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# Adenosine Deaminase Deficiency: Genotype-Phenotype Correlations Based on Expressed Activity of 29 Mutant Alleles

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## Summary

**Adenosine deaminase (ADA) deficiency causes lymphopenia and immunodeficiency due to toxic effects of its substrates. Most patients are infants with severe combined immunodeficiency disease (SCID), but others are diagnosed later in childhood (delayed onset) or as adults (late onset); healthy individuals with "partial" ADA deficiency have been identified. More than 50 ADA mutations are known; most patients are heteroallelic, and most alleles are rare. To analyze the relationship of genotype to phenotype, we quantitated the expression of 29 amino acid sequence–altering alleles in the ADA-deleted *Escherichia coli* strain SØ3834. Expressed ADA activity of wild-type and mutant alleles ranged over five orders of magnitude. The 26 disease-associated alleles expressed 0.001%–0.6% of wild-type activity, versus 5%–28% for 3 alleles from "partials." We related these data to the clinical phenotypes and erythrocyte deoxyadenosine nucleotide (dAXP) levels of 52 patients (49 immunodeficient and 3 with partial deficiency) who had 43 genotypes derived from 42 different mutations, including 28 of the expressed alleles. We reduced this complexity to 13 "genotype categories," ranked according to the potential of their constituent alleles to provide ADA activity. Of 31 SCID patients, 28 fell into 3 genotype categories that could express ⩽0.05% of wild-type ADA activity. Only 2 of 21 patients with delayed, late-onset, or partial phenotypes had one of these "severe" genotypes. Among 37 patients for whom pretreatment metabolic data were available, we found a strong inverse correlation between red-cell dAXP level and total ADA activity expressed by each patient's alleles in SØ3834. Our system provides a quantitative framework and ranking system for relating genotype to phenotype.**

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## Introduction

Deficiency of adenosine deaminase (ADA; MIM 102700), a 41-kD zinc enzyme encoded in a 12-exon/32-kb gene on chromosome 20q, causes 10%–20% of all cases of severe combined immunodeficiency disease (SCID) (Giblett et al. 1972; Wiginton et al. 1984, 1986; Wilson et al. 1991; Hershfield and Mitchell 1995). Profound lymphopenia in this disorder has been attributed to toxic levels of ADA substrates, particularly deoxyadenosine (dAdo), generated from nucleic acid breakdown associated with cell turnover in marrow, thymus, and lymph nodes. Erythrocytes of deficient patients show two striking effects of dAdo, dATP pool expansion and inactivation of S-adenosylhomocysteine (AdoHcy) hydrolase. These findings and in vitro studies have implicated two pathogenic mechanisms—namely, inhibition by dATP of DNA replication and repair, inducing apoptosis, and inhibition by AdoHcy of S-adenosylmethionine–dependent methylation (reviewed in Hershfield and Mitchell 1995). These or other effects of ADA substrates also may cause hepatic injury in ADA knockout mice and in some human patients (Migchielsen et al. 1995; Wakamiya et al. 1995; Bollinger et al. 1996).

Most ADA-deficient patients have SCID, which is usually diagnosed in infancy and is often fatal, but 15%–20% are diagnosed later in childhood or as adults with insidiously evolving immunodeficiency (Giblett et al. 1972; Hirschhorn 1979; Geffner et al. 1986; Levy et al. 1988; Santisteban et al. 1993; Shovlin et al. 1994; Hershfield and Mitchell 1995; Ozsahin et al. 1997). Healthy individuals with "partial" ADA deficiency have also been identified (Jenkins et al. 1976; Daddona et al. 1985; Hirschhorn and Ellenbogen 1986; Hirschhorn et al. 1990; Santisteban et al. 1995*b;* Ozsahin et al. 1997). To date, >50 ADA mutations, including >30 amino acid substitutions, have been found in patients. Relating genotype to phenotype has been difficult because ADA deficiency is rare (~1/$10^6$ births) and most new mutations have been reported in single patients who often are heteroallelic. Although unusual, mosaicism can occur, further complicating analysis (Hirschhorn et al. 1994, 1996).

Assessment of residual ADA activity and the effects of mutations on ADA expression has also been problematic. Obtaining lymphocytes from SCID patients is rarely possible, and estimation of "percent of normal" ADA activity from these cells or cell lines is imprecise. Erythrocyte ADA activity, usually the only direct measure of residual activity available, is very low in all phenotypes (Hershfield and Mitchell 1995; Hirschhorn 1995). This may reflect low ADA expression and lack of protein turnover in red cells, favoring loss of unstable mutant enzymes. In studies defining ADA genotype, putative missense mutations have been distinguished from polymorphisms, by transfection of cell lines with cDNA or by translation of cDNA-derived transcripts in a reticulocyte lysate. However, with either approach, endogenous ADA activity has precluded quantitation of mutant activity.

If information relevant to phenotype is to be derived from genotype, it is essential to establish a uniform, quantitative, and sensitive method for assessing effects of mutations. This might be done by transfection of a cell line derived from one of a few SCID patients known to be homozygous for an ADA-gene deletion. However, since ADA is active as an unmodified monomer and is homologous to the *Escherichia coli* enzyme, we used a more convenient expression system based on the ADA-deleted *E. coli* strain SØ3834 (Chang et al. 1991; Ibrahim et al. 1995). We report an excellent correlation between the expressed ADA activity of a panel of 29 mutant alleles and the clinical and metabolic phenotypes of a diverse group of 52 patients who possessed 43 genotypes derived from 42 different mutant alleles, including 28 from the expression panel.

## Patients, Material, and Methods

### Criteria for Patient Selection and Phenotype Classification

*Reference Patients.*—Over the past 15 years, we have diagnosed or have been involved in monitoring the treatment of ~100 ADA-deficient patients. For this study, patients were included if both *genotype* (both mutant ADA alleles) and a fully developed clinical *phenotype* (as defined in next section) were known, either from clinical case reports, reports of new mutations that included information about phenotype, or from our own unpublished analysis of genotype. Affected sibs diagnosed prenatally or at birth were excluded, but for two families both sibs were included, since they were diagnosed simultaneously and had distinctive phenotypes (Shovlin et al. 1993; Umetsu et al. 1994). We also included two healthy adults who were found to have partial ADA deficiency and who are the father and an uncle of unrelated SCID patients (Santisteban et al. 1995*b;* Ozsahin et al. 1997).

*Phenotype.*—The level of dATP or total dAdo nucleotides (dAXP; >90% dATP) in erythrocytes, if measured before transfusion, enzyme replacement, or marrow transplant, is a sensitive index of metabolic severity associated with ADA deficiency (Hershfield and Mitchell 1995). Various parameters of cellular and humoral immune function may be examined prior to these treatments, but methods used and degree of documentation have not been consistent. The terms "SCID," "delayed onset," "late (adult) onset," and "partial" deficiency have been used to distinguish levels of overall clinical severity and immune dysfunction (Hershfield and Mitchell 1995; Hirschhorn 1995). Since *onset* is often difficult to pinpoint, we prefer to use *age at diagnosis,* which is definite, as a reference. The delay between onset of symptoms and diagnosis has been estimated to average 2 mo for patients with SCID (Stephan et al. 1993) and has been >10 years for some patients diagnosed as adults (Shovlin et al. 1993; Ozsahin et al. 1997). Thus, our definitions are as follows: (1) SCID—profound lymphopenia with absent cellular and humoral immune function, leading to recurrent opportunistic infections and failure to thrive in infancy, with diagnosis in the 1st year of life; (2) delayed onset—clinical deterioration due to combined immunodeficiency, leading to diagnosis at 1 year to several years of age; (3) late onset—major immunologic and clinical deterioration, leading to diagnosis, occurs beyond the 1st decade; (4) partial—healthy ADA-deficient individuals with normal immune function. Late-onset patients may be underdiagnosed, and all "partials" have been ascertained by screening.

### Bacterial Strains and Plasmids

*E. coli* SØ3834 and plasmid pZC11–containing TAC-promoted wild-type human ADA cDNA (Chang et al. 1991) were kindly provided by Dr. Rod Kellems, University of Texas–Houston Medical School. *E. coli* SØ3834 is a multiple auxotroph (rpsL, Δadd-uid-man, metB, guaA, uraA::Tn10) with a deletion of *add* (bacterial ADA gene). When grown in minimal medium, SØ3834 requires guanosine, owing to the *guaA* (guanylate synthetase gene) mutation (Jochimsen et al. 1975; Hove-Jensen and Nygaard 1989). After transformation with a functional human ADA cDNA, this guanosine requirement can be met by 2,6-diaminopurine (DAP) (Chang et al. 1991). We confirmed this and found that several mutant ADA cDNAs also conferred the ability to grow on DAP, indicating that low levels of mutant-ADA activity could supply sufficient guanine nucleotides from DAP to support colony growth (data not shown).

### *Cloning and cDNA Manipulations*

We constructed a panel of pZ/ADA cDNA transformants of SØ3834, consisting of wild-type and 29 mutant alleles (28 missense and 1 single-codon deletion), all of which have been identified in ADA-deficient patients and have been reported elsewhere (table 1). These mutations alter 24 amino acids between positions 15 and 337 (of 363) of human ADA. Mutant ADA cDNAs were subcloned into the pZC11 *Nco*I site from which wild-type cDNA had been excised (pZ). In most cases, a 720-bp *Bam*HI/*Bgl*II or a 663-bp *Pst*I fragment of wild-type cDNA was replaced with a fragment containing the mutation of interest. Mutations V129M, R149Q, A215T, E217K, and R253P were introduced by combinatorial PCR mutagenesis, essentially as described elsewhere for the A215T and P126Q mutations (Ozsahin et al. 1997). Final pZ cDNA inserts were sequenced in order to determine that only the desired mutation was present.

### *cDNA Expression*

In preliminary studies, we evaluated factors that could influence expression and quantitation of low mutant-ADA activity in pZ-transformed SØ3834. In complete medium (to avoid selection for more-active mutants) and without induction, levels of plasmids bearing wild-type and several mutant cDNAs were relatively uniform in late log phase, as were the different levels of ADA activity that each expressed; isopropylthiogalactoside induction had little effect on ADA expression. There was no effect on assayed activity when purified ADA was diluted extensively in a concentrated SØ3834 lysate, indicating the functional absence of inhibitors or reactions competing for substrate or product.

On the basis of these results, we developed the following protocol for determining ADA expression. pZ-ADA transformants of SØ3834 were grown at 37°C on Luria-Bertani (LB) plates containing carbenicillin and tetracycline (200 and 12.5 μg/ml, respectively). Several colonies of each transformant were transferred to 10 ml LB/carbenicillin/tetracycline medium and were grown at 37°C to 0.8–1.2 A600. Aliquots of each culture (and pZ vector–only controls) were tested for pZ retention. Cells harvested from 3-ml aliquots of 3–4 cultures with equivalent plasmid levels were sonicated in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5; 75 mM KCl; 10 mM $MgC1_2$; 1 mM DTT; and 1 mM AEBSF protease inhibitor [Calbiochem]). After centrifugation (15,000 *g*, 15 min, 4°C), ADA activity in supernatants (diluted as necessary to achieve linearity in lysis buffer containing 1 mg BSA/ml) was determined by radiochemical assay (Arredondo-Vega et al. 1990). Aliquots of supernatant containing 20 μg of lysate protein also were analyzed by 10% SDS-mercaptoethanol PAGE and by western blotting performed by standard techniques. For the latter, we used goat anti-human ADA antiserum (Wiginton and Hutton 1982) kindly supplied by Dr. Dan Wiginton, University of Cincinnati; a rabbit anti-goat IgG-alkaline phosphatase conjugate was the secondary antibody. A lower limit of 0.4 ng of purified human ADA could be detected when added to an untransformed SØ3834 lysate prior to immunoblotting (data not shown).

## Results

The 52 subjects who met criteria for inclusion include 49 with immune deficiency, who were from a few weeks to 39 years of age at diagnosis, and 3 healthy subjects with partial deficiency (table 2). For the 31 SCID patients, the average age at diagnosis was 4.4 mo. This is similar to the ages reported in two large retrospective studies of >200 patients with SCID due to all causes (15% of these were ADA deficient) (Stephan et al. 1993; Buckley et al. 1997). Among our reference cases were nearly all reported cases in the delayed and late-onset categories.

Consistent with their clinical heterogeneity, the 52 reference patients possessed 43 ADA genotypes, composed of 42 different mutant alleles, only 2 of which accounted for >10% of the total (R211H, 13%; G216R, 11%) (tables 1 and 2). Forty-four subjects (85%) had at least 1, and 31 (60%) had 2, of 28 alleles with mutations that altered single amino acid residues. All 28 of these alleles are included in the SØ3834 expression panel.

### *Mutant-Allele Expression in SØ3834*

ADA activity expressed constitutively by the panel of 29 SØ3834 transformants ranged over about five orders of magnitude, from a background of 3 nmol/h/mg lysate protein for the pZ vector control to 262,600 nmol/h/mg protein for wild-type human ADA (pZC11). The activities of the 29 mutants form a roughly monotonic continuum, from twice background to 74,000 nmol/h/mg protein (fig. 1). Immunoblot bands for mutant proteins ranged from undetectable to more intense than those for wild type (fig. 2). The effects of mutations on ADA folding and stability will be the subject of a separate report, but, in most cases, mutant proteins appeared to have reduced stability. However, catalytic activity usually was reduced to a greater extent than detectable protein. A striking example is the active site mutation E217K, which consistently appeared more stable than wild type, on the basis of immunoblotting, but which had only 0.001% of wild-type ADA activity.

### *Relationship of Expressed ADA Activity to Clinical Phenotype*

Half of all alleles of the reference subjects and 14 of their 28 amino acid substitutions occurred in single in-

**Table 1**

**ADA Mutations in Reference Patients**

| Mutation | Site | No. of Alleles (No. of Homozygous Patients)[a] | Reference(s) |
|---|---|---|---|
| Expressed in SØ3834: | | | |
| H15D | Exon 2 | 1 | Santisteban et al. 1995*a* |
| G74C | Exon 4 | 1 | Arredondo-Vega et al. 1997 |
| G74V | Exon 4 | 1 | Bollinger et al. 1996 |
| A83D | Exon 4 | 1 | Santisteban et al. 1995*a* |
| R101L | Exon 4 | 1 | Santisteban et al. 1993 |
| R101Q | Exon 4 | 2 | Bonthron et al. 1985 |
| R101W | Exon 4 | 1 | Akeson et al. 1988 |
| P104L | Exon 4 | 4 (2) | Atasoy et al. 1993 |
| L107P | Exon 4 | 5 | Hirschhorn et al. 1990 |
| P126Q | Exon 5 | 1 | Ozsahin et al. 1997 |
| V129M | Exon 5 | 2 (1) | Arredondo-Vega et al. 1997 |
| G140E | Exon 5 | 1 | Arredondo-Vega et al. 1997 |
| R142Q | Exon 5 | 1 | Santisteban et al. 1995*b* |
| R149W | Exon 5 | 1 | Arredondo-Vega et al. 1997 |
| R149Q | Exon 5 | ...[b] | Hirschhorn et al. 1990 |
| R156C | Exon 5 | 3 (1) | Hirschhorn 1992 |
| R156H | Exon 5 | 6 (1) | Santisteban et al. 1993 |
| V177M | Exon 6 | 1 | Santisteban et al. 1993 |
| A179D | Exon 6 | 1 | Santisteban et al. 1995*a* |
| Q199P | Exon 6 | 1 | Arredondo-Vega et al. 1997 |
| R211C | Exon 7 | 2 | Hirschhorn et al. 1990; Shovlin et al. 1994 |
| R211H | Exon 7 | 13 (4) | Akeson et al. 1987, 1988 |
| A215T | Exon 7 | 1 | Hirschhorn et al. 1990; Ozsahin et al. 1997 |
| G216R | Exon 7 | 11 (2) | Hirschhorn et al. 1991; Santisteban et al. 1993; Ozsahin et al. 1997 |
| E217K | Exon 7 | 1 | Hirschhorn et al. 1992 |
| R253P | Exon 8 | 2 (1) | Hirschhorn et al. 1993 |
| S291L | Exon 10 | 2 | Hirschhorn 1992; Santisteban et al. 1993 |
| A329V | Exon 11 | 6 (2) | Akeson et al. 1988 |
| E337del | Exon 11 | 1 | Arredondo-Vega et al. 1997 |
| Deletion: | | | |
| 3.25 kb | Prom, exon 1 | 2 (1) | Markert et al. 1988 |
| 3.2 kb | Prom, exon 1 | 2 | Shovlin et al. 1994 |
| >30 kb | Prom, exons 1–5 | 1 | Hirschhorn et al. 1992 |
| G462 | Exon 5 | 2 (1) | Arredondo-Vega et al. 1997 |
| 1050–1054 | Exon 10 | 7 (1) | Gossage et al. 1993; Santisteban et al. 1993 |
| 1114–1115 | Exon 11 | 1 | Santisteban et al. 1993 |
| Splicing: | | | |
| +1G→A | IVS 2, 5′ss | 1 | Arredondo-Vega et al. 1994 |
| +1G→A | IVS 5, 5′ss | | Santisteban et al. 1995*a* |
| +6T→A | IVS 5, 5′ss | 1 | Santisteban et al. 1993 |
| 17 bp rearranged | IVS 8, 3′ss | 1 | Arredondo-Vega et al. 1994 |
| +1G→A | IVS 10, 5′ss | 1 | Santisteban et al. 1993 |
| −34G→A | IVS 10, 3′ss | 2 (1) | Santisteban et al. 1993 |
| Nonsense: | | | |
| Q3X | Exon 1 | 3 (1) | Santisteban et al. 1995*b* |
| R142X | Exon 5 | 2 (1) | Santisteban et al. 1995*b* |

NOTE.—Prom = promotor; ss = splice site.

[a] Among 100 alleles of 50 nonsibs.

[b] Not represented in reference population.

dividuals. To simplify assessment of the relationship to phenotype, we grouped alleles, where possible, according to their potential for providing functional ADA activity. Deletion and nonsense alleles form group 0, under the assumption that they provide no activity. The 29 amino acid substitutions in our panel were divided into four contiguous groups of increasing ADA activity in SØ3834 (fig. 1 and table 3). Group I, comprising 17 alleles with 0.001%–0.05% of wild-type activity, includes E217K and H15D, which alter substrate- and zinc cofactor–binding residues (Wilson et al. 1991; Santisteban et al. 1995*a*), and the recurrent alleles R211H,

**Table 2**

**Reference-Population Characteristics**

| | No. of Patients | Age at Diagnosis (Range) [years ± SD] |
|---|---|---|
| Phenotype: | | |
| SCID | 31 | .36 ± .25 (.1–1) |
| Delayed onset | 14 | 3.3 ± 2.0 (1.2–8) |
| Late onset | 4 | 30.6 ± 10.5 (15–39) |
| Partial | 3 | …[a] |
| | No. of Mutations | |
| Mutation type:[b] | | |
| Missense | 27 | |
| Deletion | 7 | |
| Splicing | 6 | |
| Nonsense | 2 | |
| Total | 42 | |

NOTE.—The reference population comprised 52 patients with defined clinical expression (see text for discussion of selection criteria) and both alleles identified.

[a] Current age: 20–30 years.

[b] No. of different genotypes: 43 (19 homozygous).

G216R, A329V, L107P, and P104L. Group II has 6 alleles that expressed 0.06%–0.2% of wild-type activity, including the recurrent allele R156H. Groups III and IV each include 3 alleles, with 0.3%–0.6% and 4.8%–28.2% of wild-type ADA activity, respectively. Finally, splicing mutations ("spl") are in a separate group, and no assumption was made about their potential for providing some normal ADA mRNA and functional enzyme. By use of these designations, each reference subject fell into 1 of 13 "genotype categories" (table 4).

All 10 patients in genotype categories 0/0 and 0/I and 18 of 20 patients in category I/I had a SCID phenotype (i.e., 28 of 31 SCID patients [90%] were in one of these three categories). Homozygosity or "functional hemizygosity" for the group I alleles R211H, G216R, L107P, and P104L consistently was associated with an early clinical presentation and diagnosis (Bory et al. 1990; Hirschhorn et al. 1991; Atasoy et al. 1993; Weinberg et al. 1993; F. X. Arredondo-Vega, I. Santisteban, M. S. Hershfield, unpublished data). Thus, both probands in two distantly related G216R-homozygous Amish families had opportunistic infections that led to diagnosis before 1 mo of age, and four unrelated R211H homozygotes (three Hispanic and one Canadian) were diagnosed at 3, 4, 5, and 10 mo of age. Brazilian and U.S. patients homozygous for P104L were diagnosed at 3 mo of age, and two apparently unrelated French children, who were heteroallelic for L107P and a 5-bp deletion in exon 10, were diagnosed at 2 mo of age. The group I allele with the highest activity, A329V, has occurred in several SCID patients, including two unrelated homozygotes, each diagnosed at 7 mo of age (Markert et al. 1987; Hershfield et al. 1993; Bollinger et al. 1996; F. X. Arredondo-Vega, I. Santisteban, M. S. Hershfield, unpublished data).

Only 1 of 31 SCID patients carried an allele from groups II–IV; he was heteroallelic for R211H and A179D (category I/II) (Santisteban et al. 1995*a*). The other five group II alleles were associated with a delayed-onset phenotype in patients in genotype categories 0/II, I/II, II/II, and II/spl. Among these, single patients diagnosed at 1.5–3 years of age were homozygous for A253P, R156H, or V129M (category II/II); three others, diagnosed at 1.2–8 years of age, were heteroallelic for R156H and either G216R (group I), a deletion, or a splicing defect (Hirschhorn et al. 1993; Santisteban et al. 1993; Arredondo-Vega et al. 1998; F. X. Arredondo-Vega, I. Santisteban, M. S. Hershfield, unpublished data). Single patients diagnosed at 4 and 6 years of age carried V177M or Q199P (group II) and a deletion or G216R, respectively (Santisteban et al. 1993; Arredondo-Vega et al. 1998).

All three group III alleles have been associated with delayed, late-onset, and partial phenotypes in heteroallelic patients in genotype categories 0/III and I/III. Thus, R211C (with a deletion) has been found in sisters who developed immunodeficiency as adults and who were diagnosed in their mid 30s (Shovlin et al. 1994), and P126Q (with G216R) has been found in a Swiss patient diagnosed at 39 years of age (Ozsahin et al. 1997). R211C also has been found in a healthy partial whose second allele was L107P from group I (Hirschhorn et al. 1990). G74C has occurred in one patient, diagnosed at 3 years of age, whose second allele was G216R (Arredondo-Vega et al. 1998).

The three group IV alleles (R142Q, R149Q, and A215T) have been found in healthy individuals who had group I or nonsense mutations as their second alleles (Hirschhorn et el. 1990; Santisteban et el. 1995*b;* Ozsahin et al. 1997). These alleles have not been found in any patients with immunodeficiency. As we have previously noted, splicing mutations may be associated with either severe or relatively mild phenotypes (Santisteban et el. 1993; Arredondo-Vega et el. 1994). Thus, of six reference patients with at least one splicing mutation, two had SCID, three had a delayed phenotype, and one had a late-onset phenotype (table 4).

### *Relationship of "Total Expressed ADA Activity" to Metabolic Phenotype*

Pretreatment dAXP or dATP levels were available for 37 of the reference subjects (23 SCID, 8 delayed onset, 3 late onset, and 3 partial) (table 5). This subset carried 26 of the 29 SØ3834 expression-panel alleles: 25 had two panel alleles (12 were homozygous, and 13 were compound heterozygous); 10 other patients were "func-

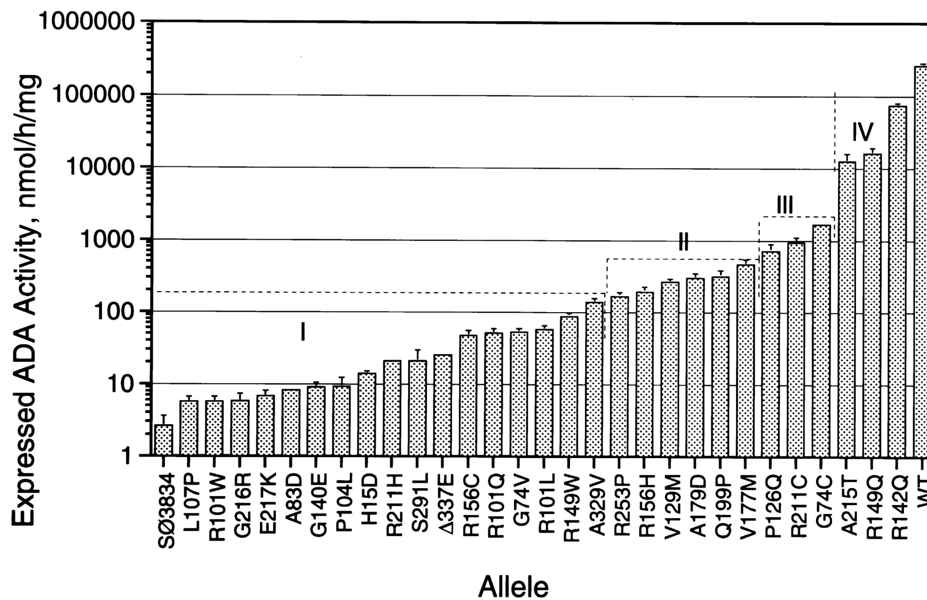


**Figure 1** Distribution of mutant-ADA activity expressed in SØ3834. The positive error bars indicate the SDs. The roman numerals and dashed lines indicate the allele groups used in establishing the genotype categories (see text and tables 3 and 4).

tionally hemizygous," with one panel allele and a second allele contributing no or negligible ADA activity; and two patients were homozygous for deletions.

On the basis of constitutive expression in SØ3834 (fig. 1), we calculated for each of these 37 subjects the total ADA activity expressed by genotype (i.e., the sum of the activity expressed by their alleles). The mean and ranges of "total expressed ADA activity" and red-cell dAXP levels for the four phenotypic groups are shown in table 5. There is probably a limit to the level to which dAXP in cells can rise when ADA is deficient, in part because the increase in dAXP is accompanied by a fall in ATP (Siaw et al. 1980; Bagnara and Hershfield 1982). Nevertheless, there was a good correlation ($r = .9$) between total expressed ADA activity and RBC dAXP levels, when data for individual patients were plotted (fig. 3).

The inverse nature of the relationship between expressed ADA activity and red-cell dAXP is apparent from figure 3 and when the means for the four phenotypes are compared (table 5). Of 23 SCID patients, 22 had dAXP >350 nmol/ml RBC and 20 had total expressed ADA activity of <200 nmol/h/mg protein (<0.04% of wild type). Conversely, all 14 patients with delayed, late-onset, or partial phenotypes had red-cell dAXP <300 nmol/ml, and 13 of 14 patients had total expressed ADA activity >190 nmol/h/mg protein. The difference in means for total expressed ADA activity and red-cell dAXP for the SCID and delayed-onset groups was significant ($P < .0001$ for each parameter; table 5). The trends for the late-onset and partial groups were

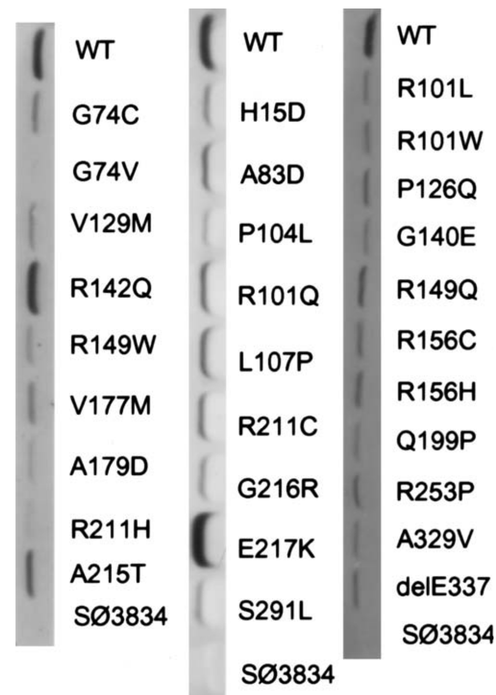


**Figure 2** Western blot of ADA expressed in lysates of SØ3834. The panels are the results of three separate experiments. WT = wild-type ADA, and SØ3834 = pZ vector control (contains no ADA cDNA).

**Table 3**

**ADA Mutants Grouped by Activity Expressed in SØ3834**

| Group | Mutations | Expressed ADA Activity (Range) [nmol/h/mg protein][a] | Percent of Wild Type (Range)[a] |
|---|---|---|---|
| I | H15D, G74V, A83D, R101L, R101Q, R101W, P104L, L107P, G140E, R149W, R156C, R211H, G216R, E217K, S291L, A329V, E337del | 30.4 ± 35.8 (3.0–134.2) | .012 ± .014 (.001–.051) |
| II | V129M, R156H, V177M, A179D, Q199P, R253P | 280 ± 104 (164–456) | .11 ± .04 (.06–.17) |
| III | G74C, P126Q, R211C | 1,093 ± 487 (711–1,642) | .42 ± .19 (.27–.63) |
| IV | R142Q, R149Q, A215T | 34,253 ± 34,443 (12,492–73,963) | 13.0 ± 13.1 (4.8–28.2) |
| | Wild type | 262,593 ± 21,590 | |

[a] Mean ± SD.

in the same direction, although the number of patients in these groups was too few to adequately evaluate significance.

## Discussion

The clinical expression of immunodeficiency, on which the diagnosis of ADA deficiency depends, can be influenced strongly by environmental factors, such as exposure to and treatment of pathogens, and also by genes (other than that for ADA) that affect responses to infection. Nevertheless, at the extremes, phenotype and residual ADA activity clearly are related: blood mononuclear cells from healthy subjects with partial deficiency have 5%–70% of normal ADA activity, versus <1%–2% in cells from patients with SCID. From reports of new ADA mutations, albeit in small numbers of patients, some splicing and missense alleles appear to be associated with delayed and late-onset phenotypes (Hirschhorn et al. 1990; Santisteban et al. 1993; Shovlin et al. 1994; Santisteban et al. 1995*b;* Ozsahin et al. 1997). This suggests that allele combinations providing more than some critical level of functional mutant enzyme (or of normal ADA from splicing mutants) (Santisteban et al. 1993; Arredondo-Vega et al. 1994) can confer a milder phenotype than SCID. To more rigorously test this hypothesis and to define this critical level of ADA activity, we systematically compared the expression of 29 patient-derived amino acid substitutions distributed throughout the ADA sequence.

**Table 4**

**Relationship of Genotype Category to Clinical Phenotype**

| Genotype Category[a] | No. of Patients | No. of Patients, by Phenotype: SCID | Delayed Onset | Late Onset | Partial |
|---|---|---|---|---|---|
| Missense, deletion, or nonsense: | | | | | |
| 0/0 | 5 | 5 | … | … | … |
| 0/I | 5 | 5 | … | … | … |
| I/I | 20 | 18 | 2 | … | … |
| 0/II | 1 | … | 1 | … | … |
| I/II | 3 | 1 | 2 | … | … |
| II/II | 3 | … | 3 | … | … |
| 0/III | 3 | … | 1 | 2 | … |
| I/III | 4 | … | 2 | 1 | 1 |
| 0/IV | 1 | … | … | … | 1 |
| I/IV | 1 | … | … | … | 1 |
| Splicing: | | | | | |
| I/spl | 2 | 1 | 1 | … | … |
| II/spl | 1 | … | 1 | … | … |
| spl/spl | 3 | 1 | 1 | 1 | … |
| Total | 52 | 31 | 14 | 4 | 3 |

[a] A 0 designates a deletion or nonsense allele; I, II, III, and IV indicate an allele in the expressed-activity groups listed in table 3. "spl" designates a splicing mutation. Among the five patients with a 0/0 genotype, three were homozygous for deletions, and two were homozygous for nonsense mutations.

ADA seemed well suited to expression analysis in *E. coli:* it is cytoplasmic and active as an unmodified monomer, and the human and *E. coli* enzymes are homologous (Chang et al. 1991). One concern was that intrinsic effects of specific mutations might be obscured by nonspecific degradation. However, western blotting of SØ3834 lysates (fig. 2) reproducibly revealed a broad range of detectable mutant ADA proteins, consistent with previous studies of patient-derived B lymphoblastoid cell lines, which used the same goat anti-human ADA antiserum (Wiginton and Hutton 1982). In most cases, less mutant than the wild-type ADA protein was detected, but catalytic activity usually was reduced to a greater degree. The mechanisms by which mutations affect ADA activity—that is, by interfering with zinc or substrate binding or with protein folding—are of interest (and will be dealt with elsewhere). However, the question posed in this study was not how mutations affect activity but whether the level of expressed mutant activity correlates well or poorly with aspects of phenotype.

For this study, the precision and sensitivity of the SØ3834 system for scaling mutant expression greatly exceed those for determining residual ADA activity in

cells that can be obtained from patients. Thus, ADA activity expressed by wild-type ADA cDNA in SØ3834 was ~ 260,000 ± 21,000 nmol/h/mg protein (mean ± SD). With the same radiochemical assay, we found ADA activity in normal red cells, blood mononuclear cells, cultured T cells, and B lymphoblastoid cell lines to be ~ 80 ± 40, 1,200 ± 500, 2,050 ± 1,360, and 7,450 ± 3,300 nmol/h/mg protein, respectively. Consistent with previous estimates that 5% of normal ADA activity is sufficient to prevent immunodeficiency, three alleles from healthy subjects with partial deficiency expressed 5%–28% of wild-type ADA activity in SØ3834. However, the range expressed by the 26 disease-associated alleles, 0.001%–0.6% of wild type, is 100-fold greater than the 0.2%–1.1% of normal ADA activity previously estimated for B cell lines derived from several immunodeficient patients (genotype unknown) (Hirschhorn et al. 1979; Wiginton and Hutton 1982).

Two of the 29 mutants examined in SØ3834 also have been expressed by mRNA translation in rabbit reticulocyte lysates (Santisteban et al. 1993; Hirschhorn et al. 1997; Ozsahin et al. 1997) and by transient transfection of Cos cells (Hirschhorn et al. 1997). Relative ADA activity (mutant vs. normal) was estimated from in situ histochemical assay of electrophoresed extracts (performed to resolve human from rabbit or monkey ADA). In Cos cells, the partial A215T mutant was estimated to have 8% of normal ADA activity, and the disease-associated R156H mutant had 1.2% of normal ADA activity (ADA protein was not evaluated) (Hirschhorn et al. 1997). The expressed activities of these mutants in SØ3834 were 4.8% ± 1.3% and 0.07% ± 0.01% of normal, respectively. The apparently higher levels in Cos cells, particularly for R156H, may reflect characteristics of the histochemical procedure. This coupled enzymatic method, developed to detect ADA isozymes, produces a colored precipitate at sites of ADA activity (Spencer et al. 1968). Use for quantitation is problematic, since linearity of color development is difficult to achieve and to document; this problem is magnified when higher protein concentrations and longer incubations are needed in order to detect mutant levels of activity, compared

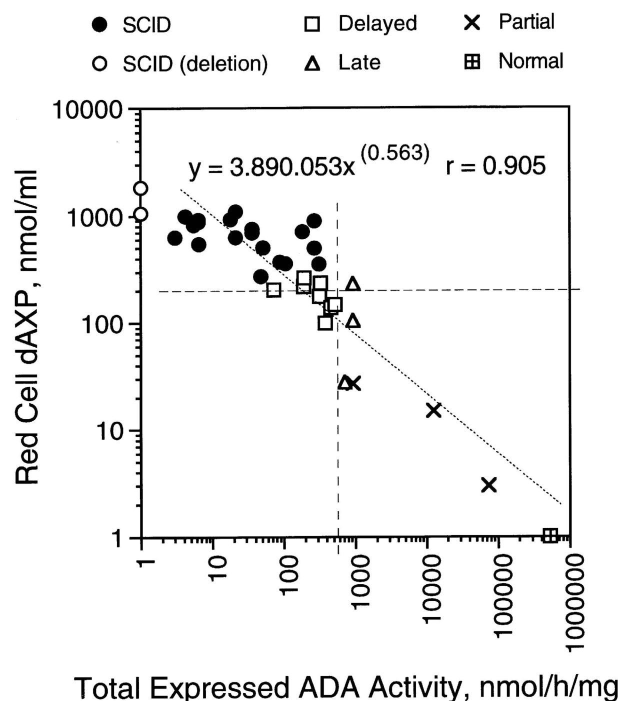


**Figure 3** Relationship between allele-determined total expressed ADA activity and erythrocyte dAXP levels. The total expressed ADA–activity value (see text) for each of 37 subjects is plotted against their pretreatment red-cell dAXP (or dATP) level. The phenotypes of the subjects are indicated by the symbols defined in the figure. To use a log scale, we assigned an expressed-ADA value of 1 nmol/h/mg protein (not 0) to the two SCID patients homozygous for deletions. ADA expression for a normal individual was assumed to be twice the ADA activity expressed from wild-type ADA cDNA in SØ3834; the normal red-cell dAXP level was assumed to be 1 nmol/ml of packed RBC. The "power" least-squares algorithm of Cricket Graph III (Computer Associates) was used for curve fitting (omitting the data points for the two patients homozygous for deletions).

with those needed to detect wild-type levels. Thus, the relative activity of mutants with low but detectable activity tends to be overestimated.

We related the SØ3834-activity data to clinical and metabolic features of 52 patients, spanning the pheno-

**Table 5**

**Total Expressed ADA Activity and Erythrocyte dAXP Levels for Phenotype Groups**

| Phenotype | No. of Patients | RBC dAXP (Range) [nmol/ml][a] | Expressed ADA Activity (Range) [nmol/hr/mg protein][a] |
|---|---|---|---|
| SCID | 23 | 766 ± 344 (273–1,839)[b] | 67 ± 96 (1–314)[b] |
| Delayed onset | 8 | 186 ± 55 (100–263) | 308 ± 149 (72–522) |
| Late onset | 3 | 122 ± 104 (28–234) | 855 ± 122 (714–926) |
| Partial | 3 | 15 ± 12 (3–27) | 29,144 ± 39,247 (927–73,963) |
| Normal | ... | <2 | 525,184 ± 43,180 |

[a] Mean ± SD.

[b] $P < .0001$, SCID vs. delayed onset.

typic spectrum, who possessed 28 of the alleles in our expression panel. These alleles were among 42 mutant alleles that combined to give 43 genotypes. We reduced this complexity by ranking genotypes, where possible, according to the potential of their constituent alleles to provide ADA activity. For this purpose, we divided the expression-panel alleles into four groups with increasing activity in SØ3834 (table 3). Group I, consisting of 17 alleles that expressed $\leqslant$0.05% of wild-type activity, includes several recurrent missense alleles that have been considered to be "severe" because of their association (when homozygous) with SCID. Groups III and IV include rare alleles that have been identified in patients with late-onset and partial phenotypes.

By using these four allele groups and by grouping true nulls (deletions and nonsense mutations) together and splicing mutations separately, we assigned each patient to 1 of 13 genotype categories. Constructing a table relating these categories to the distribution of four clinical phenotypes (table 4) revealed that 28 of 31 SCID patients fell into three genotype categories that provided $\leqslant$0.05% of normal ADA activity (i.e., the activity expressed by two copies of wild-type ADA cDNA in SØ3834). Conversely, only 2 of 21 patients with delayed, late-onset, or partial phenotypes had one of these "severe" genotypes. The SØ3834 expression system should provide a framework for evaluation of novel amino acid substitutions. For example, we would expect that the discovery of new patients with a delayed or late-onset phenotype will result in new alleles that express activity within the range for groups II and III.

In a subset of 37 patients for whom pretreatment metabolic data were available, we found a strong inverse correlation between red-cell dAXP content and the sum of the ADA activity expressed by their two alleles in SØ3834. Nearly all SCID patients had red-cell dAXP >350 nmol/ml RBC and total expressed ADA activity <200 nmol/h/mg protein (0.04% of wild type). In contrast, nearly all patients with delayed, late-onset, or partial phenotypes had red-cell dAXP <300 nmol/ml and total expressed ADA activity >190 nmol/h/mg protein. Expressed ADA activity of cloned alleles is more likely to be an index of steady-state residual ADA activity in nucleated cells (capable of protein turnover) than in erythrocytes, in which residual ADA activity usually is undetectable. Our results support the hypothesis that clinical phenotype–related red-cell dAXP levels reflect differences in total residual capacity to catabolize circulating dAdo (Cohen et al. 1978; Hershfield et al. 1987; Santisteban et al. 1993; Hershfield and Mitchell 1995). Erythrocyte dAXP levels in individual patients also may reflect rates of dAdo production, transport into red cells, and renal excretion, but differences in these factors are unlikely to be related systematically to clinical phenotype.

Our results indicate that clinical phenotype is affected significantly by a remarkably low level of residual ADA catalytic activity, that is, ~0.05% of normal. Possession of more than this activity may reduce metabolic toxicity to a degree that permits sufficient survival of functional T cells, in the 1st year of life or beyond, to protect from the kinds of severe opportunistic infections that arouse clinical suspicion of SCID and prompt testing for ADA deficiency. Patients with delayed onset often experience recurrent infections, and these infections may involve pathogens that only occasionally affect normal children. However, aggressive use of antibiotics can mask the underlying problem of immunodeficiency and delay its diagnosis.

Paradoxically, ADA-deficient patients with delayed phenotype and probably most with late-onset phenotype have persistent lymphopenia, which, if appreciated, could identify them as being at risk of having combined immunodeficiency early in life (if not at birth), before clinical disease is manifested. Indeed, mandating lymphocyte counts for all infants, as a screen for a number of primary immunodeficiency disorders, is of interest. For patients identified in this manner as being ADA deficient, predicting the course of the disorder and choosing among several treatment options could pose problems. The ability to evaluate risk, and possibly the response to enzyme-replacement therapy, on the basis of genotype-determined expression of ADA activity, could be useful in this situation.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

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