies than fusions with B cells from adults probably reflects the larger proportion of Ly-1 B cells in neonates (since Ly-1 B are known to be enriched for autoantibody-producing cells). The recovery of a relatively large number of hybridomas producing antiidiotypic antibodies from the neonatal mice is particularly noteworthy since (what appear to be) Ly-1 B cells in adults have been implicated in the regulation of allotype (34) and idiotype (35) expression in antibody responses. Perhaps the Ly-1 B lineage is specifically charged with a regulatory role that includes the production

of autoantibodies active in regulatory networks, such as those proposed originally by Jerne (36).

## Summary

Data from previous multiparameter fluorescence-activated cell sorter (FACS) analysis and sorting studies define a subset of murine B cells that expresses the Ly-1 surface determinant in conjunction with IgM, IgD, Ia, and other typical B cell markers. These Ly-1 B cells are physically and functionally distinct. They express more IgM and less IgD than most other B cells; they are not normally found in lymph node or bone marrow; they are always present at low frequencies (1–5%) in normal spleens, and, as we show here, they comprise about half of the B cells (10–20% of total cells) recovered from the peritoneal cavity in normal mice. Furthermore, most of the commonly studied IgM autoantibodies in normal and autoimmune mice are produced by these Ly-1 B cells, even though they seldom produce antibodies to exogenous antigens such as trinitrophenyl-Ficoll or trinitrophenyl–keyhole limpet hemocyanin.

Cell transfer studies presented here demonstrate that the progenitors of Ly-1 B cells are different from the progenitors of the predominant B cell populations in spleen and lymph node. In these studies, we used FACS analysis and functional assays to characterize donor-derived (allotype-marked) B cells present in lethally irradiated recipients 1–2 mo after transfer. Surprisingly, adult bone marrow cells typically used to reconstitute B cells in irradiated recipients selectively failed to reconstitute the Ly-1 B subset. Liver, spleen, and bone marrow cells from young mice, in contrast, reconstituted all B cells (including Ly-1 B), and peritoneal "washout" cells (PerC) from adult mice uniquely reconstituted Ly-1 B. Bone marrow did not block Ly-1 B development, since PerC and newborn liver still gave rise to Ly-1 B when jointly transferred with marrow. These findings tentatively assign Ly-1 B to a distinct developmental lineage originating from progenitors that inhabit the same locations as other B cell progenitors in young animals, but move to unique location(s) in adults.

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