Severe combined immune deficiency due to a homozygous 3.2-kb deletion spanning the promoter and first exon of the adenosine deaminase gene

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ABSTRACT

We have investigated the structural gene for adenosine deaminase (ADA) in a female infant with ADA deficiency associated severe combined immune deficiency (ADA⁻SCID) disease and her family by DNA restriction-fragment-length analysis. In this family a new ADA-specific restriction-fragment-length variant was detected, which involves a 3.2-kb deletion spanning the ADA promoter as well as the first exon. It was found that the patient, who was born to a consanguineous couple, was homozygous and both her parents and her brother were heterozygous for the deletion. No ADA-specific mRNA could be detected by hybridization in fibroblasts derived from this patient. Thus the patient was established to be homozygous for a true null ADA allele. In the light of the apparently normal development of most tissues except the lymphoid tissue the above finding directly questions the classification of ADA as a 'housekeeping' enzyme.

INTRODUCTION

A severe deficiency of adenosine deaminase (ADA; E.C.3.5.4.4.) in man leads to a form of severe combined immune deficiency (SCID) disease (1, see references 2,3 for reviews). In ADA⁻SCID patients the proper development of both T- and B-lymphocytes is severely affected rendering them susceptible to various opportunistic infections, most often leading to death before the age of 1 yr in the absence of proper treatment. Recently, by a selective ADA replacement using polyethylene glycol-modified ADA, Hershfield et al. (4) showed ADA deficiency to be the sole cause of the observed immune dysfunction in ADA⁻SCID patients. A consensus as to the basic metabolic pathways leading to the observed immune dysfunction in ADA⁻SCID patients still has to be reached.

The recent cloning of ADA-specific sequences enabled us and others to investigate the basic molecular defect underlying ADA deficiency. Analysis of lymphoblastoid cell lines derived from ADA⁻SCID patients revealed that they contained ADA-specific mRNA of normal length and in normal abundance (5). S1-nuclease analysis of RNAs from a number of these cell lines detected ab-

normalities in only 10% of the mRNA in some of the cell lines, suggesting the occurrence of point mutations in the remainder (6).

So far, in the ADA⁻SCID cell lines GM1715 and GM2471, 2 different point mutations have been identified that are deemed to be responsible for ADA deficiency; both mutations affected only one allele (7,8). In addition, another mutation apparently introduced during cDNA cloning was found to inactivate ADA when introduced into an expression vector (9). All three point mutations lead to different amino acid substitutions at different positions in the protein. This wide variety of point mutations all rendering the ADA gene inactive discouraged the use of recombinant-DNA techniques in the pre-natal diagnostics, as well as in carrier-detection studies in unrelated families. On the other hand, carrier status determination within a family at risk by simply determining ADA enzyme activity levels is often hampered by the fact that red cell ADA exhibits a large variation within the normal population (10). We therefore undertook a search for possible RFLPs segregating at the ADA locus to employ in studies on carrier detection and pre-natal diagnosis in families at risk.

Whilst investigating the family of an ADA⁻SCID patient we detected a novel DNA restriction variant of the ADA gene due to a 3.2-kb deletion including the ADA promoter and the first exon. The infant suffering from ADA⁻SCID disease, born to a consanguineous couple, was found to be homozygous while both her parents and her brother are heterozygous for the deletion. No aberrant ADA-specific transcripts originating from possible cryptic promoters could be detected in cultured primary skin fibroblasts derived from the patient. These data taken together clearly suggest that this patient suffers from complete deficiency of ADA due to a true null allele. Furthermore, since all but the lymphoid tissues apparently grew and developed normally in this patient, ADA does not seem to be a 'housekeeping' enzyme in the strict sense of the word.

CASE REPORT OF THE PATIENT

The patient, a girl, was presented at the age of 2 months with feeding difficulties, diarrhoea, failure to thrive and some developmental delay. Also dysostosis was observed in this patient. In the following months, she suffered from septicaemia and pneumonia caused by Pseudomonas aeruginosa and interstitial pneumonia caused by Pneumocystis carinii. Due to anaemia she received several transfusions with erythrocyte concentrates. A severe form of congenital immune deficiency was suspected.

Immunological investigations showed severe lymphopenia, an absence of in vitro proliferative response of blood lymphocytes and agammaglobulinaemia. ADA activity was found to be absent in her in white blood cells (see also table I). Therefore, the diagnosis of ADA⁻SCID disease was made.

At the age of 8 months she received a T-cell-depleted bone-marrow graft derived from her HLA-phenotypically identical but genotypically mismatched brother (relative response index = 30.5% in the mixed lymphocyte culture), after conditioning with antithymocyte globulins (ATG) 3x2mg/kg body weight (bw), Busulphan 4x4mg/kg bw and cyclophosphamide 4x50mg/kg bw. Cyclosporin A 2mg/kg bw was given to prevent Graft-versus-Host disease (GvHD) in continuous intravenous infusion from day -5 onwards. Haematological reconstitution was achieved within one month and immunological reconstitution within 7 months after bone marrow transplantation (BMT). The post-transplantation period was complicated by recurrent interstitial pneumonia caused by Pn. carinii, recurrent septicaemia via the deep indwelling intravenous catheter caused by S. epidermidis and severe feeding problems because of disturbances in gastric tonus and peristaltic motility.

Ten months after BMT, at the age of 18 months, the child was discharged in good health but still needed a formula feeding by nasogastric tube. Although there was an improvement of her mental development after BMT she was still retarded at the time of discharge and her fine motor coordination continued to be impaired.

MATERIALS AND METHODS

Enzymatic activities. Specific activities of ADA and nucleoside phosphorylase (NP) were determined essentially as described previously by Herbschleb-Voogt et al. (11).

Cell lines. ADA-deficient lymphoblastoid cell lines GM 2471, 2756, 2825, 4258 were obtained from the human genetic mutant cell repository, Cambden (N.J.), U.S.A., and were cultured, like the ALL cell line Molt-4 (12) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Primary skin fibroblasts cultures were set up and maintained in F10 medium supplemented with 15% FCS.

Isolation and blotting analysis of DNA and RNA. DNA and RNA were isolated as described by Bakker et al. (13) and Auffray and Rougeon et al. (14), respectively. DNA was analysed by restriction analysis and blotting, against GeneScreen-plus (NEN) filters, as described by Bakker et al. (13). RNA samples were processed for gel electrophoresis, size-fractionated in formalde-

Table I. Adenosine deaminase (ADA) and nucleoside phosphorylase (NP) activity levels in the peripheral blood cells of patient's family and normal controls.

Samples	Thal. ^a	HS ^a	ADA ^b	NP ^b
<u>Erythrocytes:</u>				
Patient	-	-	. ^c	. ^c
Father	+	+	0.85 $\pm$ 0.07	66.57 $\pm$ 2.83
Mother	-	-	0.66 $\pm$ 0.05	48.67 $\pm$ 1.37
Brother	+	-	0.98 $\pm$ 0.04	67.04 $\pm$ 2.03
Controls ^d	-	-	1.01 $\pm$ 0.27 (n=342)	42.90 $\pm$ 4.38 (n=227)
<u>Leukocytes:</u>				
Patient			0.0	37.6
Father			11.7	25.9
Mother			n.d.	n.d.
Brother			23.5	39.3
Control 1			14.0	33.7
Control 2			11.1	36.0
Control 3			18.4	33.4

^a The father and the brother were heterozygotes (+) for $\delta\beta$ -thalassaemia (Thal.) due to a large deletion in the haemoglobin β -gene cluster. The father was also a carrier (+) of the dominantly expressed gene for hereditary spherocytosis (HS) segregating in the family (Dr.L.F. Bernini, personal communication).

^b Expressed, in erythrocytes, as international units per gram of haemoglobin $\pm$ standard deviation (IU/gHb $\pm$ SD) (average of 10 determinations in each of the family samples) and as IU/gram of protein, in leukocytes. n.d. = not determined.

^c At the time of investigations the patient, since she was anemia, was treated with transfusions of erythrocyte concentrates. However the total white cell preparation devoid of contaminant red cells was employed initially in detecting ADA deficiency in this patient.

^d Values from Herbschleb-Voogt (1983) (n=number of samples investigated; each sample assayed in duplo).

hyde/agarose gels and blotted against nitrocellulose filters as described previously by Schrier et al. (15). Filter-bound nucleic acids were hybridized to radioactively labelled probes and washed after hybridization as essential-

ly as described previously (13). For the EcoRI-TaqI genomic probe hybridization was performed as described by Sealy et al. (16).

Probes and plasmids

Isolation and characterization of the human ADA cDNA clone pLL and the human genomic cosmid clone 4.3 were described previously (17,18). The γ -actin cDNA probe used was a gift of R.T.M.J. Vaessen.

RESULTS

ADA activities.

Table I shows the levels of ADA- and NP-activities in the extracts of erythrocytes and leukocytes of the patient and her family. Values obtained on a number of controls are also given. The patient's leukocytes showed a severe deficiency of ADA activity. Her parents and brother, however, exhibited ADA activity levels falling within the normal range. The ADA activities in the normal European population were found to range from 0.47(mean minus 2 S.D.) to 1.56(mean plus 2 S.D.) IU/g. hemoglobin (10). Remarkably, both the father and brother of the patient show significantly elevated NP-activity. It might be due to the fact that the father suffers from hereditary spherocytosis and both the father and brother also carry a δ/β -thalassaemia gene due to an extensive deletion in the globin gene cluster, which results in a high number of relatively young erythrocytes in their blood (Dr. L.F. Bernini, personal communication). However, after standardizing their NP activity to normal levels, their ADA activities are 0.64 and 0.76, respectively, i.e., still falling within the lower levels of the normal range.

Genomic organization of the mutant ADA gene.

DNA samples of the patient and her family were analysed for the presence of polymorphic PvuII sites. 20% of the Dutch population exhibit a site-specific variant for this enzyme in the ADA gene (manuscript in preparation). The variant was not found to be segregating in this family (fig.1). However, the common 2.6-kb PvuII fragment was absent in patient DNA when screened with a cDNA probe recognizing exons 1 through 11. This 2.6-kb PvuII fragment contains the first exon of the ADA gene (our own unpublished results). The absence of these sequences was shown not to be caused by the fact that, due to a RFLP, they were now contained in a fragment of altered size and, as a result, are comigrating with other fragments hybridizing with the cDNA probe. Subsequent hybridization with a genomic probe specific for exon 1 as well as for 135 bp upstream sequences did not result in any specific hybridization to patient DNA, suggesting that exon 1 is absent, probably due to a deletion

Figure 1. Southern blot analysis. 10 μ g of genomic DNA isolated from blood samples of the ADA⁻SCID patient and her family as well as of two controls, exhibiting both variants of a the *PvuII* RFLP, were digested with *PvuII*, size-fractionated on a 0.8% agarose gel, denatured and blotted against a GeneScreen-plus filter and hybridized with an ADA-specific cDNA probe covering exon 1 to 11 isolated from the human ADA cDNA clone pLL. The length of the region containing exon 1 is given in kb. Lane P = Patient, F = Father, M = Mother, B = Brother, C1 = Control 1, a homozygote for the major *PvuII* RFLP variant and C2 = Control 2, a heterozygote exhibiting the minor *PvuII* RFLP variant occurring at a frequency of about 20% in the normal Dutch population (manuscript in preparation).

(fig. 2). These hybridizations were performed as described by Sealy et al. (16) to avoid aspecific hybridization, since this genomic probe, probably due to its high GC-content (82% in the upstream region), behaves as one contain-

Figure 2. Southern blot analysis essentially as described in figure 1. The filter was hybridized according to Sealey et al. (16) with a genomic probe of 235 bp, containing both upstream as well as first exon sequences, running from the *EcoRI* site at position -135 to the *TaqI* site at position +100 relative to the cap site of the human ADA gene. DNA samples were digested with *EcoRI* and *HindIII* as indicated. Symbols are as in figure 1. Lane Ma contains *HindIII*-digested phage λ DNA for marker fragments.

ning repeated sequences (data not shown). Sibling's DNA showed a somewhat weaker hybridization signal than control DNA suggesting that here the exon 1 is present in only 1 copy. Exon 2 sequences were still present in patient's DNA because the cDNA probe has hybridized to a 2.3-kb *PvuII* fragment containing the ADA exon 2 (fig. 1).

However it is also possible that only a small portion of the first exon and or the promoter region is deleted in the patient leaving a too small stretch of homology to hybridize the *EcoRI*-*TaqI* genomic probe. To determine the size of the deleted sequences we undertook a search for single-copy probes further upstream as well as downstream from the first exon, in order to detect aberrant restriction fragments caused by the postulated deletion. Two probes indeed identified aberrant restriction fragments in the DNA of the patient and her family. The HX probe which is located about 3 kb upstream of the cap site recognized an aberrant 4.4-kb *HindIII*-fragment (instead of the normal 7.6 kb) as well as an aberrant 10.3-kb *EcoRI* fragment (instead of a 8.5 kb fragment) (fig. 3a). The same 10.3-kb *EcoRI* fragment is also recog-

nized by an AE probe located about 5 kb downstream from the first exon which recognizes a 5-kb EcoRI-fragment in the normal situation (fig. 3b).

These results combined can be explained by a homozygous 3.2-kb deletion removing the EcoRI site as depicted in figure 4a. Because the PvuII site at nucleotide (nt) -2174 and the BamHI site at nt 2056 (coordinates are according to Wiginton et al. (19) and relative to the cap site of the human ADA gene (17)) are still present in the patient's DNA (data not shown), the 3.2-kb deletion has to fall between these sites, which are 4.2 kb apart in the normal gene. Therefore the stretch of DNA deleted should at least include region A as depicted in figure 4b. This region includes all exon 1 sequences encoding the first 11 amino acids, as well as intron 1 and 5'-upstream sequences. Both parents and the brother are heterozygous for this deletion (fig. 3). Because the parents are second cousins it can be assumed that they inherited this deletion allele from one of their two great grand parents.

No ADA-specific mRNA expression detectable in primary cells derived from the patient.

Deletion analysis with the human ADA promoter linked to the bacterial chloramphenicol-acetyl-transferase gene revealed that all of the promoter sequences of the ADA gene are located between the EcoRI site at nt -135 and the cap-site (nt +1). Fragments up to 1.9 kb upstream of that EcoRI site do not harbour any sequences directing initiation of transcription (Berkvens et al., manuscript in preparation). Therefore the deletion determined in the present patient abolishes the normal ADA promoter.

To exclude the possibility that ADA-specific RNA is transcribed from potential cryptic promoters we analysed the RNA content of primary skin fibroblasts derived from the patient. No ADA-specific RNA could be detected at any position in the lanes P containing the patient's RNA sample, even after extremely long exposure (fig 5, lane P, panels ADA o/n and ADA 2 weeks). To ascertain that the same amount of intact RNA was indeed analysed in all lanes, the same filter was rescreened with a γ -actin-specific probe

Figure 3. Southern blot analysis essentially as described in figure 1. DNA samples were digested with the restriction enzymes indicated. The filters were hybridized with a with a genomic intron probe (HX, panel A) running from the HindIII site at nt -3460 to the XhoI site at nt -3209 relative to the cap site of the human ADA gene. The HX probe was isolated via subcloning from the ADA cosmid 4.3 (see materials and methods). In panel B the filter containing the EcoRI digested genomic DNA samples was also hybridized with a genomic intron probe (AE) running from the AccI site at position 4642 to the EcoRI site at position 4984 relative to the cap-site of the human ADA gene. The AE probe was also isolated via subcloning from ADA cosmid 4.3.

In summary, it can be concluded that this patient is homozygous for a true null allele. Furthermore, DNA analysis has unambiguously established the carrier status of both the parents and the brother.

We found that the ADA deficiency in an ADA⁻SCID patient was due to a homozygous deletion involving the promoter and the first exon of the ADA-gene.

Figure 5. Northern blot analysis. 40 μ g total RNA samples were size-fractionated by electrophoresis in formaldehyde/agarose gels, transferred to nitrocellulose and hybridized to an ADA-specific cDNA probe, and after stripping of this ADA signal to a γ -actin-specific cDNA probe as indicated below the panels. The RNAs analysed were isolated from a number of ADA-deficient lymphoblastoid cell lines, from APVS (a control fibroblast cell line), from Molt-4 (an ADA-overproducing ALL-cell line), and from cultured primary skin fibroblasts derived from our ADA⁻SCID patient as indicated above the lanes.

The parents are second cousins and have been confirmed as carriers of the same deletion. This report deals with the elucidation of the exact molecular basis of ADA⁻SCID disease in a patient established to be homozygous for a truly null allele. The promoter- and exon-1-deficient gene can not transcribe any mRNA, and therefore no ADA protein can be synthesized at all.

This contrasts to the situation in the ADA-deficient lymphoblastoid cell lines studied previously which were found to express ADA-specific mRNA of normal length and normal abundance (5). S1-nuclease analysis could only reveal abnormalities in 10% of the mRNA in some cell lines suggesting the presence of point mutations in the remainder (6). Furthermore, hybrid-released translation experiments using mRNAs from 2 of these cell lines yielded proteins with a molecular weight equal to that of normal ADA which, however, were difficult to precipitate with ADA-specific antisera. This was ascribed

Figure 6. Location of AluI family repeats in the region of the normal ADA gene around the first exon (based upon Wiginton et al., (19)). The borders of the region in which the 3.2-kb deletion has to map are given by the lines marked Pv (PvuII-site at nt -2174) and B (BamHI-site at nt 2056). Exon 1 is represented by the black box, AluI family repeats are indicated by the black triangles and located according to the sequence of Wiginton et al.. All repeats indicated are orientated with their A-rich region towards the right. The possible location of the observed 3.2-kb deletion is indicated by the line under the physical map. Dotted vertical lines originating from the postulated deleted fragment run to the two AluI repeats possibly involved in the etiology of the 3.2-kb deletion in the ADA⁻SCID patient. Symbols are as in fig. 4.

to either a conformational change or an increased instability of the aberrant ADA protein (5). Thusfar two point mutations have been identified that are held responsible for ADA inactivation of 1 allele each in the lymphoblastoid cell lines GM 2471 and GM 1715, respectively (7,8). These mutations abolish ADA activity most likely through destabilization of ADA protein rather than inactivation of the active site. The latter is unlikely because the inactivating point mutations found to date are scattered along the coding sequence. Moreover, Danton et al. (20) have recently isolated proteins with normal molecular weight, from several ADA⁻SCID cell lines including GM 2471, by adsorption on a coformycin affinity column. This suggests that aberrant ADA protein synthesized in these cells still harbours a structural site that can recognize its proper metabolite's three-dimensional shape.

In our patient the ADA protein can not be produced at all and hence one cannot postulate a residual activity which could be held responsible for the normal growth and development of cells that in normal individuals also express low levels of ADA. At least during her early childhood the patient had an apparently normal development of most tissues except the lymphoid system. In our opinion, this calls for a revision on the classification of ADA as a 'house-keeping enzyme' in the strict sense of the word. From the above data one can conclude that for growth and differentiation of most of the tissues except for the T-lymphocyte tissue ADA is not absolutely required. On the other hand the expression of ADA is found in all tissues from normal healthy

individuals examined so far (21). Perhaps this expression is superfluous in almost all of the tissues with the exception of the lymphoid system.

The determination of the molecular basis of ADA deficiency in this patient and the subsequent establishment of the carrier status by means of DNA analysis in the relatives at risk directly demonstrated the pitfalls encountered by determining carrier status just by measuring enzyme activity levels alone. On the basis of enzyme activity levels neither the parents nor the brother could be designated as carriers of an aberrant ADA allele whereas DNA analysis unequivocally indicates their carrier status. This also stresses the caution with which one has to interpret studies correlating ADA activity with chromosome 20 abnormalities in an attempt to localize the ADA gene through gene dosage studies (22,23,24,25). Obviously the expression of the single intact allele of the heterozygotes described in this paper may very well exceed the expression of two intact alleles in some normal individuals. Therefore it is better to correlate the presence of ADA-specific DNA sequences with chromosome 20 abnormalities to localize the ADA gene.

We have mapped the 3.2-kb deletion to a 4.2-kb region around the first exon. DNA sequence analysis of this region of the ADA gene (19), revealed the presence of 3 identically oriented full-sized Alu repeats, two of which, very suggestively, are located 3.2 kb apart (see fig. 6). Recently several groups have reported that Alu-Alu recombinational events can generate deletions as well as duplications in structural genes presumably through unequal crossing-over (26,27,28). Possibly a recombination event involving these 3.2-kb separated Alu repeats is also responsible for the observed 3.2-kb deletion described in this report. Further studies to substantiate this hypothesis are underway.

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