CLONAL DIVERSITY IN THE B CELL REPERTOIRE OF PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA

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X-linked agammaglobulinemia (XLA)1 is an inherited humoral immunodeficiency involving an intrinsic defect in B lymphocyte development (1, 2). The block in differentiation is commonly ascribed to an inability of pre-B cells to mature into B cells. While approximately normal numbers of pre-B cells are present in the bone marrow of these patients, the number of B cells present in the peripheral circulation is <1% of normal frequencies (3, 4). It has been suggested that XLA might be due to a defect in attaining complete Ig gene rearrangements (5, 6). However, there are several pieces of existing evidence against this hypothesis: (a) two EBV-transformed cell lines from an XLA patient produced native-sized μ chain protein (7, 8); (b) analysis of both functional and nonfunctional H chain alleles cloned from a single XLA B cell line indicated that VH-DJH joining can occur normally (9); (c) cell-mediated immune responses are normal in XLA patients (1), making it unlikely that the XLA defect involves Ig gene recombination because B and T cells appear to use a common recombinase system for their respective antigen receptor genes (10); and (d) the small number of B cells found in the peripheral circulation of XLA patients express a cell surface marker phenotype characteristic of immature B cells (11). XLA B cells have a high ratio of cell surface IgM to IgD, and express low levels of HLA class II gene products and CD21 antigen (4). If the defect in XLA involved only Ig gene rearrangement, one would expect those B cells having passed that point in differentiation to mature normally.

One way to study this question would be to determine the clonal diversity of B cells from patients with XLA. If there were a primary defect in Ig gene rearrangement in this disease, the B cell repertoire within each patient would be expected to be pauciclonal, with each clone representing a circumvention of the rearrangement defect. Alternatively, if significant B cell clonal diversity were found in XLA patients, this result would support the hypothesis that the XLA defect involves a

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This work was supported by an Arthritis Foundation Investigator Award, grants from the Forsyth Cancer Service and the Immune Deficiency Foundation, and National Institutes of Health grants RR-05404 and GM-40215 (to B. A. Pollok), and AI-25129 (to M. E. Conley). Address correspondence to Brian A. Pollok, Dept. of Microbiology and Immunology, Wake Forest University Medical Center, 300 South Hawthorne Road, Winston-Salem, NC 27103. M. E. Conley’s present address is the Dept. of Pediatrics, University of Tennessee School of Medicine, Memphis, TN 38103.

1 Abbreviation used in this paper: XLA, X-linked agammaglobulinemia.
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mechanism apart from Ig gene rearrangement, such as a failure in the normal expansion or activation of the pre-B or B cell population.

Our goal in this study was to define the degree of clonal diversity in the B cell repertoire in XLA by analyzing a collection of 18 EBV-transformed B cell lines derived from five patients with this disorder. Previous studies on EBV-transformed cell lines derived from normal individuals has shown extensive clonal diversity in such lines (12, 13). Differences in Ig isotype expression between the B cell lines derived from each patient provided an initial survey of clonal diversity; for the majority of XLA cell lines, clonal diversity was further assessed by determining V region gene expression in the productive H and L chain transcripts (VH and VL, respectively). This analysis also provided information on the specific sites of VHDJ and VKJK gene segment joining in several XLA B cell lines. Finally, diversity among the XLA B cell lines that expressed similar V region genes was shown by Southern blot analysis of their H chain gene rearrangement patterns.

Materials and Methods

Patients. The five patients with XLA were from three unrelated families. All had (a) the onset of recurrent bacterial infections in the first few years of life; (b) very low concentrations of serum Iggs with failure to make antigen-specific antibody; (c) markedly reduced numbers of B cells in the peripheral circulation; (d) normal T cell numbers and function; and (e) a positive family history for a similar illness in maternal male relatives. The patients were between 10 and 25 yr of age when studied. All patients have been previously reported (14, 15).

Generation of B Cell Lines. B cell lines were produced by exposing PBL (for patient BP) or T cell-depleted PBL (all other patients) to supernatant from the EBV-producing cell line B95-8. The cells were then cultured at 0.5-1.5 × 10⁶ cells/ml in 24-well culture plates with PHA (1:1,000 dilution; Difco Laboratories Inc., Detroit, MI) and Cyclosporine A (0.25 μg/ml, Sandoz Pharmaceuticals, East Hanover, NJ). The T cell-depleted lymphocytes were supplemented with irradiated T cells at 10⁶ cell/ml.

RNA Isolation and Analysis. Total cellular RNA was isolated by the method of Schibler et al. (16). The poly(A)+ fraction was enriched by affinity chromatography on oligo-dT-cellulose columns (Collaborative Research, Lexington, MA). Northern blot analysis to screen for VH3 gene family expression was done as described by Nelson et al. (17), with the exception that nylon membranes were used (Nytran; Schleicher & Schuell, Inc., Keene, NH). Northern blots using the mouse Vλ3 probes were washed under high stringency conditions (0.1 × SSC, 0.2% SDS at 52°C for 30 min). RNA blots using the Vλ Daudi probe for VJIV gene subgroup expression were similarly washed. A positive signal using either probe among the XLA lines was always comparable with the intensity of that observed using the respective C region probes, indicating that the positive signal was not due to a contaminating cell population. Direct RNA sequencing by primer extension was performed according to Geliebter et al. (18) using human Cμ1, VH, or Cκ exon-specific oligonucleotides.2

DNA Isolation and Analysis. High molecular weight genomic DNA was obtained following the procedure of Perry et al. (19). Southern blot analysis was performed as described by Schuler et al. (20) with the substitution of nonfat dry milk for Denhardt's reagent (at 1%).

Cloned DNA Probes. The mouse Vλ7183 probe was a gift of Dr. Roy Riblet (Medical Biology Institute) and has been described (21). The human VH Daudi probe has been described in detail (22). Human μ and κ C region probes were the same as used in reference 23, and the human Jκ region probe was the 4.5-kb Jκ Bam HI/Hind III fragment subcloned from the phage clone CH28-6 (24).

2 Synthesized by the DNA Synthesis Core Lab of the Cancer Center of Wake Forest University, supported by National Institutes of Health grant 12197 from the National Cancer Institute.
Results

**Ig Protein Expression among XLA B Cell Lines.** EBV transformation of PBL from five XLA patients gave rise to one cell line from patients JeB and SD, eight lines from patients JP and CP, and 22 lines from patient BP. Because our goal was to conduct a molecular analysis on Ig gene structure and expression in these cell lines, we selected only those lines that were homogeneous for Ig isotype expression; 18 of the total 40 XLA B cell lines were shown by cytoplasmic immunofluorescence to produce a single H and L chain isotype (some lines contained 0–5% of cells expressing other isotypes; these minor contaminants did not compromise our analysis). B cell lines generated from individual patients were heterogeneous for isotype expression (Table I). For example, one ακ-, one μλ-, and three μκ-producing lines were generated from patient JP, while cells from patient CP gave one γκ-, one γλ-, and two μκ-positive lines. The variability in Ig isotype expression among some of the lines parallels what has been observed by staining untransformed PBL from XLA patients (4) and suggests that at least limited B cell clonal diversity exists in certain XLA patients. However, this diversity could be due to different precursor clones forming the repertoire, or due to H chain class switching and secondary κ to λ switches (25) in a pauciclonal B cell population.

**Ig mRNA Expression among XLA B Cell Lines.** To examine the issue of clonal heterogeneity further, Ig H and L chain mRNA from the majority of XLA B cell lines was analyzed to define the diversity in V region gene use. We chose to focus on cell lines producing μ and κ chains because of the well-characterized nature of the human

| Cell line | Isotype | V<sub>μ</sub>7183 hybridization | V<sub>μ</sub>D<sub>Je</sub> rearrangement<sup>*</sup> | V<sub>μ</sub>I/V hybridization |
|-----------|---------|-------------------------------|---------------------------------|-----------------------------|
| JeB       | μκ      | +                             | V<sub>μ</sub>3 (D?/J<sub>μ</sub>4) | +                           |
| SD1       | μκ      | +                             | V<sub>μ</sub>3                 | +                           |
| JP3       | ακ      | ND                            | ND                             |                             |
| JP4       | μκ      | –                             | V<sub>μ</sub>4 (D4/J<sub>μ</sub>5) | -                           |
| JP7       | μλ      | ND                            | ND                             |                             |
| JP11      | μκ      | –                             | V<sub>μ</sub>1 (D4/J<sub>μ</sub>5) | -                           |
| JP12      | μκ      | –                             | V<sub>μ</sub>1 (D?/J<sub>μ</sub>4) | -                           |
| CP1       | μκ      | +                             | V<sub>μ</sub>3 (D21-7/J<sub>μ</sub>4) | +                           |
| CP3       | γλ      | ND                            | ND                             |                             |
| CP11      | γκ      | ND                            | ND                             |                             |
| CP101     | μκ      | –                             | V<sub>μ</sub>4 (D?/J<sub>μ</sub>5) | +                           |
| BP3       | μκ      | +                             | (D?/J<sub>μ</sub>6)            | -                           |
| BP4       | μκ      | +                             | (D?/J<sub>μ</sub>6)            | -                           |
| BP7       | μλ      | ND                            | ND                             |                             |
| BP12      | μκ      | –                             | V<sub>μ</sub>4 (D?/J<sub>μ</sub>4) | +                           |
| BP19      | μκ      | +                             | ND                             |                             |
| BP20      | δκ      | ND                            | ND                             |                             |
| BP24      | μκ      | +                             | +                              |                             |

<sup>*</sup> Rearranged V<sub>μ</sub>, D, and J<sub>μ</sub> elements are shown for those μ mRNA that were tested for V<sub>μ</sub>7183 hybridization and sequenced; tentative V<sub>μ</sub> assignment is based on >80% sequence identity with a prototype V<sub>μ</sub> gene of the respective family (insufficient sequence data for BP3 prohibited a tentative V<sub>μ</sub> designation).
μ and κ C region exon sequences to which complementary RNA sequencing primers could be made. An initial goal was to determine whether each μκ-positive line expressed μ and κ transcripts of a size corresponding to a productive mRNA (2.7 and 2.4 kb for μm and μs mRNA, and 1.2 kb for κ mRNA). Northern blotting of poly A-containing cytoplasmic RNA from each line was done using a human μ and κ C region probes (pHuCμ and pHuCκ; reference 23). All XLA B cell lines positive for μ and κ protein by immunofluorescence expressed native-sized μm, μs, and κ mRNA transcripts, and the presence of truncated or aberrant Cμ- or Cκ-hybridizing RNA transcripts was not observed (Fig. 1, A and C). Another measure of the functional nature of the μ and κ transcripts in these XLA B cell lines was a normal pattern for VnDJn and VκJκ gene rearrangement (Fig. 2, a and b, respectively). RNA sequencing of μ and κ mRNA in the region of gene rearrangement for several of the XLA lines showed that the sites of gene joining exhibited a normal degree of junctional diversity and occurred well within the usual range of codon positions reported for productively rearranged human and mouse Ig genes (26). The presence of an open translational reading frame across the V(D)J region in all cell lines examined was also consistent with their expression of Ig protein reactive with antibodies specific for the μ and κ C regions.

After establishing that the μκ-producing XLA B cell lines expressed functional μ and κ transcripts of normal primary structure, Vn gene use was examined by

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Northern hybridization analysis of Ig mRNA in μκ-producing XLA B cell lines. (A) Hybridization with human Cμ probe; (B) hybridization with mouse Vn7183 probe; (C) hybridization with human Cκ probe; and (D) hybridization with human Vκ Daudi probe. In all blots, 5 μg of poly(A)+ total cellular RNA is loaded per lane. The variability in the signal intensity between some XLA B cell lines for μ and/or κ mRNA is due to variation in the intracellular steady-state levels of these transcripts.
Northern hybridization analysis with the mouse V_{n}7183 probe, which crosshybridizes with human V_{n} region sequences belonging to the V_{n}3 gene family (27, 28). Using highly stringent hybridization and washing conditions, 7 of the 12 \( \mu \) producing lines expressed a productive \( \mu \) mRNA that hybridized with the V_{n}7183 probe (Fig. 1 B and summarized in Table I). The apparent heterogeneity in V_{n} gene use among XLA B cells was confirmed by RNA sequencing of the 3' V_{n} region for several V_{n}7183+ and V_{n}7183-XLA1A mRNA (Fig. 2 a). The V_{n}7183-hybridizing transcripts, as expected, contained sequences similar to V_{n}3 genes (76-96% sequence identity when compared with eight germline and nine cDNA V_{n}3 gene sequences; references 28 and 29). The V_{n}7183- \( \mu \) transcripts had differing sequences within this region; some cell lines such as JP4 and BP12 contained V_{n}4-like sequences (30), while JP11 and JP12 had V_{n}1-like sequences (28). The sequence data in Fig. 2 a also shows that different D and J_{n} gene segments were utilized to code.
for the productive μ RNA transcripts in most XLA lines, and that where similar
gene segments were used, the site of their rearrangement differed (e.g., JP4 vs. JP11).
Taking the Northern hybridization and RNA sequence data together, it was apparent
that various \( V_\mu \) genes were utilized by the XLA B cells, and more importantly, that
different B cell lines derived from a single patient expressed a heterogeneous \( V_\mu, D, \) and \( J_\mu \) gene repertoire.

Clonal diversity within the \( \mu \kappa \)-producing set of XLA lines was further shown by
the pattern of \( \kappa \) light V region use. \( \kappa \) mRNA from each \( \mu \kappa^+ \) line was probed with
the productive \( V_\kappa \) gene cloned from the human Burkitt lymphoma Daudi (22); a
similar \( V_\kappa \) probe hybridizes to \( \sim 75\% \) of \( \kappa \) mRNA molecules isolated from untransformed human splenic B cells (31). The Northern hybridization results using the
\( V_\kappa \) Daudi probe established that the cell lines BP3 and BP4 were clonally distinct
from lines BP19 and BP24 (Fig. 1 D). In addition, this experiment indicated that
different \( V_\kappa \) genes were utilized, and that certain \( V_\mu-V_\kappa \) gene combinations were
not selectively expressed in the B cell repertoire of XLA patients. Examination of
the partial \( V_\kappa \) sequences of the three XLA B cell lines shown in Fig. 2 b indicated
that different \( V_\kappa \) genes were represented even within the \( V_\kappa \) Daudi-hybridizing set
of XLA-derived \( \kappa \) mRNA.

**Genomic Analysis of Ig Gene Rearrangements in XLA B Cell Lines.** As a final test of
clonal diversity, we analyzed the gene rearrangement patterns for the Ig H chain
locus of the three \( \mu \kappa^+ \) JP-derived cell lines and the four \( \mu \kappa^+ \), \( V_\mu \)-like BP cell lines
by Southern blot hybridization using a \( J_\mu \) region-specific probe (Fig. 3). Because

![Figure 3. Ig H chain gene rearrangements in multiple B cell lines of two XLA patients. Southern blot analysis using a cloned human \( J_\mu \) region probe (Bam HI/Hind III fragment; reference 24) demonstrated that each B cell line had undergone distinct IgH gene rearrangement events. Restriction digests were chosen to include the entire \( J_\mu \) region on a single DNA fragment: JP DNA was digested with Xba I, BP DNA with Hind III. Note that each XLA B cell line has two \( J_\mu \)-hybridizing DNA fragments (BP 24 has two co-migrating fragments in the Hind III digest, but two separable fragments using other restriction digests), showing that both H chain alleles undergo rearrangement. The very light bands present in some of the XLA lines represent submolar amounts of DNA from the minor contaminants in these lines (0-5%).]
H chain gene rearrangement occurs early in B cell differentiation, all descendants of a single pre-B cell precursor will have the same structure of VDJ joining, and hence, the same restriction fragment hybridization pattern in this region of the genome. All three JP lines and all four \( V_n \)-3-like BP lines possessed different \( J_n \)-hybridizing restriction fragments, definitively showing that each B cell line was derived from an independent B cell precursor.

Discussion

The gene defect in XLA is intrinsic to the B cell lineage (2, 32) and results in a failure to produce B lymphocytes (1). The nature of this gene defect remains unknown. Because synthesis of a functional Ig molecule is the hallmark of normal B cell development, abnormal or inefficient rearrangement of the genes required for Ig production has been considered a possible explanation for the defect in XLA. In support of this possibility, Schwaber et al. (5) presented data demonstrating the occurrence of \( \mu \) transcripts lacking \( V_n \) regions in three bone marrow-derived pre-B cell lines from XLA patients. However, it has been shown that the earliest pre-B cells in the mouse can produce these similarly truncated \( \mu \) chains (33), suggesting that these human pre-B cell lines may represent a normal, early stage in B cell development. Among the pre-B cells that do differentiate into B cells in individuals with XLA, our results indicate that both H and L chain gene rearrangement occur in a normal fashion. The sites of \( V_n \)-D, D-J, and \( V_K \)-J gene joining all occur within the usual range of nucleotide positions, they occur normally independent of the type of \( V \), D, or J gene segment involved, and all the normal modes for introducing structural variability at the time of Ig gene joining, such as junctional diversity and generation of G/C nucleotide-rich non-DNA-encoded N segments, appear to be active. Further, our results indicated that the gene defect in XLA does not inhibit functional rearrangement of L chain genes, which is a salient point because the XLA defect is phenotypically expressed as an inability of pre-B cells to develop into B cells.

In light of these findings, can Ig gene recombination defect(s) be ruled out as the molecular basis for B cell deficiency in XLA? The obvious rejoinder to using Ig-producing B cell lines from such patients is that one is selecting for the rare cells that make it through the block in H and/or L chain gene joining. If this were the case, then we would expect to find a very limited degree of clonal diversity among the B cell lines derived from each individual patient. This situation is in fact exemplified by an animal model of lymphoid immunodeficiency, the leaky scid mouse (34). Although these mice have detectable serum Ig levels, the B cell repertoire is pauciclonal, consisting of only one to three clones of Ig-producing B cells per animal. As the scid defect appears to reflect an inability to efficiently accomplish functional rearrangement of Ig and TCR genes (20, 35), the Ig+ B cells in leaky scid mice likely represent "escapees" from the defect. The argument that XLA is an analogous situation to the leaky scid mouse is contradicted by the striking clonal diversity observed among the CP-, JP-, and BP-derived B cell lines; all five \( \mu_K^+ \) BP lines arose from different precursor clones, as did all three JP and both CP \( \mu_K^+ \) cell lines. As various \( V_K \) genes are represented in the XLA B cell lines, the ability of XLA pre-B cells to undergo L chain gene rearrangement cannot be due to a particular \( V \), gene or gene subgroup that more readily circumvents a putative rearrangement defect. This point is also relevant for H chain gene rearrangement in XLA, since extremely varied patterns of somatic rearrangements are apparent in the \( J_H \) region of the...
different cells (Figs. 2a and 3). The B cell repertoire in XLA patients, while much smaller in the total number of Ig-producing B cells, arises from multiple pre-B cell clones and is diverse in the sense that independent rearrangement events created the few B cells that are present in vivo.

A look at the fine structure of the Ig genes in the region of rearrangement for several of the XLA B cell lines indicates that B cell clonal diversity in these patients did not come at any one stage during differentiation. For the three JP- and two BP-derived lines analyzed at the sequence level: (a) the elements and sites involved in D-Jh gene joining are distinct (the first event in H chain gene rearrangement); (b) the type of Vh gene to join to a given D/Jh combination varies (e.g., JP4 is Vh4/D4/Jh5 vs. JP11 being Vh1/D4/Jh5); and (c) different L chain gene segments are utilized (e.g., BP3 differs from BP12 in Vk gene expression). The presence of an apparent somatic mutation in the Jh5 region of JP11 (the T→A transversion in codon 101 was confirmed in two separate sequences) and the occurrence of H chain class switching event in several lines indicate that structural changes in Ig genes generally associated with the mature B lymphocyte stage of differentiation also can take place in XLA B lymphocytes.

An alternative explanation for the defect in B lymphocyte differentiation in XLA may be due to a more generalized problem in B cell growth and clonal expansion. We have previously hypothesized that the defect in XLA does not lie at a single point in differentiation from pre-B cell to B cell, but instead affects B cells at several stages of maturation, resulting in decreased proliferation or activation of B cells throughout differentiation (4). Although patients with XLA are said to have “normal” numbers of pre-B cells, the actual number is somewhat decreased compared with controls. In a study by Pearl et al. (3) using age-matched children, 7.1 ± 7.5% of the lymphoid cells in the bone marrow of normals were pre-B cells, but in boys with XLA, 3.8% of the lymphoid cells were pre-B cells (range 0.7–11.2%). Compared with pre-B cells from controls, a smaller proportion of the pre-B cells from the XLA patients were synthesizing DNA as measured by autoradiography (3). Also, EBV-transformed B cell lines from XLA patients multiply at a greatly retarded rate compared with EBV cell lines from normal individuals (our unpublished observation). The greatly depressed production of B lymphocytes in patients with XLA is unlikely to be due to abnormal production of a growth or differentiation factor. Previous studies by one of us (2), demonstrating nonrandom X chromosome inactivation in B cells from obligate carriers of XLA, suggest that the gene product is not transportable between cells. Candidate mechanisms behind the XLA defect currently include problems in expression of a cell surface receptor for a growth or differentiation factor, a defect in a signal transduction pathway, or the inability of a nuclear-localized trans-acting molecule to interact at its site of action on the gene. The findings in the present study, showing normal Vh and Vk rearrangements, clear clonal diversity within patients, and expression of a varied Vh and Vk gene repertoire, provide support for the hypothesis that the defect in XLA is exerted on a B cell–specific process that is unrelated to the generation and expression of functional Ig genes, but that is nevertheless critical to the proper development of B lymphocytes.

Summary

Ig protein and mRNA expression was examined in a collection of 18 monoclonal EBV-transformed B cell lines derived from five patients with X-linked agammaglobu-
ulinemia (XLA). A diversity of H and L chain isotypes were synthesized by these lines: the majority (12 lines) expressed μκ chains, while μλ (two lines), γκ (one), γλ (one), δλ (one), and ακ (one) isotype expression was also observed. For all the μκ-producing XLA B cell lines, the μ and κ mRNA transcripts were of native size, and sequence analysis across the regions of VκDJκ and VκJκ joining showed that Ig gene rearrangements occurred in a typical manner. A variety of VκDJκ and VκJκ gene rearrangements were observed, not only within the set of μκ+ XLA B cells as a whole, but also among the cell lines derived from single patients. Southern blot analysis for genomic IgH chain gene rearrangements was done to fully assess the extent of clonal heterogeneity among multiple μκ+ XLA B cell lines derived from two patients; all the B cell lines possessed distinct gene rearrangement patterns demonstrating their clonal unrelatedness. Our findings indicate that the B cell repertoire in individual XLA patients is clonally diverse and that it is unlikely that the defect in B cell differentiation in XLA is the result of inefficient or ineffective rearrangement of Ig H or L chain genes. Rather, this study provides support for the idea that the XLA defect relates to a more generalized cellular function, such as regulating the proliferation and/or clonal expansion of cells of the B lymphoid lineage.

We thank Drs. Phil Leder, Hans Zachau, and Roy Riblet for providing probes, Persymphonie Brown Miller and Scott Palmer for technical assistance, and Teresa Hire and Melissa Jackson for preparing the manuscript.

Received for publication 18 January 1989 and in revised form 13 March 1989.

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