B-1 Cell Development: Evidence for an Uncommitted Immunoglobulin (Ig)M+ B Cell Precursor in B-1 Cell Differentiation

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Summary

Murine phosphatidyl choline (PtC)-specific B cells in normal mice belong exclusively to the B-1 subset. Analysis of anti-PtC (V_{\text{H}12} and V_{\text{H}12}/V_{\text{k}4}) transgenic (Tg) mice indicates that exclusion from B-0 (also known as B-2) occurs after immunoglobulin gene rearrangement. This predicts that PtC-specific B-0 cells are generated, but subsequently eliminated by either apoptosis or differentiation to B-1. To investigate the mechanism of exclusion, PtC-specific B cell differentiation was examined in mice expressing the X-linked immunodeficiency (xid) mutation. xid mice lack functional Bruton's tyrosine kinase (Btk), a component of the B cell receptor signal transduction pathway, and are deficient in B-1 cell development. We find in C57BL/6.xid mice that V_{\text{H}12} pre-BII cell selection is normal and that PtC-specific B cells undergo modest clonal expansion. However, the majority of splenic PtC-specific B cells in anti-PtC Tg/xid mice are B-0, rather than B-1 as in their non-xid counterparts. These data indicate that PtC-specific B-0 cell generation precedes segregation as predicted, and that Btk function is required for efficient segregation to B-1. Since xid mice exhibit defective B cell differentiation, not programmed cell death, these data are most consistent with an inability of PtC-specific B-0 cells to convert to B-1 and a single B cell lineage.

A t least two B cell subsets, B-1 and B-2, are present in the mouse periphery (1–4). One of the most intriguing aspects of these subsets is that they exhibit different repertoires (5), presumably reflecting different functions in the immune system. B-2 cells appear to be responsible for T cell-dependent responses to exogenous antigens and for generating memory B cells (4). In contrast, the B-1 subset harbors a high frequency of cells with specificities to self-antigens such as phosphatidyl choline (PtC), immunoglobulin (rheumatoid factor), DNA, as well as specificities to common bacterial carbohydrate antigens like phosphorylcholine (6–10), and may be involved in T cell-independent responses to common environmental antigens.

The distinct B-1 and B-2 repertoires are the consequence of different selective pressures (11, 12), but the nature of these differences is not known. Critical to understanding how B-1 and B-2 repertoires arise is the relationship between the cells of these subsets. The more commonly held view (the lineage hypothesis) is that B-1 and B-2 cells derive from stem cells committed to one or the other subset before Ig gene rearrangement, thereby constituting two separate lineages (13–15). An alternative hypothesis (the induced differentiation hypothesis) is that they derive from a single lineage, and that an uncommitted B cell is induced to differentiate to a B-1 cell after Ig gene rearrangement by interaction with antigen, probably T cell-independent antigens in the absence of T cell help (16, 17). Since by this hypothesis the majority of splenic B cells are uncommitted, they are referred to as B-0 cells. Thus, the B-2 cells of the lineage hypothesis and the B-0 cells of the induced differentiation hypothesis are equivalent and referred to here as B-0. Each hypothesis predicts a different means of arriving at distinct B-1 and B-0 repertoires.

We have focused on the differentiation of B cells specific for the common membrane phospholipid, PtC, as a means to understand the bases for the repertoire differences between B-1 and B-0 cells. In normal mice, PtC-specific B cells appear to be exclusively B-1 (6, 12, 18, 19). They are driven to undergo considerable clonal expansion from birth (20, 21), and in normal adults eventually account for 2–10% of the peritoneal B-1 repertoire (6). Many anti-PtC B cells express V_{\text{H}12} and V_{\text{k}4} rearrangements (11, 22). The V_{\text{H}12} H chain is restricted to CDR3s of 10 amino acids with Gly in the fourth position and Tyr, encoded by the

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Abbreviations used in this paper: Btk, Bruton’s tyrosine kinase;dbl, double; NP, nonproductive; P, productive; PtC, phosphatidyl choline;slgM, surface IgM; Tg, transgenic.

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first codon of J_{H}1, in the fifth position (referred to as 10/G4) (11). Selection for B cells of the appropriate gene rearrangements occurs at two stages during B cell development. The first results in the elimination of most non-10/G4 arrangements occurs at two stages during B cell develop-

ment. The second stage of selection is at the B cell stage where 10/G4 V_{H}12 B cells that express the appropriate V_{K}x4 L chain and bind PtC undergo antigen-driven clonal expansion in response to some ubiquitous environmental or self-antigen (24).

To understand the basis for the segregation of PtC-specific B cells to the B-1 subset, we have generated anti-PtC transgenic (Tg) mice using the V_{H}12 and V_{K}x4 gene rearrangements of the anti-PtC lymphoma CH27 (21, 22). We demonstrate here that in V_{H}12 Tg mice have been described previously (21). Double (dbl) Tg mice are generated by the inter-

crossing of 6-1 and V_{H}12 pre-BII cells and in the enrichment of 10/G4 V_{H}12 pre-B cells (23). This is probably due to positive selection of 10/G4 pre-BII cells and an absence of positive selection of non-10/G4 pre-BII cells resulting in the death of the latter. The second stage of selection is at the B cell stage where 10/G4 V_{H}12 B cells that express the appropriate V_{K}x4 L chain and bind PtC undergo antigen-driven clonal expansion in response to some ubiquitous environment or self-antigen (24).

To distinguish between these possibilities, we have combined the V_{H}12 and V_{K}x4 Tgs (21) with the xid mutation (25, 26). This mutation abolishes function of Bruton’s tyrosine kinase (Btk) (27–30), resulting in deficiencies in B cell differenti-
tation (31–33) and responsiveness to T cell-independent type II antigens (26). This mutation also blocks development of a detectable peritoneal B-1 population in CBA/N mice (34). Analysis of the cellular defect indicates that signaling through surface IgM (sgM) fails to drive xid B cells into cell cycle (35, 36). xid B cells do not appear to be deficient in induction of programmed cell death (36, 37). We demonstrate here that in xid mice, V_{H}12 pre-B cell selection is normal and that PtC-specific B cells un-
dergo modest clonal expansion. However, combining the V_{H}12 and V_{K}x4 Tgs with xid, we demonstrate that the majority of splenic PtC-specific B cells fail to segregate to the B-1 subset and instead have a B-0 phenotype. These data argue that B-0 cells are an intermediate step in B-1 cell differenti-
tion, consistent with the induced differentiation hypothesis and a single lineage of B cells.

Materials and Methods

Mice: V_{H}12 (6-1) and V_{K}x4 Tg mice have been described pre-

ciously (21) and are maintained in our colony at the University of North Carolina by backcrossing to C.B17 mice. Offspring are identified by PCR analysis of tail genomic DNA as previously described (21). Double (dbl) Tg mice are generated by the inter-

crossing of 6-1 and V_{K}x4 Tg mice. C57BL/6 xid (B6/xid) mice were obtained from the National Institutes of Health (Bethesda, M D) and bred separately with the V_{H}12 and V_{K}x4 Tg mice to ob-
tain V_{H}12 and V_{K}x4 Tg-xid/xid mice. These mice were inter-
crossed to obtain V_{H}12/V_{K}x4 dbl Tg/xid mice.
P/N P is measured to be <0.05 in the absence of clonal expansion (23).

To measure the 10/G4 and non-10/G4 P/NPs in xid mice, $V_{H12}$-D-JH1 rearrangements from genomic DNA of bone marrow, spleen, and peritoneal cells were PCR amplified. The amplified DNA was cloned, and clones were randomly selected for sequencing. As shown in Fig. 1 and Table 1, the majority of P rearrangements in the bone marrow are non-10/G4 (5 out of 8) and the non-10/G4 P/NP is 0.18, not different from that of wild-type mice, and significantly lower than the expected value of $\sim 2.3$. This value is equally low in spleen and peritoneum. Thus, as in wild-type mice (24), non-10/G4 rearrangements contribute little to the central and peripheral repertoires, indicating that pre-B cell selection is unaffected by the xid mutation.

Clonal expansion of 10/G4 B cells occurs in xid mice, but to a lesser extent than in wild-type mice. Three out of eight $V_{H12}$ rearrangements in the bone marrow are 10/G4, and the 10/G4 P/NP (0.11) is one-third that seen in wild-type mice (Fig. 1 and Table 1). However, the frequency of 10/G4 P rearrangements is higher than that observed in the absence of selection and clonal expansion (0 of 22 in $\mu$MT mice) (23), indicating that 10/G4 P rearrangements are enriched in xid bone marrow. Enrichment is greater in the spleen and particularly the peritoneum.

Table 1. $V_{H12}$ P/N P Values for B6/xid Bone Marrow, Spleen, and Peritoneum*

|                     | P (10/G4) | N P | 10/G4 | N on-10/G4 | 10/G4 | N on-10/G4 |
|---------------------|-----------|-----|-------|------------|-------|------------|
| Bone marrow         |           |     |       |            |       |            |
| Mouse 1             | 5 (2)     | 6   | 0.33  | 0.50       | 0.11  | 0.18       |
| Mouse 2             | 3 (1)     | 22  | 0.05  | 0.091      |       |            |
| Spleen              |           |     |       |            |       |            |
| Mouse 1             | 4 (4)     | 19  | 0.21  | 0          | 0.29  | 0.029      |
| Mouse 2             | 7 (6)     | 15  | 0.40  | 0.067      |       |            |
| Peritoneum          |           |     |       |            |       |            |
| Mouse 2             | 10 (10)   | 2   | 5     | 0          | 10 (32)| 0.50       |
| Mouse 3             | 11 (10)   | 0   |       |            |       |            |

*All P rearrangement data were taken from Fig. 1.

†The total number of P rearrangements is followed in parentheses by the number of P rearrangements that are 10/G4.

‡The 10/G4 and non-10/G4 P/NP values for wild-type mice are given in parentheses and are taken from studies by Ye et al. (23, 24).

§No non-10/G4 P rearrangements were observed in the peritonea of wild-type mice and therefore a non-10/G4 P/NP could not be calculated.
cells are defined as CD43 cell surface markers that distinguish B-1 and B-0 cells. B-1
model. B cells were stained for expression of a number of genes, since these transgenes exert a strong positive influ-
ence on B-1 cell development (21). We find that 6-1/Jg/Jg mice have approximately one-fourth the number of splenic
PtC-specific B cells in Tg/xid mice. PtC-specific cells can be detected by flow cytometry using as a probe fluorescein-encapsulating liposomes that contain PtC as a membrane constituent (6). As previously
published (21), all of the B-1 cells of 6-1 and dbl Tg mice bind liposomes (CD23+, CD43+, cells in Fig. 2 C).

where almost every P rearrangement is 10/G4. The 10/G4 P/NP is smaller than that observed in wild-type mice, re-
flecting a more modest clonal expansion. Nevertheless, clonal expansion of 10/G4 Vh12 peripheral B cells, presum-
ably because they are PtC-specific, occurs in B6/xid mice.

Ig Tg/xid Mice Can Develop B-1 Cells. To determine the effect of the xid mutation on B-1 cell development, we
combined the xid mutation with the Vh12 and Vk4 trans-
genomes, since these transgenes exert a strong positive influence on B-1 cell development (21). We find that 6-1/xid mice have approximately one-fourth the number of splenic B cells found in 6-1 mice (Table 2). Thus, as expected (40), the xid mutation limits B cell development in this Tg model. B cells were stained for expression of a number of cell surface markers that distinguish B-1 and B-0 cells. B-1 cells are defined as CD43+, CD23−, B220lo, IgMhi, and they may or may not express CD5. B-0 cells, on the other hand, are defined as CD43−, CD23+, B220hi, and IgMlo, and they never express CD5. Shown in Fig. 2, A and B,

and as we have previously published (21), a large fraction of the splenic B cells in 6-1 and dbl Tg mice are B-1 cells, i.e., CD5+, CD23−, B220lo, and IgM hi. A smaller number of B cells in these Tg mice are B-0, i.e., CD5−, CD23+, B220hi, and IgMlo. These cells in 6-1 mice are predominantly PtC neg (see below).

In contrast to their non-xid counterparts, B-1 cells in the spleens of Tg/xid mice are consistently a minority. Only 15–25% are B-1 cells with a CD43+, CD23−, B220lo, and IgM hi phenotype (Table 2 and Fig. 3). Most of these cells in 6-1/xid mice are CD5−, whereas most in dbl Tg/xid are CD5+ (follow the B220lo population in Fig. 3 A). The ma-

Figure 2. Analysis of spleen cells from 6-1 and dbl Tg mice. Spleen cells from 6-1 and dbl Tg mice were stained with FITC-conjugated anti-B220, PE-conjugated anti-CD43, and the biotinylated antibody indicated on the right (A and B). For C, cells were stained with carboxyfluore-
secin-encapsulating liposomes, PE-conjugated anti-CD23, and the biotinylated reagent on the left. The biotinylated antibodies were visualized with PE-conjugated streptavidin. 10,000–20,000 cells were analyzed. Percentages are of the B220lo cells.

Spleens Is Impaired. To determine the effect of the xid mutation on the segregation of PtC-specific B cells, we compared the phenotype of PtC-specific B cells from Tg/xid and Tg non-xid mice. PtC-specific cells can be detected by flow cytometry using as a probe fluorescein-encapsulating liposomes that contain PtC as a membrane constituent (6). As previously
published (21), all of the B-1 cells of 6-1 and dbl Tg mice bind liposomes (CD23+, CD43+ cells in Fig. 2 C).

DbI Tg Mice have a liposome-binding population that is intermediate in staining (liposomeint) (Fig. 2 C). These cells have a B-0 phenotype since they are CD23+, IgMlo, and B220hi (Fig. 2 C, and follow the CD23+ population in Fig. 2, A and B). The liposomeint B-0 cells account for 10–30% of the B cells in a dbl Tg spleen. These cells were not previously detected probably because the liposome probe used in this study is much brighter than that used in our earlier study (21). Since these cells likely express the Vh12 and Vk4 Tgs, we conclude that they are liposomeint by virtue of the fact that they are IgMlo. We speculate that they constitute the PtC-specific B-0 cells predicted to undergo either programmed cell death or conversion to B-1 to achieve segregation of this specificity (21). A smaller number of these cells (5.6% in Fig. 2 C) appear to be present in 6-1 mice.

The xid mutation affects the relative proportions of the PtC-specific B-0 and B-1 subsets. The number of liposome
binding cells in 6-1/xid is ~10% of that in 6-1 mice. Although their number is small, the liposome-binding B cells appear to be equally divided between the liposomeint B-0 (CD23+, CD43−) and liposomeint B-1 (CD23−, CD43+) populations (Fig. 3 C). The presence of two subsets of PtC-specific B cells in xid mice is more apparent in dbl Tg/xid mice, since the addition of the Vk4 transgene increases the number of B cells present in the xid spleen fourfold (Table 2 and Fig. 3 C). In these mice, nearly two-thirds of the PtC-specific B cells are liposomeint B-0 cells, as they are

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CD43⁻ and CD23⁺ (Fig. 3 C) and B220hi and IgMlo (follow the CD23⁺ population in Fig. 3, A and B). The remainder of the PtC-specific B cells are liposomebri B-1 cells that are CD43⁻ and CD23⁻ (Fig. 3 C), and B220lo and IgMhi (follow the CD43⁺ population in Fig. 3, A and B). Since most IgMhi B cells in these mice are CD5⁺ (Fig. 3, A and B), most of the liposomebri B-1 cells are CD5⁺. This dramatic shift in the distribution of liposome binding cells from B-1 to B-0 in xid mice indicates that the xid mutation impairs the segregation of PtC-specific B cells to the B-1 subset.

Segregation of PtC-specific B cells to the B-1 Subset Is Largely Intact in xid Peritonea. The majority of B-1 cells in normal adult mice are located in the peritoneum. B6/xid mice have ~40% of the number of B cells in the peritoneum, and on average 15% are CD5⁺ and CD23⁻, a much smaller percentage than in wild-type mice (Table 2). In addition, 2–4% of peritoneal B cells bind liposomes. Thus xid mice are not devoid of peritoneal B-1 cells or of liposome-binding B cells. This provides an explanation for our observation of enrichment of 10/G4 rearrangements in the peritonea of B6/xid mice (Fig. 1 and Table 1). This is different from previous reports that xid mice lack peritoneal B-1 cells. This is likely due to background genetic differences between B6/xid mice and the CBA/N mice used in previous studies (34).

Essentially all peritoneal B cells in 6-1 mice are liposomebri B-1 cells (Fig. 4 C and Table 2), as previously reported (21). They are CD23⁻, CD43⁺, B220lo, and IgMhi (Fig. 4, A and B). Approximately 80% of liposome binding cells are CD5⁺. In contrast to the spleen, nearly all of the peritoneal B cells in 6-1/xid and dbl Tg/xid mice are liposomebri B-1 cells (Fig. 4 C). The number of PtC-specific B cells in 6-1/xid mice is almost 10% of that in 6-1 mice, whereas that in dbl Tg/xid mice is nearly 30% (Table 2). Thus, the combination of these two transgenes does not increase the number of PtC-specific B cells in the peritoneum as dramatically as it does in the spleen (3.5x versus 46x). The liposomebri cells in 6-1/xid and dbl Tg/xid mice are mostly CD23⁻ (Fig. 4 C) and B220lo and IgMhi (Fig. 4, A and B).

### Table 2. B Cell Subpopulations in the Spleen and Peritoneum of VH12 Tg Mice

| Genotype | n | Total lymphs | % IgM⁺ | Total B cells | % CD5⁺ | Total CD23⁺ | % CD23⁺ | Total CD43⁺ | % CD43⁺ | Total Lipo⁺ | % Lipo⁺ |
|----------|---|--------------|--------|--------------|--------|------------|---------|------------|--------|------------|--------|
| Spleen   |   |              |        |              |        |            |         |            |        |            |        |
| 6-1      | 9 | 4.8 ± 24.9   | 11.6   | 46.2 ± 5.2   | 35.4   | 4.1 ± 35.9 | 4.6 ±   | 40.1 ±     | 5.4 ±  |
| 6-1/xid  | 7 | 2.7 ± 9.2    | 2.7    | 12.6 ± 0.3   | 64.9   | 1.8 ± 11.1 | 0.3 ±   | 8.8 ±      | 0.2 ±  |
| dbl Tg/xid | 5 | 4.3 ± 25.3   | 11.1   | 13.3 ± 1.1   | 60.9   | 6.5 ± 8.4  | 0.8 ±   | 84.3 ±     | 9.3 ±  |
| Normal   | 8 | 10.4 ± 56.7  | 60.5   | 10.4 ± 5.9   | 79.9   | 40.3 ± 9.3 | 5.8 ±   | 0.3 ±      | 0.2 ±  |
| B6.xid   | 7 | 5.8 ± 48.7   | 31.2   | 13.3 ± 2.9   | 73.4   | 23.6 ± 13.5| 3.4 ±   | 0.6 ±      | 0.1 ±  |

| Peritoneal cells |         |           |        |        |         |         |         |         |
|------------------|---------|-----------|--------|--------|---------|---------|---------|---------|
| 6-1              | 6       | 3.1 ± 86.3| 27.1   | 83.7 ± 22.4| 0.7 ± 0.2| 90.8 ± 25.0| 94.3 ± 21.7|        |
| 6-1/xid          | 5       | 0.6 ± 31.2| 2.1    | 54.7 ± 0.9 | 10.1 ± 0.3| 73.4 ± 1.9 | 91.3 ± 1.7 |        |
| dbl Tg/xid       | 3       | 0.9 ± 65.7| 6.1    | 71.4 ± 4.3 | 11.4 ± 0.7| 57.3 ± 2.5 | 96.3 ± 6.0 |        |
| Normal           | 6       | 2.2 ± 78.0| 17.1   | 43.4 ± 7.7 | 12.6 ± 2.3| 69.9 ± 10.0| 43.8 ± 0.8 |        |
| B6.xid           | 6       | 1.2 ± 55.8| 6.9    | 14.3 ± 0.9 | 42.9 ± 2.9| 15.3 ± 1.0 | 3.6 ± 0.4 |        |

* ×10⁶ for spleen cells and ×10⁵ for peritoneal cells
† ×10⁶ for spleen cells and ×10⁵ for peritoneal cells
‡ Standard deviation.
About half express CD5 (Fig. 4, A and B), but only about one-third express CD43, giving them a somewhat unusual phenotype relative to those in 6-1 mice. Thus, the peritoneum contains PtC-specific B-1 cells and few, if any, of the PtC-specific B-0 cells that predominate in the spleen.

**Discussion**

We demonstrate here that xid mice can generate PtC-specific B cells. Although their number is small in non-Tg xid mice, when they are provided the V_12 and V_4 Tgs, which encode anti-PtC antibodies (11) and exert strong selective pressure on B-1 cell generation and clonal expansion (21), their numbers increase substantially. However, two-thirds of the splenic PtC-specific B cells in 6-1/xid and dbl Tg/xid mice are B-0. There appears to be an equivalent but smaller B-0 population in dbl Tg non-xid mice. Thus, the V_12 and V_4 transgenes reveal a deficit in the ability of xid mice to segregate PtC-specific B cells to the B-1 subset, indicating a role for Btk in this process. That some PtC-specific B cells are B-1 indicates that either the mutant Btk has residual function, or that its function is compensated for by other signals, as may occur for others of its functions (31, 41). These data establish that PtC-specific B-0 cells are generated, and that segregation to B-1 is achieved by their subsequent elimination, consistent with our previous conclusion that PtC-specific B cells segregate to the B-1 subset by a mechanism operating after Ig gene rearrangement (21).

xid mice are also deficient in B-1 cell clonal expansion. The large number of the PtC-specific B-1 cells in 6-1 mice is due to clonal expansion (21); 6-1 mice are free to use the endogenous V_k repertoire and the majority of the developing B cells will be PtC^neg. Indeed, at birth only 4% of 6-1 splenic IgM^+ cells are liposome binding. But clonal expansion is so significant that by day 6 >80% of the splenic IgM^+ cells are liposome-binding B-1 cells. 6-1/xid mice generate B-1 cells, but the fact that their numbers remain small in adult mice indicates that these cells are unable to undergo significant clonal expansion. This is corroborated by the analysis of 10/G4 rearrangements in B6/xid mice; the 10/G4 P/NP in the spleens and peritonea of B6/xid mice, although high, is lower than in wild-type mice, indicating modest clonal expansion of PtC-specific B cells (Table 1). Thus, B-1 cell clonal expansion is impaired in xid mice, consistent with the known function of Btk in IgM-mediated signaling (35, 36). This defect is probably a major contributor to the absence of detectable numbers of B-1 cells in xid mice. Larger numbers of PtC-specific B-1 cells are seen in dbl Tg/xid mice, but this would not require clonal expansion, since most newly generated B cells will express both Tgs and bind PtC.

Segregation of B cells to the B-1 subset must be antigen driven, as PtC^neg V_12- or V_4-expressing B cells are B-0 in 6-1 and V_4-only Tg mice (21). Although the identity of the antigen that drives segregation is not known, it is not likely that the number of cells in Tg/xid mice exceeds its availability; dbl Tg/xid have only 1.5 times the number of

**Figure 3.** Analysis of spleen cells from V_12 Tg/xid mice. Spleen cells from 6-1/xid and dbl Tg/xid mice were stained as described in the legend to Fig. 2. As a control and for reference, analysis of 6-1 mice is included. Percentages are of B220^+ cells.
PtC-specific B cells as 6-1 mice, where segregation is intact, and 6-1/xid mice have only 4% the number (Table 2). Thus, the defect in segregation is likely to be related to the responsiveness of PtC-specific B cells to antigen, consistent with the known function of Btk in IgM mediated signal transduction. This leaves two possible mechanisms; either PtC-specific B-0 cells are eliminated by apoptosis after stimulation by antigen, or they convert to B-1 cells.

Signaling of programmed cell death through sIgM in xid B cells appears to be intact. Anti-IgM stimulation induces xid B cells to undergo apoptosis as it does wild type B cells (36). In addition, xid B cells are less resistant to radiation induced apoptosis (37), indicating that programmed cell death proceeds normally, but that these cells are deficient at transducing environmental signals to turn off the cell death program. Moreover, xid mice are not autoimmune, arguing that negative selection of autoreactive B cells is normal in these mice. In fact, the xid mutation prevents the development of autoimmunity in New Zealand black mice (42, 43). These findings cast doubt on apoptosis as the mechanism of segregation to B-1.

On the other hand, it is well established that the xid mutation affects B cell differentiation. xid mice are unable to respond to TI-2 antigens and have impaired responses to some T cell-dependent antigens, particularly at suboptimal doses (26), and they are unable to generate a normal number of peritoneal B-1 cells (34). Even naive B-0 cells appear to not differentiate fully, as they do not downregulate sIgM to wild type levels (44, 45). xid B cells, in cotransfer experiments with non-xid B cells, are at a competitive disadvantage in survival (32), and are similarly disadvantaged in xid heterozygous female mice (33). Studies of the biochemical defect in xid B cells indicate that xid B cells fail to enter cell cycle and differentiate upon anti-IgM signaling due to a deficiency in cyclin induction (46). Therefore, we propose that PtC-specific B-0 cells in xid mice bind antigen, but are unable to differentiate in response to it, and consequently remain B-0. This would place B-0 as an intermediate stage in B-1 cell differentiation.

This mechanism is consistent with the induced differentiation hypothesis in which commitment to the B-1 lineage occurs after Ig gene rearrangement, is dependent on the specificity of the B cell, and follows antigen stimulation. It is incompatible with the two-lineage model of B cell development, which requires commitment to one cell lineage or the other before Ig gene rearrangement, and which does not accommodate movement of cells from one subset to the other. Thus, these data argue for a single B cell lineage that can give rise to B-1 cells upon activation. An implication of this conclusion is that the B-1 repertoire includes only antigen-selected B cells, and that at no time is it equivalent to the B-0 repertoire.

The induced differentiation hypothesis offers an explanation for the existence of the liposome-induced B-0 population in non-xid dbl Tg mice. We suggest that these cells are newly generated B-0 cells that have not yet converted to B-1, either because the large number of PtC-specific B cells produced by the bone marrow reveals a population that is nor-

Figure 4. Analysis of peritoneal cells from V_{H}12 Tg mice. Cells were stained as described in Fig. 2. Percentages are of B220^+ cells.
mally too small to detect, or because the number produced exceeds the available antigen, leaving PtC-specific B-0 cells unconverted for an extended length of time. Consistent with these possibilities is that there are fewer liposome-converted cells in 6-1 mice (Fig. 2 C) in which the production rate of PtC-specific B cells is lower than in dbl Tg mice because of the available use of multiple Vx genes.

The proposal that B-1 cells derive from B-0 cells after antigen stimulation is consistent with the findings of Ying-zi et al. (47), demonstrating that stimulation of B-0 cells in vitro with anti-CD51 and IL-6 induces a B-1 cell phenotype (i.e., CD51+, CD23-, IgDlo, B220hi). It is also consistent with in vivo studies indicating that the formation of the B-1 subset is dependent on the action of coreceptors that deliver activation signals in concert with surface IgM, as well as on the presence of an intact signal transduction pathway from surface IgM. For example, mice deficient in the IgM coreceptor CD19 lack B-1 cells, whereas mice that overexpress CD19 have large numbers of B-1 cells (48–50). Likewise, mice deficient in the complement receptor CD21 lack B-1 cells (51). Both these receptors amplify the IgM-mediated signal delivered by antigen and play important costimulatory roles in responses to T cell-dependent antigens (48, 49, 51, 52). Deficiencies in the cytoplasmic protein kinases Vav and protein kinase C-βI/II (PKC-βI/II) also result in the absence of B-1 cells (53–55), and deficiency in phosphatase SHP-1 results in an excessive number of B-1 cells (56). Like Btk, these molecules are involved in signal transduction from IgM. Both Vav- and PKC-βI/II-deficient mice also exhibit impaired responses to antigen (53, 54), further evidence that signals initiated by IgM are essential for B-1 cell formation. Thus, the same signaling pathways used to respond to antigen are required for the generation of B-1 cells, consistent with an essential role for antigen in B-1 cell formation and the induced differentiation hypothesis. Based on our findings with xid mice, we predict that PtC-specific B cells in mice deficient for these receptor and signal transduction molecules will be similarly impaired in their ability to segregate PtC-specific B cells to B-1. Indeed, recent analysis indicates that PtC-specific B cells in dbl Tg, CD19-deficient mice are B-0 (Rickert, R., and S.H. Clarke, unpublished observation).

It is notable that PtC-specific B-0 cells are dominant in the spleen and lymph nodes (data not shown), but not in the peritoneum of dbl Tg/xid mice. This is most likely an indication that PtC-specific B cells home to the peritoneum only after differentiation to a B-1 cell. Since PtC-specific B cells in xid Tg mice are deficient in signal transduction, they remain in the spleen and lymph nodes as B-0 cells. The number of B cells in the peritoneum of dbl Tg/xid mice is nearly as low as it is in 6-1/xid mice, suggesting that only differentiated B-1 cells are able to migrate to the peritoneum, consistent with this possibility. However, it cannot be excluded that peritoneal B cells are more able to overcome the deficit in signal transduction because of some unique aspect of the peritoneal microenvironment, such as antigen or cytokine availability. Such factors might overcome the absence of wild-type Btk, thereby permitting a greater degree of differentiation in the peritoneum than anywhere else. Regardless of the reason, the peritoneum is an environment in which segregation is largely intact in xid mice.

This analysis also indicates that pre-B cell selection resulting in the loss of most Vx12 pre-BI1 cells and consequent enrichment for Vx12 10/G4 rearrangements is normal in xid mice. This is in accord with the findings that pre-B cell proliferation and production is normal in xid mice (57), and that a defect is not observed until the IgM stage, as evidenced by the observation that xid B cells are at a survival disadvantage relative to non-xid B cells in xid/+ female mice (33). Thus, pre-B cell development is not measurably affected by the xid mutation, as others have noted previously (32, 33, 47), in contrast to Btk deficiencies in humans (58).

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