International Immunology, Vol. 7, No. 1, pp. 55–68

De novo development and self-replenishment of B cells

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Key words: B cell, development, self-replenishment

Abstract

Previous studies distinguished two murine B cell lineages: the conventional lineage, which comprises the majority of B cells, and the Ly-1 B lineage (B-1a), which represents a small percentage of total adult B cells. A third subset, B-1b cells, shares many properties with B-1a cells, including the characteristic ability to self-replenish, but does not express Ly-1 (CD5). Reconstitution studies presented here show that (i) although the B220$^-$ population in adult spleen and bone marrow contains very little progenitor activity for B-1a cells, it can reconstitute roughly half the normal number of B-1b cells; (ii) B-1 progenitors present in adult bone marrow and spleen function at low levels in adult animals; (iii) peritoneal B-1 cells (principally B-1b) that develop following bone marrow transfer, like B-1 cells from normal animals, are capable of substantial self-replenishment; and (iv) conventional B cells do not expand (self-replenish) in adoptive recipients, although they can persist for long periods. Collectively, these progenitor and self-replenishment characteristics provide a developmental base for distinguishing B-1a, B-1b and conventional B cells.

Introduction

Reconstitution studies by Hayakawa *et al.* (1) identified two functionally and developmentally distinct B cell lineages in the mouse: conventional B cells, also designated B-2 cells (2), which predominate in adult spleen and lymph node, and Ly-1$^+$ (CD5$^+$) B cells, now designated B-1a cells (2), which are rare in spleen and lymph node but predominate in the peritoneal (3,4) and pleural cavities (5,6). Recently, we have also proposed a third B cell lineage, designated B-1b, that shares many of the B-1a phenotypic and physiological properties but does not express surface CD5 (7–10). Studies presented here provide a firmer base for these lineage distinctions by clarifying the progenitor and self-replenishment characteristics that distinguish B-1a, B-1b and conventional B cells.

Progenitors that reconstitute conventional B cells in irradiated recipients are readily detectable both in adult bone marrow and in fetal liver. Progenitors that reconstitute B-1a cells, in contrast, are readily detectable in fetal (and neonatal) liver but are very rare in adult bone marrow (1,9). Co-transfer of fetal liver and adult bone marrow neither enables development of B-1a cells from the bone marrow nor blocks the development of these cells from fetal liver, thus demonstrating conclusively that the ability to give rise to B-1a cells is an intrinsic property of a progenitor population that is present in 14 day fetal liver (9). In fact, looking at earlier sources of lymphoid progenitors, Solvason *et al.* and Godin *et al.* showed that fetal omentum (day 13) and para-aortic splanchnopleura (day 9) contain progenitors for B-1a cells but not for conventional B cells (11–14). This anatomical separation confirms the separate identity of the progenitors for B-1a and conventional B cells, and supports the idea that the progenitors for B-1a cells arise earlier in ontogeny.

The lineage commitment of the B cell progenitors is clearly defined at the pro-B cell stage of the B cell development pathway. Hardy and Hayakawa (15,16) showed that FACS-sorted pro-B cells from adult bone marrow largely give rise to conventional B cells whereas pro-B cells isolated from fetal liver mainly generate B-1a cells. More recently, Lam and Stall and Hayakawa *et al.* have shown that although adult bone marrow pro-B cells give rise to pre-B cells that universally

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Transmitting editor: K. L. Knight

Received 28 July 1994, *accepted* 26 September 1994

express MHC class II (17), class II is not expressed until well after the pre-B cell stage in B cells that develop from fetal/neonatal pro-B cells (18,19).

The lack of progenitors for B-1a cells in adult bone marrow initially was surprising since B-1a cells do not decrease in number as animals age. However, Hayakawa *et al.* showed that the mature (IgM^+) B-1a cells in the peritoneal cavity are self-replenishing in that transfers of whole PerC or FACS-sorted B-1a cells fully reconstitute B-1a populations in irradiated recipients (20). Similarly, B-1a cells populate unirradiated neonates (21). Lalor *et al.* confirmed that B-1a cells maintain themselves by self-replenishment in adults by showing that depleting the B cells that emerge during the first few weeks of life engenders a severe B-1a depletion that lasts throughout life (10,22). Conventional B cells, in contrast, are continually replenished from their bone marrow progenitors and their depletion during the neonatal period results only in a transient decrease in frequency. Thus, a broad series of developmental differences distinguish the B-1a and conventional B cell lineages.

B-1b cells are so similar to B-1a cells at the phenotypic and physiological level that, except when their differences are at issue, they are collectively referred to as B-1 cells (2,7,8,23). However, although the two types of cells show essentially the same expression of all other known surface markers, B-1a express CD5 whereas B-1b do not. Furthermore, although both cell types are found in the peritoneal and pleural cavities and, to a lesser extent in spleen, they do not appear to inter-convert or to substitute functionally for one another. Transfer of FACS-sorted B-1a and B-1b cells demonstrates the fidelity of the B-1a and B-1b cell commitment. Within experimental limits, data from these transfers show that each of these sorted B-1 populations replenishes itself but not the other (7,10).

Properties of the progenitors for the two types of cells also indicate a lineage commitment. Progenitors for B-1a and B-1b cells are similar in that they are found in fetal liver (9,11) and in fetal omentum (which does not give rise to conventional B cells) (11). In addition, published data from transfers of fetal para-aortic splanchnopleura indicate that this site contains progenitors for both types of cells (although the authors do not explicitly make this point) (14). However, while B-1a progenitors are lost from adult bone marrow, current evidence (9), coupled with data from studies presented here, demonstrates that functional progenitors for B-1b cells are present throughout life.

The evidence outlined above (reviewed in 24,25) suggests the existence of three murine B cell lineages. However, this conclusion has been challenged by several studies that raise questions concerning the use of self-replenishment capabilities of these putative lineages as a defining characteristic. First, although cells with the B-1a phenotype constitute ~1% of total spleen cells, transfers of spleen cells failed to reconstitute detectable numbers of B-1a cells (1). This could mean that despite their phenotypic similarity to peritoneal B-1 cells, the splenic B-1 cells are not capable of self-replenishment and hence do not meet this key criteria for B-1 cells. Similarly, although we have shown that transfers of bone marrow from adult animals reconstitute cells that have the B-1b FACS surface marker phenotype, we did not confirm this identification by directly demonstrating that the reconstituted B-1b cells are capable of self-replenishment. Therefore, the nature of B-1b cells derived from adult bone marrow cannot yet be considered to be firmly established. Finally, the entire issue of self-replenishment as a functional criterion for B cell lineage distinctions has been called into question by recent evidence demonstrating that conventional B cells are detectable in SCID recipients many months after transfer of splenic B cells (26) and thus may be as capable of self replenishment as B-1 cells.

In this publication, we show (i) that splenic B-1 populations are indeed self-replenishing, (ii) that the B-1b cells derived from adult bone marrow progenitors also meet the self-replenishment criterion and (iii) that conventional splenic B cells persist for a very long time when transferred into irradiated recipients, but have little or no capacity for self-replenishment. In addition, we show that the B-1b progenitors that are present in bone marrow are functional in that they continue to give rise to small numbers of B-1b cells in adults. These data may be interpreted as indicating that B-1a and B-1b cells belong to closely related lineages derived from distinct progenitors; however, we also consider alternative hypotheses in our discussion.

Methods

Mice

Two pairs of Igh-C allotype congenic mice were used: BALB/cHz (Igh-C^a) with BAB/25 (Igh-C^b) and CBA/Ca (Igh-C^a) with CBA/bb (Igh-C^b). Most animals were bred and raised in the Stanford Department of Genetics animal facility. For one experiment, BALB/cN mice were kindly provided by Dr H. McDevitt (Stanford).

Irradiation and transfer

The 3–7 month old female donors and 3–5 month old female recipients were placed on acidic water (pH 3) containing neomycin sulfate (1 mg/ml; Pharma-Tek, Huntington, NY) 4–7 days prior to irradiation. The mice were maintained on this water following transfer for either 2 weeks or until sacrifice at 8 weeks. No differences in the reconstituted B cell populations were observed between the two protocols. The recipients were X-ray irradiated with 650 rad for the BALB congenic mice and 850 rad for the CBA congenic mice 1 day prior to cell transfer. The number of cells to be injected was determined in a preliminary experiment where various amounts of spleen or bone marrow cells were transferred. The amounts used rescue all animals from the irradiation and approach a plateau of B cell recovery 8 weeks after transfer. The irradiated mice were injected i.v. with unfractionated preparations equivalent to 2×10^7 spleen, 2×10^6 fetal liver (embryonic day 14 based on the last observed vaginal plug and confirmed by characteristics of the fetus; ref. 27) or 2×10^6 bone marrow (femur and tibia) live mononuclear cells. Peritoneal (3×10^6) and lymph node (2×10^7) were transferred with 2×10^6 syngeneic bone marrow mononuclear cells as a hematopoietic source. Secondary transfers used peritoneal cells (containing

$0.5–1\times10^6$ B-1 cells in a total of 3–6 x 10^6 PerC cells, with syngeneic bone marrow) from animals which had received primary transfers of either PerC (with syngeneic bone marrow) or bone marrow 8 weeks earlier. Cell counts are for live cells based on staining with ethidium bromide–acridine orange (28). For some experiments FACS-purified populations, equal in amount to what is present in unseparated cells, were transferred. Sorted IgM^+, $B220^+$ spleen or $B220^+$ bone marrow were transferred with an appropriate amount of $B220^-$ syngeneic bone marrow. In all transfers without a syngeneic hematopoietic source, <1% of the splenic lymphocytes in the recipient were recipient-allotype B cells, indicating successful elimination of host progenitor cells by the irradiation.

Reagents and stains

Mouse monoclonal anti-Igh-6a (IgM of the a allotype, DS1) (29), anti-Igh-6b (IgM of the b allotype, AF6–78.25), anti-Igh-5b (IgD of the b allotype, AF6-122.2), anti-Igh-5a (IgD of the a allotype, AMS 9.1) (29) and rat monoclonal anti-IgM (331.12) (30) , anti-B220 (RA3-6B2)(31), anti-Mac-1 (CD11b, M1/70) (32) and anti-Ly-1 (CD5, 53-7)(33) were purified from serum-free medium (HB101; Hana Biologics, Berkeley, CA) by standard methods. Antibody conjugation to FITC (F-1906; Molecular Probes, Eugene, OR), biotin (*N*-hydroxy-succinimidobiotin; Pierce, Rockford, IL) and preparation of Texas Red (Molecular Probes)–avidin have been described previously (34). Allophycocyanin (APC) was purified from *Spirulina platensis* (34) and cross-linked with SMCC (succinimidyl 4-(N-maleimidolmethyl cyclohexane-1-carboxylate; Pierce) to the hinge region sulfhydryls of dithiothreitol reduced antibodies (method modified from 35).

Cell preparation and staining

Single cell suspensions were prepared in deficient RPMI 1640 as described (22). EDTA (1 mM) was added to the medium for staining in microtiter plates. This results in less cellular aggregation and firmer pellets during the washing, especially for the peritoneal cells, which contain large numbers of macrophages.

FACS analysis and sorting

Cells were analyzed and sorted on 'Flasher', an extensively modified dual laser (488 and 595 nm excitation) FACS II (36) (Becton Dickinson, Mountain View, CA) interfaced with a VAX 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software (W. Moore, Stanford University). Bone marrow cells were sorted on a single laser (488 nm excitation) FACStar Plus (Becton Dickinson). The probability contour plots, which contain an equal number of cells between each pair of contour levels, are gated on forward and obtuse scatter to show mostly lymphocytes (37). The number of lines in each region of the map is essentially proportional to the number of cells in that region. Direct comparison of the lines among different plots is possible only if there are an equal number of population peaks. All plots presented here have 5% probability contours.

In some experiments we needed to detect small numbers of a-allotype B cells derived from various sources in the presence of many b-allotype B cells derived from the syngeneic bone marrow used to rescue the animal. This was done by simultaneously staining with allotype-specific anti-IgD and anti-IgM (anti-Igh-6a and anti-Igh-5a) reagents, which allows detection of <1% of a-allotype B cells. Probability plots with outliers allows visualization of the small populations of donor-derived B cells. These plots display probability contours (5%) and dots which represent the last level (5%) of cells. Each dot corresponds to one cell. For example, a data set which contains 10,000 events and is displayed as a 5% probability plot with outliers will have 500 dots outside the last contour level. Dead cells were excluded with propidium iodide (38).

FACS identification of B cell populations

The reagents used for all the figures are: FITC–anti-Igh-6a, APC–anti-Ly-1, APC–anti-Mac-1 and biotin–anti-Igh-5a followed by Texas Red–avidin. Percentages of cells reported in the figures are per total number of live lymphocytes. Three B cell populations from a normal (untreated) BALB/c PerC are identified in Fig. 1(left). Conventional B cells are identified by a broad, positive IgM and tight, IgD^{hi} FACS profile (IgM^+ IgD^{hi}; Fig. 1, top panel). Both B-1a and B-1b cells are bright for IgM and dull to moderate for IgD (IgM^{hi}, IgD^{lo}). These cells are also $Mac\text{-}1^+$ and duller for B220 than conventional B cells (7,10,24). As indicated, gating on the IgM, IgD plot and the IgM, Mac-1 plots yield comparable values. The B-1 cells are quantified as B-1a ($CD5^+$) and B-1b ($CD5^-$) cells as follows: the number of B-1a cells from the IgM, CD5 stain (Fig. 1, middle panel) is subtracted from the total number of IgM^{hi}, IgD^{lo} B-1 cells to obtain the number B-1b cells in each transfer. Direct gating on the $CD5^-$, IgM^{hi} cells is avoided because of overlap with conventional B cells. Counts are based on the number of cells in 7 ml of peritoneal wash and percentages determined by FACS.

Criteria for recipient PerC analysis

Two important control criteria are applied before each recipient PerC is considered in the analysis. First, the number of host-derived B cells must be low because B-1 have a feedback mechanism via which mature B-1 cells can inhibit further development from Ig^- precursors (10). Less than 4% of the lymphocytes in each peritoneum of the animals included in the analyses are host-allotype B cells, indicating that the irradiation was successful in depleting the radiation-resistant host B-1 cells. Two of the ~80 mice examined had large host-derived B-1 cell populations (both >10% of the lymphocytes) and were excluded from the analysis. Second, donor-derived conventional B cells and T cells must be re-populated in the recipient. This is a fundamental indicator of successful transfer. One recipient had poor reconstitution of all B cell populations and was excluded.

Anti-μ treatment of Igh-C allotype heterozygote mice

Ig heavy chain allotype heterozygous mice, F_1(BALB/c/Igh-$C^a\times$BAB/Igh-C^b), were injected i.p. from birth with 100 μg of DS-1 (mouse anti-mouse Igh-6a, IgM of the a allotype) on days 1, 7 and 14. This treatment eliminates all B cells of the a allotype until days 35–45, but not B cells of the b allotype.

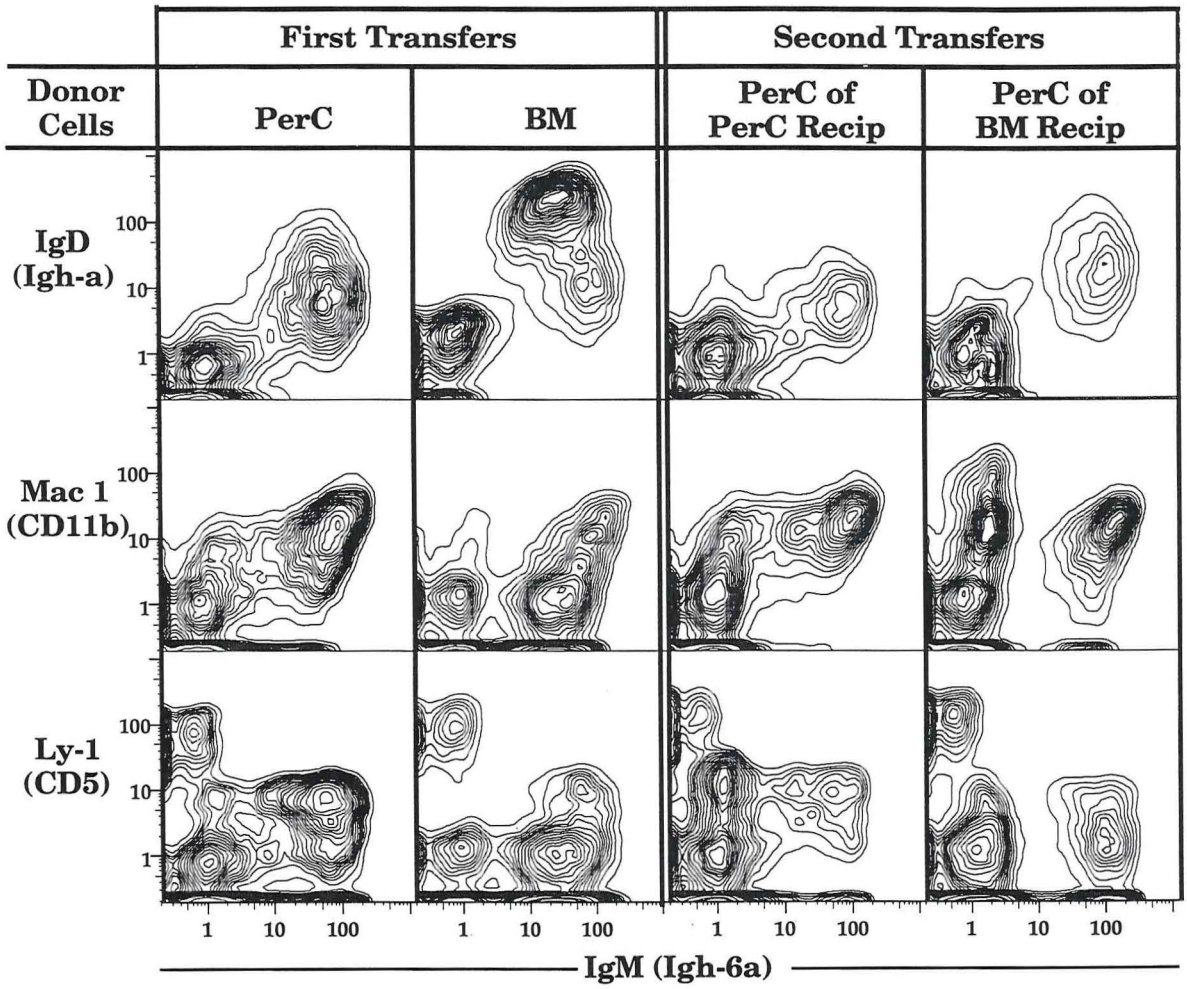

Fig. 3. Peritoneal B cells following primary and secondary transfers. Recipient peritoneal cells were stained as described above following primary transfers (8 weeks post-transfer) of either BALB/c (a allotype) PerC or bone marrow and secondary transfers (8–10 weeks post-transfer) of PerC from either the PerC recipient or the bone marrow recipient. For PerC transfers, BAB (b allotype) bone marrow was also transferred as a hematopoietic source. In all cases, stains for the a-allotype donor cells are shown. Cells derived from the BAB bone marrow were revealed with b-allotype specific stains and are mostly B-2 (data not shown). BAB-derived B-1b cells are also present (data not shown). Here, they appear as Mac-1$^+$, IgM (Igh-6a)$^-$ cells, most notably in the secondary transfers.

B-1 cells, which typically number 0.5–1×10^6 (6,39), or B-1-derived plasma cells in the spleen and gut (40). Therefore, the expansion of the transferred B-1 population is likely to be considerably greater than the 3- to 5-fold that we estimate.

The secondary transfers demonstrate that the self-replenished PerC-derived B-1 cells in the first recipients can be further transferred and expanded. In the secondary transfers, a-allotype B cells expand again and are recovered in the PerC of the second recipient (average = 2.3 ± 0.5 times the injected number of B-1 cells, n = 3). Additionally, a-allotype B-1 cells are found in the recipient spleen, although the total recovery is still slightly less than observed with primary transfers (data not shown). Some mice developed B-CLL like clones (41) following multiple transfers and were excluded from the analysis.

Most strikingly, B-1 cells derived from adult bone marrow also self-replenish when transferred to adoptive recipients (Fig. 3). Bone marrow-derived B-1 cells from the first recipient (a-allotype IgD^{lo}, IgM^{br}) were transferred and recovered in the PerC of the second recipient at two to three times the number of injected B-1 cells (average = 2.7 ± 1.5 times the number of injected B-1 cells from the PerC of the recipient of BALB/c bone marrow, n = 11). An approximately equal number of a-allotype B cells were also recovered in the spleen of the second recipient. Note that although a-allotype conventional B cells constitute 80% of the peritoneal B cells in the bone marrow recipients, few a-allotype conventional B cells are detected in the peritoneum following the second transfer (i.e. the transfer of bone marrow derived PerC B cells).

The B-1a and B-1b cells in the peritoneum of the second recipient are replenished in proportion to their representation in the donor pool. For example, when bone marrow-derived PerC B-1 cells (which have a higher proportion of B-1b cells relative to B-1a cells) are transferred, the PerC B-1 cells in the (second) recipient will similarly have a higher proportion of B-1b. Thus, the self-replenishment capacity of the peritoneal

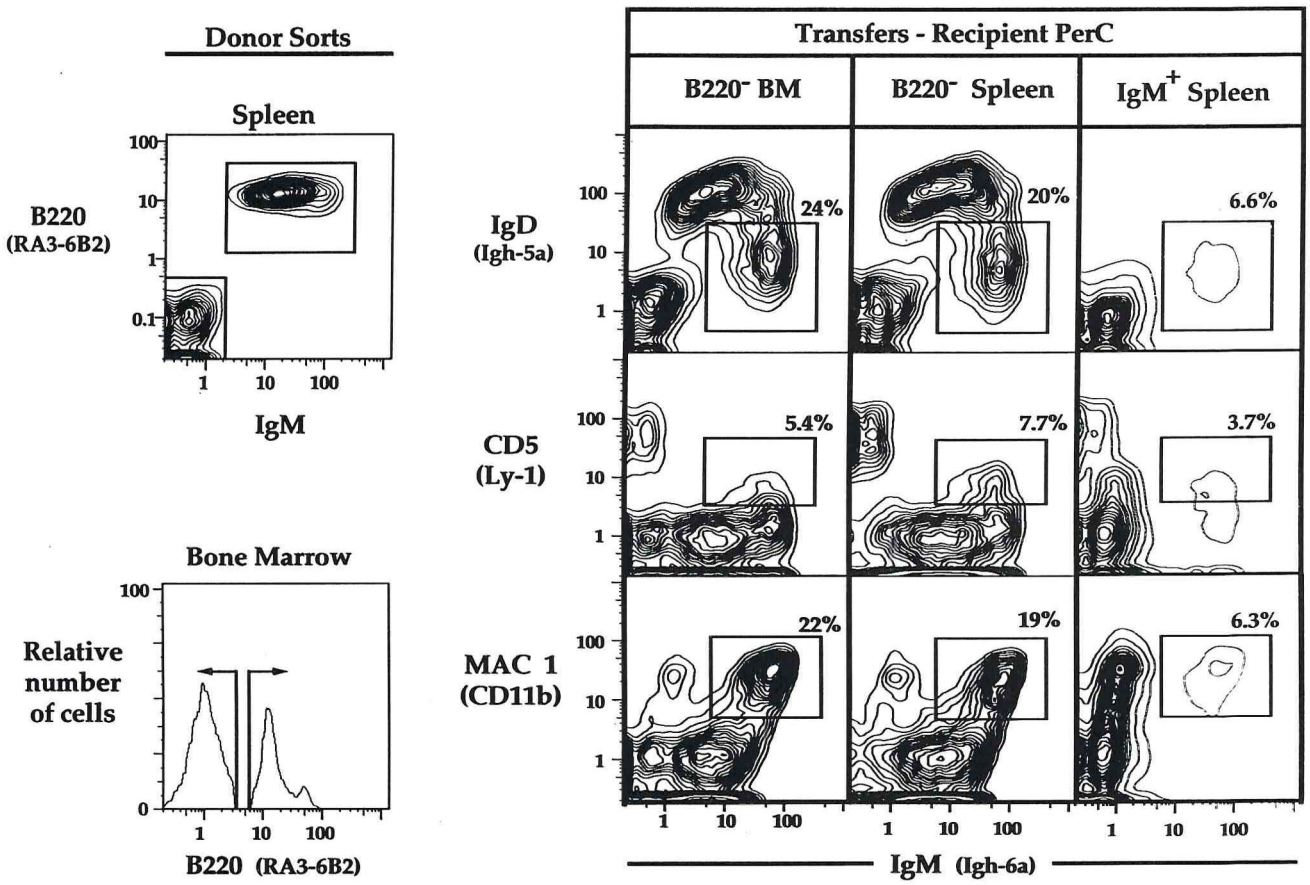

Fig. 4. Transfer of sorted spleen and bone marrow. BALB/c splenic lymphocytes were sorted on the basis of B220 (RA-3/6B2), IgM double-positive or double-negative as shown at the top. Re-analysis of the sorted populations yielded 97% pure double-positives and >99% pure double-negatives. BAB recipients received 7.0×10^6 double-positives, with $B220^-$ syngeneic bone marrow, or 7.6×10^6 double-negatives, which are the equivalent of ~2×10^7 unsorted spleen cells. Bone marrow was sorted into $B220^+$ (97% pure) and $B220^-$ (98% pure) fractions. Cells equivalent to that found in 2×10^6 bone marrow cells were transferred. PerC analyses were done 10 weeks after transfer. $B220^+$ bone marrow, which was transferred with $B220^-$ syngeneic bone marrow, did not yield resolve any detectable B cells (data not shown). As a control, spleen, bone marrow and PerC cells with syngeneic bone marrow, from the same donor pool, were transferred separately; all re-populated normally.

B-1 cells derived from bone marrow is equivalent, on a per cell transferred basis, to the self-replenishment capacity of B-1 cells in the peritoneal cavity of normal mice.

Adult spleen is better than bone marrow at reconstituting B-1a cells

The major difference between the bone marrow and spleen transfers is the higher proportion of B-1a cells compared with B-1b cells in the spleen cell recipients (compare the bottom panels in Fig. 1). B-1a cells represent 40–60% of the B-1 cells in recipients of adult spleen cells but only 20–30% of the B-1 cells in recipients of bone marrow (Fig. 2A and B). This difference, which reflects the additional presence of self-replenishing B-1 cells in the spleen but not the bone marrow (see below), is very reproducible: a one-to-one comparison of matched transfers from 11 individual donors reveals that spleen transfers always yield more B-1a cells in the peritoneum than bone marrow transfers. However, the B-1b cells are still a larger percentage of the lymphocytes in spleen recipients than in normal animals. Overall, the fraction of B-1b cells in the total peritoneal B-1 population is lowest for untreated mice and PerC recipients (data not shown), low for fetal liver recipients (9), intermediate for spleen recipients and greatest for bone marrow recipients (Fig. 2A and B).

The moderate reconstitution of B-1a cells, to 10–15% of normal levels in BALB/c mice and 20–25% of normal levels in CBA mice, by spleen transfer differs from earlier progenitor studies which indicated that adult spleen could not transfer B-1a cells (1). In those studies, however, recipients received half the dosage of spleen cells and were analyzed at 1 month rather than 2 months. We find that reconstitution is closer to completion at 2 months (data not shown).

$B220^-$ progenitors are responsible for all of the bone marrow reconstitution and much of the spleen reconstitution of B-1 cells

In order to distinguish the relative contributions of self-replenishment and progenitor activity to the reconstitution of peritoneal B-1 cells, sorted $B220^-$ and $B220^+$ cells from spleen and bone marrow were individually transferred (Fig. 4). $B220^-$ bone marrow reconstitution of peritoneal B-1a and B-1b cells is equivalent to whole bone marrow reconstitution

Table 1 Peritoneal B cell reconstitution from sorted spleen and bone marrow transfers

Cells transferred	*n*	Peritoneal B cell reconstitution (% of lymphocytes)			
		B-1a	B1-b	Conventional B	Ratio B-1b/B-1a
Spleen					
$B220^-$, IgM^-					
experiments 1 and 2	6	6.0 ± 2.0	11 ± 2.8	48 ± 4.8	1.8
$B220^+$, IgM^+					
experiment 1	3	3.2 ± 0.9	2.4 ± 0.3	<1	0.80
experiment 2	3	clone(s)	—	—	—
Unsorted					
experiment 1	4	12 ± 1.4	11 ± 4.0	44 ± 4.0	0.92
experiment 2	4	clone(s)	—	—	—
Bone marrow					
$B220^-$					
experiments 1 and 2	6	6.9 ± 1.3	15 ± 3.5	49 ± 4.5	2.2
$B220^+$					
experiment 1	2	<1	<1	<1	—
Unsorted					
experiments 1 and 2	6	6.4 ± 1.5	15 ± 3.5	54 ± 4.5	2.3
PerC:					
Unsorted					
experiment 1	3	29 ± 6.7	16 ± 4.3	<1	0.52
experiment 2	3	clone(s)	—	—	—

The BALB-derived (a allotype) peritoneal B cell populations are reported as a per cent of total live lymphocytes. The last column reports the average ratio of B-1b to B-1a cells for the recipients in each row. Control transfers of unsorted spleen, bone marrow and PerC from the same donor pools are also reported. Experiments 1 and 2 are different donor pools which were sorted at different times. See the text and Fig. 6 for a discussion of the clones in experiment 2.

in all respects (Fig. 1 versus Fig. 4; Table 1). Furthermore, $B220^-$ spleen reconstitution of PerC B cells is more similar to whole bone marrow and $B220^-$ bone marrow transfers than whole spleen transfers, i.e. there is a lower frequency of B-1a cells than observed following whole spleen transfers. The reduction of the B-1a cell population can be seen by comparing the representative IgM versus CD5 plots (Fig. 4, left and center; Fig. 1, right).

$B220^+$ bone marrow did not reconstitute any detectable B cells in irradiated recipients (Table 1). Hardy and Hayakawa have previously demonstrated that the $B220^+$ bone marrow fraction contains pro-B cells capable of reconstituting conventional B cells in lightly irradiated SCID recipients (15). This expansion of >10-fold peaks at 3 weeks (16,42,43). The transfers into SCID mice were done in the absence of any competition from $B220^-$ B cell progenitors (stem cells). In the sorted transfers presented here, the $B220^+$ bone marrow fraction did not contribute to the total, long-term (10 weeks) reconstitution by bone marrow. $B220^-$ progenitors are responsible for essentially all of the B cell reconstitution which, like the bone marrow pro-B cell transfers into SCID (15), yields mostly conventional B cells.

Transfer of IgM^+ B cells

The difference in B-1 cell reconstitution between total and $B220^-$ spleen cells, suggested that splenic IgM^+ B-1 cells were contributing to the overall reconstitution from spleen. Therefore, we undertook a series of experiments to further evaluate the contribution of IgM^+ B cells to the reconstitution of conventional and B-1 cells. Previous studies showed that B-1 cells are reconstituted from peritoneal cells that express surface IgM (20). Additional transfers of FACS-sorted peritoneal B-1a and B-1b cells further demonstrated that each population replenishes itself, but not the other (7,10). Since other groups had reported the survival of conventional B and B-1 cells in SCID recipients (26,44,45) we also wanted to compare donor B cell persistence versus expansion in irradiated recipients. Recipient PerC and spleen were both evaluated since B-1 cells are predominant in the PerC while conventional B cells are predominant in the spleen.

Splenic B-1 cells self replenish

FACS-sorted IgM^+ spleen cells, which were injected with sorted $B220^-$ syngeneic bone marrow as a hematopoietic source, yield a low but consistent level of B-1 cells (IgM^{hi}, IgD^{lo}) in the recipient peritoneum. Note that most spleen-derived (a-allotype) cells in the recipient PerC are IgM^{hi}, IgD^{lo} (Fig. 5). The few a-allotype IgD^{hi} cells are conventional B cells which persist after the transfer (see the next section for further discussion). Consistent with the B-1 phenotype, these cells are also Mac-1^+ (Fig. 4). Thus, we show that B-1 cells which are Mac-1^- in the spleen (8,23) become Mac-1^+ when they migrate to the peritoneum following transfer. IgM^+ spleen-derived cells, which are ~6% of the total PerC lymphocytes, include both B-1a ($CD5^+$) and B-1b ($CD5^-$) cells (Fig. 4 and Table 1). Thus, B-1b cells are present in the spleen and can self-replenish. Lymph node, which contains essentially no B-1 cells, does not yield B-1 cells in these recipients (Fig. 5).

The donor-derived B-1 cells in these recipients are all attributable to self-replenishment from the sorted IgM^+ B cells. The minimal reconstitution of conventional B cells indicates that the recipient B-1 cells do not derive from stem

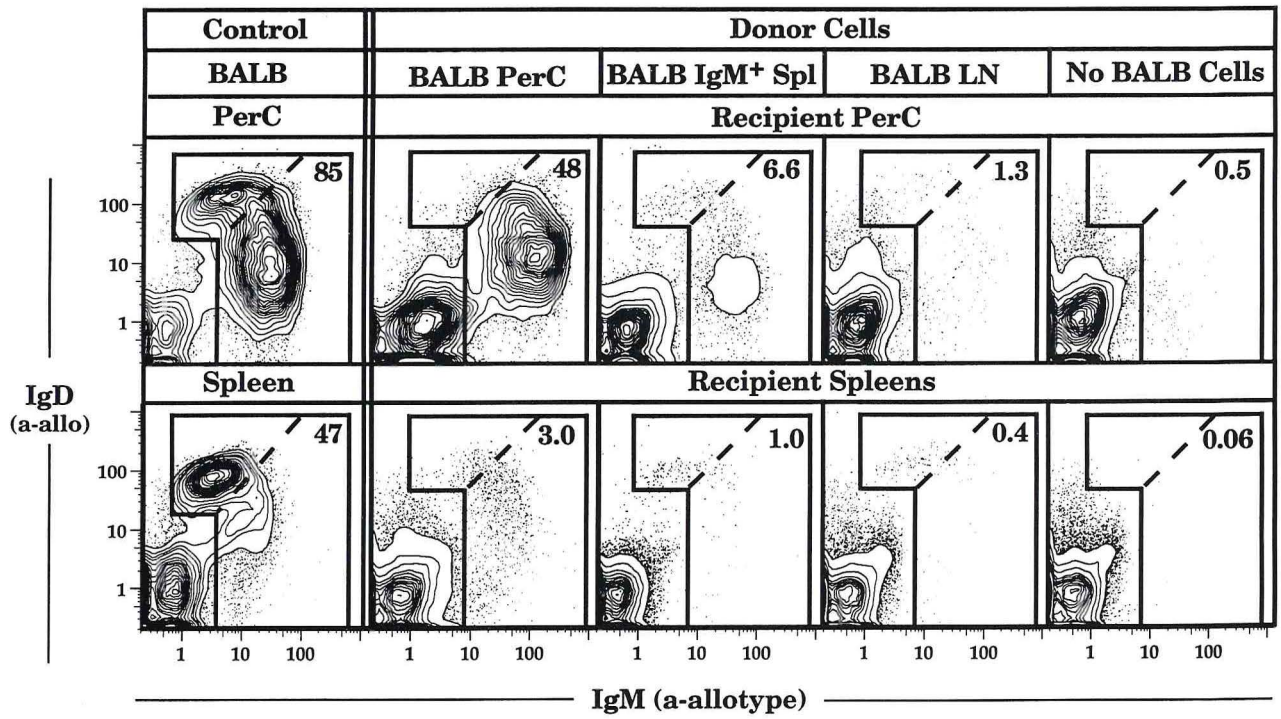

Fig. 5. Self-replenishment and persistence of B cell populations. Recipient PerC and spleen were analyzed 8–10 weeks after the transfer of BALB/c (a allotype) PerC (3×10^6 cells), FACS-sorted spleen as described in Fig. 4, lymph node (2×10^7 cells) or no BALB/c cells. For comparison, a normal BALB/c control is shown on the left. Transfer of 3×10^6 lymph node cells did not yield detectable a-allotype cells above the control (no BALB/c cells) background. In all cases BAB (b allotype) bone marrow was transferred as a hematopoietic source. Probability plots (5%) with outliers are shown after gating on live lymphocytes. The dashed diagonal line is provided as a visual reference for distinguishing conventional (IgM^{lo}, IgD^{hi}) cells and B-1 (IgM^{hi}, IgD^{lo}) cells. The numbers are the per cent of total a-allotype cells among total lymphocytes.

cells that could have been present in the small $B220^-$, IgM^- population (3%) which contaminated the sort. Furthermore, B-1 cells from the spleen and PerC are equally efficient on a per cell basis at self-replenishing. Assuming a 1% B-1a cell population in the spleen (7,23), each recipient of $B220^+$, IgM^+ spleen cells received ~10^5 B-1a cells and an even smaller population of B1-b cells; the total number and percentage of B-1 cells generated in recipient PerC by these transferred cells is equivalent to that obtained after the transfer of unsorted PerC containing 10^5 B-1 cells (data not shown).

Conventional B cells persist, but do not expand, following transfer from lymph node and IgM^+ spleen

Conventional B cells, in contrast to B-1 cells, cannot be identified in either the recipient spleen or peritoneum following PerC transfer (Fig. 5). However, since there are few conventional B cells in the donor peritoneum, we also evaluated transfers from lymph node and IgM^+ spleen. Conventional B cells were not detected above background when 3×10^6 lymph node cells were transferred (data not shown). However, a small number of a-allotype conventional B cells could be detected among a much larger b-allotype population when 2×10^7 lymph node cells were transferred. Similarly, transfer of FACS-sorted IgM^+ spleen yields a small but discernible conventional B cell population in addition to the B-1 population described above. This conventional B cell population (IgM^{lo}, IgD^{hi}) is clearest in the recipient spleens (Fig. 5).

In contrast to the transferred B-1 cells, the conventional B cells do not expand in the recipient. The a-allotype conventional B cells represent only a tiny fraction of the total conventional B cell pool, which is mostly b allotype in these recipients. Only 20–40% of the injected conventional B cells can be detected in recipient spleen 2–4 months following transfer. Transfers of unstained lymph node cells yield a similarly small conventional B cell population, thus demonstrating that the antibody staining and FACS sorting did not stimulate the IgM^+ spleen cells and lead to their persistence. In contrast to the IgM^+ spleen transfers, which yielded B-1 cells, essentially no B-1 cells are detected in the PerC of lymph node recipients at 2–4 months (Fig. 5). There is no evidence in these transfers that either splenic or lymph node conventional B cells convert to B-1 cells *in vivo*.

In addition to spleen and peritoneum, low levels of a-allotype conventional B cells are also detected in peripheral lymph nodes (data not shown). Consistent with these findings, Sprent *et al.* have shown that mature B cells preferentially migrate to the spleen, with only 6–8% detectable in the lymph node following similar transfer experiments (26,46) . Sprent *et al.* also detected ~35% of injected lymph node conventional B cells in SCID recipients (26). Our findings are in general agreement with these results.

B-1 cell clones transfer from the IgM^+ spleen population

In transfers from one pool of donors (experiment 2 in Table 1), a distinctive pattern of reconstitution was observed in the recipients. The PerC, spleen and $B220^+$, IgM^+ spleen

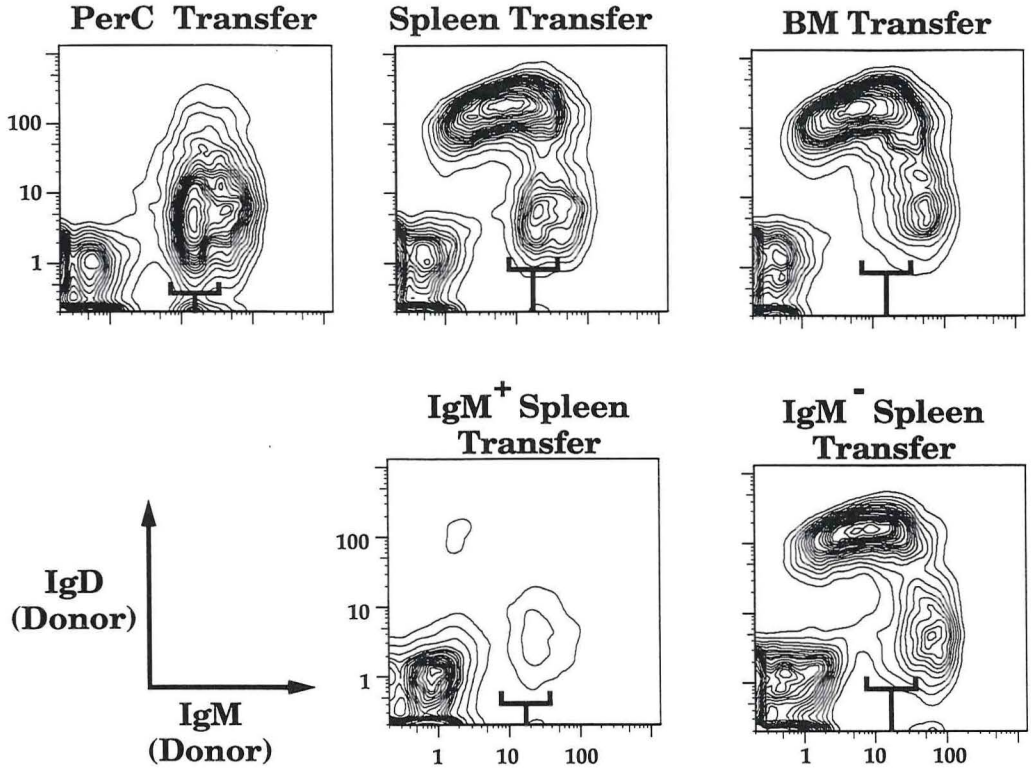

Fig. 6. Transfer of a clonal population. Pooled BALB/c spleen, bone marrow and PerC were transferred with syngeneic bone marrow and the recipient PerC analyzed at 10 weeks (top). B220/6B2, IgM double-positives or double-negatives, with $B220^-$ syngeneic bone marrow, were also sorted from the same donor pool and transferred (bottom). Re-analysis indicated that the double-positives were 97% pure and the double-negatives were 99% pure. Brackets indicate the clonal populations present in the peritoneum of PerC, spleen and IgM^+, $B220^+$ spleen recipients.

transfers gave rise to a phenotypically homogeneous population, indicative of B-1 cell clone(s) (41) within the B-1 cell phenotype. In this transfer, the clone is revealed by the homogeneous IgM surface staining as indicated by brackets in Fig. 6. The clonal population which arises from each donor tissue source appears at identical IgM levels in the recipients. There is no evidence of this homogeneous population from the transfer of either $B220^-$, IgM^- spleen cells or whole bone marrow. Thus, the clone(s) only arose from sources which contain IgM^+ B-1 cells and presumably existed in the original Ig^+ spleen population at levels undetectable by FACS analysis when the cells were sorted and transferred. Finally, we note that clone development 2–3 months after transfer is relatively rare (<5%) for PerC donors < 5 months old (41).

Some B-1 cells enter the peripheral pool during adulthood

The bone marrow reconstitution of B-1 cells (mostly B-1b) in irradiated recipients suggests that some B-1 cells might be routinely produced in normal adult animals. Previously, we showed that neonatal treatment of Igh-C allotype heterozygous mice with antibodies to IgM of one-allotype permanently depletes B-1a cells of the treatment allotype without inhibiting the development of B cells of the non-treatment allotype (22). In order to determine if B-1 cell progenitors function in adults, we reopened these studies to determine the frequencies of B-1 cells in anti-6a (specific for a-allotype μ) treated heterozygotes at various times after the treatment was terminated. This experimental approach has the advantage that normal numbers of total B cells (B-1a, B-1b and conventional) are present at all times during the procedure. Here, we analyze the return of both B-1a and B-1b cells in detail, with FACS reagents specific for both a and b allotypes.

Under the conditions used in these experiments, anti-6a treatment eliminates all a-allotype B cells until ~6 weeks of age (22) (Watanabe *et al.*, in preparation). Examined at various intervals after this time, conventional a-allotype B cells begin returning to the spleen within 1 month; however, few B-1a cells of the treatment allotype are detectable in the PerC of anti-6a treated $(BALB/c \times BAB)F_1$ mice, even at 6 months of age. In fact, outlier plots are needed to reveal the small Igh-$6a^+$, $CD5^+$ population (Fig. 7) since only ~4% of the total B-1a cells are a allotype. B-1a cells of the (untreated) b allotype, in contrast, are quite abundant. Thus, in normal animals, B-1a cells develop almost exclusively from progenitors which are active in the first ~6 weeks of life, with little *de novo* development thereafter.

B-1b cell recovery is more extensive than B-1a cell recovery. B-1b cells are quantified by the difference between total B-1 cells and B-1a cells for each allotype. Among the a-allotype B-1 cells ($6a^+$, $5a^+$ and $6a^+$, Mac-1^+), more appear to be

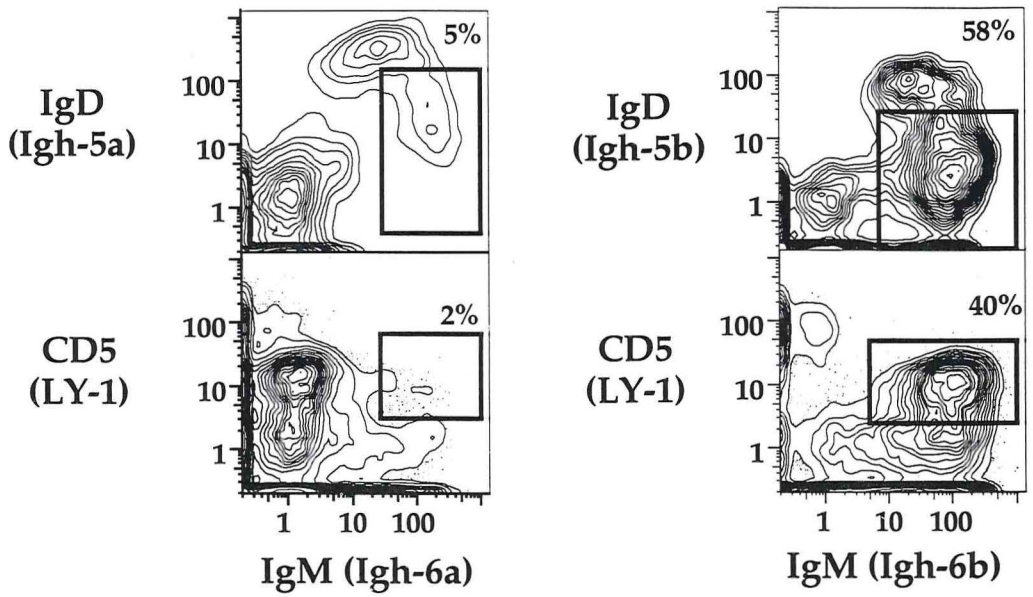

Fig. 7. Recovery of B cells in Igh-C allotype heterozygotes following neonatal treatment with anti-6a antibody. PerC cells from a 6 month old (BALB/c×BAB)F_1 which was treated with anti-6a (IgM of the a-allotype) as a neonate were analyzed for both a- and b-allotype B cells. In general the a-allotype B-1 cells are a small proportion of the total B-1 cells. Gates are drawn to avoid possible inclusion of b-allotype cells. The relative proportions of a-allotype cells at 6 or 8 months are: aB-1a/total B-1a cells = 4 ± 2 %, aB-1b/total B-1b cells = 8 ± 3%, aB-2/total B-2 cells 38 ± 4% for n = 7. Among conventional B cells in the spleen, ~40% are a allotype at this time (data not shown).

B-1b ($CD5^-$) than B-1a ($CD5^+$). In contrast, among the b-allotype B-1 cells ($6b^+$, $5b^+$ and $6b^+$, Mac-1^+), which were not depleted by the neonatal antibody treatment, most are $CD5^+$. Precise measurements of the B-1a and B-1b cells are difficult because of the overall low frequency of a-allotype B-1 cells; however, we estimate that 8% of the total B-1b cells in the PerC at 6 months are a allotype. Given equal development of a- and b-allotype cells after the treatment antibody disappears, 15–20% of all B-1b cells and 5–10% of the B-1a cells enter the peripheral pool after ~6 weeks of age. It should be noted that compared with both B-1a and B1-b cells, conventional B cells are approaching equal proportions of both allotypes by 6 months and thus have recovered substantially better than either B-1 population (compare $6a^+$, $5a^{br}$ and $6b^+$, $5b^{br}$ populations in Fig. 7).

The recovery characteristics described above for B cell populations in animals treated from birth with anti-μ antibodies are consistent with the increase in the proportion of B-1b cells to B-1a cells observed as normal animals age (23). These data are also consistent with the B cell recoveries observed in irradiated recipients of adult bone marrow, i.e. good reconstitution of conventional B cells, poor reconstitution of B-1a cells and the moderate reconstitution of B-1b cells. Thus, the B-1 progenitor activity detectable in bone marrow transfer recipients also appears to function at a low level *in situ.*

Discussion

Data presented here characterize differences in the reconstitution of B-1a, B-1b and conventional B cells following transfer of progenitors and mature B cells from various sources into irradiated adult recipients. In essence, we establish that B-1 cells are markedly better at self-replenishment than conventional B cells, that progenitor activity for conventional B cells is substantially greater than progenitor activity for B-1 cells in adult bone marrow and, furthermore, that progenitor activity for B-1b cells is greater than progenitor activity for B-1a cells in adult bone marrow.

Self-replenishment

Previous studies have shown that transfers of peritoneal B-1 cells fully reconstitute B-1 populations in irradiated recipients (20). Here we show that splenic B-1 cells, which do not express Mac-1, are also capable of self-replenishment and express Mac-1 when they localize to the recipient peritoneum (Fig. 4).

Recent studies, in which conventional (lymph node) B cells were shown to persist in SCID recipients (26), have raised questions as to whether the capacity for self-replenishment distinguishes B-1 cells from conventional B cells. Our results are in general agreement with data from the SCID transfers. However, by contrasting the fate of B-1 and conventional cells in the same type of (irradiated) recipients, we demonstrate that the number of B-1 cells in the recipients substantially *increases* (at least 3- to 5-fold) in comparison with the number of cells injected whereas the number of conventional B cells *decreases* (as much as 3- to 5-fold) (Fig. 5). The differences in expansion, while not overwhelming like the expansion of transferred stem cells, verify the characterization of B-1 cells as uniquely self-replenishing.

The maintenance of *in situ* B-1 populations by self-replenishment is consistent with the extensive expansion of

B-1 population in the transfer recipients. Data from BrdU incorporation studies with total PerC B cells (47,48) and with histologically identified B-1a cells (49) demonstrate that B-1 cells turnover at ~1% per day. The rate at which B-1 cells divide, as judged by the rate at which they reconstitute adoptive recipients, is consistent with this figure. Conventional B cells in normal animals have a similar turnover rate (~1% per day) (26,47,48); however, they turnover considerably more slowly in SCID recipients (26). Furthermore, as we and Sprent *et al.* have shown, they do not increase their numbers in adoptive recipients (in fact, they decrease with time). Thus, unlike B-1 cells, conventional B cells require *de novo* development to maintain their numbers in normal animals.

De novo generation from bone marrow progenitors

Data presented here contrast the progenitor characteristics of B-1a, B-1b and conventional B cells. In keeping with earlier findings (1,9,22), we show that progenitors in adult bone marrow reconstitute conventional B cells completely, B-1b cells moderately well and B-1a cells poorly (Fig. 2). Fetal liver, in contrast, reconstitutes B-1a cells much better in these same recipients (9). $B220^-$ progenitors (which include stem cells) are entirely responsible for this long-term B cell reconstitution by bone marrow (Fig. 3). $B220^+$ bone marrow, which includes cells ranging in development from pro-B cells to mature conventional B cells, does not contribute significantly to the long-term reconstitution in these recipients (Table 1). Qualitatively equivalent $B220^-$ progenitors are present in adult spleen (which also contains self-replenishing B-1a and B-b cells).

Bone marrow usually reconstitutes a small number of B-1a cells (on average <10% their level in normal controls). This low level B-1a cell reconstitution might have been due wholly or in part to rare self-replenishing B-1a cells located in the bone marrow. However, as we have shown, transfers of the $B220^+$ bone marrow cell population, which should include any mature B-1a cells that are present, do not yield detectable numbers of B-1a cells. Transfers of FACS-sorted $B220^-$ bone marrow (progenitor) cells, in contrast, yield levels of B-1a reconstitution similar to unsorted bone marrow cells. These findings demonstrate that a small number of B-1a progenitors survive into adulthood and are revealed when bone marrow cells are transferred to adoptive recipients.

Other groups report substantially higher levels of B-1a cell reconstitution from bone marrow than we obtain; however, as we have discussed elsewhere (24), some of these studies appear to have technical flaws (50,51) and others are too incomplete to fully evaluate (44,45,52). Nevertheless, as the recent experiments by Elliot suggest (53), an appropriate combination of environmental factors in recipients, e.g. age, levels of particular cell types and levels of cytokines such as IL-10 or IFN-γ (54), might selectively increase the number of B-1a cells reconstituted, particularly if these environmental conditions affect the yield per progenitor.

Properly controlling these kinds of experiments requires comparison of the relative levels of B-1a, B-1b and conventional B cell reconstitution obtained when various progenitor sources are transferred (singly and, ideally, in combination) into appropriate environments. The fetal liver–bone marrow mixture-transfer studies that we have completed with irradiated recipients (55) are controlled in this way and provide a basis for comparing B cell progenitor activities. The transfers of FACS-sorted pro-B cells into SCID mice that Hardy and Hayakawa have reported (16), which are similarly well controlled, agree with our findings. Thus, we conclude that B-1a progenitors are rare in adult bone marrow, but can function in irradiated recipients.

B-1b progenitors, in contrast, are readily detectable in $B220^-$ adult bone marrow. Bone marrow transfers routinely yield cells with the B-1b phenotype which, on average, reach half the normal B-1b frequency 8 weeks after transfer and persist at this level or increase slightly in the months that follow. Are these cells actually B-1b cells, or are they conventional B cells that mimic the B-1b phenotype? Our previous studies show (i) that the phenotype of the B-1b cells, as defined by four-color FACS analysis with a variety of surface markers, is identical to the phenotype of the B-1b cells in normal animals (9,23); and (ii) that the anatomical localization of the bone marrow reconstituted B-1b cells is indistinguishable from the localization of the normal B-1b cells. Sequential-transfer experiments presented here add that the self-replenishment capability of the bone marrow-derived B-1b cells is essentially the same as the self-replenishment capability of native peritoneal B-1b cells. Thus, by three key criteria (phenotype, localization, self-replenishment), data now demonstrate that the B-1b cells reconstituted *de novo* from progenitors in bone-marrow transfers are equivalent to native B-1b cells.

The B-1 progenitor activity observed in bone marrow transfers also appears to function at low levels in normal animals since some B-1 cells are found in adult Igh-C-allotype heterozygotes in which B-1 cells of one allotype were depleted by treatment with allotype-specific anti-μ during the first 6 weeks of life (Fig. 7). The *de novo* generation of B-1b cells during adulthood appears greater than the generation of B-1a cells; however, precise quantification of B-1a and B-1b frequencies is difficult with the small numbers of cells involved. This low level *de novo* B-1b development during adulthood is consistent with the increase in the proportion of B-1b cells that occurs with age within the overall B-1 population (~3-fold from 1 to 6 months of age; ref. 23). The relative increase in B-1b representation could also be due to preferential expansion (self-replenishment) of mature B-1b cells. However, data from numerous transfers of mature peritoneal B-1 cells show that B-1a and B-1b cells self-replenish in proportion to their representation in the donor pool and thus suggest that *de novo* generation of B-1b cells explains the increase in this population with age.

The question of whether B-1b cells constitute a lineage that is distinct from both the B-1a and conventional B cell lineages is still open. Our findings support this hypothesis; however, one could also reasonably believe a model in which cells with the B-1b cell phenotype arise from two different progenitors: one that is primarily active early in ontogeny and gives rise to B-1a and B-1b cells, but not to conventional B cells; and one that becomes active as the animals mature and gives rise to B-1b and conventional B cells, but not to B-1a cells (9,24). Only the identification and isolation of independent progenitors for B-1a and B-1b cells will resolve this issue.

On the other hand, B-1a progenitors are clearly distinct from conventional B cell progenitors and have been separated from them according to anatomical, ontological and phenotypic differences (24). However, there is still some question as to whether cells with the B-1a phenotype can develop from conventional B cells. Wortis and colleagues report that CD5 expression and other characteristics of the B-1a phenotype can be induced *in vitro* by co-stimulation with anti-IgM and certain cytokines (56). This result has led them and others (57) to suggest that B-1a cells arise from conventional (IgD^{hi}) B cells following T cell independent (type II) stimulation. These *in vitro* findings may reveal an inherent potential in conventional B cells; however, if so, this potential does not appear to be manifest *in vivo*. The minimal reconstitution of B-1 cells obtained in transfers of mature (IgM^{+}) spleen cells is readily explained by the self-replenishment of the splenic B-1a cells. It is unlikely to come from conversion of conventional B cells since B-1a cells are not reconstituted from adoptively transferred lymph node cells, which contain few (if any) B-1a cells. Furthermore, there is poor recovery of B-1a cells following neonatal anti-μ treatment. Whereas conventional B cells recovery fully, with a half-life of 2–3 months, B-1a cells recover minimally, with a half-life >1 year. In conclusion, we have not yet found any evidence that conventional B cells routinely convert to B-1a cells *in vivo*.

The separation of the B cell lineages led us to propose, some time ago, that the immune system has evolved a series of layers in which stem cells that give rise to evolutionary primitive lymphoid cells (such as those in the B-1a lineage) have remained functional despite the later evolution of stem cells giving rise to lymphoid components with more highly evolved functions (1,10,58). This hypothesis, which is supported by data from T cell, erythrocyte and myeloid development studies, is discussed in detail elsewhere (9,24,58,59). The results presented here are consistent with the layered immune system model.

Acknowledgments

We thank O. Herman for technical assistance, K. Seidl and Len Herzenberg for reading the manuscript, and the Stanford Shared FACS Facility for technical support. This work is supported by NIH grants HD-01287 and LM-04836 to L. H., and NRSA awards AI-07937 and AI-07290 to A. B. K. A. M. S. is a Irma T. Hershill Scholar and is supported by ACS JFRA-338.

Abbreviation

APC allophycocyanin

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