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Characteristics and Development of the Murine B-1b (Ly-1 B Sister) Cell Population

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The classic adoptive transfer studies of Hayakawa and Hardy first demonstrated the existence of two distinct lineages of B cells: conventional B cells (also designated B-2 cells) and Ly-1/CD5$^+$ B cells (now designated B-1a cells).[1] We have described a third distinct population of B cells previously referred to as CD5$^-$ Ly-1 B cells or the "Ly-1 B sister" population and now designated B-1b cells.[2,3] In this paper we review the studies that have defined this population. We further phenotype these cells and present adoptive transfer experiments that show that B-1a and B-1b cells are in fact developmentally distinct lineages. The data show that with respect to phenotype, tissue distribution, function, and development B-1b cells are closely related to the classic B-1a cells and are equally distinct from conventional B cells. The two populations differ with respect to their temporal appearance and loss of progenitor activity from B220$^-$ cells. These relationships form the basis for the designation of three mature B-cell lineages: B-1a, B-1b, and conventional B cells.

PHENOTYPE OF B-1b CELLS

Multicolor FACS analysis of Ly-1$^+$ B cells show that, in addition to the unique expression of the CD5 antigen, B-1 cells are characterized by the distinctive expression of a variety of cell-surface antigens when compared to conventional B cells (FIGURES 1-4). B-1 lineage cells express high levels of IgM, low levels of IgD, low levels of B220 (when measured with the monoclonal antibody RA3-6B2),[4,5] and are negative for CD23 ($Fc_\epsilon R$).[6] The other distinctive feature of B-1 cells is the expression of CD11b (Mac-1, C3bR).[7] Interestingly, this antigen is expressed on B-1 cells present in the peritoneal or pleural cavities but is absent from splenic B-1 cells (FIGURES 3 and 4). No single antigenic marker is sufficient to identify B-1 cells. It is the surface phenotype *in toto* and the developmental characteristics that currently best define cells of the B-1 lineage.

The phenotypic resolution of the B-1 lineage into two subpopulations was made possible by the development of three- and four-color FACS analyses using new (and

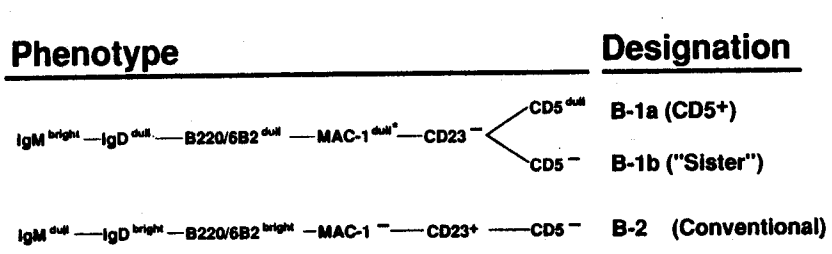

FIGURE 1. Surface phenotype of B-cell populations. See text for explanation.

more importantly, brighter) fluorochromes, such as allophycocyanin (APC). In the early studies it was not possible to fully resolve the $CD5^+$ from $CD5^-$ B cells. It was possible that all IgM^{bright}, IgD^{dull} cells might be $CD5^+$ but not bright enough to be seen as positive with the reagents available. The first indication that the B-1 lineage was not a homogeneous population came from analyses of peritoneal B-1 cells such as those shown in FIGURE 2. These analyses show that all IgM^{bright}, IgD^{dull} cells were in fact $CD11b^+$ and that this population contained resolvable $CD5^+$ and $CD5^-$ B cells.

At a minimum, one three-color staining (*e.g.*, IgM, IgD, and CD5) or two two-color stainings (*e.g.*, IgM, IgD and IgM, CD5) are needed to measure all three populations in the peritoneum: B-1a, B-1b, and conventional B cells. In the spleen the situation is more problematic. For example, splenic marginal-zone B cells and immature B cells share many phenotypic characteristics with B-1b cells, such as high levels of IgM, low levels of IgD, and no CD23 or CD5.

FIGURES 3 and 4 show the phenotype of peritoneal and splenic B cells specific for phosphatidylcholine (PtC). PtC-liposome-binding cells are equivalent to plaque-forming cells (PFC) specific for bromelain-treated mouse red blood cells (BrMRBC)[8,9] and use a restricted set of V genes.[10,11] Transfer experiments have shown that PtC-liposome-binding cells are essentially all derived from B-1 lineage cells[12] and are thus a good population for further characterizing B-1 cells. This is particularly important for splenic analyses where B-1 cells constitute only a small percentage of the B lymphocytes and are essentially impossible to cleanly identify. PtC-liposomes, which have a fluorescein derivative encapsulated in their lumen, cleanly reveal this population of B cells. In the peritoneum, the PtC-liposome binders show the classic B-1 phenotype: IgM^+, IgD^{dull}, and $B220/6B2^{dull}$. Although most of the PtC-liposome binders are $CD5^+$, a distinct population is $CD5^-$, as emphasized after gating on PtC binders (FIG. 3). In addition, the PtC-liposome binders in the peritoneum express the CD11b antigen.

Because splenic B-1 cells comprise only a small percentage of the lymphocytes, the PtC-binding subset is not revealed in the typical 5% probability contour plots. Logarithmic contour plots[13] are displayed in order to reveal this small population (0.2% of lymphocytes) of PtC-liposome-binding cells. The phenotype of the splenic B-1 cells is identical to those in the peritoneum with one major exception: the expression of CD11b (MAC-1) (FIG. 4). Neither B-1a nor B-1b cells express CD11b in the spleen. CD11b is a marker of B-1 lineage cells only in the peritoneal and pleural cavities. This lack of CD11b expression severely complicates the general identification of B-1b cells

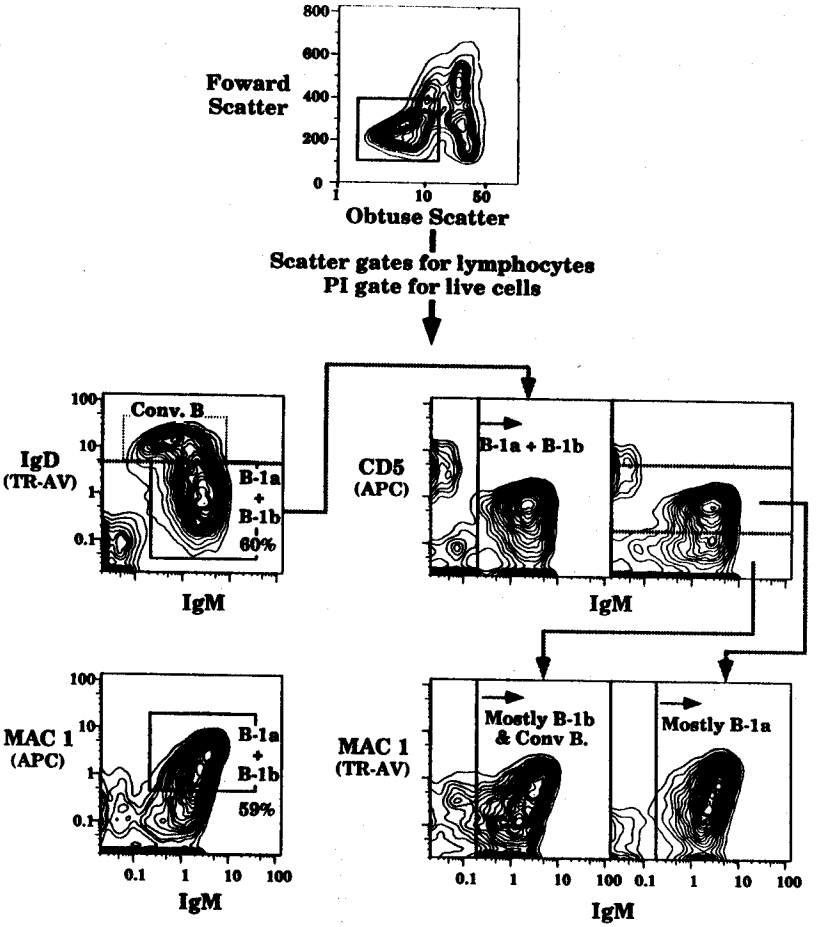

FIGURE 2. Six-parameter FACS analysis of BALB/c peritoneal B cells. B-1a, B-1b, and conventional B cells are identified by their FACS phenotype. After gating on live lymphocytes (as determined by forward and obtuse scatter and propidium iodide), conventional B cells are identified by a broad, positive IgM and tight, bright IgD FACS profile (IgM^{+} IgD^{bright}). All B-1 cells are bright for IgM and dull to moderate for IgD (IgM^{bright}, IgD^{dull}). Gating for IgD^{dull} cells better reveals the B-1a ($CD5^{+}$) and B-1b ($CD5^{-}$) cells. Back-gating on CD5 indicates that both B-1a and B-1b cells are Mac-1^{+} (note: the Mac-1 TR stain is duller than the APC stain). As indicated, gating on the IgM-IgD plot and the IgM-Mac-1 plot yields comparable values. In this mouse 44% of the lymphocytes are B-1a cells. For analysis, the number of B-1a cells is subtracted from the total number of IgM^{bright}, IgD^{dull} B-1 cells to obtain the number B-1b B cells. Direct gating on the $CD5^{-}$, IgM^{bright} cells is avoided because of potential overlap of the population with conventional B cells (see text).

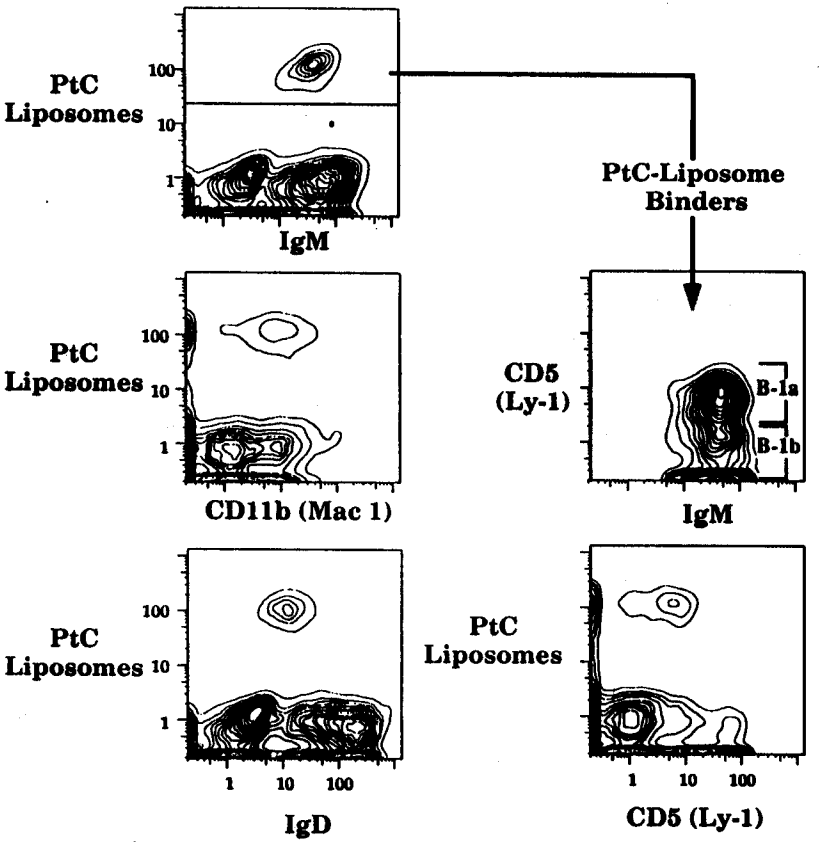

FIGURE 3. Phenotype of PtC-liposome binders in the peritoneal cavity. Cells obtained from a CBA/Ca mouse were stained with fluorescein-PtC-liposomes and a combination of the following stains: monoclonal antibodies, bi-anti-IgM (331); bi-anti-IgD (AMS 9.1); bi-anti-CD11b (M1/70); and APC-anti-CD5 (53-7). The CD5-IgM plot is gated to show only PtC-binding cells. See text for explanation.

in the spleen. As noted above, immature or marginal-zone B cells share similar patterns of IgM, IgD, and CD23 expression with B-1 cells. Thus on a purely phenotypic basis splenic B-1b cells cannot be resolved from immature or marginal-zone B cells. Functional tests, such as specificity for PtC or adoptive-transfer experiments, must be used to identify splenic B-1b cells.

B-1b CELLS ADOPTIVELY TRANSFER

As stated above, the B-1 cells are not only defined by their phenotype but also (and more importantly) by their unique developmental characteristics. One of the hallmarks

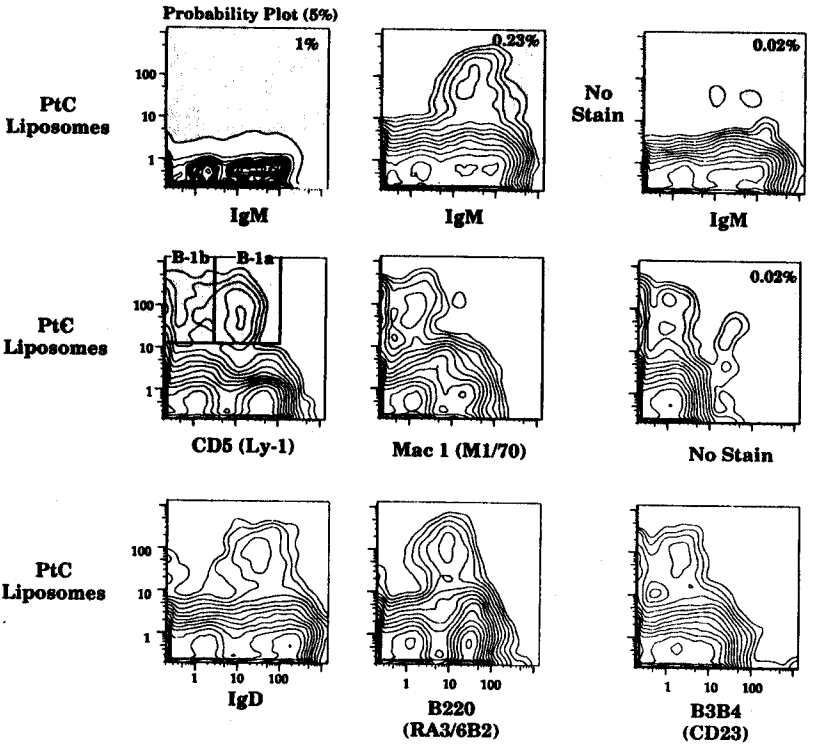

FIGURE 4. Detection and phenotype of rare PtC liposome binders in the spleen. Cells are from a CBA/Ca spleen. PtC-liposome binders, which constitute < 1% of the splenic lymphocytes, are not revealed in the standard 5% probability contour plot (upper left). Logarithmic contour plots (50%), which readily reveal these antigen-specific B cells, are shown in the remaining seven plots. Their presence at 0.2% of the lymphocytes is 10 times greater than the background stains (upper right plots). Cells were stained as in FIG. 3 and with bi-anti-CD23 (FcεR) and bi-anti-B220 (RA3-6B2).

of the B-1 lineage is the ability of mature Ig^+ cells to reconstitute the peritoneal B-1 population in irradiated recipients.[14] Similar adoptive-transfer experiments demonstrated that B-1a and B-1b cells have equal capacity for self-replenishment.[3] In these experiments CBA/bb (Igh^b) PerC were sorted into $CD5^+$ and $CD5^-$ fractions and transferred with CBA/N bone marrow into irradiated CBA/N (Igh^a) recipients, which were analyzed for donor Igh^b cells at 2 and 5 months post-transfer. Although both populations gave rise to IgM^{bright}, IgD^{dull}, B220/6B2^{dull}, Mac-1^+ cells characteristic of B-1 cells, each population reconstituted itself with respect to its CD5 phenotype. $CD5^+$ cells gave rise to $CD5^+$ cells, whereas the $CD5^-$ cells reconstituted $CD5^-$ cells. Thus in the adult B-1a and B-1b, cells are not simply two forms of the same cell population in equilibrium with each other; rather, they are developmentally distinct populations.

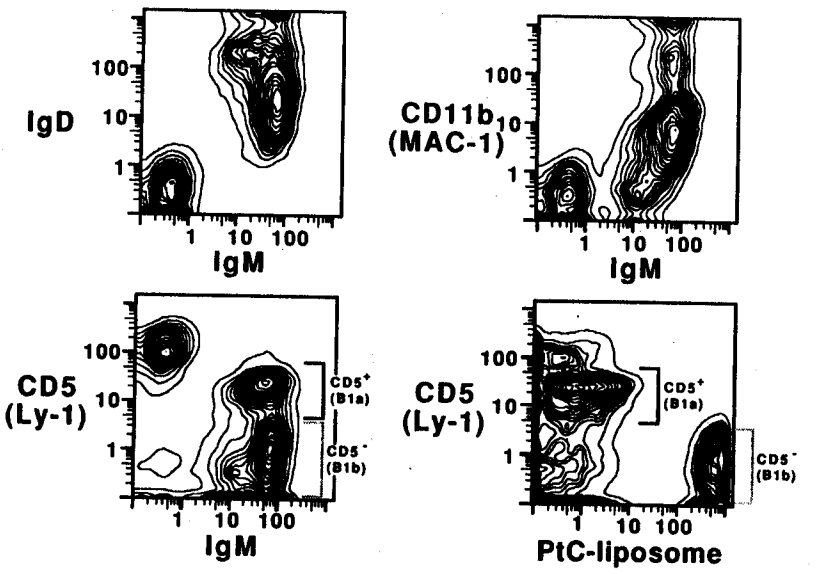

FIGURE 5. Clonal populations are derived from both B-1a and B-1b cells. Peritoneal cells from a 24-month-old CBA/Ca mouse were analyzed by FACS. Cells were stained with fluorescein(fl)-PtC-liposomes and a combination of the following fluorochrome-conjugated monoclonal antibodies: fl-anti-IgM (331); biotin(bi)-anti-IgD (AMS 9); bi-anti-CD11b (M1/70); and APC-anti-CD5 (53-7). Biotin-labeled antibodies were revealed with Texas Red-avidin. See text.

B-1b CELLS DEVELOP CLONAL POPULATIONS

A unique feature in the ontogeny of B-1 cells is the development of B-CLL-like clonal populations. We have shown that in every mouse strain studied B-1 cells inevitably give rise to oligoclonal and eventually monoclonal populations that are detectable by FACS as phenotypically homogeneous populations and by Southern analysis by a restricted set of VDJ rearrangements.[15] Again, this property is found in both B-1a and B-1b cells. FIGURE 5 shows the FACS analysis of a peritoneal washout of a two-year-old CBA/Ca in which clonal populations are clearly evident. With respect to IgM, IgD, and Mac-1 expression, it is clear that this animal has a clonal population with a typical B-1 cell phenotype. Staining with PtC-liposomes and CD5 reveals the presence of two distinct clones: a B-1b clone with specificity for PtC and a B-1a clone of unknown specificity.

The propensity for B-1a and B-1b cells to produce clones appears to be essentially equivalent. A survey of clonal populations in NZB $\times$ NZW (B/W), BALB/c, and CBA/Ca mice showed a correlation in the characteristic B-1a/B-1b ratio of the strain and the relative numbers of B-1a and B-1b clones. That is, in B/W mice in which $< 5\%$ of the B-1 cells are B-1b, no B-1b clones have been detected in over 100 animals examined. By contrast, in CBA mice in which 45-55% of the peritoneal B-1 cells are

B-1b, 30% of the observed clones were B-1b, and in BALB/c (20-30% B-1b) 10% of the clones were B-1b. Thus, those factors that drive the generation of clones operate on both B-1a and B-1b cells.

FEEDBACK REGULATION OF B-1b CELLS

Lalor *et al.*, in a series of studies involving neonatal anti-IgM treatment of $Igh^{b/b}$ homozygous and $Igh^{a/b}$ heterozygous mice, showed that the presence of mature B-1 cells could suppress the development of new B-1 cells from progenitors.[3,16] This feedback regulation of B-1 cell development was best demonstrated in adoptive transfer of anti-IgM-suppressed mice. B cells were temporarily depleted from neonatal C.B-17 mice (Igh^b) by injection with anti-IgM^b for the first four weeks of life. When the treatment antibody disappeared and the mice recovered, they developed both B-1a and B-1b cells in addition to conventional B cells. If sorted peritoneal B-1a cells or a B-1a clone was injected into the treated mice, however, endogenous B-1 cell development was completely inhibited, whereas conventional B-cell development was unaffected for at least eight months. This feedback suppression was equally effective on B-1a and B-1b cells. Although it is not known whether B-1b cells are equally capable of inducing the feedback suppression *in vivo*, both populations are regulated by the presence of mature B-1a cells.

DEVELOPMENT OF B-1b CELLS FROM PROGENITORS

The ontological relationship between B-1a and B-1b cells is dramatically demonstrated in the experiments of Solvansen *et al.*[17] They showed that day-13 fetal omentum cells give rise to B-1a and B-1b cells, but not conventional B cells. Thus, progenitors of B-1 (B-1a and B-1b) lineage cells can be physically separated from conventional B cells. We have also shown the existence of independent B-cell progenitors by the cotransfer of 14-day fetal liver (BAB, Igh b-allotype) and adult bone marrow (BALB, a-allotype) into irradiated recipients. Although fetal liver is capable of reconstituting both B-1 cells and conventional B cells, the development of these B-cell lineages from progenitors was shown to proceed independently. In some cotransfer recipients, all conventional B cells were derived from the bone marrow source. The fetal liver reconstituted B-1 cells in these recipients but failed to reconstitute conventional B cells.[16a]

The most striking difference between B-1a and B-1b cells is seen in the development of the two populations from Ig^- precursors in the adult. The first evidence for this difference came from the studies of Lalor *et al.*[3] in which neonatal mice were treated with anti-IgM for four weeks. This treatment eliminated all B-1 and conventional B cells during the first four weeks of life, at which point the animals were released from suppression and allowed to generate new B cells. In the absence of any feedback suppression from mature B-1 cells, as described above, the mice produced B-1 cells (presumably from Ig^- precursors in the bone marrow). In contrast to control mice, however, the majority of the B-1 cells in the suppressed mice five months following release were B-1b (TABLE 1). This indicates that by as early as four weeks of age progenitor capacity for B-1a cells is preferentially lost in comparison to B-1b cells.

Recently we have examined in detail the generation of B-1a and B-1b cells in reconstitutions using fetal liver and adult bone marrow.[16a] These experiments clearly demonstrate that progenitors of B-1a and B-1b cells can be distinguished by the fact that progenitor activity for B-1b but not B-1a cells is present in adult bone marrow. These experiments are analogous to the first transfer experiments of Hayakawa *et al.*

TABLE 1. B-1 Lineage Cells Recovering following Neonatal Anti-IgM Treatment Are Predominantly B-1b Cells[a]

Animals	Antibody[b] treatment	Peritoneal cells from 8-month-old mice			
		Total cells	Conventional B lineage	B-1 lineage	
				B-1a	B-1b
		$\times 10^6$	(Percent)	(Percent)	(Percent)
C.B-17	None	9.5	16	34	16
C.B-17	Anti-Igh-6b	4.0	23	17	30

[a] Data from Lalor *et al.*[3] In that paper the values for B-1a and B-1b, no treatment, were switched in the table. This table corrects that printing error.

[b] Mice were injected with 2 mg of anti-Igh-6b (IgM^b) during the first 4 weeks of life.

showing that bone marrow could reconstitute conventional B cells but not B-1a cells.[1] FIGURE 6 shows a comparative FACS analysis of recipients reconstituted with day-14 fetal liver and adult bone marrow. In the animal reconstituted with day-14 fetal liver, both B-1a and B-1b cells are generated. Moreover, the ratio of B-1a to B-1b (~2:1) cells is similar to control animals, which also favor B-1a over B-1b cells (~3:1). In striking contrast, the majority of B-1 cells in adult bone marrow recipients are B-1 cells, with few B-1a cells present in the peritoneum. Cotransfers of fetal liver and adult bone marrow prove that there are no accessory cells in the bone marrow that block B-1a cell development from the fetal liver; likewise, fetal liver does not enhance bone marrow reconstitution of B-1a cells.

FIGURE 7 shows the cumulative results from a series of transfers. These data show that day-13 or -14 fetal liver is able to fully reconstitute the B-1b cells, whereas B-1a cells are reconstituted to about half of normal levels. By contrast, adult bone marrow yields < 10% of the normal level of B-1a cells but reconstitutes 50% of the normal levels of B-1b cells. In all animals the conventional B cells are fully reconstituted.

In additional transfers of FACS-sorted populations, we observe that the $B220^-$ fraction of adult bone marrow reconstitutes the B-1a, B-1b, and conventional B cells the same as unfractionated bone marrow; $B220^+$ cells do not contribute to the long-term (> 8 weeks) reconstitution from adult bone marrow. Previous transfers with day-1 Ig^- neonatal spleen cells showed an intermediate level of reconstitution, with more B-1b (15%) than B-1a (8%) cells generated. Ig^+ cells from neonatal spleen preferentially reconstitute B-1a cells over B-1b cells, suggesting that B-1a cells are more prevalent in the neonate.[16]

What emerges is a model of three lineages of B cells, each arising from distinct progenitors at sequential, overlapping times during ontogeny. B-1a cells arise first and B-1a progenitor activity starts to shut down around birth. B-1b progenitors seem to arise second and function into adulthood. Conventional B cells arise last and are generated *de novo* from progenitors throughout the life of the animal. The loss of progenitor activity is not an instantaneous event but progresses over a period of time. Thus B-1a progenitor activity is diminished in neonatal spleen and is rare in adult bone marrow. Similarly B-1b progenitor activity, normal in fetal liver and neonatal spleen, is present, albeit somewhat diminished, in most adult bone marrow. The fact that the period over which this loss occurs can vary is seen in the variability of bone marrow transfers from animal to animal. In some bone marrow transfers significant numbers of B-1a cells are generated, and on occasion no B-1 cells are produced. We have never

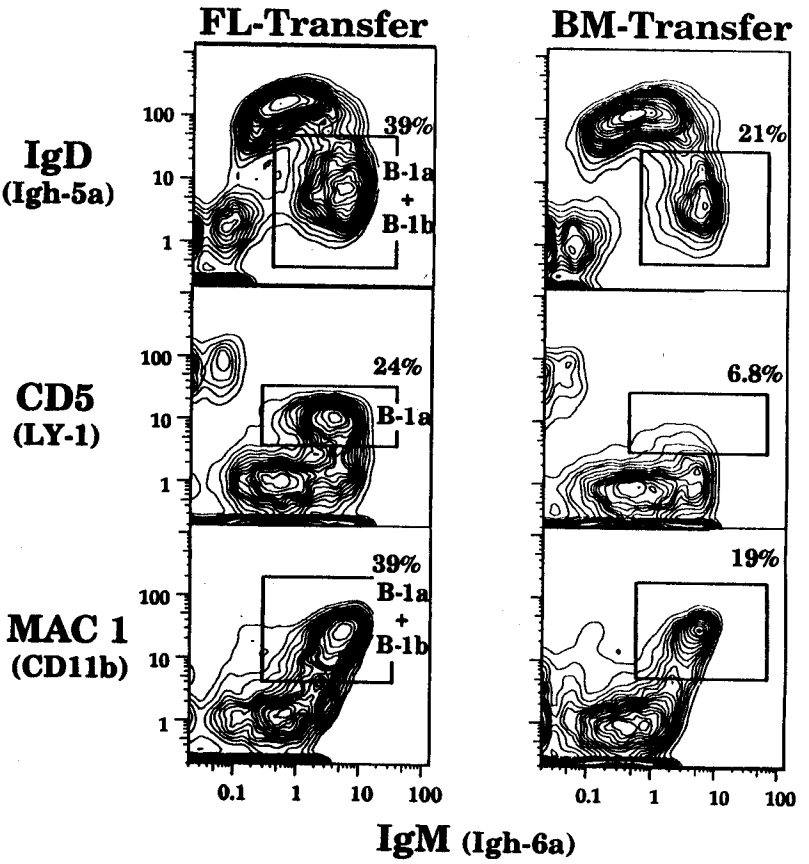

FIGURE 6. Peritoneal B cells from a fetal liver recipient and a bone marrow recipient. Representative plots are shown for BAB (Igh-b) recipients that received 14-day BALB fetal liver (left) or adult BALB bone marrow (right) eight weeks prior to analysis. Allotype-specific anti-δ (IgD) and anti-μ (IgM) reagents are used to identify the donor-derived B cells. The percentages of cells within the boxed (solid line) populations are given per total number of live lymphocytes. All the plots presented here have 5% probability contours.

observed, however, a bone marrow transfer in which B-1a cells cells have been generated in the absence of B-1b cells. Thus, although the time span may vary, B-1a progenitor activity is always lost before B-1b progenitor activity. The factors that regulate the timing of this progressive loss of B-1a and B-1b progenitor activity in a given animal are not known.

SUMMARY

In this paper we have outlined the evidence for two distinct branches of the B-1 cell lineage. The data show that phenotypically B-1a and B-1b cells are essentially

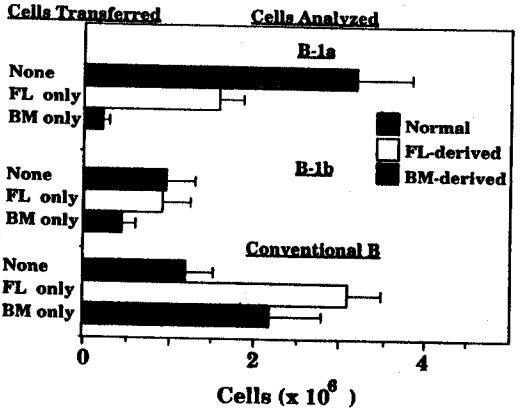

FIGURE 7. Reconstitution of B-cell populations from different progenitor sources. The total number of cells in each B-cell population is shown for normal BALB/c mice, BALB 13- and 14-day fetal liver transfer (no difference observed between the two sources), and adult BALB bone marrow transfers into irradiated BAB recipients. Transfers from BAB donors to BALB recipients yield equivalent results. The average number of cells from 8 to 14 recipients is presented in each bar; error bars are one standard deviation. The mice were analyzed eight weeks following transfer.

identical, distinguished only by the presence or absence of the CD5 antigen. Functionally no differences between the two populations have yet been identified. Both produce anti-PtC antibodies, a specificity not observed in conventional B cells. Both produced high levels of IgM as measured in adoptive transfer experiments.[2] Developmentally, B-1a and B-1b cells are indistinguishable with respect to generation from progenitors present in fetal liver and omentum,[16a,17,18] feedback regulation of new B-1a and B-1b cells from bone marrow,[3,16] self-replenishment from Ig^+ cells following adoptive transfer,[2] and the generation of clonal populations.[15] The major difference in the two populations is seen in the development of B-1a and B-1b cells from $B220^-$ progenitors in the adult bone marrow. Although $B220^-$ B-1a progenitors are rare in adult (> 6 weeks) bone marrow, the progenitors for B-1b cells persist well into adulthood.

Our understanding of B-1b cell ontogeny is at a stage similar to that of B-1a cells five years ago. We have evidence from transfer experiments that strongly suggests the existence of two distinct progenitors for B-1a and B-1b, but we have yet to physically separate these progenitors as Solvansen *et al.* have done for B-1 and conventional B cells. Furthermore we must determine whether the B-1b cells that develop from fetal liver and bone marrow are functionally and developmentally equivalent to those that develop from adult bone marrow.

As with B-1a cells, the role of B-1b cells in the immune system is unclear. Although we have not yet discerned functional differences between B-1a and B-1b, given the recent identification of CD72 (Lyb-2) as the ligand for CD5,[19] it is tempting to speculate that B-1a cells are more involved in B-B cell interactions such as idiotype-anti-idiotype regulation of the early B-cell repertoire and that B-1b cells are more involved in B-T cell interactions. Whatever their function, it is clear that in trying to understand the role of the B-1 lineage it is important to consider both the B-1a and B-1b lineages.

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