

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 V_HDJ_H 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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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 (V_H and V_L , respectively). This analysis also provided information on the specific sites of V_HDJ_H and V_KJ_K 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 Igs 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 $0.5\text{--}1.5 \times 10^6$ cells/ml in 24-well culture plates with PHA (1:1,000 dilution; Difco Laboratories Inc., Detroit, MI) and Cyclosporine A (0.25 $\mu\text{g}/\text{ml}$, Sandoz Pharmaceuticals, East Hanover, NJ). The T cell-depleted lymphocytes were supplemented with irradiated T cells at 10^6 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 V_H3 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 V_H7183 probes were washed under high stringency conditions ($0.1 \times \text{SSC}$, 0.2% SDS at 52°C for 30 min). RNA blots using the V_K Daudi probe for $V_{K1/IV}$ 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 $C\mu 1$, V_H , or $C\kappa$ 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 Denharts' reagent (at 1%).

Cloned DNA Probes. The mouse V_H7183 probe was a gift of Dr. Roy Riblet (Medical Biology Institute) and has been described (21). The human V_K 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 J_H region probe was the 4.5-kb J_H^+ 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 $\alpha\kappa$ -, one $\mu\lambda$ -, and three $\mu\kappa$ -producing lines were generated from patient JP, while cells from patient CP gave one $\gamma\kappa$ -, one $\gamma\lambda$ -, and two $\mu\kappa$ -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

TABLE I
Diversity of Ig Expression among XLA B Cell Lines

Cell line	Isotype	V _H 7183 hybridization	V _H DJ _H rearrangement*	V _κ I/IV hybridization
JeB	$\mu\kappa$	+	V _H 3 (D [?] /J _H 4)	+
SD1	$\mu\kappa$	+	V _H 3	+
JP3	$\alpha\kappa$	ND		ND
JP4	$\mu\kappa$	-	V _H 4 (D4/J _H 5)	-
JP7	$\mu\lambda$	ND		
JP11	$\mu\kappa$	-	V _H 1 (D4/J _H 5)	-
JP12	$\mu\kappa$	-	V _H 1 (D [?] /J _H 4)	-
CP1	$\mu\kappa$	+	V _H 3 (D21-7/J _H 4)	+
CP3	$\gamma\lambda$	ND		
CP11	$\gamma\kappa$	ND		ND
CP101	$\mu\kappa$	-	V _H 4 (D [?] /J _H 6)	+
BP3	$\mu\kappa$	+	(D [?] /J _H 6)	-
BP4	$\mu\kappa$	+		-
BP7	$\mu\lambda$	ND		
BP12	$\mu\kappa$	-	V _H 4 (D [?] /J _H 4)	+
BP19	$\mu\kappa$	+		+
BP20	$\delta\lambda$	ND		
BP24	$\mu\kappa$	+		+

* Rearranged V_H,D, and J_H elements are shown for those μ mRNA that were tested for V_H7183 hybridization and sequenced; tentative V_H assignment is based on >80% sequence identity with a prototype V_H gene of the respective family (insufficient sequence data for BP3 prohibited a tentative V_H 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 $\mu\kappa$ -positive line expressed μ and κ transcripts of a size corresponding to a productive mRNA (2.7 and 2.4 kb for μ_m and μ_s 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 (pHuC μ and pHuC κ ; reference 23). All XLA B cell lines positive for μ and κ protein by immunofluorescence expressed native-sized μ_m , μ_s , and κ mRNA transcripts, and the presence of truncated or aberrant C μ - or C κ -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 V_HDJ_H and V_KJ_K 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 $\mu\kappa$ -producing XLA B cell lines expressed functional μ and κ transcripts of normal primary structure, V_H gene use was examined by

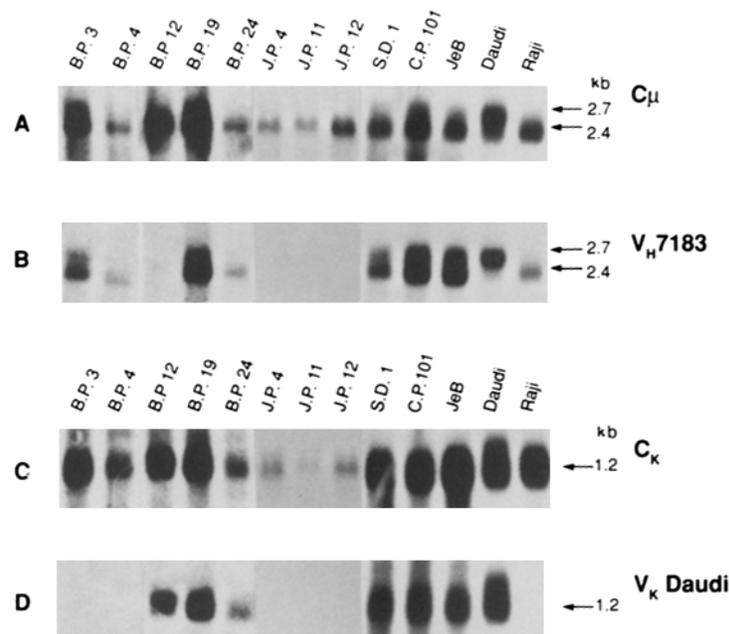


FIGURE 1. Northern hybridization analysis of Ig mRNA in $\mu\kappa$ -producing XLA B cell lines. (A) Hybridization with human C μ probe; (B) hybridization with mouse V_H7183 probe; (C) hybridization with human C κ probe; and (D) hybridization with human V κ Daudi probe. In all blots, 5 μ g of poly(A)⁺ 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.

a V_H-D_H-J_H sequence

a T GGG GGA GGC GTA

20

a GTG CAG CCT GGG AGG TCC CTG AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC

40

a TTC AGT AGC TAT GGC ATG CAC TGG G-T CG- -AG GCT CCA GGG AAG GGG CTG

60

a GAG TGG GTG GCA CTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT CGA GAC
b G TGG G-C TCA CCT A-G TGG T-- G-- G-T -G- ACC ACA TAC TAC GCA GAC

V_H region

a TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAC TCC AAG AAC ACG CTG TAT
b TCC GTG AAG GGC CGA --C ACC -TC TCC AGA GAC -AT TCC -AG AAC ACA CTG TAT
c AC ACG TCC AAG AAC --A TTC TCC
d GTA GGC ACG TCC AAG AAC CAC TTC TCC
e A TCC A-G ACG ACA GCC TAC
f TCC GCG ACG ACA GCC TAC
g TCC AAG AAC CAG TTC TCC

80 94

a CTG CAA ATG AAC AGC CTG AGG ACC GAG GAC ACG GCT GTG TAT TAC TGT GCG AGA
b CTA CAA ATG AAC AGC CTG AGG ACC GAG GAC ACG GCC GTA TAT TAC TGT GCG AAA
c CTG AGG CTG ACC TCT GCC ACC G-C -CG GAA ACG GCT GTG TAT TAC TGT GCG AGA
d CTG AAC CTC ACC TCT GTG ACC GGC AAG GAA CCG GCC GTG TAT TAC TGT GCG AGA
e ATG GAG CTG AGC AGC CTG AGA TCT GAA GAC AAG GCG GTC TAT TAC TGT GCG AGA
f ATG GAG CTG AGC AGC CTG AGA TCT GAA GAC ACG GCT GTG TAT TAC TGT GCG AGA
g ATG GAG CTG AGC AGC CTG AGA TCT GAA GAC ACG GCT GTG TAT TAC TGT GCG AGA
h CTG AAG CTG AGC TCT GTG ACC GGC GCG GAC ACG GCT GTG TAT TAC TGT GCG AAG

D region

a GAC CCT ATA GCA GAT GGT GGG GGG
b ACC TCG GGC TCC TAT GGT TCG GGG AGT TAT CCC
c GGC CCG GGC TCC TAT GAC AAT AGT ACT TAT
d GCG G-A GGC TAT TGT AGT AGT ACC ACT GCG CGA GGG
e AAG GCC TCT GTA TGT AGT AGT ACC ACG TGT TAT CCC CGG
f GAG GGC CCT AGC AGT GGC TCG GCG TTA ACC C-A GGG ATC GGG
g CGA CTC CBT TAT GGG AAG GAG GCT ACG TGT GTC TGG CBT
h GGC AAG GGG CCG TAT TAC TAT GAT AGT AGT GGC CAA ACC AAG TCC TCC GGG TCG ACG

J_H region 101

a TAC TAC TGG G
b TAC TTT GAC TAC TGG GGC
c TAC TAC GG
d AAC TGG TTC GA
e AAC TGG ATC
f TTT GAC TAC TGG GGC
g TAC TAC TAC TAC TAC GGT AT
h TTT GAC TA- TGG GGC

b V_K-J_K sequence

50

a ACG GAA TCC -GG -TC CCT GAC CGA --C AGT GGC A-C GGG
b TAT GCT GCA TCC AGT TTG CAA AGT GGG GCC CCA TCA AGC TTC AGT GGC AGT GGA

70

V_K region

a TCT GGG ACA A-A TTC ACT CTC ACC ATC ACG ACG CTG CAG CCT GAT GAT TTT GCA ACT
i TCT GGG ACG AGA TTC ACT CTC ACC ATC A-- ACG GTG CAG GCT GAA GAT GTG GCA GTT
b TCT GGG ACA GAT TTC ACT CTC ACC ATC ACG AGT CTG CAA CCT GAA GAT TTT GCA GCT

90

J_K region

a TAT TAC TGC CAA CAG TAT AAT AGT TTT TGG ACG TTC GGC C-A GGG AC
i TCT TAC TGT -A- CAA TAT TAT AGT ACT CCT CTA ACG TTC GGC CAA GGG AC
b TAC TAC TGT CAA CAG ACT TAC AGA ACC CCC CGG CAG ACG TTC GGC CAA GGG

FIGURE 2. (a) RNA sequence of the productive μ chain mRNA in seven XLA B cell lines within the region of V_HD_HJ_H gene rearrangement. A C μ 1 exon-specific oligonucleotide (5'-GTT-GGGGCGGATGCACTCC-3') was used to prime cDNA synthesis for this region of the μ transcript; a V_H-specific oligonucleotide was used for completion of the 5' end of the sequence in the case of CP1 and JeB. The V_H codons are numbered according to the amino acid residue in the mature form of the H chain protein (as according to Kabat et al. [26]). (b) RNA sequences of three functional XLA κ transcripts across the V_KJ_K junction. A C κ exon-specific oligonucleotide (5'-GACAGATGGTGCAGCCAC-3') was used. Numbering is as in a. Individual sequences are designated as follows: a, JeB; b, CP1; c, CP101; d, JP4; e, JP11; f, JP12; g, BP3; h, BP12; i, SD1.

Northern hybridization analysis with the mouse V_H7183 probe, which crosshybridizes with human V_H region sequences belonging to the V_H3 gene family (27, 28). Using highly stringent hybridization and washing conditions, 7 of the 12 μ K-producing lines expressed a productive μ mRNA that hybridized with the V_H7183 probe (Fig. 1 B and summarized in Table I). The apparent heterogeneity in V_H gene use among XLA B cells was confirmed by RNA sequencing of the 3' V_H region for several V_H7183⁺ and V_H7183⁻ XLA μ mRNA (Fig. 2 a). The V_H7183-hybridizing μ transcripts, as expected, contained sequences similar to V_H3 genes (76–96% sequence identity when compared with eight germline and nine cDNA V_H3 gene sequences; references 28 and 29). The V_H7183⁻ μ transcripts had differing sequences within this region; some cell lines such as JP4 and BP12 contained V_H4-like sequences (30), while JP11 and JP12 had V_H1-like sequences (28). The sequence data in Fig. 2 a also shows that different D and J_H gene segments were utilized to code

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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_H 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_H , D, and J_H gene repertoire.

Clonal diversity within the $\mu\kappa$ -producing set of XLA lines was further shown by the pattern of κ light V region use. κ mRNA from each $\mu\kappa^+$ line was probed with the productive V_κ gene cloned from the human Burkitt lymphoma Daudi (22); a similar V_κ probe hybridizes to $\sim 75\%$ of κ mRNA molecules isolated from untransformed human splenic B cells (31). The Northern hybridization results using the V_κ 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_κ genes were utilized, and that certain V_H - V_L gene combinations were not selectively expressed in the B cell repertoire of XLA patients. Examination of the partial V_κ sequences of the three XLA B cell lines shown in Fig. 2 b indicated that different V_κ genes were represented even within the V_κ Daudi-hybridizing set of XLA-derived κ 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_H3 -like BP cell lines by Southern blot hybridization using a J_H region-specific probe (Fig. 3). Because

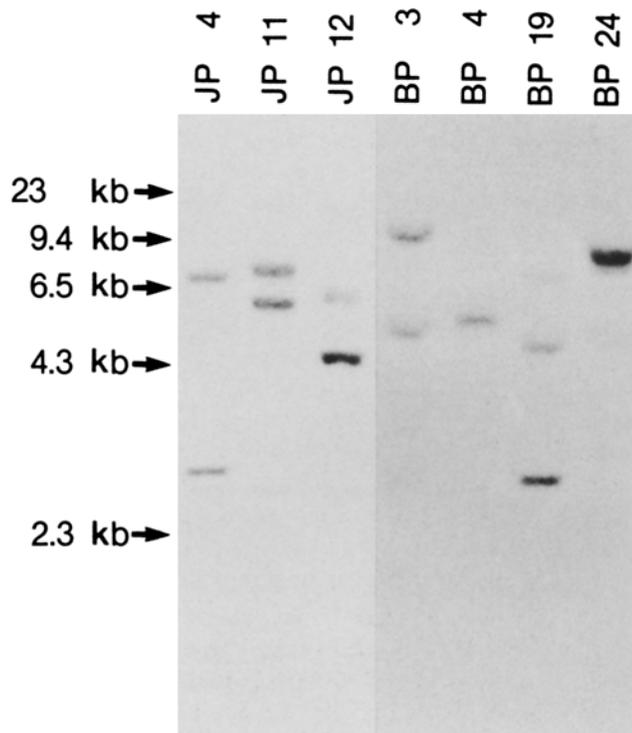


FIGURE 3. Ig H chain gene rearrangements in multiple B cell lines of two XLA patients. Southern blot analysis using a cloned human J_H region probe (Bam HI/Hind III fragment; reference 24) demonstrated that each B cell line had undergone distinct IgH gene rearrangement events. Restriction digests were chosen to include the entire J_H 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_H -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_H -3-like BP lines possessed different J_H -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 μ transcripts lacking V_H 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 μ 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_H -D, D- J_H , and V_K - J_K 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 $\mu\kappa^+$ BP lines arose from different precursor clones, as did all three JP and both CP $\mu\kappa^+$ 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_L 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

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different cells (Figs. 2 *a* 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-J_H gene joining are distinct (the first event in H chain gene rearrangement); (*b*) the type of V_H gene to join to a given D/J_H combination varies (e.g., JP4 is V_H4/D4/J_H5 vs. JP11 being V_H1/D4/J_H5); and (*c*) different L chain gene segments are utilized (e.g., BP3 differs from BP12 in V_κ gene expression). The presence of an apparent somatic mutation in the J_H5 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 V_H and V_L rearrangements, clear clonal diversity within patients, and expression of a varied V_H and V_L 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 nonetheless 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 agammaglob-

ulinemia (XLA). A diversity of H and L chain isotypes were synthesized by these lines: the majority (12 lines) expressed $\mu\kappa$ chains, while $\mu\lambda$ (two lines), $\gamma\kappa$ (one), $\gamma\lambda$ (one), $\delta\lambda$ (one), and $\alpha\kappa$ (one) isotype expression was also observed. For all the $\mu\kappa$ -producing XLA B cell lines, the μ and κ mRNA transcripts were of native size, and sequence analysis across the regions of V_HDJ_H and V_KJ_K joining showed that Ig gene rearrangements occurred in a typical manner. A variety of V_HDJ_H and V_KJ_K gene rearrangements were observed, not only within the set of $\mu\kappa^+$ XLA B cells as a whole, but also among the cell lines derived from single patients. Southern blot analysis for genomic Ig H chain gene rearrangements was done to fully assess the extent of clonal heterogeneity among multiple $\mu\kappa^+$ 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.

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References

1. Rosen, F., M. Cooper, and R. Wedgwood. 1984. The primary immunodeficiencies. *N. Engl. J. Med.* 4 311:235.
2. Conley, M. E., P. Brown, A. Pickard, R. Buckley, D. Miller, W. Raskind, J. Singer, and P. Fialkow. 1986. Expression of the gene defect in X-linked agammaglobulinemia. *N. Engl. J. Med.* 315:564.
3. Pearl, E., L. Vogler, A. Okos, W. Crist, A. Lawton, and M. Cooper. 1978. B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficient states. *J. Immunol.* 120:1169.
4. Conley, M. E. 1985. B cells in patients with X-agammaglobulinemia. *J. Immunol.* 134:3070.
5. Schwaber, J., H. Molgaard, S. Orkin, H. Gould, and F. Rosen. 1983. Early pre-B cells from normal and X-linked agammaglobulinemia produce $C\mu$ without an attached V_H region. *Nature (Lond.)* 304:355.
6. Schwaber, J., and R. Chen. 1988. Premature termination of variable gene rearrangement in B lymphocytes from X-linked agammaglobulinemia. *J. Clin. Invest.* 81:2004.
7. Fu, S.-M., J. Hurley, J. McCune, H. Kunkel, and R. Good. 1980. Pre-B cells and other possible precursor lymphoid cell lines derived from patients with X-linked agammaglobulinemia. *J. Exp. Med.* 152:1519.
8. McCune, J., and S.-M. Fu. 1981. Ig biosynthesis in a human pre-B cell line. *J. Immunol.* 127:2609.
9. Mensink, E., R. Schuurman, J. Schot, A. Thompson, and F. Alt. 1986. Immunoglobulin heavy chain gene rearrangements in X-linked agammaglobulinemia. *Eur. J. Immunol.* 16:963.
10. Yancopoulos, G., T. Blackwell, H. Suh, L. Hood, and F. Alt. 1986. Introduced T cell receptor variable region gene segments in pre-B cells: evidence that B and T cells use a common recombinase. *Cell.* 44:251.

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11. Tedder, T., M. Crain, H. Kubagawa, L. Clement, and M. Cooper. 1985. Evaluation of lymphocyte differentiation in primary and secondary immunodeficiency diseases. *J. Immunol.* 135:1786.
12. Gregory, C., C. Kirchgens, C. Edwards, L. Young, M. Rowe, A. Forster, T. Rabbits, and A. Rickinson. 1987. Epstein-Barr virus-transformed human precursor B cell lines: altered growth phenotype of lines with germline or rearranged but nonexpressed heavy chain genes. *Eur. J. Immunol.* 17:1199.
13. Kubagawa, H., P. Burrows, C. Grossi, J. Mestecky, and M. Cooper. 1988. Precursor B cells transformed by Epstein-Barr virus undergo sterile plasma-cell differentiation: J-chain expression without immunoglobulin. *Proc. Natl. Acad. Sci. USA.* 85:875.
14. Levitt, D., H. Ochs, and R. Wedgwood. 1984. Epstein-Barr virus-induced lymphoblastoid cell lines derived from the peripheral blood of patients with X-linked agammaglobulinemia can secrete IgM. *J. Clin. Immunol.* 4:143.
15. Conley, M. E., and J. Puck. 1988. Carrier detection in typical and atypical X-linked agammaglobulinemia. *J. Pediatr.* 112:688.
16. Schibler, U., K. Marcu, and R. Perry. 1978. The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells. *Cell.* 15:1495.
17. Nelson, K., J. Haimovich, and R. Perry. 1983. Characterization of productive sterile transcripts from the immunoglobulin heavy chain locus: Processing of μ_m and μ_s mRNA. *Mol. Cell. Biol.* 3:1314.
18. Geliebter, J., R. Zeff, R. Melvoid, and S. Nathenson. 1986. Mitotic recombination in germ cells generated two major histocompatibility complex mutant genes shown to be identical by RNA sequence analysis: κ^{bm9} and κ^{bm6} . *Proc. Natl. Acad. Sci. USA.* 83:3371.
19. Perry, R., D. Kelley, U. Schibler, K. Huebner, and C. Croce. 1979. Selective suppression of the transcription of ribosomal genes in mouse-human hybrid cells. *J. Cell. Physiol.* 98:553.
20. Schuler, W., I.-J. Weiler, A. Schuler, R. Phillips, N. Rosenberg, T. Mak, J. Kearney, R. Perry, and M. Bosma. 1986. Rearrangement of antigen receptor genes is defective in mice with severe combined immunodeficiency. *Cell.* 46:963.
21. Brodeur, P., and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) in the mouse. I. One hundred IghV genes comprise seven families of homologous genes. *Eur. J. Immunol.* 14:922.
22. Klobeck, H., G. Combriato, and H. Zachau. 1984. Immunoglobulin genes of the κ light chain type from two human lymphoid cell lines are closely related. *Nucleic Acids Res.* 12:6995.
23. Pollok, B., R. Anker, P. Eldridge, H. Hendershot, and D. Levitt. 1987. Molecular basis of cell surface expression of immunoglobulin μ chain without light chain in human B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:9199.
24. Ravetch, J., U. Siebenlist, S. Korsmeyer, T. Waldmann, and P. Leder. 1981. Structure of the human immunoglobulin in μ locus: characterization of embryonic and rearranged J and D genes. *Cell.* 27:583.
25. Kleinfeld, R., R. Hardy, D. Tarlington, J. Dangi, L. Herzenberg, and M. Weigert. 1986. Recombination between an expressed immunoglobulin heavy chain gene and a germline variable gene segment in a Ly-1⁺ B-cell lymphoma. *Nature (Lond.)* 322:843.
26. Kabat, E., T. Wu, and H. Bilofsky. 1979. Sequences of Immunoglobulin Chains, National Institutes of Health, Bethesda, MD. 80:2008.
27. Berman, J., S. Mellis, R. Pollock, C. Smith, H. Suh, B. Heinke, C. Kowal, U. Surti, L. Chess, C. Cantor, and F. Alt. 1988. Content and organization of the human Ig V_H locus: definition of three new V_H families and linkage to the Ig C_H locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:727.
28. Kodaira, M., T. Kinashi, I. Vmemua, F. Matsuela, T. Noma, Y. Ono, and T. Honjo. 1986. Organization and evaluation of variable region genes of the human immunoglobulin heavy chain. *J. Mol. Biol.* 190:529.

29. Schroeder, H., J. Hillson, and R. Perlmutter. 1987. Early restriction of the human antibody repertoire. *Science (Wash. DC)*. 238:791.
30. Lee, K., T. Matsuda, T. Kinashi, M. Kodaira, and T. Honjo. 1987. A novel family of variable region genes of the human immunoglobulin heavy chain. *J. Mol. Biol.* 195:761.
31. Bentley, D. 1984. Most κ immunoglobulin mRNA in human lymphocytes is homologous to a small family of germline V genes. *Nature (Lond.)*. 307:77.
32. Fearon, E., J. Winkelstein, C. Civin, D. Pardoll, and B. Vogelstein. 1987. Carrier detection in X-linked agammaglobulinemia by analysis of X-chromosome inactivation. *N. Engl. J. Med.* 316:427.
33. Reth, M., and F. Alt. 1984. Novel immunoglobulin heavy chains are produced from DJ_H gene segment rearrangements in lymphoid cells. *Nature (Lond.)*. 312:418.
34. Bosma, G., M. Fried, R. Custer, A. Carroll, D. Gibson, and M. Bosma. 1988. Evidence of functional lymphocytes in some (leaky) *scid* mice. *J. Exp. Med.* 167:1016.
35. Malynn, B., T. Blackwell, G. Fulop, G. Rathbun, A. Furley, P. Ferrier, L. Heinke, R. Phillips, G. Yancopoulos, and F. Alt. 1988. The *scid* defect affects the final step of the immunoglobulin VDJ recombination mechanism. *Cell*. 54:453.