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Research Article

The X chromosome-linked antibody deficiency disease, X-linked agammaglobulinemia (XLA), results from failure of B lymphoid development. In the minor form of XLA, B lymphoid development terminates at the stage of immature B lymphocytes that produce truncated Ig heavy (H) chains composed of D-J-C(μ/δ), resulting from failure of VH gene rearrangement. Fusion of B cells from a patient with the minor form of XLA with mouse myeloma results in complementation of this defect; hybrid cells produce full-length H chains composed of VH-D-JH-C. The VH gene is of human origin. Complementation occurs independent of retention or loss of the human X (XLA) chromosome in the hybrid cells. These results indicate that the D-JH-C structure of the XLA B cells is fully functional for the subsequent rearrangement of a VH gene element, and that failure of immunoglobulin expression is susceptible to correction.

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Correction of the Molecular Defect in B Lymphocytes from X-linked Agammaglobulinemia by Cell Fusion

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Abstract

The X chromosome-linked antibody deficiency disease, X-linked agammaglobulinemia (XLA), results from failure of B lymphoid development. In the minor form of XLA, B lymphoid development terminates at the stage of immature B lymphocytes that produce truncated Ig heavy (H) chains composed of D-J-C(μ/δ), resulting from failure of V_H gene rearrangement. Fusion of B cells from a patient with the minor form of XLA with mouse myeloma results in complementation of this defect; hybrid cells produce full-length H chains composed of V_H -D-J_H-C. The V_H gene is of human origin. Complementation occurs independent of retention or loss of the human X (XLA) chromosome in the hybrid cells. These results indicate that the D-J_H-C structure of the XLA B cells is fully functional for the subsequent rearrangement of a V_H gene element, and that failure of immunoglobulin expression is susceptible to correction.

Introduction

X-linked agammaglobulinemia (XLA)¹ is a congenital antibody deficiency disease resulting from failure of B lymphoid development (1). There is variability in the stage at which the arrest of development occurs: the major phenotype is arrested at the stage of pre-B cells, while a minor phenotype is arrested at the stage of immature B lymphocytes (1–4). The failure of B lymphoid development is associated in both phenotypes with failure of Ig heavy (H) chain variable region rearrangement (5, 6). Creation of H chains results from ordered, somatic rearrangement of variable (V_H), diversity (D), and joining (J_H) gene segments (7–10). A single D segment is first recombined with a J_H segment, followed by recombination of a V_H onto the formed D- J_H element (8, 9, 11). The immature B cells of a patient with the minor phenotype of XLA produce truncated μ and δ H chains composed of D- J_H -constant (C)(μ/δ), resulting

from failure to rearrange a V_H segment (6). All H chains identified in this patient's cells are of this truncated form. X chromosome linkage implies that the failure of V_H recombination does not result from an inherited defect of the structural genes for H chain, which are encoded on chromosome 14 (12). We proposed that the failure of V_H gene rearrangement in the XLA B cells results from the absence of a regulatory element required for V_H recombination (6). Alternatively, the failure could result from production of an aberrant D- J_H structure that does not serve as a substrate for V_H to D- J_H recombination (13). Premature termination of rearrangement due to the absence of a regulatory element should be susceptible to complementation by a cell expressing a normal form of the gene that causes XLA. We report that fusion of D-J-C(μ/δ) XLA B cells with mouse myeloma complements the failure of V_H gene rearrangement. H chains produced by such hybrid cells are composed of V_H -D- J_H -C. The genes encoding each of these elements are of human parental origin, indicating that the mouse myeloma provides a *trans*-acting regulatory element necessary for V_H rearrangement which the XLA B cells lack. Complementation occurred in all hybrid cells examined, regardless of whether the human X chromosome was retained.

Methods

The patient has been identified as 4–8 in previous publications (3, 4, 6, 14, 15). B cell lines and cell hybrids with peripheral blood from this patient have been previously described (4, 6, 14). Cell hybrids with clone C6 of the LAZ 166 cell line were prepared by fusion with the RPC 5.4 mouse myeloma cell line (14) as previously described (16). Cells were grown in the alpha modification of MEM, supplemented with 10% fetal bovine serum.

To select for retention of the human (XLA) X chromosome, hybrid cells were cloned and maintained in hypoxanthine aminopterin thymidine (HAT) medium from the time of fusion. To select for loss of the human (XLA) X chromosome, hybrid cells were selected for 3 d after fusion in HAT medium, washed free of HAT, cultured 24–48 h without selection, and then grown in medium containing 6-thioguanine. All culture and selection conditions were as previously described (17). Karyotypic examination of the hybrid cells selected in HAT medium identified two clones of six that retained an intact human X chromosome.

Hybrid cell proteins were metabolically labeled with [³⁵S]-methionine. Ig proteins in hybrid cell supernatants were immunologically precipitated using H chain isotype specific antisera, with goat antiserum to rabbit IgG at equivalence as second antibody (18). Because of the formation of interspecies hybrid molecules by these hybrid cells, we used H chain-specific antisera from which L chain reactivity had been removed by absorption. Precipitated proteins were electrophoresed in 12.5% acrylamide slab gels (19) and autoradiographed. Supernatant from the RPC 5.4 mouse myeloma cell line was used to ensure species specificity, and supernatant from OO7 C4.4 and SMI 4, normal B cell lines producing IgG and IgM, respectively, were used for internal molecular weight markers.

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1. *Abbreviations used in this paper:* C, constant; D, diversity; H, heavy; HAT, hypoxanthine aminopterin thymidine; J_H , joining; V_H , variable; XLA, X-linked agammaglobulinemia.

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RNA was isolated from frozen cell pellets by the guanidine thiocyanate method and poly A-containing sequences were enriched as previously described (6). Northern blots were run as previously described (20). A cDNA library was constructed by the RNase H method of Gubler and Hoffman (21), without *Escherichia coli* DNA ligase in second strand synthesis. cDNA was ligated into lambda gt 10 (22) and infected into C600 Hf1. The library (3×10^6 independent phage) was screened for γ H chain with a 2-kb genomic clone of the human C γ 4 gene (23) and phage clone 4-1 of the C6 1-3 hybrid (referred to as C6 1-3 here forward) was isolated. The sequence of this clone was determined by sequential application of Maxam-Gilbert chemical degradation (24) of a Nar I-Eco RI fragment isolated from the phage clone, and dideoxy chain termination (25) on an Eco RI-Bgl II fragment subcloned in pUC 18 and 19.

Genomic DNA, isolated from frozen cell pellets as previously described (15), was digested to completion with Eco RI or Hind III and Southern blotted (26). The filters were probed with a 330-bp restriction fragment resulting from Eco RI-Bgl II (5' terminus to amino acid position 86) digestion, representing the V_H cDNA of C6 1-3 hybrid including most of framework region 3. Filters were washed to 0.2 \times standard saline citrate, 0.1% SDS, at 60 $^\circ$.

Selection of V_H sequences for comparison with the C6 1-3 cDNA was made by searching the Genbank data base. The 51P1 human VH1 sequence had not been entered at the time of comparison and was found by reference to the literature (27).

Results

Fusion of peripheral blood B lymphocytes from this XLA patient, limited to low level expression of δ and μ H chains, with mouse myeloma induces synthesis and secretion of IgM and IgG (14). In light of our finding that B cell lines from this patient produced truncated H chains composed of D-J_H-C (μ/δ) (6), we reexamined hybrid cell clone PBL 1-3 (resulting from fusion of peripheral blood B lymphocytes with RPC 5.4 mouse myeloma) to determine whether cell fusion induced production of full-size, mature H chains. μ and γ H chain proteins from this hybrid clone migrated as full-size molecules in SDS acrylamide gels (Fig. 1). mRNA encoding μ and γ H chains was also full-size (data not shown). These results suggest that cell fusion complemented the failure of V_H gene rearrangement, as well as an isotype switch from μ/δ to μ/γ .

Production of full-size H chains by XLA B cell hybrids might result from fusion with a rare mature V_H-D-J_H-C-producing XLA B lymphocyte. To ensure that we were testing for complementation, we fused an XLA B cell line clone with mouse myeloma. Clone C6 from the LAZ 166 parental line produces truncated δ chain composed of D-J_H-C δ , without V_H sequence (6). We have been unable to identify a second D-J_H-C allele in this clone. cDNAs isolated from clone C6 all had the same D-J_H rearrangement, and Southern blot analysis revealed only a single band hybridizing with J_H probe (data not shown). The B allele of this clone might have the same rearrangement as the A allele, or might have been deleted. Fusion of this clone with mouse myeloma resulted in a switch in H chain isotype, from δ to γ , observed by immune fluorescence (data not shown). Mouse parental γ chain was co-expressed. Three cell hybrid clones were selected for analysis of the human γ chains.

γ Chain protein from the hybrids of C6 migrated as full-size molecules of 55,000 D (Fig. 1). In contrast, δ chain protein of the parental clone was reduced by 14,000 D compared with normal (6). mRNA encoding γ chains from C6 cell hybrids were also full-length (data not shown).

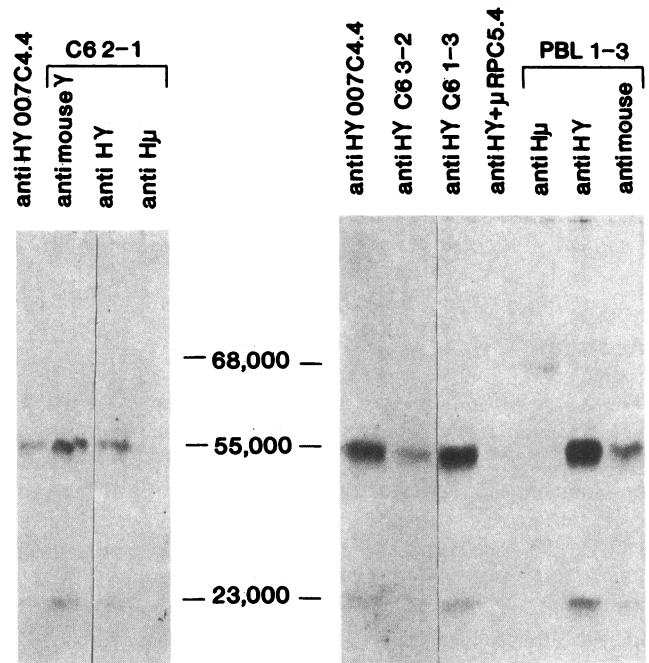


Figure 1. SDS-acrylamide gels of Ig proteins produced by XLA B lymphocyte and XLA cell line clone C6 hybrid cells. Hybrid clones were isolated from fusion of RPC 5.4 mouse myeloma cells with either peripheral blood lymphocytes of the XLA patient (PBL 1-3) or from fusion with clone C6 of the LAZ 166 cell line derived from this patient (clones C6 2-1, C6 1-3, and C6 3-2). Clone C6 has been shown to produce δ chain of 49,000 D compared with 63,000 for normal δ chain, composed of D-J_H-C δ , resulting from failure to rearrange V_H (6). Specificity of the precipitations was shown with supernatants from the mouse and human parent cell lines, as well as supernatant from OO7C4.4, a normal human B cell line clone which produces IgG. *Anti H γ* , precipitation with antiserum specific for human γ chains; *anti H μ* , antiserum specific for human μ chains; *anti mouse γ* , antiserum specific for mouse γ chains. The anti H γ and anti H μ were isotype specific and did not precipitate truncated δ chain from LAZ 166 clone C6 parent cells. Molecular mass markers included labeled and unlabeled normal human L (23,000 D), γ (55,000 D), and μ (68,000 D) electrophoresed in the same slab gels. Electrophoresis of cellular proteins from cell lysates yielded the same molecular masses for hybrid cell Igs.

To determine whether the increased size of the H chain mRNAs resulted from expression of V_H-D-J_H-C, a γ chain cDNA clone was isolated from the cDNA library of C6 hybrid clone 1-3. The 5' terminal sequence of cDNA clone C6 1-3 includes a human V_H, followed by D, J_H, and C γ 4 (Fig. 2). The clone begins with a leader sequence, followed by a V_H gene. This V_H gene is probably a human sequence of the V_HI family because (a) it has 94% identity to the human V_HI gene 51P1 in the framework regions (27), (b) it has all 10 invariant residues of human V_HI genes, and (c) the derived amino acid sequence of this V_H is 93% identical to the derived amino acid sequence of 51P1 through the framework regions. The complementarity-determining regions are less similar, with 56 of 66 nucleotides identical. In contrast, there is 77% identity of this V_H gene with the mouse V_H 124 gene (28), a member of the mouse J558 V_HII family that has most similarity to human V_HI genes (29). There are only 38 of the 49 mouse V_HII invariant residues in the derived amino acid sequence of the hybrid

sequence (30), and the derived amino acid sequence has only 68% identity with the mouse V_{H124} sequence.

The V_H sequence is followed by nine nucleotides of N insertional nucleotides, with preservation of only three nucleotides of D gene segment. Replacement of most of the D region has been reported in previous H chains (9, 10). The same J_H sequence identified in the D- J_H -C δ truncated cDNA of the C6 parent (6) forms the fourth framework region. The H chain isotype switch, previously identified in hybrids with peripheral blood cells (14), and identified in these hybrids by antisera and Northern blotting, is confirmed by the downstream sequence which is identical to human C γ 4 through the Nar I site at nucleotide 36 (amino acid position 126). There are two nucleotide differences in the sequence of human C γ 2 through this region, and four nucleotide changes from human C γ 1, which lacks the Nar I site. Mouse C γ sequences lack the Nar I site used for isolation of the 5' fragment for sequence analysis, and show at most 55% identity to the first 36 nucleotides of the hybrid cell C γ sequence.

We examined the V_H gene pools of genomic DNA from the mouse and human parental cells to confirm the human origin of the recombined V_H gene element. The V_H probe isolated from the C6 1-3 hybrid cell cDNA hybridized with multiple bands in DNA from the 166 C6 human parent cell line, while no bands were identified in DNA from the RPC 5.4 mouse myeloma parent cell line (Fig. 3). This confirms the identification of this V_H gene as a human V_{H1} gene element. Genomic DNA from the hybrid clone retained only a limited selection of the V_{H1} genes found in the human parental cell, indicating that V_H gene deletion accompanied recombination. The array of V_{H1} genes in 166C6 was essentially identical to that identified in the T cell line CEM, which has not undergone any rearrangements affecting its Ig H chain locus.

We tested whether retention or loss of the human X (XLA) chromosome from the hybrid cells affected complementation. Six hybrid clones derived from fusion with 166 C6 were grown from the time of fusion in HAT medium, forcing retention of the human (XLA) X chromosome. All six clones switched to production of full-length human γ H chains, identified by immune fluorescence and by gel electrophoresis (data not

shown). Another six clones were isolated under selection for loss of the human X (XLA) chromosome. All six clones switched to production of full-length human γ H chains. This suggests that the human (XLA) X chromosome plays no role in the observed complementation.

Discussion

These data show that RPC 5.4 mouse myeloma cells complement the failure of V_H gene rearrangement in B cells from a patient with the minor phenotype of X-linked agammaglobulinemia. B lymphocytes from this patient are immature forms, with the phenotype of limited expression of L chains, limitation to expression of μ/δ isotypes, and production of truncated H chains composed of D- J_H -C (μ/δ), as a result of failure to rearrange a V_H gene (4, 6, 15). Fusion of these cells with mouse myeloma results in recombination of a human V_H gene element with the formed D- J_H . This complementation is independent of retention or loss of the human (XLA) X chromosome. We have proposed that the arrest of B lymphoid development in this patient results from a central failure of V_H gene rearrangement (6, 15). The failure of V_H gene rearrangement could result from premature termination of normal V_{H1} -D- J_H gene rearrangement, or from abortive rearrangement that leaves a D- J_H structure not susceptible to V_H recombination (13). Sequence analysis of the D- J_H -C(μ/δ) produced by the XLA B cells suggests that the D- J_H rearrangement is normal, including the 5' 9-12-7 consensus sequence (6). Production of V_{H1} -D- J_H -C γ by the hybrid cells demonstrates that arrest at the stage of D- J_H -C(μ/δ) results from premature termination of normal V_{H1} -D- J_H gene rearrangement.

Cell fusion complemented the XLA phenotype in both peripheral blood B lymphocytes and the B cell line clone. Complementation was indicated by production of full-size H chains, V_H gene rearrangement, and switch in H chain isotype. Fusion of XLA minor form peripheral blood lymphocytes has been previously shown to result in a switch of H chain isotypes from μ/δ to μ , γ , and α H chains (14). We have confirmed our previous observation with peripheral blood cells and shown that B cell line clone C6 switched from δ to γ . 21 hybrid clones have now been examined, and complementation occurred in all of them. The H chain proteins from hybrid cells were full-sized, suggesting production of mature V_{H1} -D- J_H -C. Hybrid clone C6 1-3 was shown by sequence analysis to produce V_{H1} -D- J_H -C γ 4. The V_{H1} -D- J_H appears to have resulted from recombination of a V_{H1} gene with the preformed D- J_H structure of the A allele of C6. V_H gene rearrangement in the XLA B cell hybrids results directly from regulatory elements supplied by the myeloma parental cell. Whether the switch in H chain isotype also results from cell fusion, or is secondary to completion of V_H gene recombination, could not be determined by these experiments.

Complementation of the failure of V_H gene recombination indicates that the arrest of rearrangement in XLA results from premature termination of the normal rearrangement process. X chromosome linkage of XLA implies that there is not an inherited defect of the structural genes for H chain, which are encoded on chromosome 14 (12). Complementation occurred regardless of whether the hybrid cells were selected to force retention or loss of the human (XLA) X chromosome. This indicates that this XLA phenotype results from the absence (or a defective product) of a *trans*-acting gene element that can be

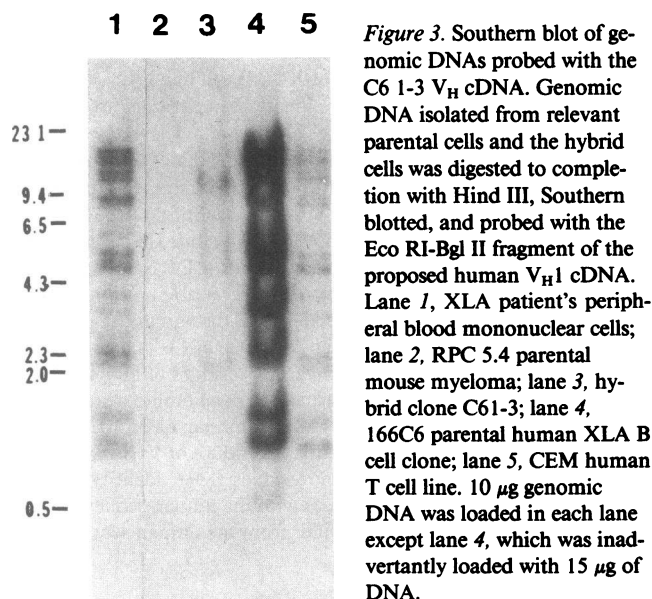


Figure 3. Southern blot of genomic DNAs probed with the C6 1-3 V_H cDNA. Genomic DNA isolated from relevant parental cells and the hybrid cells was digested to completion with Hind III, Southern blotted, and probed with the Eco RI-Bgl II fragment of the proposed human V_{H1} cDNA. Lane 1, XLA patient's peripheral blood mononuclear cells; lane 2, RPC 5.4 parental mouse myeloma; lane 3, hybrid clone C61-3; lane 4, 166C6 parental human XLA B cell clone; lane 5, CEM human T cell line. 10 μ g genomic DNA was loaded in each lane except lane 4, which was inadvertently loaded with 15 μ g of DNA.

supplied by the mouse myeloma. Recent evidence for lyonization of the XLA gene in B lymphocytes from carriers of this form of XLA indicates that the failure of V_H rearrangement is intrinsic to the B lymphocytes (15).

Premature termination of V_H -D- J_H rearrangement in XLA could result from a failure of the enzymes that regulate V_H -D- J_H rearrangement. B cell lines producing IgM with full-length μ chain have been established from rare B lymphocytes in peripheral blood of patients with the major (pre-B cell) form of XLA (31, 32). Failure of enzymatic catalysis of variable region gene rearrangement would result in a rate limiting step, with accumulation of precursors and decreased development of B lymphocytes. Given the short half-life of B lymphocytes in peripheral circulation (33), reduced production of B lymphocytes would result in the apparent absence of more mature forms. In the minor form of XLA, we have not identified B cells producing V_H -D- J_H , either because of a predominance of D(μ/δ) B cells, which are susceptible to transformation by Epstein-Barr virus (4, 6), or because the rate constant for V_H rearrangement is unmeasurably low in this form of XLA. To ensure that our experiments were a test of complementation, we fused clone C6 derived from the XLA B cell line LAZ 166, in which spontaneous V_H gene rearrangement has not been observed in more than 10^3 generations involving more than 10^{10} cells. Further, we continued an aliquot of the cells used for fusion with mouse myeloma cells in culture for 6 mo, and found no evidence for spontaneous V_H gene rearrangement.

Alt and co-workers (34, 35) have proposed a common mechanism for rearrangement of the variable genes of B and T cell antigen receptors. Conserved nonamer-heptamer consensus sequence pairs at the 5' and 3' ends of the gene elements provide a structural basis for transient pairing of the gene elements (8, 9). Yancopoulos et al. (34) have shown that a common recombinase acts on D- J_H recombination in T and B cells, requiring *trans*-acting elements to specify the site of action of the recombinase. Blackwell et al. (35) have reported that recombination is enhanced in cells that are actively transcribing V_H gene elements in their embryonic configuration. This suggests that the specification element may act by altering the conformation of the V_H gene locus, coincidentally permitting transcription of the locus. T cell function is normal in XLA (1), suggesting that recombination of T cell antigen receptors is normal. Thus, the recombinase common to T and B cell antigen receptor rearrangement is unlikely to be defective in XLA. Rather, there could be a defect in the element that provides access for rearrangement of V_H onto the preformed D- J_H . These access elements might then be encoded on the X chromosome.

Failure of V_H to DJ_H recombination could result in truncation of B cell development in this XLA patient. The disparity between the phenotype of his B cells (δ chain expression, membrane Ig expression, and peripheral circulation) (3, 4, 6, 25) and arrest of variable region recombination at the pre-B cell stage of DJ_H strongly suggests such a failure (6). Alternatively, a higher level regulatory gene might cause failure of B lymphoid development in this patient with the failure of VDJ gene rearrangement as a secondary effect. We prefer the hypothesis that sequential recombination of VDJ gene elements is regulated by the products of the intermediate stages of gene rearrangement, without reference to external events, until the complete antigen receptor is formed. Whether the affected gene in XLA regulates B lymphoid development in general or

variable region rearrangement specifically, complementation of the failure of V_H gene rearrangement independent of X chromosome retention and induction of H chain isotype switch suggests that this form of XLA will be susceptible to gene replacement therapy.

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