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Research Article

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IgG Antibody Response to Polyethylene Glycol-modified Adenosine Deaminase in Patients with Adenosine Deaminase Deficiency

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Abstract

Polyethylene glycol (PEG)-modified bovine adenosine deaminase (ADA) is used for replacement therapy of severe combined immunodeficiency disease due to inherited ADA deficiency. We monitored IgG anti-ADA antibody in 17 patients treated by intramuscular injections of PEG-ADA for 1 to > 5.5 yr. ELISA-detectable anti-ADA IgG appeared in 10 patients, usually between the third and eighth months of treatment. Anti-ADA levels did not correlate with trough plasma ADA activity, which averaged 1.8–5 times normal blood (erythrocyte) ADA activity, depending on dose (15–60 U/kg per wk). ELISA-detectable anti-ADA antibodies were directed primarily at bovine-specific peptide (rather than PEG-containing) epitopes. Enhanced enzyme clearance, mediated by antibody that directly inhibited native and PEG-modified bovine ADA, and native, but not PEG-modified human ADA, occurred in two patients. In one, tolerance was induced; in the second, twice weekly injections of PEG-ADA compensated for accelerated clearance. We speculate that inhibitory antibodies recognize conserved, relatively PEG-free epitope(s) encompassing the active site, and that in human, but not bovine, ADA a PEG-attachment site “shields” the active site from immune recognition. We conclude that PEG-modification largely prevents the development of high affinity, or high levels of, clearing antibodies to bovine ADA, and that PEG-modified human ADA should be further investigated as a possible treatment for ADA deficiency. (*J. Clin. Invest.* 1992. 89:1643–1651.) Key words: ELISA • inhibitory antibody • severe combined immunodeficiency disease • enzyme replacement therapy • immune tolerance

Introduction

The technique of modifying proteins by covalent addition of polyethylene glycol (PEG), aimed at slowing degradation and reducing immunogenicity, was developed to improve the characteristics of enzymes and other proteins for parenteral therapy (1–3). The approach has first been tested in the treatment of severe combined immunodeficiency disease (SCID)¹ due to in-

herited deficiency of adenosine deaminase (ADA) (4–6). Initial studies of patients treated for several months with PEG-modified bovine ADA (PEG-ADA, ADAGENTM) showed that weekly intramuscular injections produced high levels of ADA activity in plasma, corrected metabolic abnormalities, and led to improved immune function (6–9).

Successful replacement therapy of this usually fatal disorder requires continuous maintenance of high circulating levels of ADA activity, which could be jeopardized by the development of clearing antibodies to PEG-ADA. Before clinical trial, PEGylation had been shown to reduce both the antigenicity and the immunogenicity of bovine ADA in mice (10). Nevertheless, there were concerns that residual immunogenicity would limit the use of PEG-ADA in humans as chronic replacement therapy of ADA deficiency (11). Indeed, treatment with PEG-ADA entails significant, repeated antigenic challenge: 2.5 ml of the clinical preparation ADAGENTM, which several patients receive weekly, has the ADA activity of $\sim 10^{12}$ normal T cells or 4.5 liters of normal erythrocytes. Antibodies could arise towards “nonhuman” regions of PEG-ADA, either bovine-specific peptide segments or epitopes involving PEG. Conserved regions might also be immunogenic in patients who may never have developed tolerance to the native human ADA gene product: in addition to their immune deficit, most cell lines from ADA-deficient SCID patients have lacked or had very low levels of immunologically detectable ADA (12, 13). We have tried to address these issues and to define the evolution of IgG anti-ADA antibodies and their impact on therapy in 17 of the first 18 patients, who have been under treatment with PEG-ADA for 1.5 to > 5.5 yr.

Methods

PEG-modified bovine ADA (PEG-ADA, ADAGENTM) and disuccinyl-monomethoxyPEG, *M*, 5,000 (14) were provided by Enzon, Inc. (South Plainfield, NJ). The PEG enzyme was supplied as a sterile solution in 0.05 M potassium phosphate buffer, pH 7.4, containing 250 U of ADA activity per ml measured at 25°, ~ 550 U/ml measured at 37° (1 U = conversion of 1 μ mol of adenosine to inosine per min). Lots of PEG-ADA were changed according to the schedule of production. Except for brief periods, all patients were treated with the same lot of enzyme at any time.

Calf intestinal ADA was obtained from Sigma Chemical Co. (St. Louis, MO) and from Enzon. The preparation and characterization of PEG-modified *Escherichia coli* PEG-modified purine nucleoside phosphorylase (PNP) have been described (15). Purified recombinant human ADA was generously provided by Dr. Fred Rudolph, Rice University. It was treated with disuccinyl-monomethoxy PEG as described for preparation of PEG-PNP (15), resulting in modification of 40% of primary amino groups, as estimated by reaction with fluorescamine (16).

Enzyme assays and measurement of erythrocyte nucleotides. Fractionation of heparinized blood, radiochemical assays for plasma ADA and erythrocyte *S*-adenosylhomocysteine hydrolase (AdoHcyase) activities, and HPLC analysis of total erythrocyte adenine deoxyribonucleotides (dAXP) were performed as previously described and referenced in

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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; AdoHcyase, *S*-adenosylhomocysteine hydrolase; dAXP, total adenine deoxyribonucleotides; IVIg, intravenous Ig; PNP, purine nucleoside phosphorylase; SCID, severe combined immunodeficiency disease.

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(6, 17). Protein was determined by the BCA method (Pierce Chemical Co., Rockford, IL).

Preparation of IgG from plasma. Plasma was diluted 1:1 with 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.0, 5 mM EDTA (PBS/EDTA) and centrifuged for 20 min in a microcentrifuge; then 0.45 ml of supernatant was applied to a 1-ml column of Protein A Sepharose CL-4B (Sigma Chemical Co.) equilibrated with PBS/EDTA. After 30 min the column was washed with 21 ml of PBS/EDTA (ADA activity eluted in this step). IgG was then eluted with 6 ml of 0.1 M sodium citrate, pH 3.0. After dialysis against PBS, the IgG fraction was brought to 40% saturated ammonium sulfate and kept on ice for 1 h; the precipitate was then collected by centrifugation, washed with 40% saturated ammonium sulfate, dissolved in 0.2–0.3 ml of PBS, and dialyzed overnight against PBS. The final volume was brought to 0.45 ml with PBS and stored at -70° .

ELISA. Polystyrene 96-well microtiter plates (Immulon 2; Dynatech Laboratories Inc., Chantilly, VA) were treated overnight at 4° with 0.1 ml of purified, unmodified bovine ADA ($10 \mu\text{g/ml}$ in 0.1 M sodium carbonate, pH 9.8), or with buffer alone (no-antigen control). After washing with PBS, wells were blocked with 0.2 ml of 2% goat serum (Gibco BRL, Gaithersburg, MD) in PBS for 1 h at room temperature, then washed with PBS containing 0.1% Tween 20. Patient plasma (0.1 ml) sequentially diluted threefold from 1:10 to 1:2,430 in PBS, 2% goat serum, was added to antigen-coated and antigen-free wells in parallel rows. After 1 h at 37° plates were washed with PBS containing 0.1% Tween 20 and bound human IgG was detected with a biotinylated goat anti-human IgG (gamma chain specific)-avidin-horseradish peroxidase detection system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), using *o*-phenylenediamine as peroxidase substrate. After 25–30 min at room temperature the peroxidase reaction was stopped by addition of 0.1 ml/well of 1 N HCl and absorbance at 490 nm (A_{490}) was measured with a V_{max} Kinetic Microplate Reader (Molecular Devices Corp., Palo Alto, CA). After subtracting values from no-antigen wells, A_{490} vs. μl of plasma was plotted; the level of anti-ADA was estimated from the linear region of the plot and expressed as the theoret-

ical A_{490} per $10 \mu\text{l}$ of plasma. The same protocol was used in some experiments to detect antibody binding to immobilized PEG-ADA and to human ADA.

Competition ELISA. Aliquots (0.05 ml) of a predetermined dilution of patient plasma (sufficient to give an absorbance of 0.5–1.5 in the anti-ADA ELISA) were mixed with 0.05 ml of 2% goat serum (control), or with 2% goat serum containing increasing amounts (0.0016–20 μg) of unmodified or PEG-modified ADA, or PEG-modified *E. coli* PNP. After overnight incubation at 4° , these mixtures were tested in the ELISA as above (i.e., using unmodified bovine ADA as the immobilized antigen). A decrease in ELISA response relative to the control indicated binding of antibody by the antigen in solution.

Assay for ADA-inhibitory antibody. Duplicate aliquots (usually $10 \mu\text{l}$) of patient plasma or plasma IgG fraction (fraction B above), or an equal volume of 0.1% bovine serum albumin in PBS (PBS/BSA), were combined with PEG-ADA (0.1–0.3 mU at 37° , prepared by dilution in PBS/BSA) and brought to $50 \mu\text{l}$ with PBS/BSA. After 30 min, $5 \mu\text{l}$ aliquots were removed for radiochemical assay of ADA activity (final vol $50 \mu\text{l}$) (17). Reactions performed without PEG-ADA were used to correct for ADA activity in the plasma or IgG fraction (the latter was always negligible). Percent inhibition of ADA activity was determined relative to activity obtained with PBS/BSA. The same procedure was used to measure inhibition of unmodified bovine ADA and human ADA (native and PEG-modified).

Patient characteristics. We evaluated anti-ADA antibody responses during 12–58 mo of treatment in 17 of the first 18 ADA-deficient SCID patients to receive PEG-ADA (designated by number according to the order in which they began treatment). Patient 15, who has been under treatment for 16 mo, is not included because antibody was followed for only the first 6 mo of therapy (none was detected). Six patients began treatment at 3.8–14.9 yr of age, the other eleven at 6 wk–26 mo (Table I A). No patient had an HLA-matched sibling bone marrow donor. Before PEG-ADA, patient 1 had twice received HLA-haploidentical bone marrow transplants, but without stable engraftment (18); patients 2–4 had been treated for 9–10 yr with monthly (approximately) transfu-

Table I. Patient Profile (A) and ELISA Response to Unmodified Bovine ADA (B)

A. Patient* (sex)	Age at start of therapy	Months on of therapy†	Maintenance dose of PEG-ADA	B. Month onset +ELISA	Anti-ADA ELISA ELISA response		
					Before therapy	During treatment with PEG-ADA	
	mo		U/kg per wk		U	U	mo
1 (F)	45	67	20	5–6	27.4	60.2±11 (43–88)‡	9–58
2 (F)	117	62	20	—	0.5	1.2±1 (0–3)	9–53
3 (M)	144	57	20	5–6	3.8	33.7±9 (20–48)	8–48
4 (M)	115	56	15	6–8	0.7	34.0±10 (20–52)	10–46
5 (F)	69	55	20	1–2	1.1	53.1±15 (29–87)	8–45
6 (M)	1.6	52	30	24–28	0.6	18.1±9 (8–28)	28–43
7 (M)	22	39	20	4–6	4.9	11.4±5 (6–22)	9–26
8 (F)	178	39	20	4–5	2.8	54.8±16 (36–87)	10–29
9 (F)	26	36	25	2–4	24.8	38.4±16 (6–56)	8–26
10 (F)	22	34	60	—	0.7	4.3±3 (2–9)	7–25
11 (M)	1.7	34	60	—	0.2	2.4±2 (0–5)	8–24
12 (F)	16	29	60	4–5	2.5	52.1±23 (29–99)	9–20
13 (M)	19	21	30	—	0.3	2.2±1 (1–4)	6–17
14 (F)	8	18	30	6–7	3.6	7.8±5 (2–17)	6–14
16 (M)	11	16	30	—	2.4	1.7±1 (1–3)	7–14
17 (M)	2	13	30	—	1.9	3.5±2 (0–5)	7–12
18 (M)	6	12	30	—	2.2	3.8±1 (3–5)	6–12

* Numbered according to order of starting treatment with PEG-ADA; patient 15 is not included because complete data were not collected. ‡ As of November 1, 1991. § Mean±SD (range). || Treatment period (months) used for determining mean ELISA level.

sions of irradiated erythrocytes, used as a form of enzyme replacement therapy (19); patients 1, 5, 7, and 10–12 had received transfusions for shorter periods. At the time PEG-ADA therapy was initiated all patients were lymphopenic and had decreased lymphocyte mitogen responses. Their clinical characteristics and responses to PEG-ADA therapy will be summarized elsewhere. From April, 1986 until its approval by the U.S. Food and Drug Administration in March, 1990, PEG-ADA therapy was experimental and was performed with informed parental consent and approval of Investigational Review Boards.

PEG-ADA administration. The intramuscular dose of PEG-ADA was adjusted to maintain preinjection (trough) plasma ADA activity at $>12 \mu\text{mol/h}$ per ml (approximately equivalent to total erythrocyte ADA activity in normal individuals), sufficient to lower red cell dAXP to $<10\text{--}15 \text{ nmol/ml}$ packed cells and increase red cell AdoHcyase activity into the normal range (6–8). In most cases these conditions were achieved with one weekly injection of 15–30 U/kg. Some newly diagnosed patients who were malnourished or severely ill initially required twice weekly injections of 30 U/kg; this was changed to one injection a week when clinical status and T cell function improved. The weekly maintenance doses that patients received during the period when average ELISA levels were determined (see Results) are listed in Table I A. The effect of higher doses on immune function is being evaluated in some patients, but here we will consider only the relationship of dose, plasma ADA activity, and anti-ADA antibody response.

Results

ELISA-detectable anti-ADA antibody. The standard ELISA measures IgG antibody to unmodified bovine ADA; specificity was established by the ability of antigens in solution to inhibit the ELISA. Pretreatment values for 15 patients averaged 2.11 ± 1.6 (SD) U (range 0.5–4.7) (Table I B), vs. 2.94 ± 2.4 U for 26 controls. Pretreatment levels of 25–30 U for patients 1 and 9 were nonspecific, i.e., inhibited neither by unmodified ADA

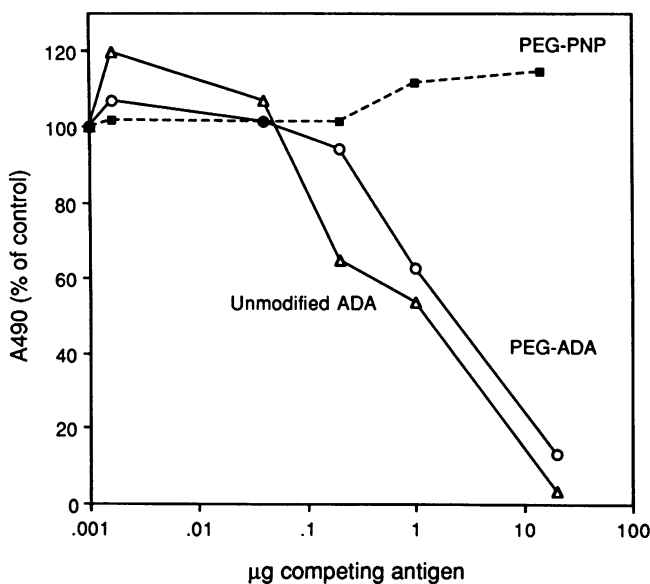


Figure 1. Specificity of anti-bovine ADA ELISA. Aliquots of diluted plasma (containing $0.37 \mu\text{l}$ of plasma) obtained from patient 4 after 18 mo of therapy were mixed with PBS (control) or with the indicated amounts of unmodified bovine ADA (triangles), PEG-ADA (circles), or PEG-modified *E. coli* PNP (squares). As described in Methods, these mixtures were incubated overnight and then tested by ELISA for IgG antibody to unmodified bovine ADA.

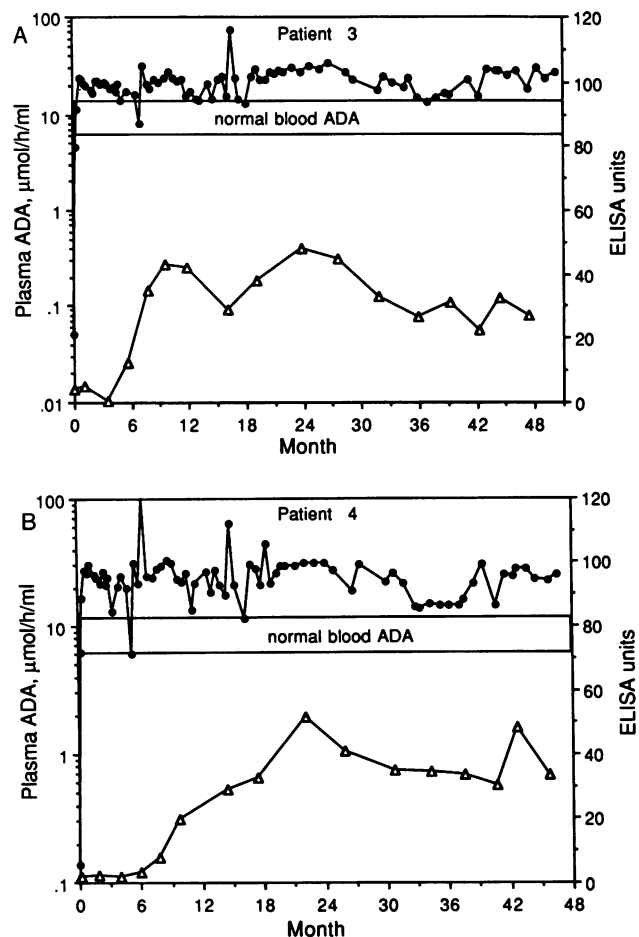


Figure 2. Evolution of anti-ADA antibody and relationship to trough (preinjection) plasma ADA activity. Filled circles, plasma ADA activity; open triangles, anti-ADA ELISA. Data are shown for patients 3 (A) and 4 (B).

nor by PEG-ADA (data not shown). As illustrated in Fig. 1 with plasma obtained from patient 4 after 18 mo of therapy, the specific ELISA response was inhibitable by preincubation with either unmodified bovine ADA or PEG-ADA, but not by PEG-modified purine nucleoside phosphorylase.

For each patient we determined a mean treatment ELISA value by testing 6–18 samples covering a 6–50-mo period beginning with the sixth to tenth month of therapy (Table I B). The mean ELISA value has remained negative (<7.8 units, 2 SD above the mean for controls) in 7 patients; 10 have developed specific anti-ADA IgG responses ranging from 8 to 55 ELISA U (average values). In most cases, the ELISA became positive between the third and eighth month of therapy (Table I B) and remained positive thereafter, as illustrated for patients 3 and 4 (Fig. 2). There was no correlation between mean ELISA values and mean trough plasma ADA activity (determined from biweekly to bimonthly measurements over the period used to determine average ELISA response) (Fig. 3 A). Mean plasma ADA activities ranged from 21.2 to 62.7 $\mu\text{mol/h}$ per ml (mean \pm SD = 29.6 ± 12), or $\sim 1.8\text{--}5$ times the normal level of total erythrocyte ADA activity, and correlated fairly well with the weekly dose of PEG-ADA (Fig. 3 B).

Fig. 4 compares inhibition of the standard ELISA by un-

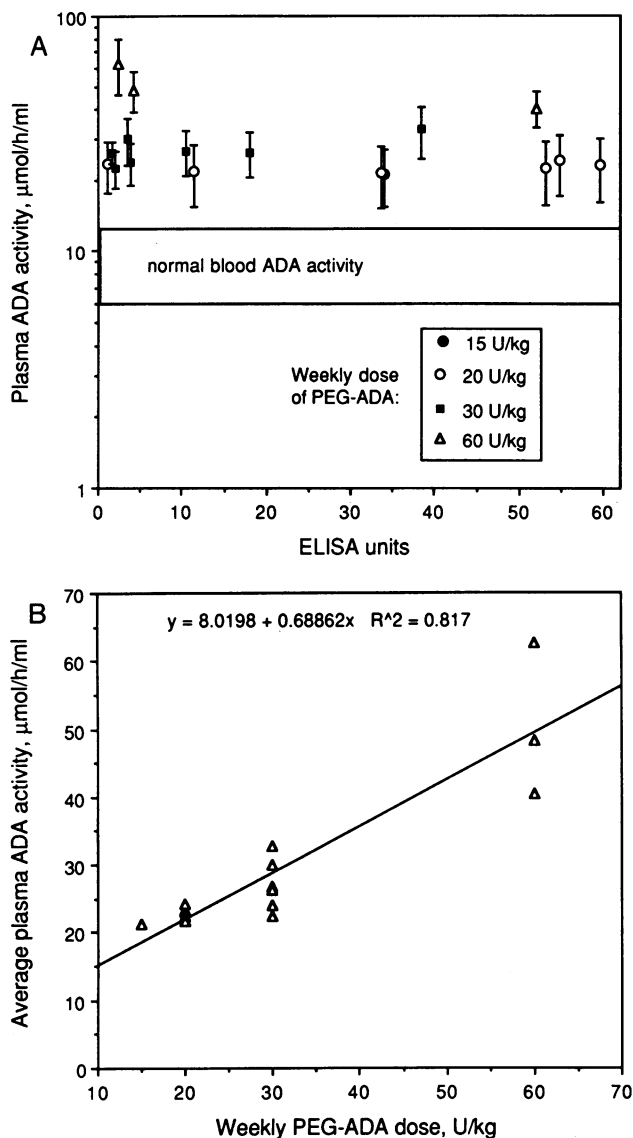


Figure 3. Relationships among anti-bovine ADA ELISA values, trough levels of plasma ADA activity, and weekly dose of PEG-ADA. (A) The average treatment ELISA values for patients (horizontal axis) are plotted against their mean preinjection (trough) plasma ADA activity (vertical axis). Vertical bars attached to symbols are 1 SD; the different symbols represent the weekly dose of PEG-ADA, as indicated in figure. (B) Weekly dose of PEG-ADA (horizontal axis) is plotted against average trough plasma ADA activity (vertical axis). The line was determined by least squares fit. The period of treatment evaluated is defined in Table I.

modified ADA and PEG-ADA in a study of recent plasma samples from seven anti-ADA positive patients. At 200 µg/ml, each ADA species caused 80–100% inhibition. The concentration of competing antigen causing a 50% decrease in ELISA response (IC_{50}) ranged from ~2 to 120 µg/ml (~10³- to 10⁴-fold higher than the concentration of PEG-ADA present in the diluted patient plasma samples tested in the ELISA). The lowest IC_{50} occurred in patients 8 and 12. In this experiment the IC_{50} for PEG-ADA was approximately twofold lower than for unmodified ADA, but no consistent difference between the two species was found in other similar experiments. Neither

PEG-modified *E. coli* PNP at up to 200 µg/ml, nor free PEG at up to 20 mg/ml, inhibited the ELISA in any case (not shown).

In agreement with a preliminary report (20), ELISA responses were considerably lower when PEG-ADA was substituted for unmodified bovine ADA as immobilized antigen (each ELISA was performed at saturating antigen concentration). Values of 20–25 ELISA U were obtained with plasma from patients 8 and 12, 10 U with patient 5, and 0–6 U with the other 7 patients (who all had significant responses to unmodified ADA). In the three patients with significant positive responses, the anti-PEG-ADA ELISA was inhibited equally by unmodified ADA and PEG-ADA (not shown). The insensitivity of the ELISA with immobilized PEG-ADA may reflect inaccessibility of some epitopes of the immobilized PEG enzyme to antibody.

ADA-inhibitory antibody. In 2 of the 17 patients (8 and 12), antibody to ADA did affect plasma ADA activity, requiring

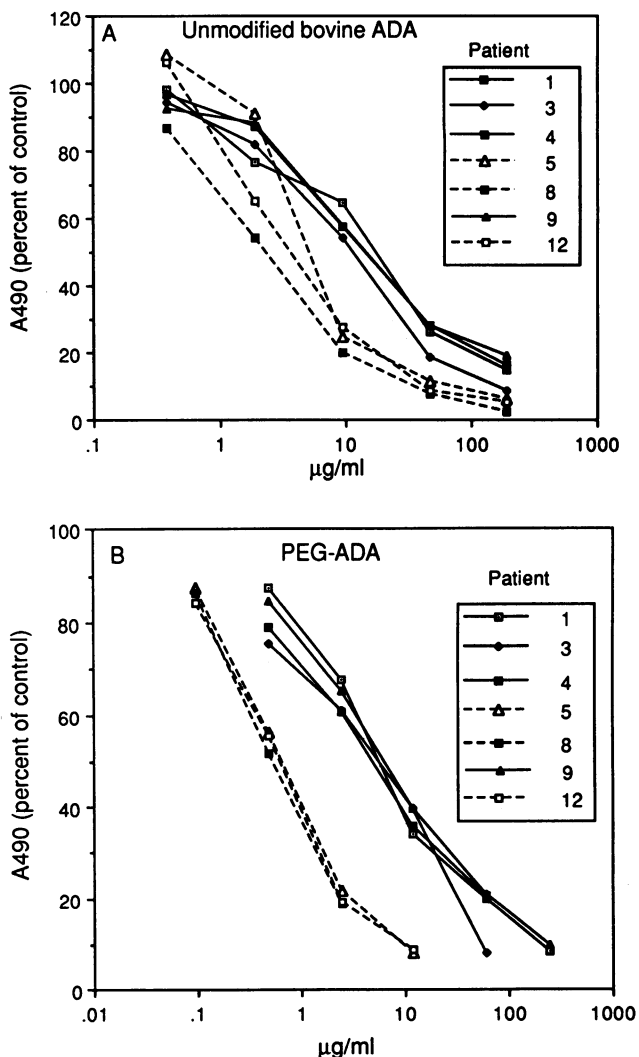


Figure 4. Inhibition of anti-bovine ADA ELISA. Diluted patient plasma samples were mixed with the indicated concentrations of unmodified bovine ADA (A) or PEG-modified bovine ADA (B), incubated overnight, and then tested by ELISA for IgG antibody to unmodified bovine ADA. The experimental protocol is described in Methods and in the legend to Fig. 1.

treatment modification. We will review studies of patient 8, which were aimed at defining and suppressing the effects of anti-ADA antibody. For the first 4 mo of therapy, a weekly PEG-ADA dose of 15 U/kg maintained trough plasma ADA activity at 13–23 $\mu\text{mol/h}$ per ml. Between weeks 21 and 25 the level fell to < 0.1 $\mu\text{mol/h}$ per ml, associated with development of a positive ELISA (Fig. 5), accelerated enzyme clearance (Fig. 6 A), and an increase in red cell dAXP (Fig. 6 B). Thus, during the first two weeks of treatment plasma ADA activity peaked 48–72 h after injections of 10 and 15 U/kg, then declined with a half-life of > 6 d. At week 26, following a dose of 20 U/kg, plasma ADA peaked at 24 h at about half the level achieved during week 1, then fell with a half-life of < 2 d to 0.1 $\mu\text{mol/h}$ per ml by day 7.

Plasma samples obtained from patient 8 between weeks 25 and 33, which had very low ADA activity, directly inhibited both PEG-ADA and unmodified bovine ADA; no inhibition was observed with pretreatment or control plasma (which in some cases stimulated slightly the activity of highly diluted ADA) (Table II). In mixing experiments week 26 plasma from patient 8 inhibited the ADA activity in plasma of other PEG-ADA-treated patients (data not shown). The ADA inhibitor was not dialyzable and could be recovered in the IgG fraction, separated from PEG-ADA, when the patient's plasma was fractionated on a protein A Sepharose column. Neither the preparation of IVIg that the patient was receiving, nor IgG prepared from the plasma of other ELISA-positive patients, appreciably inhibited ADA (Table II). The evolution of ADA inhibitory activity during the first 6 mo of treatment paralleled the development of ELISA-detectable anti-ADA IgG (Fig. 5).

In an attempt to suppress the effects of antibody (21), PEG-ADA was withheld between weeks 27 and 34, then resumed with three injections of 20 U/kg during week 35, then continued at twice weekly injections of this dose for four months (Fig. 5). The patient also received high dose infusions of IVIg weekly

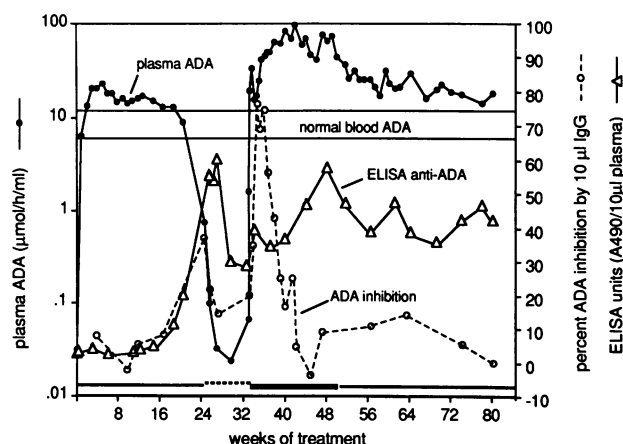


Figure 5. Evolution of anti-ADA antibody, effect on plasma ADA activity, and induction of tolerance to PEG-ADA in patient 8. Plasma ADA levels (filled circles) were determined in preinjection samples. IgG antibody to unmodified bovine ADA was determined by ELISA (open triangles), and by ability of IgG isolated from patient plasma samples to inhibit PEG-ADA (open circles), as described in Methods. The horizontal lines above the x axis indicate dose of PEG-ADA: thin line, one injection per week; dashed line, no injections; thick line, two injections per week.

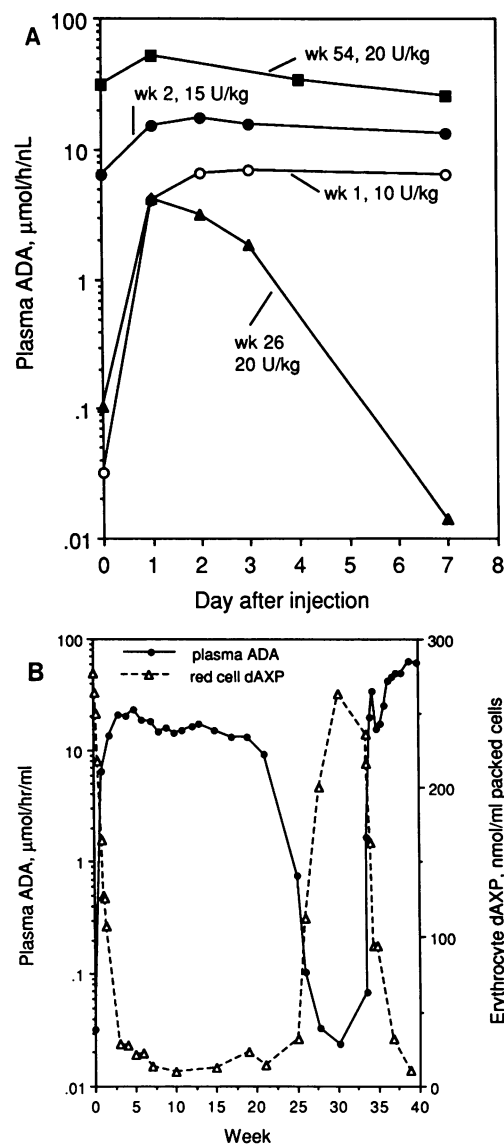


Figure 6. (A) Clearance of PEG-ADA. Plasma ADA levels were determined following intramuscular injection of PEG-ADA; dose and week of treatment are indicated in the figure. (B) Relationship between plasma ADA activity and erythrocyte dAXP in patient 8.

for a month and a course of prednisone tapered over four months (this regimen and the patient's clinical response to PEG-ADA therapy will be described elsewhere). Six days after PEG-ADA was reinstated, plasma ADA activity reached 34 $\mu\text{mol/h}$ per ml but then, despite the more frequent injection schedule, the ADA level fell to 15–17 $\mu\text{mol/h}$ per ml on days 9–12. This coincided with a sharp, apparently anamnestic increase in IgG ADA-inhibitory activity, which was not reflected in the ELISA (Fig. 5, weeks 34–36). Over the next eight weeks ADA inhibitory antibody (but not ELISA-detectable anti-ADA) declined to the pretreatment baseline and plasma ADA activity increased to 41–96 $\mu\text{mol/h}$ per ml, resulting in depletion of red cell dAXP (Fig. 6 B). At week 52, PEG-ADA was returned to a single weekly dose of 20 U/kg. The kinetics of PEG-ADA clearance examined during week 54 resembled the pattern observed at the beginning of treatment (Fig. 6 A). Trough plasma ADA activity has ranged from 14 to 39 $\mu\text{mol/h}$

Table II. Inhibition of PEG-ADA Activity by Anti-ADA Antibody from Patient 8

Sample type	Source	Week(s) of treatment	Percent inhibition of PEG-ADA activity
Plasma	Controls (n = 4)	—	-14.4±11.7* (0.4 to -27.9)†
Plasma	Patient 8	Pretreatment	-15.5
Plasma	Patient 8	25	28.4
Plasma	Patient 8	27	79.4
IgG	Pooled IVIg	—	2.8
IgG	Control	—	1.1
IgG	Patient 8	Pretreatment	-1.6
IgG	Patient 8	4-17	2.8±3.6
IgG	Patient 8	25	22.1
IgG	Patient 8	36	75.9
IgG	Other ELISA + patients [‡] (n = 5)	20-141	4.5±5.1 (-2.2 to 11.2)

* mean±SD; † (range). ‡ Patients 1, 3, 4, 5, and 6.

per ml for the > 24 mo since week 55, even though anti-ADA antibody detectable by ELISA has persisted at levels > 50 U. Maintenance of adequate ADA levels has been accompanied by improvement in immune function and sustained freedom from serious infections (J. D. Chum et al., manuscript in preparation).

In the experiment shown in Fig. 7, inhibition of ADA activity was measured when increasing amounts of PEG-ADA were incubated with a fixed amount of ADA-inhibitory IgG. The units (in moles) of ADA inhibited (i.e., bound by inhibitory antibody) increased in a saturable manner as a function of ADA concentration (Fig. 7 A). A Scatchard plot of the ratio of inhibited (bound)-to-active (free) enzyme vs. inhibited (bound) enzyme gave a straight line (Fig. 7 B), indicating a single species of inhibitory antibody. From this plot we estimate a K_d of ~ 0.5 nM for the antibody-PEG-ADA complex (this is similar to an approach used by Ehle et al. [22] to estimate the affinity of enzyme-inhibitory antibodies). Similar studies with unmodified bovine ADA gave an estimated K_d of ~ 0.7 nM (data not shown).

Patient 12 also experienced a fall in plasma ADA after about four months of therapy, which coincided with appearance of a positive anti-ADA IgG ELISA (Fig. 8) and plasma ADA inhibitory activity (see below). PEG-ADA was stopped for two weeks, then restarted at 30 U/kg twice weekly, along with two high dose IVIg infusions and a tapering course of prednisone. From months 7 to 14, two weekly PEG-ADA injections of 25 U/kg maintained plasma ADA activity at $25.1 \pm 7.3 \mu\text{mol/h}$ per ml. When a once weekly injection schedule (30 U/kg) was resumed at month 14, plasma ADA activity declined to 3-4 $\mu\text{mol/h}$ per ml and red cell dAXP increased from 6 to 12 to ~ 50 nmol/ml packed cells (Fig. 8). Twice weekly injections of PEG-ADA (30 U/kg) were resumed, which has maintained plasma ADA activity between 30 and 50 $\mu\text{mol/h}$ per ml. As reported elsewhere (23), each decline in plasma ADA activity was accompanied by a fall in red cell AdoHcyase activity and blood lymphocyte count, which reversed with increase in PEG-ADA dose. Patient 12 remains clinically well on continuing therapy with PEG-ADA.

Species specificity of anti-ADA antibodies. We have recently examined the ability of patient anti-ADA antibodies to recognize recombinant human ADA and its PEG-modified derivative. Plasma from 13 patients showed insignificant reaction with unmodified human ADA (ranging from undetectable to 5.8 U) when it was substituted for bovine ADA in the ELISA; patient 6 had a positive response of 11.8 U, vs. 21 U with bovine ADA (Fig. 9 A). When preincubated with patient plasma at 200 $\mu\text{g/ml}$, unmodified and PEG-modified human ADA failed to inhibit or showed weak inhibition of a standard anti-bovine ADA ELISA compared with their bovine counterparts (data not presented). In contrast to the ELISA results, plasma samples from patients 8 and 12 inhibited the activity of unmodified human ADA, the former to the same degree and the latter ~ 50% of the inhibition of bovine ADA and PEG-ADA preparations (Fig. 9 B). An important finding was that neither patient plasma inhibited PEG-modified recombinant human ADA.

Discussion

Specific IgG antibody to ADA reached significant levels (> 2 SD above the mean for controls in the standard ELISA) in 10 of the 17 patients studied, usually between three and eight months of treatment. This time course parallels improvement of impaired immune function, although some patients who have developed specific cellular and humoral responses to other antigens have not developed anti-ADA antibody (a separate report will deal with immunologic and clinical responses to treatment). Anti-ADA ELISA levels did not correlate with trough (preinjection) plasma ADA activity and, except in two patients, enzyme levels have been stable in anti-ADA positive patients over periods ranging from 24 to 60 months. We suspect that stable clearance reflects low affinity or low absolute levels of the population of antibodies generated towards epitopes incompletely masked by PEG strands (see below).

In two patients appearance of anti-ADA IgG was accompanied by a striking fall in plasma ADA activity, enhanced enzyme clearance, and the appearance in plasma of an IgG inhibitor of both PEG-ADA and unmodified ADA. An empirical approach (21) (details to be described elsewhere) was successful in inducing apparent tolerance to PEG-ADA in patient 8: PEG-ADA was withheld for several weeks, during which metabolic abnormalities and immune deficiency recurred; twice-weekly injections of PEG-ADA were then instituted, along with high dose IVIg and a tapering course of prednisone. ELISA-detectable anti-ADA IgG persisted, but after an abrupt anamnestic rise in level, ADA-inhibitory IgG disappeared over approximately two months and has not reappeared. Prolonged circulating life of PEG-ADA was restored, allowing treatment with a single weekly dose to be resumed. A similar regimen did not fully suppress the effects of antibody in patient 12, but a twice-weekly injection schedule compensated for enhanced ADA clearance. Patients 8 and 12 have now received PEG-ADA for > 3 yr and > 2.5 yr, respectively, and each has done well clinically (reference 23 and J. D. Chum et al., manuscript in preparation).

The reciprocal relationship between plasma ADA levels and ADA-inhibitory IgG in patient 8 indicates that the latter was responsible for enhanced enzyme clearance. Scatchard

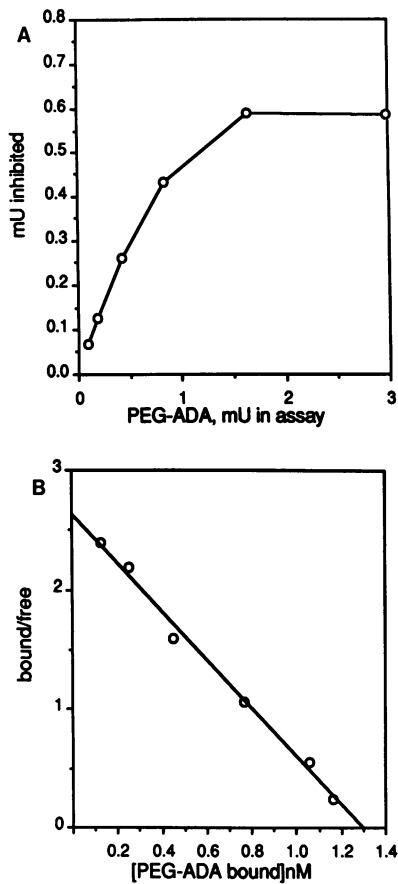


Figure 7. Relationship between ADA concentration and inhibition of ADA activity by IgG from patient 8. Aliquots (40 μ l) of dilutions of PEG-ADA, prepared in PBS/BSA, were mixed with 10 μ l of IgG prepared from plasma obtained at week 36 of treatment, or with 10 μ l of PBS/BSA (no-antibody control). After 20 min, 5 μ l aliquots were assayed for ADA activity. (A) Relationship between ADA activity present in the assay and units of ADA activity inhibited (difference between activity found in the absence and presence of antibody). The percentage of activity inhibited ranged from 19.8 to 73%. (B) Scatchard analysis of the data in A. The concentration of ADA present in the assay and the amounts bound (inhibited) and free (uninhibited) were calculated from the measured specific activity and the concentration of ADA protein.

analysis of data relating ADA concentration to activity inhibition by IgG isolated from patient 8 indicated a single species of ADA-inhibitory antibody, possibly the product of one B cell

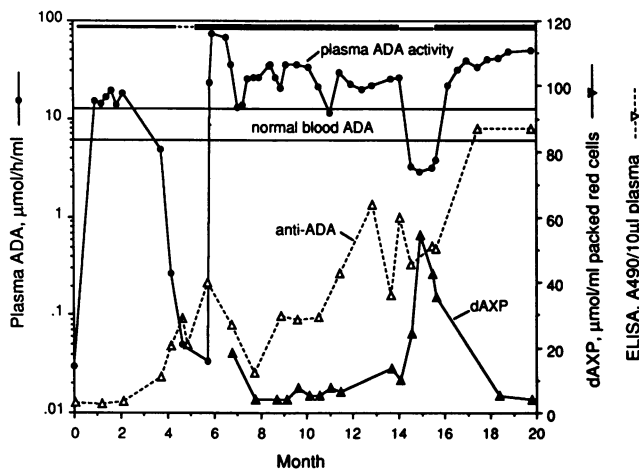


Figure 8. Anti-ADA antibody, plasma ADA activity, and red cell dAXP in patient 12. The horizontal lines at the top of the figure indicate the number of ADA injections per week: *single thickness*, one injection; *dashed*, no injections; *double thickness*, two injections. Data on red cell dAXP are shown for the period when the schedule of PEG-ADA administration was changed from two to one per week, and then returned to twice weekly.

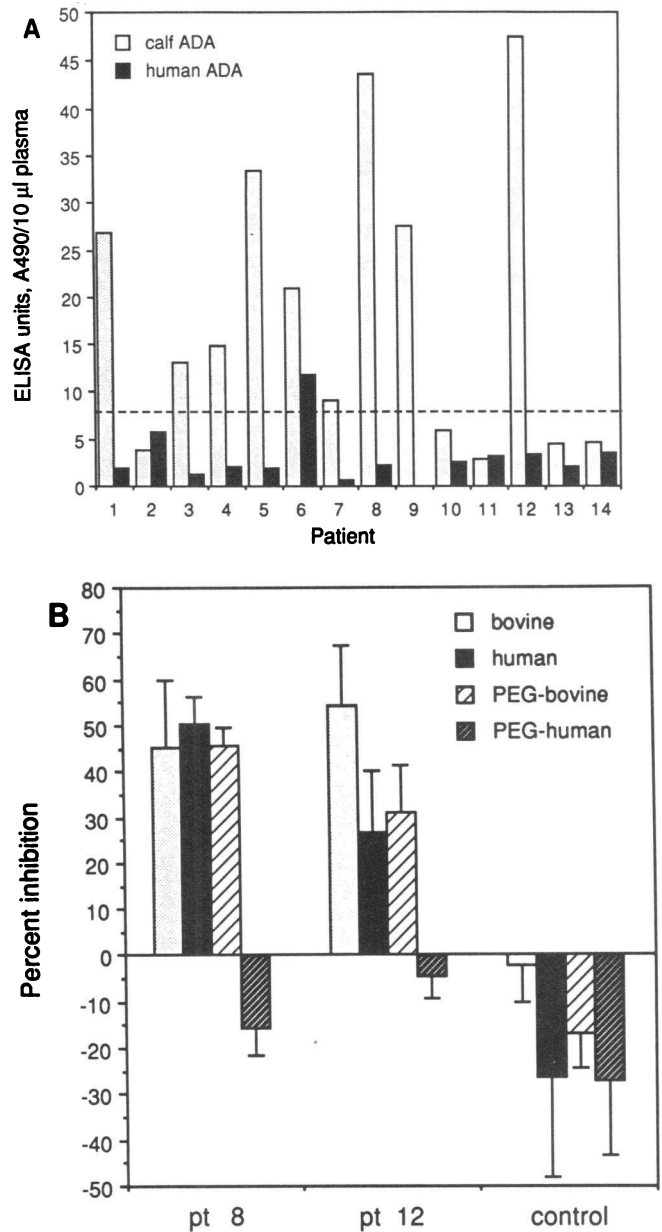


Figure 9. Comparison and antibody recognition of human and bovine ADA. (A) ELISA performed with unmodified calf (light bars) or human (dark bars) ADA as immobilized antigen. (B) Inhibition of bovine and human native ADA and PEG-ADA by plasma from patients 8 and 12. Plasma samples were from weeks 27 (patient 8) and 20 (patient 12) of therapy. Control is a plasma sample from a normal adult; the stimulation (negative inhibition) of purified ADA diluted in PBS/BSA was also observed with pretreatment plasma samples from patients and with plasma from other normal individuals (see Table II).

clone. The basis for the selective arrest in production of inhibitory antibody in this patient is unknown, but may be related to her underlying disorder as well as to properties of PEGylated antigens. In addition to the effects of prednisone and IVIg, immunosuppressive effects of metabolic abnormalities that recurred when ADA levels declined may have played a role in aborting the evolving inhibitory antibody response. PEG-modified allergens were toleragenic in mice, apparently by a

mechanism that involved T cell suppression (24). On the other hand, the response in patient 8 resembles the experimental abrogation of primary immunization by high doses of soluble antigen, which may have resulted from direct effects on B cells (25). Although the use of steroids in a patient with SCID should be carried out with caution, the tolerizing regimen used in the present patients was much less intensive than that employed in hemophilia patients with high levels of inhibitory antibodies to Factor VIII:C, which included cyclophosphamide and plasmapheresis or protein A adsorption to remove circulating antibody (26).

Certain conclusions can be drawn regarding the specificity of anti-ADA IgG antibodies. (a) Protein epitopes rather than PEG are recognized: in competition experiments, native and PEG-modified bovine ADA were equally effective in blocking ELISAs performed with either species as the immobilized antigen; in contrast, neither a nonspecific PEG-protein nor free PEG had any effect. (b) Most anti-ADA antibodies recognize bovine-specific rather than conserved epitopes: ELISA responses to immobilized human ADA were low, and human ADA (native or PEG-modified) blocked the anti-bovine ADA ELISA weakly. (c) In contrast to ELISA-detectable antibodies, the inhibitory antibodies of patients 8 and 12 recognize conserved region(s), since they inhibited human as well as bovine ADA. Both of these patients have detectable residual ADA activity so that development of inhibitory antibody is not related to a complete absence of the human ADA gene product. Rather, the active site of the PEG-modified enzyme may be a favorable site for immune recognition and antibody binding.

To preserve activity, PEG strands, which are linked to lysines, cannot be attached to an essential residue or block the active site. Consequently, a relatively PEG-free region around the active site may be an immunologic "Achilles heel." It is significant that while plasma from patients 8 and 12 inhibited native human ADA and native and PEG-modified bovine ADA, PEG-modified human ADA was not inhibited. We postulate that in human (but not bovine) ADA, a site for PEG attachment exists within the active site-related epitope, effectively shielding it from antibody. The postulated PEG-attachment site might also prevent an inhibitory antibody response to human PEG-ADA. The underlying hypothesis, that antibodies to PEG enzymes are generated against and bind to relatively PEG-free regions, is supported by recent studies in mice. We showed that the immunogenicity and antigenicity of PEG-modified wild type purine nucleoside phosphorylase from *E. coli* could be reduced substantially by using a mutant form of the enzyme in which arginine-to-lysine substitutions had been selectively introduced to provide additional sites for PEG attachment (15). We are currently investigating the epitope recognized by inhibitory anti-ADA antibody and we are pursuing the possible use of PEG-modified human ADA as a treatment for ADA deficiency.

Treatment with PEG-ADA has been a safe and effective way of maintaining the high levels of circulating ADA activity required to prevent toxic metabolic abnormalities caused by ADA deficiency. In more than five years of use (now in 25 patients) there have been no allergic reactions to PEG-ADA and in no patient has PEG-ADA been discontinued because of immunogenicity. Enhanced enzyme clearance, mediated by antibody, has occurred in two patients. However, it has been relatively easy to induce tolerance or to compensate for acceler-

ated clearance by increasing the frequency of injections. Based on the present and ongoing studies, we recommend monitoring of preinjection plasma ADA levels every other week during the first six to eight months of therapy with PEG-ADA, then monthly; assay for anti-ADA antibodies by ELISA and by enzyme inhibition should be performed promptly if a persistent decline in enzyme levels occurs so that measures can be taken to provide appropriate prophylaxis against infection and to restore adequate enzyme levels.

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