MUTATIONS IN THE MU HEAVY-CHAIN GENE IN PATIENTS WITH AGAMMAGLOBULINEMIA

LEMAN YEL, M.D., YOSHIYUKI MINEGISHI, M.D., ELAINE COUSTAN-SMITH, M.SC., REBECCA H. BUCKLEY, M.D., HUBERT TRÜBEL, M.D., LAUREN M. PACHMAN, M.D., GEOFFREY R. KITCHINGMAN, PH.D., DARIO CAMPANA, M.D., PH.D., JURG ROHRER, PH.D., AND MARY ELLEN CONLEY, M.D.

ABSTRACT

Background Most patients with congenital hypogammaglobulinemia and absent B cells are males with X-linked agammaglobulinemia, which is caused by mutations in the gene for Bruton's tyrosine kinase (Btk); however, there are females with a similar disorder who do not have mutations in this gene. We studied two families with autosomal recessive defects in B-cell development and patients with presumed X-linked agammaglobulinemia who did not have mutations in *Btk*.

Methods A series of candidate genes that encode proteins involved in B-cell signal-transduction pathways were analyzed by linkage studies and mutation screening.

Results Four different mutations were identified in the mu heavy-chain gene on chromosome 14. In one family, there was a homozygous 75-to-100-kb deletion that included D-region genes, J-region genes, and the mu constant-region gene. In a second family, there was a homozygous base-pair substitution in the alternative splice site of the mu heavy-chain gene. This mutation would inhibit production of the membrane form of the mu chain and produce an amino acid substitution in the secreted form. In addition, a patient previously thought to have X-linked agammaglobulinemia was found to have an amino acid substitution on one chromosome at an invariant cysteine that is required for the intrachain disulfide bond and, on the other chromosome, a large deletion that included the immunoglobulin locus.

Conclusions Defects in the mu heavy-chain gene are a cause of agammaglobulinemia in humans. This implies that an intact membrane-bound mu chain is essential for B-cell development. (N Engl J Med 1996;335:1486-93.)

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THE development of B cells proceeds through a series of well-defined stages characterized by sequential rearrangements of immunoglobulin genes and by the expression and extinction of enzymes and structural proteins required for presentation of the immunoglobulin molecule on the cell surface and signal transduction through this molecule.[1-3] Defects in B-cell development, both spontaneous defects in humans and those created by homologous recombination in mice, have clarified the importance of many of the genes involved in this process.

Patients with X-linked agammaglobulinemia have severe congenital hypogammaglobulinemia, and although they have normal numbers of pro-B cells, they have a marked reduction in the number of pre-B cells[4] and less than 1 percent of the normal number of B cells.[5] In 1993 two groups showed that X-linked agammaglobulinemia was caused by mutations in the gene for Bruton's tyrosine kinase (Btk), a cytoplasmic tyrosine kinase.[6,7] The substrates phosphorylated by Btk have not yet been identified; however, it is clear that Btk is activated by cross-linking of a variety of cell-surface receptors, including, perhaps most importantly, surface IgM on B-lineage cells.[8-11]

Although over 100 different mutations in *Btk* have been identified, some patients with the clinical and laboratory characteristics of X-linked agammaglobulinemia have not demonstrated mutations in this gene.[12-16] In addition, approximately 5 to 10 percent of patients with early-onset hypogammaglobulinemia and absent B cells are girls.[17-20] Together, these findings suggest that there may be autosomal recessive disorders that are phenotypically identical to X-linked agammaglobulinemia. To investigate this possibility, we studied two consanguineous families in which both boys and girls had panhypogammaglobulinemia and markedly reduced numbers of B cells (Fig. 1).

METHODS

Patients

The members of Family A, who live in Appalachia, are of Scottish–Irish ancestry and have received their specialty medical care at Duke University Medical Center in Durham, North Carolina. Patient 1 was evaluated in 1973, at nine months of age, because of a six-week history of fever, weakness, and rashes. Immunologic studies showed hypogammaglobulinemia and absent B cells. He was given the diagnosis of X-linked agammaglobulinemia and treated with plasma therapy to provide gamma globulin. At 4½ years of age, he died of chronic enteroviral encephalitis. Patient 2, a cousin of Patient 1, had bilateral pneumonia at four months of

From the Departments of Immunology (L.Y., Y.M., J.R., M.E.C.), Hematology/Oncology (E.C.-S., D.C.), and Virology (G.R.K.), St. Jude Children's Research Hospital, Memphis, Tenn.; the Department of Pediatrics, Duke University School of Medicine, Durham, N.C. (R.H.B.); the Department of Pediatrics, University of Mainz, Mainz, Germany (H.T.); the Department of Pediatrics, Northwestern University Medical School, Chicago (L.M.P.); and the Department of Pediatrics, University of Tennessee, Memphis (L.Y., D.C., M.E.C.). Address reprint requests to Dr. Conley at St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105.

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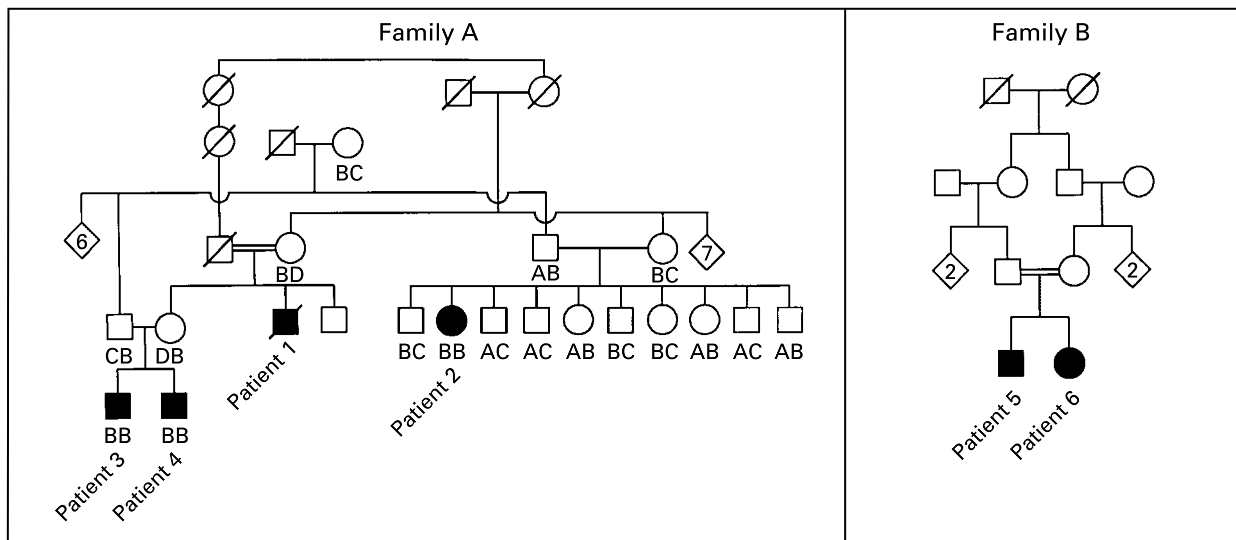

Figure 1. Pedigrees of the Study Families.

The immunoglobulin haplotypes inherited by the members of Family A are shown below the symbol for each family member. The haplotypes were detected by Southern blotting with a 2.2-kb probe from the mu-chain switch region to analyze *Sac*I-digested DNA. Circles denote female family members, squares male family members, and symbols with a slash deceased family members.

age and was evaluated in 1979 at six months of age, when she had persistent infection and failure to thrive. Laboratory studies demonstrated hypogammaglobulinemia and reduced numbers of B cells, but normal cellular immunity. Chronic enteroviral encephalitis developed, and the girl was treated with high-dose intravenous immune globulin and a course of intrathecal immune globulin, which resulted in the resolution of most signs of infection at six years of age. She is currently receiving intravenous immune globulin and is doing well, with mild mental retardation. (Patients 1 and 2 have been previously described as Patients 22 and 25 by McKinney et al.[18]) Patient 3, a nephew of Patient 1, had chronic otitis and had an episode of bronchopneumonia and gastroenteritis at seven months of age. In 1991, at one year of age, he was evaluated for recurrent infections and was found to have hypogammaglobulinemia. He has done well since that time with intravenous immune globulin treatment. Patient 4, the brother of Patient 3, was examined at one month of age in 1991 because of the family history of immunodeficiency. Treatment with intravenous immune globulin was begun when he was found to have hypogammaglobulinemia. He has not had noteworthy infections.

The members of Family B are of Turkish descent and have received their specialty care at Mainz University Hospital in Mainz, Germany. Recurrent respiratory tract infections developed in Patient 5 at three months of age. At seven months of age, in 1993, he was hospitalized for septic shock due to *Pseudomonas aeruginosa.* He was found to have hypogammaglobulinemia and absent B cells, and he was treated with intravenous immune globulin and given the diagnosis of X-linked agammaglobulinemia. Since that time he has done well, except for an episode of aseptic arthritis at two years of age. Patient 6, the sister of Patient 5, was hospitalized in 1994, at six months of age, with pneumonia. She was recognized to have hypogammaglobulinemia, and treatment with intravenous immune globulin was begun. She has had recurrent episodes of perirectal abscesses.

Patient 7 is the son of a Korean mother and a white father, born in 1983. At 15 months of age, two weeks after he had received oral poliovirus vaccine, fevers, weakness, rashes, and neutropenia developed. At 20 months of age, he was hospitalized at Children's Memorial Hospital, Chicago, for persistent fevers and weakness. He was found to have hypogammaglobulinemia and absent B cells, and treatment with intravenous immune globulin was begun. Although he has some residual weakness and recurrent otitis, he has had normal growth and development.

Polymerase Chain Reaction

Genomic DNA was isolated from peripheral-blood leukocytes. The polymerase chain reaction (PCR) was carried out in a 20-μl volume containing 100 ng of genomic DNA, 100 μM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 U of Taq DNA polymerase. For single-strand conformation polymorphism (SSCP) analysis, 3 μCi of [^{32}P]α-deoxycytidine triphosphate was added to the reaction mix. The samples were denatured at 95°C for 5 minutes, followed by 30 cycles at 95°C for 45 seconds and annealing at the temperature indicated in Table 1 for 30 seconds and at 72°C for 30 seconds, with a final 5-minute extension at 72°C.

Single-Strand Conformation Polymorphism

SSCP analysis was performed as previously described,[12] except that some PCR samples were digested with restriction enzymes before analysis. Labeled amplified DNA was mixed with loading buffer (95 percent formamide, 20 nM EDTA, 0.05 percent bromophenol blue, and 0.05 percent xylene cyanole FF) in a 1:5 ratio, denatured for five minutes at 90°C, placed on ice, loaded onto an MDE gel (AT Biochem, Malvern, Pa.), and electrophoresed at 4°C in 0.6× TBE buffer (1× TBE buffer is 89 mM TRIS, 89 mM borate, and 2 mM EDTA) at 2 to 4 W overnight. Gels were transferred to 3MM paper (Whatman, Clifton, N.J.), dried, and exposed to Kodak X-OMAT film (Kodak, Rochester, N.Y.).

Short-Tandem-Repeat Analysis

The primer pairs for microsatellite-repeat polymorphisms near the genes for Syk (D9S257, D9S910, and D9S922), mb-1 (D19S178 and D19S246), and EBF (D5S1471, D5S820, and D5S1456) and the mu heavy-chain gene (D14S611 and D14S118) were obtained from Research Genetics (Huntsville, Ala.). PCR was used to amplify ^{32}P-labeled DNA, which was analyzed on a denaturing 6 percent polyacrylamide gel.

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TABLE 1. PRIMER PAIRS USED TO SCREEN GENOMIC DNA FOR MUTATIONS IN THE MU HEAVY-CHAIN GENE.

PRIMER PAIR	SENSE	ANTISENSE	EXON	RESTRICTION ENZYME	SIZE OF FRAGMENTS	ANNEALING TEMPERATURE
					bp	°C
1a	ACTCAGAACGCCACTCAG	TGAGGTGGCTGCGTACTT	1	*Eco*RI	162 and 129	60
1b	CTTCCCATCAGTCCTGAG	ACGAAGACGCTCACTTTG	1	*Hae*III	157 and 106	56
2	ACTGCAGTGATTGCCGAG	AGTGGACCCCTGTGTGTC	2	*Bsa*HI	223 and 167	60
3a	CAGAGGCAGTTGCTACTC	TCACAGCTTCGCCATTCT	3	—	180	60
3b	ATGACAGCGTGACCATCT	GGGGTGCTTTCCACCATG	3	*Eco*RI	160 and 134	60
4a	CATGGTGGAAAGCACCCC	TGGCAGCAAGTAGACATC	4	—	157	58
4b	GATGTCTACTTGCTGCCA	GGTTTACCGGTGGACTTG	4	*Rsa*I	187 and 130	60
4c	TACTTCGCCCACAGCATC	GCACTCAGGACCAGTATC	4	*Pvu*II	169 and 159	60
m1	CTGCACTTGCTCTCCCCA	CTGTTGGGATCATTTCAC	Membrane exon 1	*Rsa*I	210 and 152	56
m2	CGTCACCTTGTTCAAGGT	CAGGGCAACAAGCGTATC	Membrane exon 2	*Mbo*I	230 and 194	60

Southern Blot Analysis

Standard methods were used in Southern blot analysis. The probes used to examine the genes for VH6 and JH4 and the constant-region genes for mu, delta, and gamma have been previously described.[21-23] To obtain a probe for the DH region, a 500-bp PCR product based on the sequence reported by Ichihara et al.[24] was produced with use of the forward primer 5′CAGGTACAGCTGTAGAGA3′ and the reverse primer 5′AGACAGCAGCCTTGAGAG3′.

Cloning and Sequencing

PCR products from the patients with visible band shifts on SSCP analysis were cloned into TA vector (Invitrogen, San Diego, Calif.) and sequenced with use of M13 primers or oligonucleotides from the human mu heavy-chain gene. All mutations were confirmed by a second, independent PCR reaction.

Immunofluorescence Staining

Peripheral-blood lymphocytes were incubated with monoclonal anti-CD19 antibody and with goat antihuman IgM, both conjugated to phycoerythrin. Bone marrow cells were stained with anti-CD19, anti-CD34, and polyclonal antibodies against human light chains conjugated to phycoerythrin, peridinin chlorophyll protein, and fluorescein isothiocyanate, respectively. Nuclear terminal deoxynucleotidyl transferase (TdT) and cytoplasmic mu heavy chain were detected with specific antibodies conjugated to phycoerythrin and fluorescein isothiocyanate applied after cells had been rendered permeable with OrthoPermeafix (Ortho Diagnostics, Raritan, N.J.). Immunophenotypes were analyzed with a FACScan flow cytometer with Lysis II software (Becton Dickinson, San Jose, Calif.).

RESULTS

Linkage Analysis

The patients in Family A were related through the maternal lineage; however, linkage analysis showed that the defect in this family did not map to the X-linked agammaglobulinemia locus at Xq22, and genomic DNA from Patient 2 did not demonstrate mutations in *Btk*. Therefore, a series of candidate genes that encode other proteins involved in signal transduction through the surface immunoglobulin receptor was chosen for analysis. Particular emphasis was placed on genes that are expressed early in B-cell differentiation and genes that are specific to the B-cell lineage. Linkage analysis was performed with highly polymorphic short tandem repeats located near the genes for Syk, a cytoplasmic tyrosine kinase encoded at 9q22[25]; CD79a (also known as mb-1 or Igα), an invariant component of the B-cell antigen–receptor complex that has been mapped to 19q13.2[26]; EBF, a transcription factor required for CD79a transcription that is encoded at 5q34[27]; and the immunoglobulin heavy-chain genes at 14q32.3.[28] The region showing the best linkage with the disease gene in Family A was near the immunoglobulin heavy-chain locus on the long arm of chromosome 14; when haplotypes derived from the polymorphisms at D14S611 and D14S118 were used, only a single crossover was seen. In Family B the parents shared a haplotype at this locus, and both children were homozygous for the shared haplotype.

Mutation Detection

A probe from the mu switch region, at the 5′ end of the exons for the mu constant-region gene, cross-hybridizes to polymorphic switch regions at the 5′ end of the genes for alpha 1 and alpha 2, revealing over 25 immunoglobulin haplotypes in genomic DNA digested with *Sac*I.[29] This probe demonstrated complete linkage with the defect in Family A, an event that would be expected to occur by chance with a likelihood of less than 0.1 percent. The mu switch-region probe revealed a deletion in the affected children in Family B. The extent of this deletion was determined with probes for VH6, DK1, JH4, and the mu, delta, and gamma heavy-chain genes. A deletion of 75 to 100 kb, encompassing

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the D-region genes, the J-region genes, and the mu constant-region gene, including the membrane exons, was identified (Fig. 2). Using the signal intensity of the *VH6* band to control for the amount of DNA loaded in each lane, we found that the signal intensity of the $C\mu$ band in the DNA samples from the parents was approximately 50 percent of that of the control band.

To screen for mutations in the mu constant-region gene in Family A, PCR primers that flank each exon were designed for use in SSCP analysis (Table 1). Genomic DNA from the affected girl in Family A and from patients who were presumed to have X-linked agammaglobulinemia, but in whom mutations in *Btk* had not been identified, was screened. Analysis of exon 4, the exon that encodes the CH4 domain, demonstrated the loss of normal bands and the gain of different aberrant bands in the DNA from Patient 2 and from a boy with presumed X-linked agammaglobulinemia without a mutation in *Btk* (Patient 7) (Fig. 3). The loss of the normal bands suggested that the alterations in both patients were homozygous or hemizygous. The same primer pair, pair 4c, was used to examine DNA from 100 unrelated people (200 chromosomes) and did not demonstrate the pattern seen in either patient. All three affected children in Family A had the same aberrant pattern, and each family member who inherited the B haplotype (Fig. 1) at the mu switch locus was heterozygous for the normal and the aberrant SSCP pattern.

DNA from both patients with altered band patterns in exon 4 was amplified by PCR, cloned, and sequenced. A single-base-pair substitution, a G-to-A transition at nucleotide 1831 (according to the num-

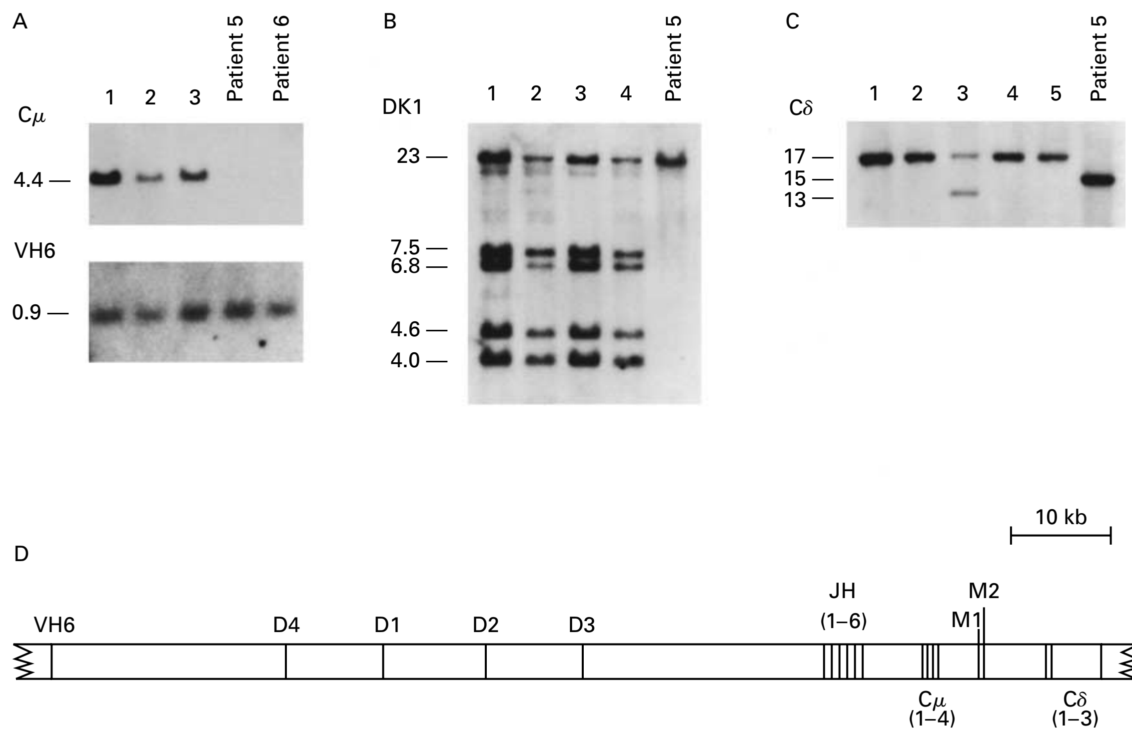

Figure 2. Southern Blot Analysis Demonstrating a Deletion That Includes the Mu Heavy-Chain Gene in Family B.

Genomic DNA from a control (Panel A, lane 1), from the parents (lanes 2 and 3), and from Patients 5 and 6 was digested with *Sac*I and analyzed with a $C\mu$ probe. The blot was stripped and rehybridized with a VH6 probe. DNA from four controls (Panel B, lanes 1, 2, 3, and 4) and from Patient 5 was digested with *Bam*HI and analyzed with a probe for DK1. This probe identifies five fragments in control DNA; four of these fragments correspond to genes within 9-kb tandem repeats in the DH region, and the fifth identifies a gene in a DH region that is on chromosome 15.[30] The four fragments from the DH region on chromosome 14 were absent in the DNA sample from the patient, localizing the 5′ border of the deletion to a region between VH6 and the first DK sequence in the DH region. In Panel C, DNA from five controls and from Patient 5 was digested with *Xba*I and analyzed with a $C\delta$ probe from the second hinge exon, which detects a 17- or 13-kb polymorphism.[31] The patient was homozygous for an aberrant 15-kb band, localizing the 3′ border of the deletion to a region between the mu membrane exons and the $C\delta$ constant-region gene. Panel D is a diagram of the immunoglobulin locus, with the extent of the deletion in Family B indicated below. The 5′ and 3′ borders of the deletion are represented by dashed lines. Values in Panels A, B, and C are kilobases.

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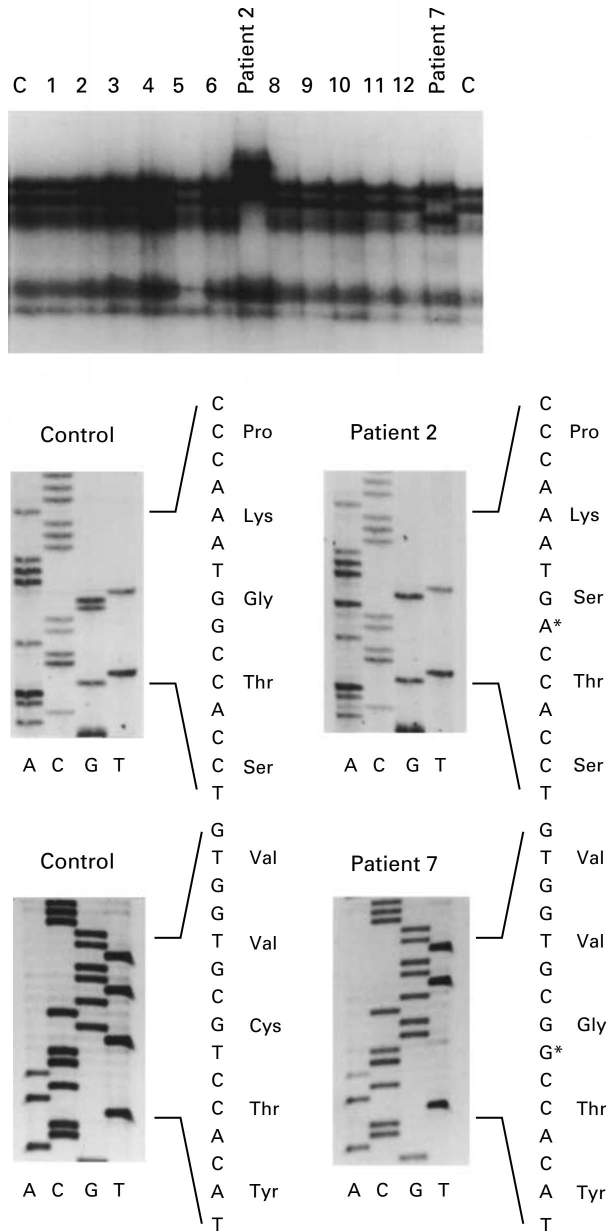

Figure 3. SSCP Analysis and Sequencing of Exon 4 of the $C\mu$ Gene, Demonstrating Mutations in DNA from Patients 2 and 7.

In the top panel, SSCP analysis with primer pair 4c (Table 1) shows altered patterns for Patients 2 and 7 as compared with controls (C) and other patients with reduced numbers of B cells (lanes 1 to 6 and 8 to 12). In the middle and bottom panels the partial DNA sequences of $C\mu$ exon 4 from Patients 2 and 7 are compared with the normal sequence. The base-pair substitutions in the patients are indicated by asterisks.

bering system of Friedlander et al.[32]), was found in the DNA of the affected girl. This alteration, which destroys an *Msp*I restriction site, was confirmed in the DNA of Patients 2, 3, and 4 by digesting amplified DNA with this enzyme. This single-base-pair replacement is at the −1 position of the alternative splice-donor site that is used to produce the membrane rather than the secretory mu transcript (Fig. 4). A mutation at this critical site would be expected to have three effects. First, this change would cause a substitution of serine for glycine in the secreted form of the mu chain. Second, in the membrane form of the mu chain, a positively charged lysine would be substituted for the wild-type, negatively charged glutamic acid. Finally, because the alternative splice-donor site has only weak homology to the consensus splice-donor sequence (Shapiro–Senapathy score, 71.5),[33] the loss of the consensus G at the −1 position would be expected to markedly reduce efficient splicing at this site, leading to an absence of the membrane form of the mu heavy chain.

The DNA from Patient 7 showed a T-to-G transition at nucleotide 1768 (Fig. 4); this alteration, which creates an *Msp*I restriction site, was confirmed by digesting PCR-amplified DNA with this enzyme. This nucleotide change results in the substitution of glycine for the wild-type cysteine at codon 536 in the carboxy-terminal immunoglobulin domain of the mu chain.[34] The cysteine at this site is the 3′ cysteine involved in the intrachain disulfide bridge that is characteristic of all immunoglobulin domains.[35] This mutation would be expected to result in an unstable form of both membrane and secreted mu chain.[36] To determine whether Patient 7 was homozygous or hemizygous for this mutation, his genomic DNA was digested with *Sac*I and examined by Southern blot analysis with a probe for the mu constant-region gene. A single fragment of the expected size was detected; however, the intensity of the signal was 50 percent of that of the control, suggesting a deletion of the mu-chain gene on one chromosome. Further studies using the VH6 probe and a probe for the gamma constant-region genes also showed a 50 percent decrease in signal intensity, indicating that the deletion was greater than 260 kb. The karyotype was normal.

Functional Studies

To determine the physiologic consequences of mutations in the mu-chain gene, we compared the phenotype of peripheral-blood lymphocytes from Patients 2, 3, 4, and 7 with those from patients with known mutations in *Btk.* The number of CD19+ B cells, as determined by flow cytometry, was markedly decreased in patients with mutations in *Btk;* however, 38 of 44 patients had detectable B cells (between 0.01 and 1.0 percent of peripheral-blood lymphocytes[5]). By contrast, none of the patients with mutations in the mu-chain gene had detectable

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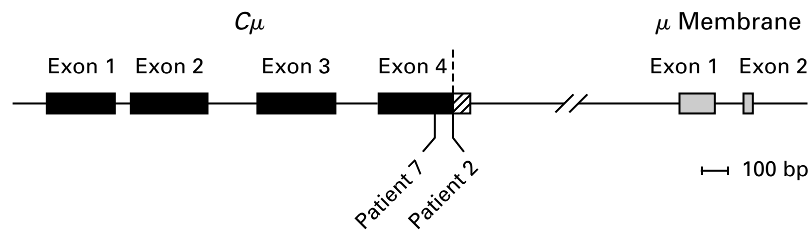

Figure 4. Diagram of the Mu Heavy-Chain Gene Showing the Four Exons of the *Cμ* Constant-Region Domains and the Two Exons of the Membrane Domain.

The alternative splice site, marked by the dashed line at the end of exon 4, allows the *Cμ* constant-region domains (solid blocks) to be spliced to the membrane exons (gray blocks). If this splice site is not used, a transcript for the secretory form of the mu heavy chain is produced that includes the secretory carboxy-terminal end (hatched area). The sites of the mutations in Patients 2 and 7 are shown.

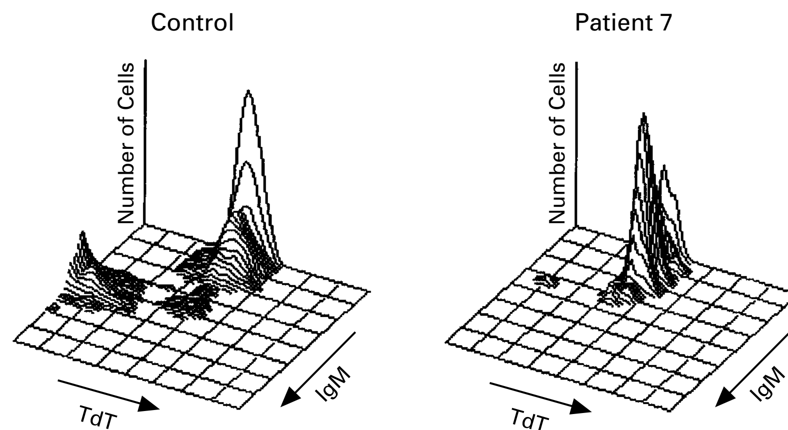

Figure 5. Evaluation of CD19+ Cells from the Bone Marrow of Patient 7 for the Expression of Terminal Deoxynucleotidyl Transferase (TdT) and Mu Heavy Chain.

The isometric contour plot shows that Patient 7 had normal percentages of pro-B cells that expressed TdT. However, there was a marked decrease in the next stages of B-cell differentiation: early pre-B cells that are TdT+ and IgM+, and late pre-B cells that are TdT− and IgM+.

B cells (less than 0.01 percent of peripheral-blood lymphocytes).

Bone marrow was obtained from Patient 7 to determine the point in B-cell differentiation at which maturation was blocked. The results demonstrated that the patient had normal percentages of the earliest precursors, pro-B cells that express CD19 and CD34 on the cell surface and terminal deoxynucleotidyl transferase in the nucleus. However, there was a marked decrease in the number of cells at the next stage in B-cell differentiation, the stage at which mu heavy chain is first expressed in the cytoplasm (Fig. 5). There was a small number of IgM-positive cells, but the staining for IgM was dim (the mean fluorescence intensity for IgM was 34.65 in Patient 7, as compared with 120.35 in the control), a finding consistent with the hypothesis that the amino acid substitution in the mu heavy chain in Patient 7 resulted in an unstable protein.

DISCUSSION

Many proteins are required for the assembly and expression of the immunoglobulin molecule; however, it is the mu heavy-chain gene itself that is at the center of this process. Our studies show that several different types of mutations in this gene are a cause of profound immunodeficiency in humans. Homologous recombination has been used to "knock out" the J-region genes or the membrane exons of the mu-chain gene in murine models of immunodeficiency.[37-39] In contrast to alterations in *Btk,* which result in a mild B-cell abnormality in mice,[40-42] but a much more severe defect in humans,[4,5] the B-cell phenotype of mice that have mutations in the mu constant-region gene is identical to that of patients with mutations in this gene. In both there is a complete absence of B-cell production and profound hypogammaglobulinemia.

Heterozygous deletions of the mu heavy-chain

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gene have been reported in two patients with complex chromosomal rearrangements involving chromosome 14.[28] These patients had multiple morphologic defects but no immunodeficiency. It is notable that the heterozygous parents, siblings, and aunts and uncles included in our study were also free of immunodeficiency. Deletions of the gamma, alpha, or epsilon heavy-chain gene, or of a combination of these genes, have been described by several groups.[43] Persons with these deletions have subclass deficiencies but usually have minimal or no signs of immunodeficiency.

The two families in this study represent the only families that we have analyzed in which both males and females have lacked B cells. Although we have identified mutations in either the mu heavy-chain gene or *Btk* in 79 unrelated persons referred for genetic analysis, we have also studied an additional 4 patients with sporadic disease who did not have mutations in either gene. This suggests that the immunodeficiency in these patients is due to a combination of genetic and environmental factors or that there may be additional forms of autosomal recessive disease resulting in the absence of B cells.

The identification of genes that cause immunodeficiencies has both clinical and biologic implications. As improved therapies for immunodeficiencies become available, it may be critical to know the exact nature of the genetic defect. Specific mutations also provide clues to the normal development of the B-cell lineage. The findings in our patients support the hypothesis that expression of a surface mu chain is essential for the progression of B-cell differentiation beyond the pre-B-cell stage.

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