Spontaneous in vivo reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency

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Somatic mosaicism in genetic disease generally results from a de novo deleterious mutation during embryogenesis. We now describe a somatic mosaicism due to the unusual mechanism of in vivo reversion to normal of an inherited mutation. The propositus was an adenosine deaminase-deficient (ADA-) child with progressive clinical improvement and unexpectedly mild biochemical and immunologic abnormalities. Mosaicism due to reversion was evidenced by absence of a maternally transmitted deleterious mutation in 13/15 authenticated B cell lines and in 17% of single alleles cloned from blood DNA, despite retention of a maternal 'private' ADA polymorphism linked to the mutation. Establishment of significant somatic mosaicism following reversion to normal could modify any disorder in which revertant cells have a selective advantage.

Deficiency of the purine salvage enzyme adenosine deaminase (ADA) results in a rare, autosomal recessive immunodeficiency disorder of varying severity. In general, the degree of retention of residual ADA activity correlates inversely with both clinical severity and extent of accumulation of the toxic metabolites deoxyadenosine and deoxy ATP. Individuals with mutations at the ADA locus that result in complete absence of the enzyme exhibit neonatal onset of severe combined immunodeficiency (SCID), a disorder that, in the absence of therapy, is lethal prior to two years of age. Mutations resulting in retention of 1–5% of ADA activity are associated with progressive immunodeficiency including a delayed infantile onset immunodeficiency with retention of humoral (B cell) immunity, a later childhood onset immunodeficiency and an adult onset disorder that is clinically indistinguishable from the relatively frequent syndrome common variable immunodeficiency. All of these patients accumulate considerable amounts of deoxy ATP in erythrocytes and excrete markedly elevated amounts of deoxyadenosine. Individuals who totally lack ADA in erythrocytes, but retain residual ADA ranging from 10–40% of normal in non-erythroid cells and only minimally accumulate deoxyadenosine and deoxy ATP, have been ascertained by screening of healthy populations or by newborn screening. A number of such individuals, originally designated as 'partially' ADA deficient, have remained healthy into early adult life (refs 1–7; unpublished data).

It is well recognized that somatic mosaicism due to de novo mutations during embryogenesis is an additional basis for milder phenotype in many X-linked and autosomal dominant inherited disorders. We have previously described somatic mosaicism as a modifier of phenotype in a young adult who presented at two years of age with complete ADA deficiency and immunodeficiency but who is currently alive at 20 years of age without therapy. Parental DNA was unavailable, and we therefore could not determine whether or not somatic mosaicism had resulted from the usual mechanism of a de novo mutation on a normal allele during embryogenesis.

We have now studied another patient from an unrelated family who also had an unexpectedly mild phenotype. A prior sib had presented at 14 months with classic delayed onset SCID and died of the disorder before three years of age. By contrast, the sib studied here had a progressively milder course and is currently essentially normal without therapy at over 12 years of age (ref. 10 and case history). We now show that he is a somatic mosaic for a missense mutation (Arg156His), previously identified as deleterious in several unrelated ADA- immunodeficient patients11. The mutation was heteroallelic with a splice site mutation (IVS5 +1 GT to AT), which was recently reported in another unrelated ADA- immunodeficient patient12, and has been observed in two additional unrelated ADA- SCIDs (R.H. et al., unpublished data). We demonstrate here that both mutations were inherited; the IVS5+1 GT to AT mutation from his father and the Arg156His from his mother. We further demonstrate that the unexpected basis for somatic mosaicism was an in vivo spontaneous reversion to normal of the maternally transmitted mutation.
Residual ADA activity and metabolites

Adenosine deaminase (ADA) activity was not detectable in erythrocytes (RBCs) at age 5 but concentrations of deoxy ATP in RBCs and deoxyadenosine in urine were only minimally elevated, as compared to concentrations found in patients with early onset ADA-SCID (Tables 1 and 2). Both parents exhibited approximately 50% of normal erythrocyte ADA as did two young adult healthy sibs. Enzyme activity in lymphocytes was diminished to approximately 15% of normal in the proband and 20–25% of normal (within the heterogeneity range) in both the father and mother. Lymphoid cell lines established from the proband and both parents also exhibited markedly diminished ADA. Surprisingly however, enzyme activity in the cell line from the proband was indistinguishable from that in the parental cell lines. The considerable residual enzyme activity in non-erythroid cells and low concentrations of metabolites were similar to findings in partially ADA deficient children ascertained by population screening who have remained healthy during the first years of life (ref. 13, references therein and unpublished data).

By contrast, the death in infancy due to immunodeficiency of a prior sib and the abnormal immunologic findings in the proband during the first years of life were more consistent with complete ADA deficiency (ref. 10 and case history). To clarify the basis for the apparent dichotomy, we undertook a molecular analysis of this family.

A paternally transmitted mutation (IVS5 +1G→A)

Analysis of cdNA clones from the proband’s lymphoid cell line revealed deletion of the 116-nt sequence contained in exons 5, with seven of the 19 cdNA clones smaller than normal size. A splice site mutation at the invariant G of the 5′ donor site in IVS 5 (IVS5 +1GT to AT transition) was identified as the molecular basis for deletion of exon 5 from cdNA by sequence analysis of the proband’s lymphoid line and peripheral blood (PB) genomic DNA. The splice site mutation was present in the father’s genomic DNA from both lymphoid line and peripheral blood, as detected by loss of a Bsr site (Fig. 1a) and confirmed by sequence analysis (data not shown).

Absence of maternal mutation in B cell line

To identify the maternally transmitted mutation, we examined the 12/19 additional cdNA clones that were of grossly normal size and which presumably represented the maternal allele. Two clones contained a G to A transition at nt 467, predicting an Arg156His substitution—a deleterious mutation previously reported in ADA deficient immunodeficient patients11. (As a probably incidental finding, one of these two clones also showed mismapping from mid exon 7 to exon 8 with a deletion of 27 bp in exon 7, a splicing error we have

Fig. 1a. Detection of a paternally transmitted splice site mutation (IVS5 +1 GT to AT) by loss of a Bsr site a 254-bp fragment containing exon 5 was amplified from genomic DNA. The mutation abolishes the only Bsr site, resulting in a 254-bp fragment in the proband and father. Identical results were obtained with PB and lymphoid line DNA. b, Detection of a maternally transmitted missense mutation (G467A, Arg156His) by loss of a HindIII site. The Arg156His mutation abolishes the only HindIII site in the 254-bp fragment amplified, resulting in the mutant 254-bp fragment. The absence of the mutation in the proband’s lymphoid line but presence in his peripheral blood, as well as in both lymphoid line and PB DNA of the mother was confirmed by sequence analysis (data not shown). A faint 254-bp band in the proband’s lymphoid line was shown to contain the mutation by purification and sequence analysis (data not shown). c, Single strand conformational polymorphism (SSCP) analysis of lymphoid line (LL) and peripheral blood (PB) DNA consistent with absence of maternal mutation in proband’s lymphoid line and presence in his PB DNA. Arrows indicate abnormal bands due to maternal and paternal mutations, FA=father, Mo=mother, PRO=proband, NL=normal.

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 Authentication of proband's B lymphoid cell line

The virtual absence of the maternally transmitted mutation in the proband's lymphoid cell line and its clear demonstration in PB DNA may be explained by an in vivo reversion of an inherited mutation to normal in lymphoid cells. The findings could also result from two trivial causes: a major contamination of the proband's lymphoid line with the paternal cell line or an in vitro chance reversion to normal of the maternally transmitted mutation with selective growth in tissue culture of revertant cells. We first established the authenticity of the proband's cultured lymphoid cell line by comparison of DNA from the maternal, paternal and proband's cell lines for polymorphic markers and presence of a Y chromosome. We examined the highly polymorphic D20S19 VNTR on chromosome 20 with over 10 alleles, the D16S85 VNTR on chromosome 16 with over 30 alleles and multiple intragenic ADA RFLPs that included a maternally transmitted MspI RFLP in ADA IVS 6 that is unique to this family (Fig. 2 a-e). Most significantly, the mother and child were both heterozygous for an additional site for MspI in IVS 6, resulting in two additional restriction fragments (Fig. 2c). This maternally transmitted MspI RFLP appears to be 'private' since we have not found this RFLP in over 100 chromosomes from patients or normals. Analysis of PB DNA from the proband and his parents for the ADA IVS 2 and exon 6 RFLPs as well as the private RFLP in ADA IVS 6 gave results identical to those for the respective lymphoid cell lines. Moreover, the two sibs who had inherited the maternal mutation also exhibited the maternally derived private ADA IVS6 MspI RFLP. The combined data establish the authenticity of the proband's cell line lacking the maternally transmitted mutation and also linkage of the maternally transmitted Arg156His mutation in exon 5 and the ADA IVS 6 'private' RFLP.

Absence of mutations in some PB sibs

To determine if the reversion to normal of the maternally transmitted mutation had occurred in vivo, we initially analysed 89 clones, each containing a single allele, generated by PCR of PB DNA from two separate patient blood samples (Table 3). As expected, approximately 50% of clones carried the paternal mutation, detected by loss of a Bst site. By contrast, only 34% carried the maternal mutation, detected by loss of a HindII site. Most significantly, 17% of clones did not carry either mutation. None of the clones carried both mutations. Absence of both mutations was confirmed.
Table 3 Reversion to normal in individual alleles cloned from proband PB DNA: absence of both parental mutations

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*see Methods for differences between experiments 1 and 2."

Fig. 3 Sequence analysis of individual cloned alleles lacking both maternal and paternal mutations. Left panel = sequencing for G only; right panel = sequencing for A only. Lane P = clone carrying paternal mutation (upper arrow), lane M = clone carrying maternal mutation (lower arrow), lanes 1-11 = clones lacking both mutations (Expt 1, Table 3). Both the paternal and maternal mutations are G to A substitutions and therefore presence of the mutation is shown by loss of a normal G in the left panel and appearance of an A in the right panel. The four clones (from Expt 2, Table 3) lacking a mutation by restriction digest were sequenced conventionally using all 4 deoxynucleotides, also confirming absence of both mutations (data not shown).

by single lane sequence analysis (Fig. 3) or complete sequence analysis (not shown). All 11 clones lacking a mutation and containing exons 5 and 6 carried the common allele for the Msd RFLP in exon 6, consistent with heterozygosity of the parents and homozygosity of the child for this RFLP in PBL and lymphoid line DNA (Fig. 1b). To determine whether the reversion had occurred on the maternally or paternally inherited chromosome, we analysed clones containing IVS 6 for the 'private' Mpl RFLP carried by the mother and child. Clones carrying the maternally derived mutation carried this private RFLP in ADA IVS6, as did all four clones analysed that lacked both mutations (Fig. 4). Therefore these single allele clones that lacked both mutations must have resulted from reversion to normal of the maternal mutation.

Absence of maternal mutation in new B cell lines

To discern in vivo reversion to normal of a transmitted mutation, we newly established multiple EBV transformed B cell lines and T cell pools from a blood sample obtained from the proband at age 11. Mutation analysis of 15 independent B cell lines revealed presence of the paternally derived mutation in all 15 but absence of the maternal mutation in 13/15 (Table 4). The maternal mutation was detected in two B cell lines, but in only trace amounts, as judged by the relative intensities of the undigested (maternal mutation)/digested HinfI1 bands. All 23 T cell pools contained both mutations, although the relative intensity of the undigested band suggested that there was a subpopulation of cells lacking the maternal mutation. B cell lines, whether lacking or containing the maternal mutation, as well as T cell pools, showed the maternally derived 'private' RFLP that is linked to the maternal mutation.

Discussion

Several observations forced us to test the unusual hypothesis that in vivo reversion to normal of an inherited mutation in a human gene had resulted in somatic mosaicism. These findings were: 1) the virtual absence of a maternally transmitted mutation in the child's original lymphoid cell line despite its presence in PB DNA; 2) the clear authenticity of this cell line; 3) the considerable residual ADA and low metabolite concentrations; and 4) the progressively milder clinical course in contrast to the early demise of a previously affected sibling. By two separate approaches we have demonstrated in vivo reversion to normal of the maternally transmitted missense mutation (CGC677AC, Arg156His). PB DNA contained individual alleles that did not carry a mutation and that were shown (by presence of a 'private' RFLP linked to the maternal mutation) to derive from the maternally transmitted allele. Secondly, 13 of 15 newly established B lymphoid cell lines totally lacked the maternal mutation, although this mutation was present in multiple T cell pools. Both B cell lines and T cell pools carried the maternally transmitted private RFLP, as well as the maternal mutation. The relatively high residual ADA enzyme activity in lymphoid cells, low concentrations of metabolites and survival without therapy to the current age of 12 would appear to be primarily due to the demonstrated back mutation and resulting somatic mosaicism, rather than to possible 'leakiness' of the splice site mutation and/or expression of considerable residual ADA by the Arg156His mutation. While this work was in progress, both the IVSS +1G→A and the Arg156His mutations were reported in several additional unrelated ADA* immunodeficient patients (heteroallelic with several different mutations). All of these other ADA* immunodeficient patients had less than 1% of normal enzyme activity in lymphoid cells and accumulated ten fold greater deoxy ATP than the child studied here. Additionally, the deleterious nature of the Arg156His..."
missense mutation was confirmed by expression stud-
ies11. In confirmation of these prior results, we have
also directly compared the expression of 18 differ-
type ADA mutations, including the Arg156His mutation by
more sensitive method of transient expression and
in situ histochemistry (manuscript in preparation).
The Arg156His mutation expressed activity less than
1-2% of normal, similar to what we found for several
other deleterious mutations and considerably less than
that of any of the 'partial' ADA mutations. Therefore,
based on findings in other patients carrying the same
mutations, as well as in vitro expression studies, 'leaki-
ess' cannot explain the 15% of normal ADA activity in
the proband's lymphoid cells.
The mechanism for this site-specific reversion to
normal of an inherited point mutation is undefined.
Gene conversion appears unlikely in this case since one
end of the conversion event would have to be within
the 12 nt separating the sites of the maternal and
paternal mutations. Apparent reversion to normal of
mutations on one allele has been reported in regener-
ating liver nodules in four patients with the autosomal
recessive disorder, tyrosinaemia12. Formal demonstra-
tion that the mutations was transmitted from the par-
ents of the patients was not provided. However, it
would seem unlikely that all four patients were mosaic
for de novo mutations, supporting the hypothesis of
back mutations. Two of these patients were homozy-
gous for the mutation in non-regenerating liver, clear-
ly excluding gene conversion. ADA deficiency and
tyrosinaemia share several features of possible signifi-
cance. Both disorders have increased concentrations of
metabolites with potential to cause DNA damage, the
alkylating agents fumaryl and malonyl-acetate in
tyrosinaemia and deoxyxanacose, which has been
reported to induce DNA strand breaks in vitro, in ADA
deficiency14,15. In both disorders, the abnormal
metabolites also provide an in vivo selection systems poten-
tially conferring a selective advantage on cells
that have reverted one allele to normal. Lastly, both
involve rapidly dividing cells (regenerating liver and
lymphocytes). Since an increased mutation rate has
not been actually demonstrated, a selective advantage
alone could have allowed for selective survival and
proliferation of sufficient spontaneous revertants
derived from progenitor cell(s) to result in a detectable
sonic mosaicism.

Our finding that a patient with ADA deficiency had
site-specific reversion to normal of an inherited muta-
tion may have important implications for evaluation
and/or design of current trials of gene therapy that are
combined with enzyme therapy. Such enzyme therapy
might well tend to abolish the selective advantage and
expansion of recombinant cells containing a normal
allele, a hypothesis supported by a recent report of rela-
tive expansion of transfused cells carrying recombi-
nant retroviral ADA following reduction in the
amount of enzyme therapy18.
Significant somatic mosaicism due to reversion to
normal could be present in any disorder where normal
cells have a selective advantage. The most obvious
examples of a demonstrated selective advantage are
the several X-linked immunodeficiency disorders
where female carriers show selective survival of cells with the
normal X chromosome active in the cell types affected
by the disease (for example ref. 19). An additional can-
didate is X-linked HGPS deficiency, where similar
selective advantage is seen in haematopoietic cells of
carrier mothers20. Differences in XIC/IXIE between
sibs carrying single gene defects may therefore result
not only from environmental differences or modifying
cis or trans elements but also to chance reversion to
normal of inherited mutation, selection and resulting
somatic mosaicism.

**Methods**

**Case history.** The case history of the child used in our study
to age 5 has been reported10. A prior sib had presented at 14
months with pneumonitis, failure to thrive, diarrhea and
marked lymphopenia affecting both T and B cells (2% lymph-
ocytes with 8% T cells and 5% B cells). He died before three
years of age with a diagnosis of 'Neelof syndrome', now recog-
nized as a variant of SCID with retention of some humoral
immune function. The child studied here presented during the
first 5 years of life with short stature, recurrent cutaneous
staphylococcal abscesses, sinusopulmonary infections, mucocu-
taneous candida infections, eosinophilia, marked hyper IGE
and lymphopenia, particularly of CD4 lymphocytes. At age 5
years he was found to lack ADA in erythrocytes but to exhibit
15% of normal ADA in mononuclear cells (see Table 1). Between
the ages of 8 and 12 years, he has been clinically
healthy, with normal growth and development despite the per-
sistent findings of hyperimmunoglobulinemia E, decreased
absolute numbers of CD4 lymphocytes and B lymphocytes
CD19, and increased absolute numbers of CD8 lymphocytes.
In addition, he has had repeated infections and on the most recent
testing, the appearance of somewhat diminished in vitro lymphocyte response to mitogens. For reli-
gious reasons the child has not received transfusion of blood
products, including gammaglobulin, PEG-ADA enzyme thera-
py, bone marrow transplants, nor undergone skin biopsy for
establishment of fibroblasts. The parents are not consan-
genous and 2 older sibs are healthy.

**Biochemical methods.** Adenosine deaminase (ADA) and
purine nucleoside phosphorylase (PNP) activity and metabol-
line concentrations were determined as described21,22.

**Genotyping and cDNA analysis.** Southern analysis of lymph-
oid line DNA for VNTRs, Y chromosome and PCR of PB
and lymphoid line genomic DNA for ADA intragenic RFLPs
were performed as described (ref. 9, 13, and 24, and refs there-
in). mRNA was extracted and cDNA libraries generated both
by classical methods and by RT/PCR using ADA specific primers
as described25. Sequence analysis used either Sequenase (US
Biomedical) or cycle sequencing with a Bilo kit (Promega) as
described25.

**Cloning and analysis of individual alleles from PB.** Genomic
DNA from two independent samples of PB from the proband
was amplified by PCR and cloned into the TA vector pCR/II
Luminogen). DNA was extracted from PB leukocytes obtained
at age 10 y (Table 3, exp 1), or from PB mononuclear cells
sep-
ared by standard Hypaque-Ficol purification from blood drawn at age 11 years (Table 3, exp. 2). A 1.65-kb genomic DNA fragment containing exons 5 and 6 was amplified by PCR from sample 1 (Table 3, exp. 1) with primers BG5299 (5′-CAGTCGGAGACTCTGCTTTATGGCCTC-3′) and BG5148 (ref. 23). A 1.42-kb fragment containing exons 5–7 was amplified from sample 2 (Table 3, exp. 2), using primers BG5399 x BG4545 (5′-CGTGAAGATGGTAAGCTGCGCCTC-3′). For analysis of mutations by restriction enzyme digest, a 254-bp fragment containing exon 5 was amplified from the clones using primers BG5399 x BG5292 (5′-CAGTCGGAGACTCTGCTTTATGGCCTC-3′). Exon 6 (ref. 23) or a 1.42-kb fragment containing exons 6–7 was amplified from the clones using primers BG4149 (ref. 23) x BG4545 for analysis of restriction variants. For the latter, the Bgl II/bial (Bgl II) enzyme digest in isosmotic lysates of the proband (PMII), mother (OM), father (OMII) and brother of normal (NL) 1 and NL 2, digested with MspI and methylated in 3% agarose. The order and size of fragments for the ‘private’ RFLP were determined by double enzyme digests as well as analysis of Southern blots hybridized with a 6-kb specific genomic probe which also demonstrates the ‘private’ RFLP.

Detection of mutations in genomic DNA. Genomic DNA from EBV-transformed B cell lines and T cell pools or PB DNA was amplified using nested PCR of exon 5 (primers BG5151 x DG747) (ref. 23) followed by BG5399 x 5298. Individual cloned alleles derived by PCR of PB DNA were tested by enzyme digestion for presence of the paternal and maternal mutation and clones lacking both mutations were then sequenced. SSCP was performed and analysed on MDE gels as described by the manufacturer (J.T. Baker), based on described methods24.

Cell lines. The original EBV transformed cell lines from the patient and his parents were established when the patient was 5 years old, at the time of enzyme and metabolic studies. These cell lines (GM 10077, 10078 and 10079) are deposited with the NIH Mutant Cell Repository in Camden, NJ. Multiple EBV transformed B cell lines and T cell pools were newly established from the blood sample obtained at age 11 years. Fifteen independent EBV B cell lines were generated in microtitre plates by standard methods25 and expanded for 3–5 weeks for analysis of DNA. Purified activated T lymphocyte cultures were prepared as described by stimulation with PHA for 3 days followed by expansion of non-adherent cells for 2–4 weeks in the presence of recombinant human IL-2 (ref. 28).

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