CBA/N mice have an x-linked immunodeficiency that is mainly expressed by poor antibody production to some T-independent antigens, called TI-2 antigens (1-4) and to a few T-dependent antigens (5-8). This functional defect is associated with a failure in development of a late-appearing B lymphocyte subset (9) that can be defined in normal animals by distinctive surface antigens such as Lyb-3 (10), Lyb-5 (11), and others (12-14). Because many TI-2 antigens induce IgG antibody largely of the IgG3 subclass (15, 16), a normally minor subclass of IgG, and because CBA/N mice are deficient in IgG3 production (17), we proposed that the Lyb-3+, -5+ B cell subpopulation may be responsible for the bulk of IgG3 production and that IgG3 subclass response to any antigen would be defective in CBA/N (16). This has been confirmed for both T-independent and T-dependent antigens (5, 6, 16). Antibodies of other isotypes are less regularly influenced by the immunodeficiency (5).

The mutation in CBA/N mice, called xid, is linked to the x chromosome (1). Because of x chromosome inactivation, females heterozygous for xid will be mosaics, with some cells expressing the xid-bearing x chromosome, others not. Further, the recessive nature of the xid trait results in heterozygous females that are phenotypically normal (1). By examining x chromosome expression in the cells of these animals, it should be possible to determine whether the effect of xid on B cell development is direct or indirect. Direct effects, whereby the normal counterpart of the xid gene product(s) is expressed within the B cell and permits its development, would result in unbalanced x chromosome mosaicism among B lymphocytes from xid heterozygous females, because only B cells expressing the normal x chromosome could develop. Conversely, indirect effects, where the xid gene product(s) is expressed by non-B cells that subsequently influence B cell development or function, would result in balanced mosaicism among B lymphocytes. Here we examine x chromosome expression in xid heterozygous female mice using alloenzymes of phosphoglycerate kinase-1 (PGK-1)1 as x chromosome markers.

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Abbreviations used in this paper: G6PD, glucose-6-phosphate dehydrogenase; HAT, hypoxanthine, aminopterin, and thymidine; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PGK-1, phosphoglycerate kinase-1; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid).
Materials and Methods

Animals. AT-10 female mice bearing \( Pgk-1^a \) allele were obtained from Dr. V. Chapman (Roswell Park Memorial Institute, Buffalo, NY). Since AT-10 is congenic to C3H/HeHa, males with \( Pgk-1^a \) were obtained by mating female AT-10 with male C3H/HeHa (Seneca Laboratory, West Seneca, NY). These males with \( Pgk-1^a \) allele were used to produce the experimental animals by mating with female CBA/N (maintained in our facilities from original stocks obtained from the National Institutes of Health), CBA/J (animal F), and C57BL/10SnJ (animals G, H) (The Jackson Laboratory, Bar Harbor, ME). Animals D, E, and Q were produced by mating (AT-10 × C3H/HeHa)F1 females with CBA/N males.

The selection of x chromosomes for inactivation is not random (18–20). Cattanach et al. (18) have found the inactivation to be influenced by an x-linked gene called \( Xce \). Three alleles of \( Xce \) have been described (20) according to their relative preference in determining x chromosome expression: \( Xce^c \) (AT-10 mice) is most preferred; \( Xce^b \) (C57BL/10SnJ) is intermediate; and \( Xce^e \) (CBA and C3H) is least preferred.

Preparation of Tissue and Hematopoietic Cells. PGK enzyme was extracted from various hematopoietic cells by lysing them in phosphate-buffered saline (PBS) containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Whole blood lysate was used as erythrocyte lysate. Using petri dishes coated with antibody to mouse IgM (21), splenocytes were separated into B cell-enriched (~81% DNLL1.9 + [22]) and B cell-depleted (~5% surface Ig +) populations. The enzyme was extracted from various tissues by mincing samples in PBS followed by freeze and thaw cycles. Hybridomas were produced from heterozygous females after stimulation with 25 µg of lipopolysaccharide (LPS) (Escherichia coli 0128:B12; Difco Laboratories, Detroit, MI) intraperitoneally. 5 d later their splenocytes were hybridized with SP2/0-Ag14 cells as described elsewhere (23). The fused cells from each animal were distributed in 5–10 96-well plates. The supernates of the wells were screened for IgG1 and IgG3 production using solid-phase radioimmunoassay (17). Cells from fusions with >12% of wells showing cell growth were cloned in soft agar (24). The clones were again tested for IgG1 and IgG3 production. All the cells were maintained in culture medium containing hypoxanthine, aminopterin, and thymidine (HAT) (25) to minimize loss of the x chromosome.

Assay of PGK-1 Activity. Because the enzyme activities of different tissue extracts were variable, tissue extracts were diluted until 10 µl of the extract gave 0.1–0.15 absorbance unit changes per minute at 340 nm when 400 µl of substrate solution was used. The substrate solution contained 2 mM ATP, 15 mM 3-phosphoglycerate, 12 mM MgSO4, 0.7 mM disodium EDTA, 0.34 mM NADH, and 2.2 U/ml of glyceraldehyde-3-phosphate dehydrogenase (Sigma Chemical Co.) in 100 mM Tris buffer, pH 7.6 (25). The hybridoma cell lysates were obtained by lysing ~1 × 10⁶ cells in 100 µl of 1% Triton X-100 in a Tris-citrate buffer (225 mM Tris and 75 mM citrate, pH 7).

Electrophoresis of tissue extracts was performed on cellulose acetate paper (Gelman Sciences, Inc., Ann Arbor, MI) at 20 V/cm for 90 min using a pH 6.9 buffer containing 15 mM Tris, 5 mM Pipes (piperazine-N, N'-bis[2-ethanesulfonic acid]; Sigma Chemical Co.), and 2.4 mM citrate. Samples (0.25 µl) were applied to the membrane using a sample applicator (Sepratek-8; Gelman Sciences, Inc.). The enzyme activity was visualized by overlaying the cellulose acetate paper with a reagent strip (Gelman Sciences, Inc.) soaked with substrate solution that contained 25% sucrose but was otherwise identical to that described above. A photographic record was made with Polaroid film (type 667) in a long wave UV box (Fotodyne, Inc., New Berlin, WI). The enzyme activity was detected as a nonfluorescent spot in the strip. Relative amounts of enzyme allelic forms were determined with this method by estimating the dilutions of the sample at which the enzyme activity in each band became undetectable.

Alternatively, electrophoresis of hybridoma cell lysates was performed in 1% agarose (equal mixture of medium and low electroendosmosis agarose [Sigma Chemical Co.]) at 10 V/cm for 6 h using the pH 7 Tris-citrate buffer over a flat-bed cooling plate (LKB, Sweden). The enzyme was located by overlaying the electrophoresis gel with a filter paper (Whatman-1; Fisher Scientific Co., St. Louis, MO) impregnated with the substrate solution without sucrose. After 15–30 min, the filter paper was dried and photographed as above.
Results

*X Chromosome Expression in Hematopoietic Cells.* Relative expression of the two alleles of *Pgk* among hematopoietic cells was studied. Fig. 1 shows examples of the PGK-1 isoenzyme patterns of cell lysates of splenocytes either enriched for (E) or depleted of (D) B cells. The upper and lower bands represent the variant PGK-1A and more common PGK-1B isoenzymes, respectively. By determining the dilutions of the cell lysate at which the enzymatic activity of each isoenzyme becomes undetectable, a semiquantitative estimate of the relative expression of the enzyme can be made. Thus, cells from two animals heterozygous for *Pgk* but lacking *xid* (Fig. 1, control) show either equal representation of the enzymes or slight preference of the PGK-1 alloenzyme. These patterns of mosaicism were seen in spleen cell populations depleted of or enriched for B cells as well as in thymocytes and erythrocytes (Table I). In fact, the degree of x chromosome mosaicism is largely shared among diverse hematological cell populations (27). On the other hand, cells from animals heterozygous for *Pgk* and for *xid* (Fig. 1, experimental animals and Table I) show different patterns of PGK expression. Although erythrocytes, thymocytes, and B-depleted splenocytes resemble each other, B-enriched populations of splenocytes are nearly devoid of the isoenzyme encoded by the X chromosome bearing the *xid* gene. This strongly suggests that

![Figure 1: Estimation of isoenzyme activity by serial dilution. Experimental mice are heterozygous for *xid* and *Pgk-1*, whereas the control mice are heterozygous for *Pgk-1* only. S, position standards; E, splenocyte sample enriched for B cells; D, splenocyte sample depleted of B cells. Serial dilution is in the direction shown with horizontal arrows.]
Table I

| Cell types                      | Experimental animals | Control animals |
|---------------------------------|----------------------|-----------------|
|                                 | A  | B  | C  | D  | E  | F  | G  | H  |
| Erythrocytes                    | 2  | 4  | 3  | 3  | 3  | 2  | 0.5| 1  |
| Thymocytes                      | 3  | 3-9| 3  | 3  | 1  | 1-3| 0.5| 1  |
| Splenocytes                     | 9  | 9  | 9  | 9  | 9  | 5  | 1  | 1  |
| B cell-depleted splenocytes     | 3  | 3  | 3  | 5  | 3  | 1-3| 0.5| 1  |
| B cell-enriched splenocytes     | >3 | >9 | 27 | >9 | >9 | 1-3| 0.5-1| 1 |

Numbers represent the ratio of enzyme activity between PGK-1A and PGK-1B isoenzymes.

B lymphocytes with the xid gene defect do not develop.

The slight predominance of the Pgk-1\(^a\) allele seen in many animals is probably the result of the regulation of x chromosome inactivation by Xce, and unconnected with xid, because all animals expressing this preference (A–F) are Xce\(^a\)/Xce\(^c\) heterozygotes, while the two with more equal expression (G and H) are Xce\(^b\)/Xce\(^c\) heterozygotes.

Most studies of animals expressing the xid trait have identified B cells as the principal target of the immunodeficiency, but only a subpopulation, approximately half of the total B cells, are thought to be involved (2, 4, 10, 11). The very marked changes in isoenzyme expression we find in isolated B cells suggests either that most B lymphocytes are affected by xid or that the amount of enzyme per cell produced by Pgk-1\(^a\)- and Pgk-1\(^b\)-bearing cells are different. To distinguish these alternatives, individual B cells must be examined. However, because of limitations in sensitivity of the enzyme assay in which \(10^5\)–\(10^6\) cells per determination are required, individual B lymphocytes were clonally expanded by somatic cell hybridization with myeloma cells. This technique is known to preserve the status of the inactive x chromosome (28, 29), unless a stringent selection is used (30).

We chose to compare x chromosome expression in IgG3- and IgG1-secreting hybridomas, because the xid trait is known to depress IgG3 production uniformly (5, 6, 16, 17), but has only variable effects on IgG1 (5, 17). We obtained 93 IgG3-producing hybridomas and 43 IgG1-producing hybridomas from nine animals; their phenotypes are summarized in Table II. Since the SP2/O-Ag14 myeloma cells have a Pgk-1\(^b\) allele, a hybridoma generated from fusion with a splenocyte bearing Pgk-1\(^b\) allele would express only one slow-moving enzyme band. This phenotype is labeled as CBA/N type in Table II. However, if a hybridoma was formed from a splenocyte that expressed a Pgk-1\(^a\) allele, the hybridoma would have both Pgk-1 alleles and show two bands upon electrophoresis. These hybridomas are labeled as AT-10 type in Table II. If an AT-10 type hybridoma discards the active splenocyte x chromosome, then phenotypically the hybridoma would express only the slow moving PGK-1B enzyme and be indistinguishable from the CBA/N type. Even though all the hybridomas were maintained in HAT selection media to minimize this conversion, there may be examples of the splenocyte x chromosome loss.
As with splenocyte populations, the AT-10 x chromosome is preferentially active among both IgG1- and IgG3-producing hybridomas. ~70% of IgG1-secreting and ~93% of IgG3-secreting hybrid cell lines express both PGK-1 isoenzymes. Given the possibility of chromosome loss, these values may be underestimates. To rule out multiple representation of the same B cell clone among the hybridomas, only one clone was derived from a single well and electrophoretic mobilities of the IgG1 hybridoma proteins from animal M were shown to be different. However, it is clear that not all B cells express the AT-10 phenotype, because in two animals (M and O) all of the IgG1-secreting hybridomas were of the CBA/N phenotype. Interestingly, in these animals, nearly all of the IgG3-secreting cells expressed the AT-10 phenotype. These results thus suggest that the number of bone marrow stem cells for B cells is small and that at least occasionally the stem cells that generate IgG3-secreting cells are different from those that generate IgG1 secretors.

Isoenzyme Patterns in Tissue. To determine whether other cell types in addition to B lymphocytes expressed the unbalanced x chromosome mosaicism as a result of \textit{xid} gene expression, a survey was made of various tissues for PGK-1 isoenzyme expression in \textit{xid} heterozygous mice. Extracts of brain, kidney, liver, muscle, and peritoneum were examined (Fig. 2). No consistent differences were seen between control and experimental groups of animals; most animals expressed both isoenzymes in similar amounts although occasional animals expressed one or the other enzyme dominantly. Further, most tissues from an individual animal had the same pattern, although striking exceptions are evident. This is clearly seen in the brain of animal B as well as in the liver and peritoneum of animal C. Further, all parts of the liver from animal C had the extreme PGK-1\texttextsuperscript{a} predominance (data not shown). These examples are consistent with the notion that various organs arise from limited numbers of precursor cells (31–33). However, in this limited survey of tissues no consistent trend in unbalanced X-chromosome expression was seen between experiments and control animals. Study of individual cell types within the various tissues is required to determine whether only certain cell types express unbalanced mosaicism.
FIGURE 2. Electrophoretic patterns of PGK-1 isoenzymes from various tissues. Position standards are marked with vertical arrows. Source of tissues: B, brain; K, kidney; L, liver; M, muscle; P, peritoneum; R, erythrocytes. Experimental animals A–E are heterozygous for \textit{xid} and \textit{Pgk-1}. Control animals F, G, and H are heterozygous for \textit{Pgk-1} only.

Discussion

The nature of the lesion(s) induced by \textit{xid} has been studied extensively. Defects identified have included Lyb-3, -5, and -7-bearing B cells (10–12), Ia.W39-bearing B cells and/or macrophages (13), as well as some (34, 35), but not all (36, 37), T helper cells. These defects produce poor response to many polysaccharides (1, 3, 6, 16) and some proteins and polypeptides under immune response gene control (5), and a tendency to develop tolerance in vitro more easily than
normal (38). Because the functions involved depend on several cells, and even
the development of a class of lymphocyte may depend on other cells (39, 40), it
is not clear which if any of the defects is the initial lesion and which are secondary.
Analysis of x chromosome mosaicism in heterozygous females is a more direct
approach to this question. Cell populations directly affected by the xid mutation
should not express the xid-bearing x chromosome, whereas those indirectly
affected or unaffected should express this chromosome randomly.

The preliminary results reported here show clearly that B lymphocytes express
unbalanced x chromosome mosaicism. Other cell populations do not, but this
may reflect the failure to examine homogeneous subpopulations. Until this is
possible, we cannot conclude that B lymphocytes are the only cell type directly
affected by xid.

Examination of B cell subpopulations by hybridoma technology showed that
IgG3-secreting cells nearly always expressed the normal x chromosome, while
many IgG1-secreting cells expressed the xid-bearing x chromosome. These data
support the idea that xid preferentially affects a subset of B lymphocytes (10, 11)
that is associated with IgG3 production (16, 17). Whether the population not
associated with IgG3 is unaffected by xid cannot be stated with assurance because
only small numbers of cells have been examined. It does seem certain, however,
that B lymphocyte subpopulations may express xid-bearing x chromosomes to
different degrees.

Other examples of unbalanced x chromosome mosaicism exist. Lesch-Nyhan
syndrome in man is caused by a deficiency in the x-linked enzyme, hypoxanthine-
guanine phosphoribosyl transferase (41). Carriers of this recessive trait have
erthrocytes exclusively derived from stem cells expressing the normal x chro-
mosome; individual fibroblasts may express either (42). Similarly, carriers of the
trait for Wiskott-Aldrich syndrome, an x-linked recessive disease involving
immunodeficiency and thrombocytopenia, have unbalanced mosaicism of glucose-
6-phosphate dehydrogenase (G6PD, an x-linked enzyme) among T lymphocytes
and platelets particularly, and less marked effects among other hematopoietic
cell types (43, 44).

Unbalanced mosaicism can occur normally because the bulk of many organs
or cell subpopulations are often derived from small numbers of precursor cells.
For instance, study of G6PD mosaicism suggests that human erythrocytes are
derived from 8–19 precursor cells (31). Also mosaicism in skin showed mouse
melanocytes are derived from 34 cells (33). Further, because x chromosome
inactivation occurs early in embryogenesis and is permanent (45–47), topo-
graphic mosaicism is a normal feature in heterozygous females (18). Thus, small
samples of organs or cell populations such as a patch of skin are unrepresentative
of the whole, and may express unbalanced mosaicism. Our results with several
organs (brain, peritoneum, and liver, Fig. 2) are consistent with either mecha-
nism. Nevertheless finding 10 randomly selected but different CBA/N IgG1
hybridomas from one animal (Table II) supports the idea of limited precursor
cells. Also the imbalance throughout the entire liver in animal C clearly supports
the view that hepatocyte precursors are few. However, unlike the situations with
the xid-mediated immunodeficiency, Wiscott-Aldrich syndrome and Lesch-Ny-
han syndrome, this imbalance of normal mosaicism arises due to random expres-
sion of x chromosomes.

Having established that B lymphocytes are most likely a direct target of \textit{xid} gene effects, is it possible to determine when in hemopoietic development that \textit{xid} acts? We have found no gross effect of \textit{xid} in T cells. The fact that marrow grafting from normal to nonirradiated CBA/N establishes B cell but not T cell chimerism (48) is also consistent with the notion that B cell precursors, but not T cell precursors, are defective; thus, common lymphoid stem cells are probably not affected. Kincade and co-workers (49, 50) have determined that CBA/N mice appear to develop pre-B cells with cytoplasmic $\mu$ and the 14.8 determinant (known to be expressed on B cell precursors) at the appropriate time in development (49), but that cells which give rise to B cells capable of growth in soft agar (clonable B cells) fail to develop (50). Clonable B cells appear simultaneously with surface Ig-bearing B cells at $\sim 16$–$17$ d of gestation. Thus, \textit{xid} operates at least at an early stage of B cell development. Another x-linked immunodeficiency in DBA/2Ha mice has been localized to B lymphocytes. This gene appears to encode for a B cell receptor for T cell replacing factor (51). Thus, x-linked genes are involved in early and later stages of B cell differentiation.

These results are consistent with several models of B cell development. \textit{xid} may block the development of more mature B cells such as Lyb-5$^+$ cells and clonable B cells from Lyb-5$^-$ cells. Alternatively, there may be several pathways of B cell development that diverge early and give rise to different functional subsets. The development of subsets responsive to T1-2 antigens is sensitive to \textit{xid}. These models would suggest that the subset of B cells which develop in CBA/N animals would be normal. There is evidence, however, that the Lyb-5$^-$ B cells of CBA/N mice may not be equivalent to Lyb-5$^-$ B cells of normal animals. DeFranco and colleagues (52) noted that CBA/N B cells respond differently than normal cells to anti-$\mu$ stimulation. Although both defective and nondefective cells increase in size, defective cells fail to proliferate. Thus, it may be that even the B cells which develop in CBA/N mice may not be normal. Our results with x chromosome expression are consistent with this notion, although we do not wish to draw too fine a distinction because our data are limited. B cells from \textit{xid} heterozygous females are more unbalanced in their x chromosome expression that would be expected if only a subpopulation were affected. It may be that \textit{xid}-expressing B cells are at a proliferative disadvantage when compared with normal cells and that heterozygotes reflect this selection. Considerably more data are required to be confident about the extent of skewed expression of x chromosomes in heterozygotes; it is clear that it is substantial.

Finally, attention should be called to the utility of x-linked isoenzyme expression in the dissection of x-linked diseases, particularly immunodeficiencies. The x-chromosome plays an important role in the normal immune system. At least five immunodeficiency diseases in humans (53) and two in mice involve x-linked genes. The determination of the pattern of x chromosome expression in these diseases should be particularly informative.

Summary

The immunodeficiency in CBA/N mice is reflected by abnormal development of a subset of B lymphocytes. However, it is not clear how \textit{xid}, the mutant gene
in CBA/N mice, affects the development of this subset. Specifically, it is not
known if the xid gene influences the development of the B cell subset directly or
indirectly by providing the improper developmental milieu through effects on
other cells. We investigated this question using female mice heterozygous for
two x chromosomal genes, xid and Pgd-1 (phosphoglycerate kinase-1). Since
females are mosaic because of x chromosome inactivation, their lymphocytes can
be studied for the choice of the x chromosome, using the two PGK-1 isoenzymes
as the cytological marker. We find that B lymphocytes in the spleen prefer the x
chromosome without xid while the remaining splenocytes and cells from other
tissues do not. This suggests that xid affects B lymphocytes directly and not
through their developmental milieu. Furthermore, our data suggest that the
precursors for IgG1- and IgG3-producing cells may be both few and different.

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