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Somatic Mosaicism for a Newly Identified Splice-Site Mutation in a Patient with Adenosine Deaminase–deficient Immunodeficiency and Spontaneous Clinical Recovery

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Summary

Absent or severely reduced adenosine deaminase (ADA) activity produces inherited immunodeficiency of varying severity, with defects of both cellular and humoral immunity. We report somatic mosaicism as the basis for a delayed presentation and unusual course of a currently healthy young adult receiving no therapy. He was diagnosed at age 2½ years because of life-threatening pneumonia, recurrent infections, failure of normal growth, and lymphopenia, but he retained significant cellular immune function. A fibroblast cell line and a B cell line, established at diagnosis, lacked ADA activity and were heteroallelic for a splice-donor-site mutation in IVS 1 (+1GT→CT) and a missense mutation (Arg101Gln). All clones (17/17) isolated from the B cell mRNA carried the missense mutation, indicating that the allele with the splice-site mutation produced unstable mRNA. In striking contrast, a B cell line established at age 16 years expressed 50% of normal ADA; 50% of ADA mRNA had normal sequence, and 50% had the missense mutation. Genomic DNA contained the missense mutation but not the splice-site mutation. All three cell lines were identical for multiple polymorphic markers and the presence of a Y chromosome. In vivo somatic mosaicism was demonstrated in genomic DNA from peripheral blood cells obtained at 16 years of age, in that less than half the DNA carried the splice-site mutation ($P<.002$, vs. original B cell line). Consistent with mosaicism, erythrocyte content of the toxic metabolite deoxyATP was only minimally elevated. Somatic mosaicism could have arisen either by somatic mutation or by reversion at the site of mutation. Selection in vivo for ADA normal hematopoietic cells may have played a role in the return to normal health, in the absence of therapy.

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Introduction

Mutations at the adenosine deaminase (ADA) locus result in a spectrum of immunologic dysfunction. The majority of patients initially described with ADA deficiency had the full-blown classic syndrome of severe combined immunodeficiency (SCID) with infantile onset of overwhelming infections with fungal, viral, and bacterial agents and retardation of growth and development. Immunologic evaluation typically demonstrated lymphopenia and absence of both in vitro cellular immune function and non-maternally derived immunoglobulin. A smaller group of initially described patients presented with a slightly more delayed onset of disease and retention of some degree of humoral immune function as manifested by presence of immunoglobulins. In both phenotypes, the disorder was rapidly fatal in the absence of either definitive therapy with bone marrow transplantation or ameliorative therapy provided by enzyme replacement. More recently a small but increasing number of ADA-deficient patients are being ascertained with much milder and often other more subtle immunologic defects, including autoimmunity, hyper-IgE, and asthma, with onset of serious disease in late childhood and even in late adolescence (Shovlin et al. 1993). Last, a number of children have been ascertained with "partial" ADA deficiency by screening for ADA in erythrocytes of normal adults and newborns. Such individuals have absence of enzyme in erythrocytes but retention of variable amounts of ADA (5%–90% of normal) in such cultured cells as Epstein-Barr virus–immortalized B lymphoid line cells and fibroblasts. Although the majority of such partially ADA-deficient individuals are still in childhood, partial deficiency in these children has not been associated with immunologic abnormalities. In general, the amount of residual ADA (in tissues other than erythrocytes) correlates inversely with severity of disease, with ~5% of normal ADA appearing to be compatible with normal immune function. Similarly, the extent of accumulation of the toxic metabolite, deoxyATP, and/or urinary excretion of the substrate deoxyadenosine, shows gross correlation with severity of disease (reviewed in Kredich and Hershfield 1989; Hirschhorn 1993*a*, 1993*b*).

Both the ADA structural gene, encoded on the long arm

of chromosome 20, and the expressed cDNA have been isolated and sequence determined. The cDNA contains 1,089 bp of coding sequence, distributed in 12 exons within ~32 kb of DNA (Daddona et al. 1984; Valerio et al. 1984; Wiginton et al. 1984, 1986). Multiple different mutations have now been reported. These are primarily missense mutations, but splice-site and nonsense mutations, small deletions, and two different large deletions have also been reported (Bonthron et al. 1985; Valerio et al. 1986; Akeson et al. 1987, 1988; Markert et al. 1988; Hirschhorn et al. 1989, 1990, 1991, 1992*a*, 1992*b*, 1993*a*, 1993*b*; Berkvens et al. 1990; Hirschhorn 1992; Kawamoto et al. 1993; Yang et al. 1994). Several of these mutations have been found in more than one unrelated patient, either through independent recurrence or apparently from a common ancestor (Akeson et al. 1987; Markert et al. 1989; Hirschhorn et al. 1991; 1992*a*; 1993*a*)

This genetic heterogeneity is believed to be a major factor in the clinical heterogeneity. We now report somatic mosaicism for a newly identified splice-site mutation at the ADA locus, as the basis for delayed presentation and a highly unusual course in a patient, initially diagnosed during the 3d year of life because of life-threatening infections, who is currently a healthy young adult despite the absence of therapy.

Patient, Material, and Methods

Case History

A brief initial case history and results of subsequent lymphocyte proliferation studies were reported elsewhere (Wolf et al. 1976; Uberti et al. 1978, 1979), and are included below. The patient, born in 1972, was the 2,750-g product of a pregnancy complicated by maternal heroin addiction. He was hospitalized at age 9 mo with gastroenteritis, dehydration, and oral moniliasis and at age 15 mo with tracheobronchiolitis, gastroenteritis, and oral moniliasis. He recovered both times with standard therapy but had somewhat diminished lymphocytes (1,518 cells/mm^3) at age 9 mo and lymphopenia (840/mm^3) at age 15 mo. At age 26 mo he was hospitalized with pneumonia, failure to thrive (height and weight <3d percentile), marked lymphopenia (148/mm^3), paucity of tonsillar tissues, and absence of palpable lymph nodes. He improved with standard therapy but was readmitted 3 wk later with bilateral pneumonia, dyspnea, and oral moniliasis. The first 3$^1/_2$ mo of this 4$^1/_2$ mo hospital course were characterized by a slowly progressive bilateral pneumonia with shifting areas of diffuse infiltrates and increasing hypoxia (oxygen saturations falling to 30%–40%), despite multiple courses of antibiotics. Immunologic studies showed a variable but persistent lymphopenia (172–1,336 cells/mm^3), low numbers of T lymphocytes (E-rosetting; 8%–12%), relatively increased percentage of B lymphocytes (EAC rosetting; 34%) and a bone marrow aspirate with few lymphocytes or plasma cells. Lymph node biopsy revealed a "hypoplastic node with follicles, but largely absent T-dependent areas and sinus histiocytosis." Skin tests for delayed hypersensitivity with tetanus, SK/SD, and candida were repeatedly negative despite a recent DT booster. Four attempts to sensitize the patient with the potent immunogen dinitrochlorobenzene (DNCB) were unsuccessful. However, lymphocyte stimulation by phytohemagglutinin (PHA) or by allogeneic lymphocytes was reported as normal while that with tetanus and candida was low. Quantitative immunoglobulins remained normal (IgG = 670; IgM = 121; and IgA = 46). Daily thymosin injections were started on the 74th hospital day, with an increase in T lymphocytes (E-rosetting cells) to 60% in the peripheral blood, but without clinical improvement or alteration in DNCB sensitization and delayed skin tests. With diagnosis of ADA deficiency (table 1), partial exchange transfusions with irradiated packed red blood cells (RBCs) were started on the 99th day to provide some degree of enzyme replacement (Polmar et al. 1976). Treatment with fresh frozen plasma

Table 1

Adenosine Deaminase Activity

	Patient	Normal	Percent Normal
Peripheral blood cells:			
RBCs:			
2 years	.9[a]	85.0[a]	1.0[a]
16 years	< .5[a]	85.8[a]	< 1.0[a]
	(.16[b])	(60.9[b])	(.3[b])
Mononuclear cells:			
2 years	240[a]	1,291[a]	18.6[a]
16 years	...	...	...
	(44[b])	(665[b])	(6.6[b])
Cell Lines:[c]			
GM2445 (fibroblasts)	50.4[a]	806[a]	6.2[a]
	(18.4[b])	(499[b])	(3.7[b])
GM 1715 (lymphoid line)	22.0[a]	2,003[a]	1.1[a]
	(14.8[b])	(1,782[b])	(.8[b])
MS1267 (lymphoid line)	2,307.0[a]	3,986[a]	57.9[a]

[a] Method of assay also detects ADA activity due to a second isozyme (adenosine aminohydrolase; see Patient, Material, and Methods) not affected in ADA$^-$ SCID and can be significant with respect to residual ADA activity. For comparison, ADA in mixed mononuclear cells from five other patients with ADA$^-$ immunodeficiency, determined concurrently by the same method was 5%–12% of normal (67–156; average = 100 nmol/mg protein/min) (Hirschhorn et al. 1979).

[b] More specific method of assay; EHNA-inhibitable ADA activity excludes activity of the nonrelevant isozyme (see Patient, Material, and Methods). ADA in mononuclear cells of seven patients with ADA$^-$ SCID, assayed by this method, were <1%–3.8% of normal (0.3–24.9 nmol/mg protein/h; average = 8.0) (Hirschhorn et al. 1979).

[c] Cells from GM2445 and GM1715, cultured for several passages in media supplemented with horse serum, which does not contain any endogenous ADA that could potentially be endocytosed or bind to ADA combining protein on the cell surface. Both had <1% normal ADA activity (data not shown).

was added 1 wk later. His condition stabilized and then began to improve, although he remained lymphopenic (200–800 cells/mm^3). Skin tests became weakly reactive by the 117th hospital day; the intermittent fever resolved; and he began to gain weight and was discharged after 133 hospital days. Shortly after discharge, more extensive and repeated testing of lymphocyte proliferation revealed diminished proliferative responses to PHA (30% of normal) but normal responses to Con A and pokeweed mitogen (PWM) when performed with media containing human serum. However, significantly diminished responses to all three mitogens were found when such testing was performed in media containing horse serum (devoid of exogenous ADA) (Uberti et al. 1978).

Over the next 4 years the patient's height and weight gradually increased from <3d percentile to the 25th–50th percentile, but he remained lymphopenic (429–630 cells/mm^3). He was briefly hospitalized twice for respiratory infections, but symptoms resolved both times in <7 d. There were no further hospitalizations during age 7–20 years. Absolute blood lymphocytes had increased (1,100/mm^3) at age 9 years, with 62% T cells (E-rosetting), and the plasma and red cell transfusions were discontinued, although monthly thymosin injections (1 mg/kg) were maintained until age 13 years. The absolute lymphocyte count was essentially normal (average = 1,878/mm^3; range = 1,092–3685) during age 11–14 years, with a normal percentage of T cells (T11) (average = 81%; range = 67%–88%). Response to mitogens at ages 13 and 14 years were within the normal range, except for a slightly diminished response to PHA at age 13 years (percentages of control stimulation at ages 13 and 14 years, respectively, were as follows: PHA = 38% and 90%; Con A = 157% and 130%; and PWM = 164% and 142%; normal response >50% of control). He had developed varicella at age 11 years, with a normal presentation and a 10 d course without any specific therapy, attesting to normal in vivo cellular immunity. Total immunoglobulins (IgM, IgG, and IgA) remained normal throughout. However, IgE was markedly elevated (431 U/ml) when first determined at 5 years and remained elevated over the next 10 years (average = 510; n = 14). The patient was last examined at 16 years of age, at which time he was a healthy-appearing adolescent with height and weight in the 25th–50th percentile (171 cm and 56.3 kg). There were no specific medical concerns or recent history of intercurrent illness. Telephone follow-up when the patient was 20 years old revealed absence of any medical problems.

General Methods

Isolation of DNA, digestion, electrophoresis, Southern transfers, and hybridization were by standard methods as described elsewhere (Tzall et al, 1989).

Amplification of Reverse-Transcribed RNA

Isolation of RNA, reverse transcription, amplification by PCR (RT/PCR), subcloning into pUC18, and sequence analysis were as described elsewhere (Hirschhorn et al. 1991), but we used a 5′ sense primer in exon 1 (MS033 5′ GCCCAGACGCCCGCCTTCGACAAGCCCAAA).

Amplification of Genomic DNA, Direct Sequencing, and Analysis of Digested DNA on Polyacrylamide Gels

ADA exons 1, 2, 4, 6, 7–9, and 10–11 were amplified by PCR from genomic DNA as described elsewhere (Hirschhorn et al. 1992*a*, 1994). Exon 3 in a 483-bp fragment was amplified with primer BG4299 in IVS 2 and with BG4300 in IVS 3, both containing added restriction sites, and with 30 cycles of 94°C for 90 s and 72°C for 2 min, preceded by 5 min at 94°C prior to addition of *Taq* polymerase and terminated by extension at 72°C for 10 min. Exon 5 in a 560-bp fragment was amplified with primer BG5151 in IVS4 and with DG747 in IVS5 as above, with annealing at 60°C for 30 s. Primer sequences were as follows: BG4299 5′, cgagaattcGTTCTCAGTTTCCCCATCTGTCCAGTGGGAGCAG; BG4300 5′, aggaagcttCTGAGGGACAGGCCTGGTCCTAGTCATAGGGAT; BG5151 5′, GTGAACATCATGGCAGGCCCAAGCTTAGCA; and DG747 5′, TCATGAAGCCCGAAGTTCATGCCAGTGGGCTCAAG. Amplified DNA, purified on Magic PCR columns (Promega), was directly sequenced with ADA-specific primers and an fMole kit (Promega), essentially as directed but with an increase over the recommended amount of DNA and radioactivity, prolonged exposure time, and two intensifying screens. Exon 1 was amplified in the presence of ^{33}P dCTP or dATP; 7 µl of the amplification was digested with *Dde*I in 35 µl; an aliquot was fractionated by electrophoresis at 25 V overnight in 10% acrylamide, 0.5% Bis gels. The gel was analyzed by phosphoimaging and quantitation of radioactivity in each band.

Analysis for VNTRS and Y chromosome

VNTR alleles at the chromosome 20 D20S19 (CMM6) locus and at the chromosome 16 D16S85 locus (CriPat 3′ HVR) were analyzed by hybridization of Southern transfers of *Pst*I- or *Pvu*II-digested genomic DNA with the CMM6 or the CriPat 3′HVR probe (Nakamura et al. 1988; Kidd et al. 1989, pp. 783, 815; Keith et al. 1990). Y chromosome–specific amplification used primers from the 5′ and 3′ sequence in pY53.3 (Sinclair et al. 1990), with 30 cycles of 94°C for 45 s, 62°C for 75 s, and 72°C for 105 s.

Biochemical Measurements

Measurement of ADA, measurement of deoxyATP content of erythrocytes, and electrophoresis in starch gels, with in situ detection of ADA activity, were all performed as described elsewhere (Hopkinson et al. 1969; Hirschhorn et al. 1979, 1981, 1982). Two different methods were

used for determination of ADA activity. In the first, inosine produced by deamination of adenosine is converted to uric acid by addition of nucleoside phosphorylase and xanthine oxidase (Boehringer Mannheim), and the rate of increase in A_{293} is measured spectrophotometrically (Hopkinson et al. 1969). The second assay method is more specific and measures the conversion of radiolabeled adenosine to inosine (and to hypoxanthine by endogenous nucleoside phosphorylase) in the presence and absence of an inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Hirschhorn et al. 1979). EHNA inhibits only the ADA isozyme that is encoded by the ADA locus on human chromosome 20 and deficient in ADA− SCID, without inhibiting ADA activity of a second 100-kD isozyme present in mononuclear cells and not affected in ADA$^-$ SCID (Hirschhorn and Ratech 1980; Daddona and Kelley 1981; Ratech et al. 1981). EHNA-inhibitable activity therefore measures only ADA encoded by the relevant chromosome 20 locus and excludes activity of the nonrelevant isozyme, the activity of which is significant with respect to measurements of residual ADA in cells from ADA-deficient individuals.

Results

Adenosine Deaminase Activity of Erythrocytes, Mononuclear Cells, Cultured Cell lines, and Concentrations of deoxyATP

ADA activity shortly after diagnosis (using a less specific assay than subsequently was employed) was 1% of normal in erythrocytes and 18% of normal in mixed mononuclear cells, as compared with 5%–12% of normal in mononuclear cells of five other patients similarly tested (table 1). At age 16 years, (with use of a more specific assay that measures only the ADA isozyme encoded on chromosome 20 and deficient in ADA$^-$ SCID; see Patient, Material, and Methods and footnote to table 1), there was still essentially no ADA (0.3% of normal) in RBCs and appreciable amounts of ADA activity (6% of normal, as compared with <1%–3.8% of normal ADA in mononuclear cells of seven patients with ADA$^-$ SCID similarly assayed; Hirschhorn et al. 1979) in mononuclear cells. The measurements of ADA activity in mononuclear cells at ages $2^1/_2$ years and 16 years cannot be directly compared, because of both the difference in specificity of the assays used (see Patient, Material, and Methods and footnote to table 1) and the alteration in relative subpopulations of B and T cells containing differing ADA activity (Tung et al. 1976; Barton et al. 1979). Nonetheless, at both $2^1/_2$ years and 16 years, the patient, although clearly ADA deficient, had the highest residual ADA activity found in mononuclear cells from a series of patients with ADA$^-$ SCID.

ADA activity, in both cultured lymphoid and fibroblast cell lines (GM1715 and GM2445), established at diagnosis, showed deficiency of ADA, with <4% of normal ADA both in the lymphoid line and in fibroblasts cultured in media containing FCS, which contains endogenous ADA that could be endocytosed (table 1). ADA activity was <1% of normal in both cell lines when cells were cultured in media containing horse serum, which lacks endogenous ADA (not shown). These values are consistent with values reported by other laboratories for these cell lines (Dadonna et al. 1980; Daddona and Kelley 1981; Uberti et al. 1983). The residual ADA in the B cell line (GM1715), analyzed <6 mo after establishment, had a normal molecular weight and K_m but slightly increased anodal mobility on electrophoresis and on in situ staining for enzyme activity (data not shown). The alteration in charge is consistent with the subsequently described Arg101Gln missense mutation (Bonthron et al. 1985), with replacement of positively charged arginine by neutral glutamine. In startling contrast, a lymphoid line established at 16 years of age (MS1267) exhibited >50% of normal ADA activity (table 1) with a normal electrophoretic mobility (not shown).

Concentrations of the toxic metabolite deoxyATP in erythrocytes were not measured initially, since diagnosis and institution of partial exchange transfusions preceded the discovery of marked elevations of deoxyATP in erythrocytes of ADA$^-$ SCID individuals (Cohen et al. 1978; Coleman et al. 1978). RBC deoxyATP content at age 16 years, when the patient had not been receiving exchange transfusions for 7 years, was 31 nmol packed erythrocytes/ml (normal = 3±1.3; range = 1–10; n = 34). This concentration of deoxyATP is markedly lower than what we have found in eight patients with ADA$^-$ immunodeficiency (average = 893; range = 174–2,248) (Hirschhorn et al. 1991, 1992*b*; and R. Hirschhorn, unpublished data) and is within the range found in children with "partial " ADA deficiency (R. Hirschhorn, unpublished data). The lesser accumulation of deoxyATP is consistent with both the 6% residual ADA activity in peripheral blood mixed mononuclear cells (PBMs) of this child and isolation of a B cell line expressing 50% of normal ADA activity.

Molecular Analysis and Identification of Mosaicism for a Splice-Site Mutation

To elucidate the molecular basis for the differences in ADA activity in the three different cell lines, we determined the specific mutations present in the original lymphoid and fibroblast cell lines (GM1715 and GM2445), both of which lacked ADA activity, and in the newly established lymphoid line (MS1267), which expressed ADA activity. A missense mutation (Arg101Gln) due to a G→A transition at nt 302 (1 = ATG) has previously been identified in cDNA from the lymphoid line lacking ADA activity (GM1715) (Bonthron et al. 1985). The mutation creates a new site for the restriction enzyme *Bsg*I, and all three cell lines were heterozygous for the mutation, by both enzyme digestion (fig. 1) and sequence analysis (not shown). To determine the mutation on the second allele, we directly

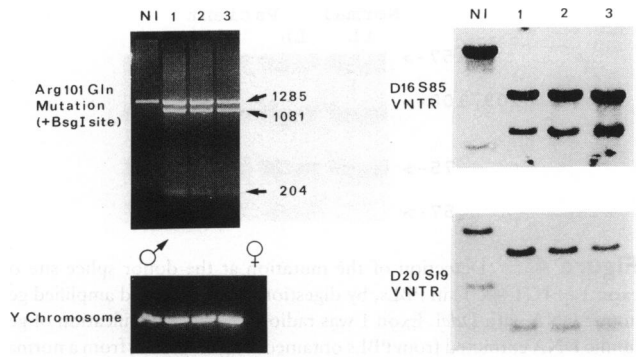

Figure 1 Identity of all three cell lines; heterozygosity for an Arg101Gln mutation (+*Bsg*I site); presence of a Y chromosome; and identity for alleles at two highly polymorphic VNTRs (CMM6 D20S19 and CriPat 3'HVR D16S81). (The lines were also identical at the ADA locus for two uncommon haplotypes defined by RFLPs and silent nucleotide changes; see text.) All three cell lines are heterozygous for the Arg101Gln mutation, as demonstrated by generation of a new site for *Bsg*I. Exons 4 and 5 were amplified from genomic DNA and were digested with *Bsg*I. Normal exons 4 and 5 do not contain a site for *Bsg*I, while the G302A transition (1=ATG) predicting the Arg101Gln substitution introduces a *Bsg*I site, resulting in generation of 1.081- and 0.204-kb fragments. Lanes Nl, Normal; Lanes 1, Patient's fibroblasts (GM2445); Lanes 2, Patient's lymphoid line not expressing ADA (GM1715); Lanes 3, Patient's lymphoid line expressing 50% normal ADA (MS1267). (Heterozygosity was confirmed by direct sequence analysis of amplified genomic DNA.) Genomic DNA was amplified by PCR with primers specific for the Y chromosome (Sinclair et al. 1990). DNA from a normal male was amplified, but that from a normal female was not (male and female are indicated by symbols). All three cell lines from the patient also contained Y-specific DNA, confirming that all were male and could not have derived from engraftment of maternal cells. Order of patient's DNA, from left to right: MS1267, GM2445, and GM 1715. All three cell lines from the patient were identical for 2 different alleles (from >30 alleles) at the D16S85 VNTR locus. Genomic DNA, digested with *Pvu*II, was electrophoresed in agarose, was transferred to nitrocellulose, and hybridized with the CriPat 3'HVR probe. All three cell lines from the patient were identical for 2 different alleles (from >10 alleles) at the D20S19 VNTR locus. Genomic DNA, digested with *Pst*I, was electrophoresed in agarose, was transferred to nitrocellulose, and hybridized with the CMM6 probe.

sequenced exons 1–11 amplified from genomic DNA. Both the fibroblast and B cell lines established at diagnosis and lacking ADA activity (GM2445 and GM1715) were heteroallelic for a newly identified splice-site mutation (+1GT→CT transversion) at the donor splice site in IVS 1 and for the previously described Arg101Gln missense mutation in exon 4 (fig. 2). By contrast, the B cell line expressing ADA activity (MS1267) contained the missense mutation but lacked the splice-site mutation (fig. 2). Both lymphoid cell lines expressed ADA mRNA, but mRNA in the cell line lacking ADA (GM1715) and carrying the splice-site mutation was reduced when compared both with a normal cell line and with the cell line expressing ADA (MS1267)(fig. 3). On the basis of the differences in ADA mRNA content, the splice-site mutation, altering the invariant GT of a donor splice-site consensus sequence, results in an unstable mRNA.

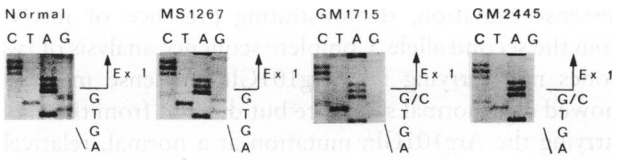

Figure 2 Sequence analysis of exon 1 amplified from genomic DNA. A splice-site mutation at the donor site of exon 1 (i.e.,+1GT→CT) was present in the two cell lines devoid of ADA activity (GM1715 and GM2445) but was not seen in the cell line expressing ADA (MS1267). ADA exon 1 was amplified from genomic DNA and was directly sequenced. The top of the figure is 5', and the bottom is 3'. First panel is normal cell line; second panel is MS1267 (patient's B cell line expressing 50% normal ADA; third panel is GM1715 (patient's B cell line lacking ADA activity); and last panel is GM2445 (patient's fibroblast cell line lacking ADA activity.

To confirm that the splice-site mutation was abolishing mRNA from the second allele, we isolated and analyzed multiple cDNA clones derived by RT/PCR of mRNA from both B cell lines. All 17 cDNA clones isolated from the original lymphoid cell line (GM1715) carried the Arg101Gln missense mutation, confirming that mRNA in this cell line is derived from the allele carrying the missense mutation, with the second allele, which carries the splice-site mutation, giving rise to an unstable mRNA. In contrast, half the mRNA (5/10 clones) from the cell line expressing 50% of normal ADA (MS1267) carried the previously reported Arg101Gln mutation, and half lacked the

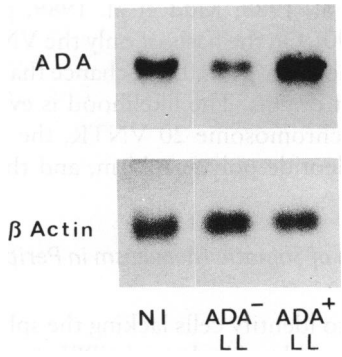

Figure 3 Comparison of ADA mRNA in the patient's B lymphoid cell line carrying the splice-site mutation and lacking ADA, the patient's B cell line expressing ADA, and a normal B cell line. PolyA mRNA was extracted, electrophoresed, and hybridized with an ADA cDNA probe (*top panel*), and then rehybridized with an actin probe (*bottom panel*). ADA mRNA in the cell line not carrying the splice-site mutation and expressing ADA (i.e., MS1267) was similar in amount to that in the normal cell line, while ADA mRNA was diminished in the cell line carrying the splice-site mutation and lacking ADA (i.e., GM1715). Actin mRNA was similar in all cell lines. Lanes Nl, Normal B cell line (GM7345); Lane ADA⁻ LL, Patient's cell line lacking ADA (GM1715); Lane ADA⁺ LL, Patient's B cell line expressing ADA (MS1267).

missense mutation, demonstrating presence of mRNA from the second allele. Complete sequence analysis of two clones not carrying the Arg101Gln missense mutation showed only normal sequence but differed from the allele carrying the Arg101Gln mutation at a normal, relatively infrequent, silent nucleotide polymorphism (G390A; 1 = ATG) (Bonthron et al. 1985). All three cell lines were heterozygous for this silent nucleotide substitution in exon 5 of genomic DNA (data not shown).

Identity of All Three Cell Lines

To exclude the possibility that the cell line expressing ADA was not derived from the patient (i.e., that it was either engrafted in utero from the mother—or, less likely, from a blood donor also heterozygous for the missense mutation—or as a result of contamination with a different normal cell line during culture), we examined DNA from all three cell lines, for presence of the Y chromosome and for multiple polymorphic DNA markers. All three cell lines were male and therefore could not represent maternal engraftment (fig. 1) (Sinclair et al. 1990). All three cell lines were identical at RFLPs at the ADA locus and at the relatively uncommon G390A silent nucleotide polymorphism in the ADA cDNA, with findings consistent with our prior demonstration of the rare IX and X haplotypes in the original B cell line (heterozygous at the *Pst*I RFLP and homozygous for the rarer allele at both the *Bal*I (*Msc*I) RFLP in exon 6 and the *Msp*I RFLP in IVS 5 and for the common allele for the *Msp*I RFLP in IVS3; data not shown) (Tzall et al. 1989). The cell lines were additionally all heterozygous with identical bands for both the highly polymorphic D20S19 VNTR (>10 alleles) on chromosome 20 and the D16S85 VNTR (>30 alleles) on chromosome 16 (fig. 1) (Nakamura et al. 1988; Kidd et al. 1989, pp. 783, 815; Keith et al. 1990). On the basis of only the VNTR on chromosome 16, there is a <1/10,000 chance that the cell lines are of different origins. The likelihood is even less if one considers the chromosome 20 VNTR, the ADA RFLPs, the single nucleotide polymorphism, and the Y chromosome.

Demonstration of Somatic Mosaicism in Peripheral Blood Cells

We sought to identify cells lacking the splice-site mutation in peripheral blood leukocytes (PBLs), as indicated by a diminution in the ratio of DNA carrying the splice-site mutation relative to total DNA. The splice-site mutation introduces a new *Dde*I site, cutting a 157-bp fragment to 100 bp and 57 bp (fig. 4). To assess the relative proportion of DNA mutant and normal at the splice site, exon 1 was radiolabeled by PCR amplification and was digested with *Dde*I, and the amount of radioactivity remaining in the 157-bp fragment or present in the abnormal 57-bp fragment was compared with that in a 75-bp invariant band (as determined by phosphoimaging and normalized for the number of labeled bases in each fragment) (table 2). (The 109-bp fragment could not be used because it comigrated with the 100-bp fragment.) In normal DNA, the ratio of 157-bp fragments to 75-bp fragments (157:75 ratio) was 1:1 (1.03±0.028), as expected. The 157:75 ratio in DNA from the patient's lymphoid line (GM 1715), heteroallelic for the splice-site mutation and lacking ADA, was 0.612 ± 0.029, slightly greater than the theoretical 0.5. The ratio in PBLs, however, was higher than that found in the patient's lymphoid line (0.776±0.022; $P<0.001$), indicating that significantly less than half of the DNA in PBLs carried the splice-site mutation. Correspondingly, the ratio of the 57-bp fragments to 75-bp fragments (57:75 ratio) proved to be significantly lower ($P<.002$) in the PBLs (0.238±0.027) when compared with that in the patient's lymphoid line (0.409±0.038). These results confirm the presence of in vivo somatic mosaicism. The 57-bp fragment was also present in the fibroblast cell line (fig. 4), where it appeared to represent 50% of the DNA (0.513±.032; n=3), but was seen neither in normal DNA nor in DNA from the patient's lymphoid cell line expressing ADA (MS1267) (data not shown).

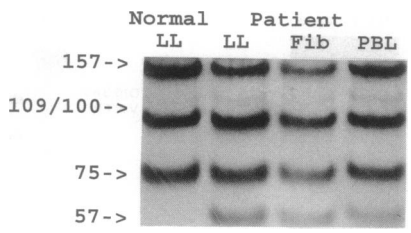

Figure 4 Detection of the mutation at the donor splice site of exon 1 (+1GT→CT) in PBLs, by digestion of radiolabeled amplified genomic DNA with *Dde*I. Exon 1 was radiolabeled by amplification of genomic DNA extracted from PBLs obtained at age 16 years, from a normal lymphoid line (i.e., Nl LL), and from the patient's lymphoid and fibroblast cell lines (both lines lacking ADA and heteroallelic for the splice-site mutation) and was digested with *Dde*I, fractionated on polyacrylamide gels, and autoradiographed (as well as quantitated by phosphoimaging (see table 2). The splice-site mutation introduces a new site for *Dde*I. Normal DNA contains 157-, 109-, and 75-nt fragments. The splice-site mutation results in digestion of the 157-nt fragment to 100- and 57-nt fragments. (The 100- and 109-nt fragments comigrate on this gel.) The presence of the 57-nt fragment indicates presence of the splice-site mutation. First lane, Normal B lymphoid line, LL; Second lane, Patient's B cell line lacking ADA (GM1715); Third lane, Patient's fibroblast cell line lacking ADA (GM1445); Fourth lane, Patient's PBLs obtained at age 16 years.

Discussion

We have found that a child with an unusual clinical course for ADA deficiency is a somatic mosaic. His initial course was one of delayed onset and persistent lymphopenia but with retention of substantial in vitro cellular immune responses and apparent response to modes of therapy, including the limited enzyme replacement that is provided by partial exchange transfusions and thymosin

Table 2

Quantitation of DNA Carrying the Splice-Site Mutation (Mosaicism in PBLs)

Ratio	Normal LL	Patient	
		ADA⁻ LL[a]	PBLs[b]
157:75[c]	.93	.49	.66
	.99	.57	.71
	1.02	.61	.73
	1.03	.60	.74
	1.06	.62	.77
	1.07	.62	.78
	1.11	.63	.79
	1.20	.76	.82
	...	...	.87
	...	...	.89
Average ± SEM	1.051 ± .003	.612 ± .026	.776 ± .022
95% CI	.984–1.119	.550–.675	.725–.827
P		<.001	
57:75[d]	<.01	.27	.11
	nd	.29	.17
	nd	.35	.20
	nd	.35	.21
	nd	.38	.21
	nd	.41	.22
	nd	.47	.25
	nd	.54	.25
	nd	.62	.37
	...	...	.39
Average ± SEM	...	.409 ± .038	.238 ± .027
95% CI	...	.320–.498	.177–.299
P		<.002	

[a] GM1715, patient's lymphoid cell line lacking ADA activity and heteroallelic for the splice-site mutation.

[b] Patient's total PBLs obtained at age 16 yers.

[c] Ratio 157:75 = the ratio of counts per minute (CPMs) (per labeled nucleotide) in the 157-bp fragment that is digested by *DdeI* in the presence of the splice-site mutation, to CPMs in an invariant 75-bp fragment. Complete absence of the splice-site mutation (NL) predicts a ratio of 1.0; presence of the splice-site mutation on one chromosome of all cells predicts a ratio of 0.5; and mosaicism predicts an intermediate ratio.

[d] Ratio 57:75 = the ratio of CPMs in the 57-bp fragment generated by *DdeI* in the presence of the splice-site mutation, to CPMs in the invariant fragment. Presence of the splice-site mutation on one chromosome of all cells predicts a ratio of 0.5, and mosaicism predicts a lower ratio.

[e] nd = not detected.

injections that are without effect in most patients. A few patients with similar initial characteristics have been previously described, but all either died at <10 years of age or are currently receiving the more efficient enzyme replacement provided by Polyethylene glycol–ADA (Hershfield et al. 1993; Hirschhorn 1993*a*, 1993*b*). Of note, he exhibited an elevated IgE, an abnormality seen in several late-onset patients (see references in Kurlandsky et al. 1993). He was also biochemically unusual for both the highest residual ADA activity in mononuclear cells of a group of seven ADA⁻ SCID individuals and the lowest concentration of deoxyATP, overlapping with concentrations found in healthy "partially" deficient children ascertained by screening of normal newborns. Most significantly, this patient was unique for his normal health in late adolescence, without any form of therapy during age 13–20 years.

The patient is heteroallelic for two different mutations: a previously reported Arg101Gln missense mutation and a newly identified +1GT→CT transition at the donor splice site in IVS 1, resulting in undetectable mRNA. He is also mosaic for cells with one ADA allele carrying the missense mutation but with the second allele lacking the splice-site mutation and normal for ADA transcription and translation. We have excluded trivial explanations for the finding of mosaicism in cultured cell lines, including maternal engraftment and contamination of cell lines. More significantly, we have provided evidence for somatic mosaicism in uncultured PBLs of the patient.

Three earlier observations are consistent with our demonstration of mosaicism. These are the phenotypic reversion of an aliquot of the original B cell line to expression of normal ADA (Uberti et al. 1983); the finding, unique to fibroblasts from this patient, of immunologically normal ADA proportional to residual ADA activity (Daddona et al. 1980); and the independent establishment of a B cell line from the patient noted to express ADA activity within the normal range (Arredondo-Vega et al. 1990). The first two observations suggest that the mosaicism was present in the original B cell line (GM1715) and possibly in the fibroblast cell line. The finding that slightly more than half of the DNA from this original lymphoid line was normal at the splice-site mutation is consistent with mosaicism in the original B cell line. The possibility of further in vivo selection is raised by the very clear mosaicism present in total PBLs obtained at age 16 years, as well as by the reported independent isolation of a second B cell line expressing ADA at 13 years of age. Such selection for ADA normal cells could be provided by the presence in vivo of elevated amounts of the toxic metabolites deoxyadenosine and deoxyATP. However, ADA activity measured in PBMs at ages $2\frac{1}{2}$ years and 16 years could not be used to assess possible in vivo selection, because of the differences in assay methods used. Additionally, in vivo selection limited to B cells would not have been detected by ADA assay of total PBMs because of the lower percentage of B cells in mononuclear cells at age 16 years.

In addition to mosaicism, two other factors could contribute to the relatively high residual ADA activity in this patient. The splice-site mutation could be "leaky" in vivo, since our results do not totally exclude presence of normal mRNA from the allele carrying the splice-site mutation (maximal value = 12% of normal from a single allele; 95% confidence limit for n=17). However, mutations at the G of the invariant GT donor splice site are usually not "leaky." Second, expression of ADA by the Arg101Gln

missense mutation could contribute to residual ADA, since we have been able to detect very low amounts of ADA activity following transient transfection into Cos cells (R. Hirschhorn and D. R. Yang, unpublished observations). However, the amount of expression cannot account for the relatively high residual ADA activity, since ADA expression was at the limits of detection and was the lowest in a series of 8/16 mutations from immunodeficient patients we have found to also express detectable ADA activity.

Somatic mosaicism is well established for chromosome abnormalities, for mitochondrial inherited disorders, for disorders due to expansion of triplet repeats, and functionally with respect to expression, in females, of genes on the X chromosomes. Somatic mosaicism has also been inferred on clinical grounds in autosomal dominant and X-linked disorders (Hall 1988) and more recently documented molecularly with increasing frequency in such disorders (see Maddalena et al. 1988; Levinson et al. 1990; Wallis et al. 1990; Taylor et al. 1991; Voit et al. 1992, and references therein). In addition, de novo somatic mutation with somatic mosaicism has been identified as the basis of McCune-Albright disease (Schwindinger et al. 1992). Ascertainment of somatic mosaics for autosomal dominant and X-linked disorders is simpler than it is for autosomal recessive disorders, since occurrence of only a single mutant allele, in any individual, results in disease. Mosaics have been identified either because of milder disease in a patient bearing an apparently de novo mutation or during further clinical investigations of germ-line mosaics. To our knowledge, there has been no previous molecular documentation of somatic mosaicism for a human autosomal recessive disorder.

The mosaicism could be due to a postzygotic somatic mutation or a reversion at the site of the splice-site mutation. Presence of the splice-site mutation in either parent would document a reversion event, while absence of the mutation in both parents would document a new somatic mutation in the child as the basis of mosaicism. Unfortunately, DNA is not available from the parents. Analysis of single or clonal ectodermal and endodermal cells (hair roots or buccal mucosa) could provide further information as to the extent and time of occurrence of mosaicism. Although a C→G reversion of a specific deleterious point mutation would be highly unusual, selection in vivo could allow for detection of such a rare event. Unlikely as a specific reversion event appears, we are currently studying a patient with two transmitted mutations who also appears to be a somatic mosaic (R. Hirschhorn, unpublished observations) that must be due to either site-specific reversion or a gene conversion event. Alternatively, interallelic somatic recombination could provide a mechanism for site-specific reversion. We have recently provided evidence for such a recombination in IVS 1 in a family with ADA^- SCID (Hirschhorn et al. 1994). However, a simple mitotic crossover event predicts retention of both the splice-site and missense mutation in genomic DNA, which is not found in genomic DNA of this patient's B cell line expressing ADA.

In summary, our studies document that the patient studied here is a somatic mosaic carrying some B cells with one normal ADA allele and a second allele carrying the Arg101Gln missense mutation. The major cell population carries both the splice-site mutation and the Arg101Gln mutation. Somatic mosaicism for the splice-site mutation would appear to have resulted both in the mild nature of his immunodeficiency and in his state of health in early adulthood without any form of therapy for the past 7 years. It is possible that there may be an increased frequency of somatic mosaicism in ADA-deficient individuals because of in vivo selection for rare events.

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