DEVELOPMENT OF B LYMPHOCYTES IN MICE HETEROZYGOUS FOR THE X-LINKED IMMUNODEFICIENCY (*xid*) MUTATION

xid Inhibits Development of All Splenic and Lymph Node B Cells at a Stage Subsequent to Their Initial Formation in Bone Marrow

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The *xid* (X-linked immunodeficiency) mutation in the CBA/N mouse strain has been used extensively in studies of the functional and lineage relationships of B lymphocyte subpopulations (1–7). Defects associated with CBA/N (*xid/xid*) mice include the inability to respond to some thymus-independent antigens, hyporesponsiveness to some B cell mitogens and unresponsiveness to others, and inability to produce B lymphocyte colonies in soft agar after mitogen stimulation (8–12). These functional defects have been associated with the absence of at least one subset of mature B cells, first detected in the spleens of normal mice at 2–3 wk of age and characterized by expression of Lyb-5 antigens (12), minor lymphocyte-activating determinants (13, 14), a high concentration of membrane Ig (mIg)[1] δ chains and a low concentration of membrane μ chains (12, 15). The successful maturation of donor B cells in CBA/N mice given normal B cell progenitors suggests that the defect is intrinsic to affected B cells, rather than humoral or microenvironmental (16).

Other subpopulations of mIg-bearing B cells and pre-B cells are present in normal numbers in CBA/N mice (17, 18), which suggests that they may be unaffected by the mutation. The defects described above have been demonstrated in either the homo- or hemizygous-recessive environment, or by comparing normal cells with *xid*-bearing cells that have matured in a recessive environment. Further insight into the defects associated with *xid* may be obtainable if ontogeny and development can be followed in a heterozygous environment.

We have studied the development of *xid* B cells in *xid*/+ heterozygous mice by exploiting the existence of a second X-chromosome gene that serves as a marker to distinguish between cells in which the normal or the *xid*-carrying chromosome is active (7, 19). This gene is *Pgk-1*, of which there are two allelic forms, *a* and *b*;

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[1] *Abbreviations used in this paper:* mIg, membrane immunoglobulin; MTT, methylthiazolyldiphenyl tetrazolium (thiazolyl blue); PGK, phosphoglycerate kinase; XLR, X-linked lymphocyte-regulated genes.

these encode forms of phosphoglycerate kinase (PGK) that can be distinguished electrophoretically (20) and measured relative to each other (21). Female mice can be produced that are heterozygous for both *xid* and *Pgk-1*. X inactivation results in only one of the X chromosomes being active per cell. In such heterozygotes, cells in which the *xid*-carrying X chromosome is active express one PGK allozyme, while activity of the putatively normal X chromosome is marked by the other allozyme. Females heterozygous at X-linked loci are normally mosaics for their respective gene products. If, however, mutations at X-linked loci (such as *xid*) are lethal to particular cells, or place them at a selective disadvantage, the population of those cells will either be nonmosaic or show disproportionately low expression of the appropriate marker gene. Relative measurement of the A and B forms of PGK-1, after electrophoresis of cell lysates has therefore been used to determine the effect of the *xid* mutation on subpopulations of B cells, as well as on pre-B cells and cells of other hematopoietic lineages.

Materials and Methods

Mice. All mice were bred and maintained in this laboratory under conventional conditions. CBA/Ca-*Pgk-1*^{a} males were mated to either CBA/N females to produce *xid*/+;*Pgk-1*$^{b/a}$ female F_1 hybrids or to CBA/Ca females to produce control +/+;*Pgk-1*$^{b/a}$ mice. Reciprocal crosses were also made to assess any parental effects on X chromosome inactivation.

Preparation of Cell Suspensions. Subcutaneous lymph nodes, thymus, and spleen were dissociated in ~1 ml RPMI 1640 medium containing 0.05% wt/vol BSA and 0.1% wt/vol sodium azide (RPMI-BSA) with the aid of a ground glass homogenizer. The resulting cell suspension was poured through a fine stainless steel sieve to remove stroma. Bone marrow cavities were flushed with RPMI-BSA, and the marrow plug was aspirated through a 25-gauge needle to obtain a single-cell suspension. Erythrocytes were removed from the spleen and bone marrow cell suspensions by hypotonic shock (22). Viability was determined by staining with a solution of acridine orange and ethidium bromide and examination under a fluorescence microscope (23).

Isolation of B-lineage Cells. Fluorochrome-labelled cell suspensions were analyzed and sorted in a modified fluorescence-activated cell sorter (FACS IV; Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with argon ion and tunable dye lasers.

All staining steps were performed at 4°C for 40 min at a cell concentration of 5×10^6 cells/ml in RPMI-BSA. Cells were washed three or four times in cold RPMI-BSA after each staining step, and were resuspended to a concentration of ~10^7 cells/ml for FACS analysis and sorting.

Fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin antiserum (Miles-Yeda, Rehovot, Israel) was used to isolate mature B lymphocytes from lymphoid cell suspensions. A total of 2.5×10^6 lymph node or spleen cells and 7.5×10^6 bone marrow cells were stained with 5 and 15 μl antiserum respectively. Pre-B cells were isolated from bone marrow by means of a rat monoclonal antibody (14.8) (17) against B220, the 220 kD B lineage–specific form of the leukocyte-common glycoprotein family (24). 6×10^6 bone marrow cells were incubated with 30 μl of 14.8 antibody (10-fold concentrated, purified culture supernatant, kindly given to us by Dr. P. W. Kincade, Oklahoma Medical Research Foundation). After washing, cells were incubated with 80 μl fluorescein-labelled goat antiserum to rat immunoglobulin (Tago Inc., Burlingame, CA). This monoclonal antibody differentially stains B and pre-B lymphocytes. Cells that stain brightly have been shown by two-color FACS analysis to express membrane Ig (our unpublished data), and are therefore considered to be B cells. Cells that stain relatively dully with 14.8 express no mIg and are considered to be B lymphocyte precursors.

Nonlymphocytic cells were isolated from blood and bone marrow on the basis of their

TABLE I

Relative X Chromosome Activity in Erythrocytes of xid/+ and +/+ Mice

Parental strains		Genotype of offspring	Mean ratio PGK1B: PGK1A‡	SD	*n*
Male	Female				
CBA/Ca*	CBA-Pgk-1^a	+/+; *Pgk-1*$^{b/a}$	28:72	6	100
CBA-Pgk-1^a	CBA/N	+/*xid*; *Pgk-1*$^{a/b}$	26:74	11	27
CBA/N	CBA-Pgk-1^a	*xid*/+; *Pgk-1*$^{b/a}$	24:76	9	27

* Reciprocal crosses of these lines showed no significant difference in X chromosome activity (25), and so were pooled.

‡ The probability of X chromosome inactivation is considered to depend on the *Xce* locus, closely linked to *Pgk-1* (40). These mice are heterozygous (*Xce*$^{a/c}$), which results in the unequal proportions of the two allozymes observed. All values shown are the means of at least two replicate electrophoretic analyses for each individual.

relatively high wide-angle light-scattering characteristics. The cells thus obtained from blood were mainly granulocytes, while those from bone marrow were an undefined mixture that included many granulocyte-lineage cells.

B lymphocyte subpopulations were isolated by staining 4×10^6 spleen cells with 200 μl Texas Red–labelled goat antiserum to mouse IgM heavy chain (E. Y. Laboratories, San Mateo, CA) and 25 μl fluorescein-labelled goat antiserum to mouse IgD heavy chain (Nordic, Tilburg, the Netherlands). Nonlymphocytes were excluded from the analysis on the basis of their wide-angle scatter characteristics. Lymphocytes were analyzed and sorted using green and red fluorescence simultaneously.

Aliquots of $1–2 \times 10^5$ (lymphocytes) or $3–5 \times 10^5$ (granulocytes) cells, were sorted on the FACS into microcentrifuge tubes (Sarstedt Ltd., Leicester, UK) and centrifuged for 5 min at 12,000 *g*. The supernatant was carefully removed and the inside of the tubes dried with tissue. A lytic buffer (21) was added to the pellet to a volume of 1 μl/5×10^4 lymphocytes. The sample was then stored at −60°C until thawed for allozyme analysis.

PGK-1 Analysis. Electrophoresis and measurement of PGK-1 allozymes was carried out as described in detail elsewhere (21). Briefly, cell lysates were electrophoresed on cellulose acetate membranes (Helena Laboratories, Beaumont, TX) for 45 min at 4°C (15 mA/membrane, constant current). Alloenzyme activity was visualised in the membranes by a series of linked enzyme reactions leading to the reduction of the dye thiazolyl blue (methylthiazolyldiphenyl tetrazolium; MTT) to its formazan derivative, or alternatively by the inclusion of ^{14}C-labelled glucose in the reaction mixture and the detection of ^{14}C-labelled products by autoradiography. Whole tissue samples were diluted to an appropriate concentration immediately before PGK-1 analyses (sorted cell samples were undiluted) and analyzed by the ^{14}C method. Blood samples for tests of mosaicism were diluted 1:1 and analyzed by the MTT method. The relative proportions of the PGK-1A and B allozymes were then measured by scanning membranes or autoradiographs on an automated scanning and integrating densitometer (Chromoscan 3; Vickers Instruments Ltd., Goteshead, UK).

Results

Erythrocyte Mosaicism. In initial experiments, erythrocyte mosaicism in CBA/N × CBA-*Pgk-1*^{a} hybrids was compared with that in control CBA/Ca × CBA-*Pgk-1*^{a} mice; reciprocal crosses were also studied. No significant differences were observed (Table I). This showed that cells in which the *xid*-carrying X chromosome was active (henceforth referred to simply as xid cells) were not at any disadvantage to non-xid cells in the erythroid lineage. Against this background, subsequent experiments, were designed to investigate the effect of *xid*

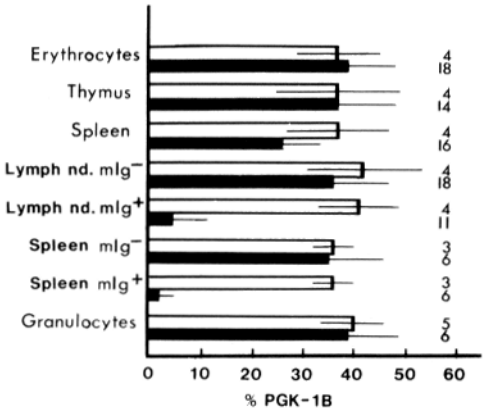

FIGURE 1. Activity of *xid*-carrying X chromosome (marked by the PGK-1B allozyme) in various cell populations obtained from female *xid*/+ (*black bars*) and control +/+ (*white bars*) mice (mean ± SD of the number of mice shown). These mice were selected for relatively high (>30%) expression of the B allozyme in erythrocytes. Granulocytes were isolated from peripheral blood by FACS sorting of cells showing high wide-angle scatter.

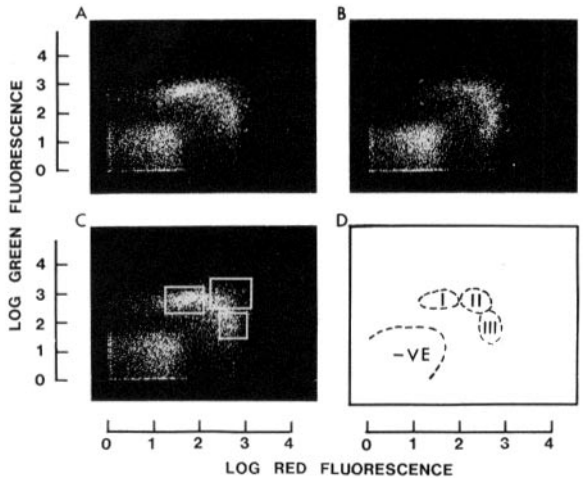

FIGURE 2. Two-color immunofluorescence analysis of spleen cells from female CBA/Ca-*Pgk-1*^{a} (+/+) (*A*), CBA/N (*xid*/*xid*) (*B*), and an F_1 hybrid between them (*xid*/+) (*C*). Cells were stained with fluorescein-labeled anti-δ and Texas Red–labeled anti-μ antisera. The positions of populations I, II, and III are shown in *D*, and the boundaries of the rectangular sorting windows that were used are marked in *C*. Total B cell numbers in *xid*/*xid* mice were about half those in +/+ animals, *xid*/+ heterozygotes being intermediate (data not shown).

on mosaicism in other hematopoietic lineages. For these experiments mice were selected that had >30% PGK-1B in their erythrocytes.

Mosaicism in Lymphocytes and Granulocytes. PGK-1 phenotypes of several cell types isolated from *xid*/+ mice were compared with those in +/+ mice (Fig. 1). There was virtually no PGK-1B allozyme in B lymphocytes isolated from lymph nodes and spleen (4 and 2%, respectively) of *xid*/+ mice, while in erythrocytes, thymocytes, granulocytes, and non-B cells of both sets of mice, and in B cells of +/+ mice, the expected proportion of PGK-1B (30–40%) was observed. These data showed that the *xid* gene inhibited the development of B cells in heterozygotes, and suggested that other lineages were unaffected.

B Lymphocyte Subpopulations. As previously reported by Hardy et al. (15), B lymphocytes were divided by two-colour FACS analysis into three subpopulations (arbitrarily designated populations I, II, and III) on the basis of their relative expression of membrane μ and δ chains (Fig. 2). In agreement with the earlier description (15), we found population I (high δ, low μ) to be reduced or missing in CBA/N (*xid*/*xid*) mice. In *xid*/+ mice, all three populations were present in similar proportions to controls. However, analysis of populations I, II, and III sorted from the spleens of *xid*/+ mice 3–18 months of age showed that all three

TABLE II

Activity of the xid-bearing X Chromosome in Erythrocytes, Splenic Non-B Cells, and Splenic B Cell Subpopulations Isolated from Four 3–18-mo-old xid/+ Mice

Cells	Percent* PGK-1B in individuals:			
	1	2	3	4
Erythrocytes	39	35	38	51
Non-B cells	28	30	32	41
Population I‡	0	0	0	0
Population II	4	0	0	0
Population III	6	10	0	0

The *xid*-bearing chromosome also carried the gene for PGK-1B; hence, the percentage of this allozyme provides an estimate of the percentage of xid cells. Control (+/+) mice showed 34–38% PGK-1B in all cell types.

* All values shown are means of at least two replicate electrophoretic analyses.

‡ Populations I, II, and III were defined by their relative expression of membrane δ and μ chains (see text).

TABLE III

Activity of the xid-bearing X Chromosome in Erythrocytes, Splenic Non-B Cells, and Splenic B Cell Subpopulations from 2- and 6-wk-old xid/+ Mice

Cells	Percent PGK-1B in individuals:*					
	2-wk-old			6-wk-old		
	1	2	3	4	5	6
Erythrocytes	33	38	46	46	37	36
Splenic non-B	36	36	44	42	36	41
Spenic population I‡	0	ND	ND	1	2	0
Splenic population II	9	17	19	24	8	8
Splenic population III	16	41	42	29	12	14

The *xid*-bearing chromosome also carried the gene for PGK-1B; hence, the percentage of this allozyme provides an estimate of the percentage of xid cells. Control (+/+) mice showed 34–38% PGK-1B in all cell types.

* All values shown are means of at least two replicate electrophoretic analyses.

‡ Populations I, II, and III were defined by their relative expression of membrane δ and μ chains (see text). There were insufficient cells in population I for analysis in mice 2 and 3.

populations were nonmosaic for PGK-1 (Table II). This shows that it was not only population I whose development was affected by the *xid* mutation.

A similar analysis of 2- and 6-wk-old mice showed that xid cells were rare or absent in population I. Such cells were present in populations II and III, but usually in subnormal proportions (Table III).

Bone Marrow B Lineage Cells. Bone marrow cells from *xid*/+ and +/+ mice were sorted into 14.8-bright, 14.8-dull, mIg^+, and mIg^- fractions. 14.8-dull cells were taken as the pre-B lymphocyte population, 14.8-bright and mIg^+ cells as mature B lymphocytes. The mIg^- fraction included high wide-angle light-scattering, nonlymphocytic cells. Differences in the proportions of PGK-1B between the various cell populations are listed in Table IV. The mosaicism in the pre-B

TABLE IV

Difference in Proportion of PGK-1B Allozyme between B, Pre-B, and Non-B Cell Populations Isolated from Bone Marrow of xid/+ and +/+ Mice

Mice	*n*	Difference between: mIg⁻ vs. mIg⁺ (non-B vs. B)		14.8-dull vs. 14.8-bright (pre-B vs. B)		mIg⁻ vs. 14.8-dull (non-B vs. Pre-B)	
		Mean ± SD	*p*	Mean ± SD	*p*	Mean ± SD	*p*
xid/+ Mice aged							
2 wk	3	2 ± 2	NS	ND		ND	
4–6 wk	6	7 ± 3	<0.01	6 ± 4	<0.05	−2 ± 5	NS
2–6 mo	8	13 ± 6	<0.01	11 ± 9	<0.01	−1 ± 2	NS
12–20 mo	7	24 ± 11	<0.001	22 ± 12	<0.01	−1 ± 5	NS
+/+ Control mice aged							
2 wk	2	−6	NS	ND		ND	
2–6 mo	3	−1 ± 3	NS	1 ± 1	NS	1 ± 2	NS

In *xid*/+ mice, PGK-1B serves as a marker for activity of the *xid*-bearing X chromosome.
All values shown are calculated from means of at least two replicate electrophoretic analyses for each individual. Statistical comparisons were done by paired Student's *t* test.

cell population of *xid*/+ mice was not significantly different from that in +/+ controls. As the mice matured, significant imbalances in mosaicism became apparent when comparing the mIg⁺ cells with either 14.8-dull or mIg⁻. In control (+/+) mice, no differences in mosaicism were observed between any of the cell populations analyzed (Table IV). These data showed that bone marrow B cells became progressively depleted of xid cells with age, but pre-B development remained unaffected by the mutation.

Discussion

The use of an assay for measuring relative quantities of X chromosome–linked allozyme markers (21) made it possible to assess the effects of the *xid*/+ mutation on defined cell populations and to identify a stage of B lymphocyte differentiation at which this mutation may act. The analysis of erythrocytes in *xid*/+ heterozygotes showed that the *xid*/+ mutation did not affect the probability of X-inactivation per se: PGK mosaicism, inferred from the relative proportions of the two allozymes measured, was similar to that seen in +/+ mice. Effects of *xid*/+ on particular cell populations were therefore assessed by comparing the mosaicism in these populations with that in erythrocytes of the same individual. A similar degree of mosaicism was assumed to show that there was no selection against (or indeed for) xid cells in the development of the population concerned; it remains possible that *xid* had other effects that were not reflected in the numbers of cells present.

The development of thymocytes, T cells, and granulocytes appeared unaffected by the mutation. This agrees with previous observations that T cell function was normal in CBA/N mice (12, 26, 27), and that after transplantation of mixtures of normal and CBA/N bone marrow to lethally irradiated hosts,

both sets of marrow contributed to the repopulated T cell pool in the expected proportions (6, 7).

In contrast, B cells isolated from the spleen and peripheral lymph nodes of *xid*/+ mice were virtually nonmosaic. This showed that xid cells, being in direct competition with normal non-xid B cells, made up, at most, a very small proportion of the B cell pool. This was confirmed by the absence of mosaicism in the separated splenic B lymphocyte subpopulations I, II, and III of adults. The fact that only xid B cells were absent in these animals confirms earlier suggestions (16, 19) that a cell-intrinsic effect is involved.

These results may be compared with previous reports that, in CBA/N (*xid*/*xid*-homozygous) mice, only population I cells (high-δ, low-μ, Lyb-5$^+$) are deficient (15). CBA/N mice are also able to give T cell–dependent antibody responses, indicating that the presence of the *xid* mutation does not necessarily prevent the differentiation of some components of the mature B cell pool. The lineage relationships between the three populations are unclear, but our data suggest that the expression of the *xid* gene blocks completely the differentiation of the population I cells, and renders the other two populations at a competitive disadvantage. The disadvantage is less marked in 2–6-wk-old mice. The splenic xid B cells in such mice cannot, however, be assumed to be the functional equivalents of B cells in adult mice; rather, they may be immature cells produced in a spleen that is still hematopoietic, and thus be analogous to newly-formed bone marrow B cells in the adult.

There was no evidence that pre-B xid cells were selected against in the bone marrow: they showed normal mosaicism for PGK. Pre-B cells are present in normal numbers in CBA/N mice (17, 18, and our unpublished observations), and pre-B and small lymphocyte production proceeds at a similar rate in CBA/N and normal mice (28). All the data suggest, therefore, that xid pre-B cells, whether in the homozygous *xid*/*xid* mouse or in competition with normal cells in the heterozygote, are unaffected by the mutation. The status of xid B cells in the bone marrow is less clear. At 2 wk of age, these cells showed normal mosaicism. By 4–6 wk of age, however, mosaicism began to be unbalanced in favor of non-xid cells. This imbalance became more marked with increasing age. The rate of B lymphocyte production in rodents is high, sufficient to replace the entire B cell pool every 4 d (29, 30). The longevity of B cells in the secondary lymphoid organs (31, 32) implies that a large proportion of the B cells produced in the marrow never become established in the peripheral pool (33). The presence of mIg$^+$ xid cells in the marrow of heterozygous mice, but not in the spleen, lymph nodes, and blood suggests that it is at the point of selection into the peripheral pool that these cells are at a disadvantage. The increasing mosaic imbalance that develops in the bone marrow B cells is readily explained by the observation that mature B cells circulate through the marrow and that the number of such cells increases with age (34).

Recent evidence suggests that the *xid* mutation has occurred within a family of genes on the X chromosome named XLR (X-linked lymphocyte-regulated genes) (35, 36). Probing various lymphocyte cell lines with cDNA clones of this region has shown that XLR mRNA is present in IgD-expressing B cell, but not pre-B cell lines. mRNA associated with this region has also been described in

mature T cells (36), where *xid* appears not to be expressed. The data of several authors suggest the existence of other X-associated mutations that affect immune responses in the mouse (37–39). The analysis of mosaic imbalance as reported in this paper may prove useful for the study of these and other mutations that affect the hematopoietic system.

Summary

CBA/N mice were crossed with CBA/Ca-*Pgk*-1^a to produce female F_1 hybrids that were heterozygous for both xid and the phosphoglycerate kinase 1 (PGK-1) allozymes. PGK acted as a quantifiable marker for the frequency of cells in which the *xid*-bearing X chromosome was active in lymphocytic and other cell populations. In adults, such cells (termed xid cells) were virtually absent in FACS-sorted splenic and lymph node B cells, and in all three splenic subpopulations distinguished on the basis of their relative expression of membrane μ and δ chains. Thus, the *xid* mutation appeared to compromise the development of all B cells. Erythrocytes, thymocytes, T cells, and granulocytes were unaffected. Selection against xid cells was less pronounced in the spleens of 2–6-wk-old mice. In the bone marrow, there was evidence for selection against xid in the production of B cells (except at 2 wk of age), but not at the pre-B cell level. These data suggest that, in competition with normal non-xid cells, newly-formed xid B cells were less likely to be incorporated into the peripheral B cell pool.

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