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A High Proportion of ADA Point Mutations Associated with a Specific Alanine-to-Valine Substitution

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Summary

In 15%–20% of children with severe combined immunodeficiency (SCID), the underlying defect is adenosine deaminase (ADA) deficiency. The overall goal of our research has been to identify the precise molecular defects in patients with ADA-deficient SCID. In this study, we focused on a patient whom we found to have normal sized ADA mRNA by Northern analysis and an intact ADA structural gene by Southern analysis. By cloning and sequencing this patient's ADA cDNA, we found a C-to-T point mutation in exon 11. This resulted in the amino acid substitution of a valine for an alanine at position 329 of the ADA protein. Sequence analysis revealed that this mutation created a new *BalI* restriction site. Using Southern analyses, we were able to directly screen individuals to determine the frequency of this mutation. By combining data on eight families followed at our institution with data on five other families reported in the literature, we established that five of 13 patients (seven of 22 alleles) with known or suspected point mutations have this defect. This mutation was found to be associated with three different ADA haplotypes. This argues against a founder effect and suggests that the mutation is very old. In summary, a conservative amino acid substitution is found in a high proportion of patients with ADA deficiency; this can easily be detected by Southern analysis.

Introduction

Severe combined immunodeficiency (SCID) is a syndrome characterized by a profound deficiency in B- and T-cell function (Bortin and Rimm 1977). Infants born with this disorder die in the first few years of life unless treated successfully by bone marrow transplantation or kept in a sterile isolation facility (Bortin and Rimm 1977; Buckley et al. 1986). In most cases of SCID the underlying molecular defect is unknown. In 15% of the patients, however, a deficiency of the enzyme adenosine deaminase (ADA; E.C.3.5.4.4) is found (Kredich and Hershfield 1983; Buckley et al. 1986) and is thought to be responsible for their immunodeficiency (Giblett et al. 1972).

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In recent years the normal ADA cDNA (Orkin et al. 1983; Valerio et al. 1983; Wiginton et al. 1983) and normal ADA gene (Wiginton et al. 1986) have been characterized, facilitating studies of the molecular defects in patients with ADA deficiency. The specific mutations found to date have been similar to those found in other inherited disorders. In most patients Northern RNA analyses have revealed normal sized ADA mRNA (Adrian et al. 1984), suggesting that most ADA mutant alleles carry a point mutation leading to an inactive or unstable ADA protein. In eight mutant ADA cDNAs sequenced to date, point mutations affecting amino acid sequence have been found (Bonthron et al. 1985; Valerio et al. 1986; Akeson et al. 1987, 1988). One patient has been described with abnormal sized ADA mRNA, and an RNA splicing defect was identified in this case (Akeson et al. 1987). In two patients, no ADA mRNA has been detected. These patients have been shown to carry deletions encompassing the first exon and promoter regions of the ADA gene (Berkvens et al. 1987; Markert et al. 1988).

We are interested in elucidating the molecular defect

in patients with ADA deficiency who are well characterized clinically and immunologically. In this study, we began our work by focusing on one patient, LB, who was previously shown to have very low levels of ADA enzyme activity (Markert et al. 1987*a*). The research described in the present paper led to the precise definition of the mutation in this patient, a point mutation leading to the substitution of valine for alanine in the ADA protein. As the point mutation created a restriction-site polymorphism, it was possible by Southern analysis to screen additional patients for this mutation. The surprising result of this study was that five of 13 patients (seven of 23 alleles) were found to carry this mutation. These patients represented a wide diversity of ethnic and racial backgrounds. This is an unusually high percentage of a particular mutation in an unselected population.

Material and Methods

Cell Lines

B lymphoblastoid cell lines were established from Ficoll-Hypaque-purified peripheral mononuclear cells by Epstein-Barr virus transformation (Markert et al. 1987*b;* Katsuki and Hinuma 1975). The patients reported in the present paper have been extensively studied clinically at Duke University Medical center (Buckley et al. 1986; Markert et al. 1987*a*). Enzymatic studies have confirmed the patients' lack of ADA enzymatic activity (Markert et al. 1987*a*). HLA typing was used to confirm the identity of the cell lines.

RNA and DNA Purification from the Lymphoblastoid Cell Lines

RNA was harvested by the technique of Chirgwin (1979; Markert et al. 1987*b*) by using guanidine isothiocyanate extraction and purification by cesium chloride gradient centrifugation. Poly A+ RNA was isolated according to a method described elsewhere (Avis and Leder 1972). DNA was purified by the method of Gustafson et al. (1987).

Southern DNA Analyses

These analyses were done using minor modifications of the standard technique of Southern (Southern 1975), as described by Markert et al. (1987*b*).

Northern RNA Analyses

These analyses were done using formaldehyde agarose gels according to a method described elsewhere (Markert et al. 1987*b*).

Probes

The ADA cDNA and genomic probes were provided by Dr. John Hutton (Cincinnati). The cDNA and first exon genomic probes used in figures 1 and 2 have been described in detail elsewhere (Markert et al. 1987*b*). The genomic probe used in figure 4 is the 849-bp-*Pst*I-*Pst*I genomic fragment whose 3′ end is in exon 11. The genomic probe mentioned in table 1 is the 299-bp *Pst*I-*Pst*I genomic fragment whose 5′ end is in exon 5 (see Wiginton et al. 1986 for sequences). The actin control probe, pHFβA.1, a cDNA for human fibroblast cytoplasmic β actin (Gunning et al. 1983), was provided by Dr. Larry Kedes (Stanford). The probes were labeled with ^{32}P to a specific activity of $>10^8$ cpm/μg by using the random primer method (Feinberg and Vogelstein 1983, 1984).

cDNA Synthesis, Cloning, and Sequencing

cDNA was prepared by the method of Gubler and Hoffman (1983). The cDNA was size-selected by the potassium-acetate-gradient method of Aruffo and Seed (1987). *Eco*RI adapters (Pharmacia) were added, and the cDNA was cloned into λgt10 (Promega). This was packaged (Promega) and titered on *Escherichia coli* C600hfl. Two hundred thousand plaques were screened by hybridization after transfer to nitrocellulose filters by Benton and Davis plaque hybridization (Benton and Davis 1977). DNA from positive clones was purified by a plate lysate method (Maniatis et al. 1982), and recombinants were confirmed by restriction-endonuclease mapping. cDNAs were subcloned into pUC and then into M13 vectors. Both strands of five cDNA isolates were sequenced by dideoxy chain-termination analysis (Sanger et al. 1977; Markert et al. 1988).

Results

ADA mRNA Levels

Figure 1 shows RNA blot hybridization (Northern blot) of poly A+ RNA isolated from the B cell lines established from the ADA-deficient patient LB, her parents, and a normal control. The probe used in panel *A* was the *Bam*HI-*Acc*I fragment of the ADA cDNA, which contains exons 2–12. Lanes 1–4 contain poly A+ RNA. All individuals show ADA mRNA of the correct size (approximately 1,500 bases). As a control, the membrane in figure 1*A* was stripped and reprobed with a human fibroblast cytoplasmic β actin cDNA as shown in panel *B*. All poly A+ RNA samples, including the RNA from patient LB in lane 3, hybridized well to the

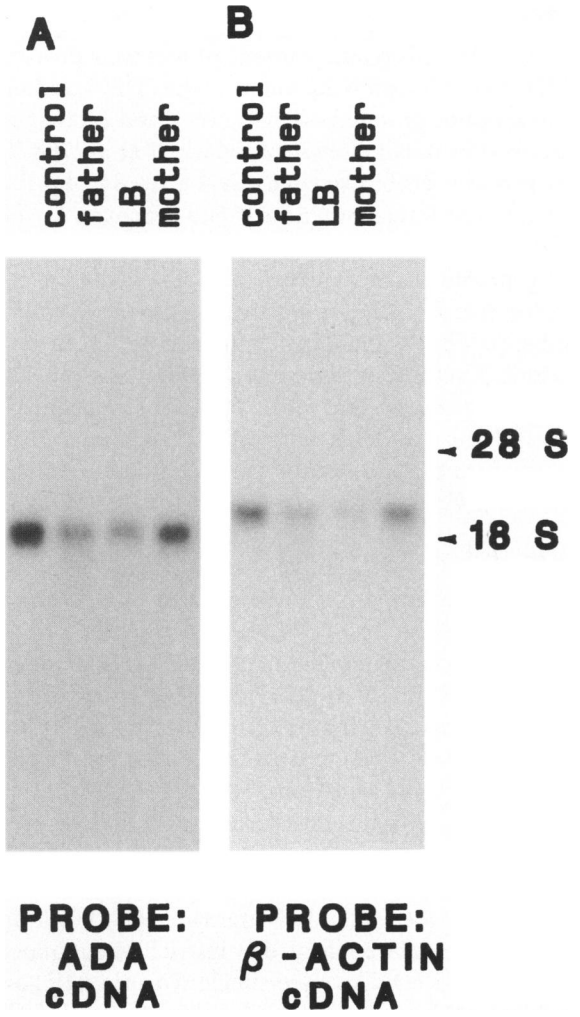

Figure 1 Northern blot of poly A+ RNA from LB family. Three micrograms of poly A+ RNA from the cell lines indicated were applied to the gel. After electrophoresis and capillary blot transfer, the filter was hybridized with radiolabeled (*A*) ADA cDNA or (*B*) actin cDNA. The same filter is shown in both panels. The positions of 18S and 28S RNA is indicated.

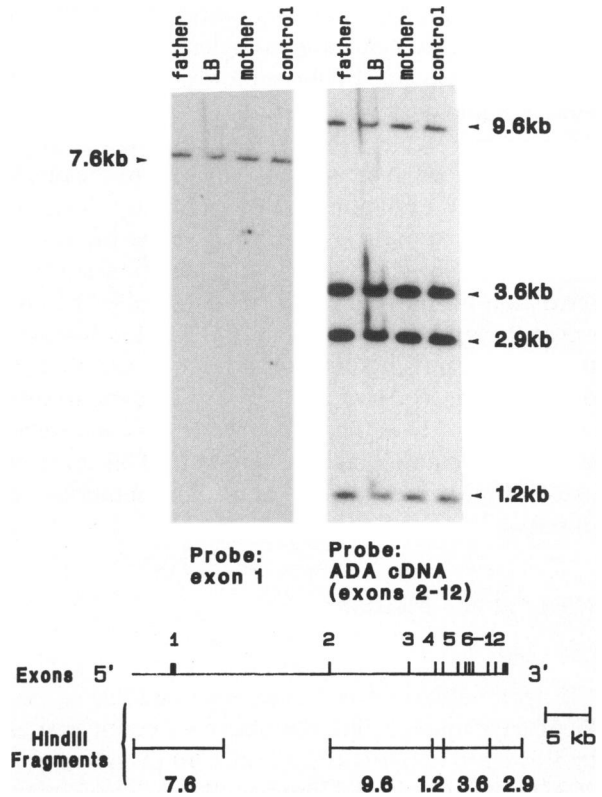

Figure 2 Southern blot analyses of DNA from LB family members. Ten micrograms DNA from family members was digested with *Hin*dIII, electrophoresed, and transferred to a membrane by capillary blotting. The filter on the left was hybridized with a genomic probe isolated from exon I, and the filter on the right is the same filter hybridized with a probe isolated from an ADA cDNA. At the bottom of the figure is a diagrammatic representation of the ADA gene. The 12 exons are indicated by vertical lines. The sizes of the normal *Hin*dIII restriction fragments are indicated.

actin probe at the expected position for β-actin mRNA, 2,000 bases. Normalization of the signal intensities, in panel *A*, with those for the actin control in panel *B* shows that all RNA samples carry equivalent amounts of ADA mRNA.

Analysis of Gene Integrity at the DNA Level

Figure 2 shows DNA blot hybridization (Southern blot) of *Hin*dIII-digested DNA from the same B cell lines examined in figure 1. The membrane was probed with an ADA genomic clone taken from the exon 1 region and with a cDNA probe encompassing exons 2–12. DNA from patient LB demonstrates the same pattern as is seen both in her parents and in the control. This demonstrates that the ADA structural gene in patient LB is intact.

cDNA Sequence Results

cDNA prepared from poly (A) RNA was cloned into λgt10, and 200,000 plaques were screened. Forty positives were identified; eight were subcloned; and five were chosen for sequence analysis. These were subcloned into M13 for sequencing. The regions of the five cDNAs that were sequenced are indicated in figure 3. Four of the five encompassed all the protein-coding portion of the ADA cDNA (positions 96–1184).

Only two differences were found in comparing the wild-type sequence to the LB cDNA. Patient LB has

a C-to-T mutation in exon 11 (position 1081) of the cDNA according to Wiginton et al. [1986]) and a G-to-A polymorphism in exon 6 (position 629 of the cDNA). The mutation at position 1081 causes an amino acid substitution of valine for alanine at position 329 of the protein sequence. The polymorphism at position 629 does not affect the amino acid sequence.

Southern Analysis of Mutation at Base Pair 1081

Analysis of the cDNA sequence around the 1081 mutation revealed that a new *BalI* restriction site had been formed. Patient LB and her parents were examined to determine whether she was homozygous for the mutation. Figure 4 (lanes 1–3) shows the results of a Southern analysis with DNA from the LB family. Patient LB demonstrates only the abnormal restriction fragment of 1.8 kb. Both of her parents carry one normal fragment of 2.6 kb and one abnormal fragment of 1.8 kb. Thus, patient LB is homozygous for this mutation. Because of the cDNA sequencing studies shown in figure 3, we feel confident that this is the only mutation in this patient's ADA mRNA.

Seven additional ADA-deficient patients (and/or their parents) followed at our institution were screened by Southern analysis in looking for this mutation. As can be seen in figure 4, two additional patients from the seven families tested carried this mutation; one (patient JB) in addition to LB is homozygous for the mutation, and another (patient BJ) is heterozygous for the mutation. (Data from the normal families are not included.) The homozygous state found in LB and JB can likely be explained by their parents being related. The LB parents come from a small town and believe it is possible that they could have an ancestor in common. The parents of JB are known to be related. Since DNA from BJ's father is not available, it is not known whether BJ inherited the 1081 mutation or whether it was a spontaneous mutation. LB, JB, and BJ are not related to each other by recent intermarriage. They, GM2756, and GM2825A come from widely scattered regions of the United States and include both white and black individuals.

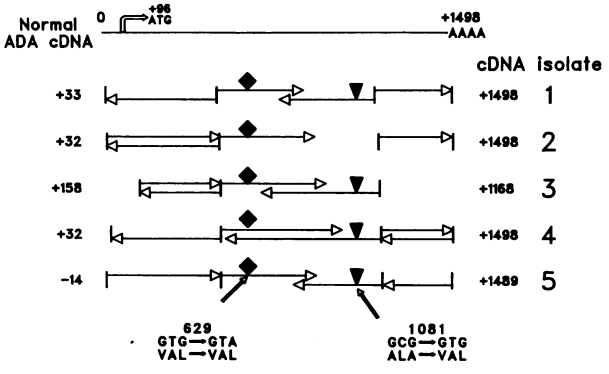

Figure 3 Sequence analysis of the ADA cDNA from patient LB. Five ADA cDNA clones were independently isolated. The 5′ and 3′ limits of the cDNAs are indicated relative to the normal ADA cDNA, shown at the top of the figure. The five ADA-cDNA clones were sequenced as indicated by the arrows (← and →). The polymorphisms (◆) and mutations (▼) found in the five cDNAs are indicated. The region missing in clone 2 was never successfully cloned into M13.

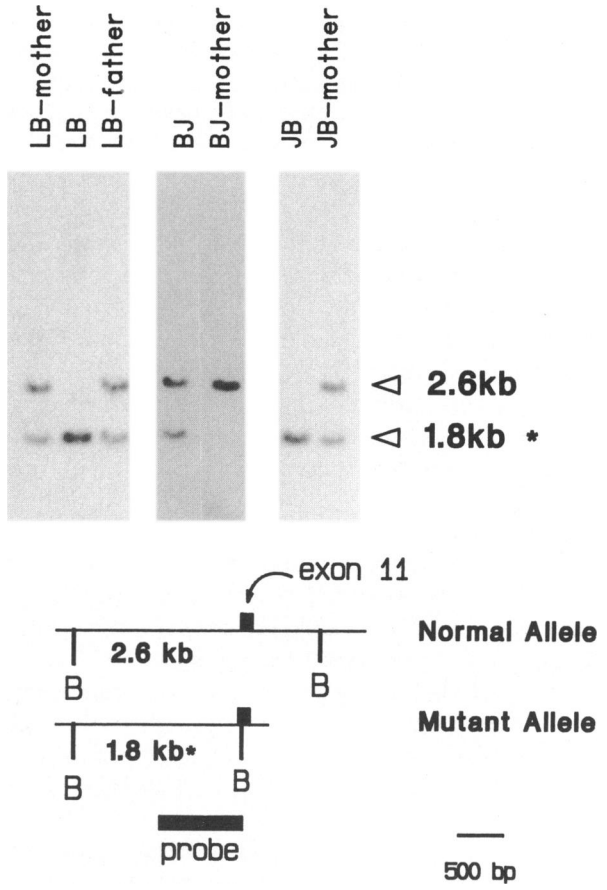

Figure 4 Examination of patient DNA for the 1081 mutation. Ten micrograms of genomic DNA from the indicated cell lines was digested with the restriction enzyme *BalI* and was analyzed by the Southern blot technique. Family-member designations are indicated above the lanes. The normal restriction fragment is 2,606 bp; the mutant restriction fragment indicated by the asterisk is 1,848 bp. At the bottom of the figure is a diagrammatic illustration of restriction fragments found in the normal and mutant alleles.

The sequences of five mutant alleles (GM1751, GM-2471, GM2756, GM2825A, and GM2606) have been published elsewhere (see Akeson et al. 1987 for previous summary; also see Akeson et al. 1988). Two of the five (GM2825A and GM2756) carry the 1081 mutation. When our data are combined with data previously published, we see that five of 13 patients and seven of 22 alleles with point mutations carry this mutation. This is a relatively high frequency of a single point mutation in this population.

In an assessment of gene frequency, it is important to determine which alleles are not included in the compilation. In this study, although 13 patients were examined, 22—not 26—alleles are included. In four individuals, only one allele is listed. For GM2471, only one cDNA was successfully cloned (Valerio et al. 1986); no comment can be made about the second allele. For GM1715, the patient had "partial" ADA deficiency (Wolf et al. 1976); one ADA cDNA was normal (Bonthron et al. 1985). For GM2825A, the second allele carries an RNA splicing problem (Akeson et al. 1987); thus, it is not included in this compilation of point mutations. For one of our ADA-deficient patients, only DNA from the mother was available, since the patient had died in 1977. Thus, only one parental allele could be examined by Southern analysis.

Our assessment of gene frequency in this population may be somewhat high because we included both alleles of patient LB and JB. The parents of JB are related, and the parents of LB probably are related. Some investigators would count each patient only once. This would lower the observed frequency of this mutation to 25%. On the other hand, we may have underestimated the frequency of this mutation, since, in the case of two patients (three alleles), no patient material was available and parent DNA was screened. One of these parents might have been normal, and the affected patient might have carried a spontaneous mutation on one allele. This would not have been detected by examination of parent DNA. Of 19 mutant alleles examined from *patient* material, seven carried the 1081 mutation. If only one allele each of LB and JB are counted, then five of 17 alleles examined from patient material carry the 1081 mutation.

A total of five other point mutations have been scattered throughout the ADA gene. None of these mutations create restriction-site changes that lend themselves to screening by Southern analysis. In contrast to the 1081 alanine-to-valine mutation described in the present paper, none of the other mutations leading to amino acid substitutions have been detected more than two times in the 10 alleles that have been fully sequenced.

Analysis of ADA Haplotypes in Patients Carrying the 1081 Mutation

Table 1 divides the seven alleles carrying the 1081 mutation into three ADA haplotypes. The first three alleles (the first haplotype) come from two white patients, GM2825A and LB. Sequence analysis has shown that these three alleles carry an "A" at position 629. The homozygosity of patient LB at position 629 was confirmed by Southern analysis (data not shown). The second three alleles (the second haplotype) comes from two black patients, GM2756 and JB. These three alleles carry a "G" at position 629. GM2756's allele was sequenced (Akeson et al. 1988). The nucleotide at 629 in patient JB's alleles was determined by Southern analysis (data not shown). The third haplotype is represented by the affected allele of patient BJ. Southern analysis reveals a restriction fragment that is of a different size than those found in patients LB and JB (see table 1). Thus, there are at least three haplotypes associated with the 1081 mutation. The possibility also exists that there may be more than three haplotypes, since DNA from GM2825A and GM2756 was not examined by Southern analysis.

Table 1

Three ADA Haplotypes Found in Association with the 1081 Mutation

Mutant Allele (race)	Restriction Fragment Size[a] 629	(kb)	1081
GM2825A-a (W)	A		T
LB-a (W).....	A	3.2	T
LB-b (W)	A	3.2	T
GM2756-a (B)	G		T
JB-a (B).....	G	1.9	T
JB-b (B).....	G	1.9	T
BJ-a (B).....	?	2.9	T

[a] A 299-bp DNA probe from intron 5 (see Material and Methods) was hybridized to a Southern blot of genomic DNA digested with *BalI*. A 1.9-kb restriction fragment is detected by Southern analysis if a G is present at position 629 of exon 6. A common polymorphism occurs when an A is present at 629. *BalI* can then not cut at 629; a longer restriction fragment of 3.2 kb is usually detected. Finding a 2.9-kb fragment is unusual. Data on GM2825A are from Akeson et al. (1987); data on GM2756 are from Akeson et al. (1988). The two alleles from each patient are designated a and b.

Discussion

The question arises as to why the 1081 mutation is found so frequently in this ADA-deficient patient population. Since three different ADA haplotypes are shown to carry the 1081 mutation, two possibilities are most likely: either this is a very old mutation or position 1081 is a hot spot for mutation. The finding of three haplotypes is not consistent with a founder effect.

It is possible that the CpG dinucleotide at position 1081 is a hot spot for mutation. The dinucleotide CpG is known to frequently be associated with C-to-T mutations and with G-to-A mutations (reviewed in Cooper and Youssoufian 1988). The vast majority of CG mutations are CG-TG or CG-CA, consistent with methylation-induced deamination of 5-methylcytosine (Coulondre et al. 1978). This type of mutation was involved not only in the 1081 mutation reported in the present paper but also in three of the other five mutations described to date (CG to TG at 396, CG to CA at 397, and CG to CA at 727; the numbers refer to the cDNA sequence of Wiginton et al. [1986]). It is not involved in the mutations at either position 334 or position 1006. Thus, four of six mutations reported for ADA deficiency involve this dinucleotide. What is unknown is why there is such a predominance of mutations in the CpG at position 1081, as opposed to any of the other CpG dinucleotides in the ADA cDNA.

Our conclusions regarding this mutation in ADA deficiency contrast with recent findings in phenylketonuria (PKU). In PKU, certain mutations are associated with particular PKU haplotypes (DiLella et al. 1986, 1987). This suggests that all individuals with a given mutation descended from a common ancestor (founder effect). With the 1081 ADA mutation, individuals carrying the same mutation have different ADA haplotypes. This argues against the founder effect.

It remains to be determined how the 1081 mutation causes a lack of ADA enzyme activity. The mutation is a conservative amino acid substitution of valine for alanine. One would not predict this mutation to significantly affect protein hydrophobicity or secondary structure (Garnier et al. 1978; Akeson et al. 1988). Some information has already been published regarding this mutation. Akeson et al. (1988) conducted in vitro transfection experiments using a cDNA whose only abnormality was the 1081 mutation. They found markedly decreased levels of ADA activity, compared with experiments using the normal ADA cDNA (Akeson et al. 1988). Western blots of cell lysates from the cell line GM2825A, which is heterozygous for this mutation (Akeson et al. 1987), showed no immunoreactive material (Wiginton and Hutton 1982). Akeson concluded from their studies that the mutant protein (Ala to Val, 1081) may be unstable or structurally altered (Akeson et al. 1988). Ultimate proof will require in vitro mutagenesis of the 1081 mutation to wild type and demonstration of restored ADA activity. To investigate these possibilities, in vitro translation experiments are currently underway with wild-type and mutant constructs.

Regardless of why the 1081 mutation is so common, this observation may prove useful in patient studies. Patients can easily be screened by Southern analysis. In a high frequency of cases, this test will reveal the underlying mutation of at least one allele.

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