The Use of Enzyme Therapy to Regulate the Metabolic and Phenotypic Consequences of Adenosine Deaminase Deficiency in Mice: Differential Impact on Pulmonary and Immunologic Abnormalities*

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Running title: Enzyme Therapy in ADA-deficient Mice
SUMMARY

Adenosine deaminase (ADA) deficiency results in a combined immunodeficiency brought about by the immunotoxic properties of elevated ADA substrates. Additional non-lymphoid abnormalities are associated with ADA deficiency, however little is known about how these relate to the metabolic consequences of ADA deficiency. ADA-deficient mice develop a combined immunodeficiency as well as severe pulmonary insufficiency. ADA enzyme therapy was used to examine the relative impact of ADA substrate elevations on these phenotypes. A “low-dose” enzyme therapy protocol prevented the pulmonary phenotype seen in ADA-deficient mice, but did little to improve their immune status. This treatment protocol reduced metabolic disturbances in the circulation and lung, but not in the thymus and spleen. A “high-dose” enzyme therapy protocol resulted in decreased metabolic disturbances in the thymus and spleen and was associated with improvement in immune status. These findings suggest that the pulmonary and immune phenotypes are separable and are related to the severity of metabolic disturbances in these tissues. This model will be useful in examining the efficacy of ADA enzyme therapy and studying the mechanisms underlying the immunodeficiency and pulmonary phenotypes associated with ADA deficiency.
INTRODUCTION

Adenosine deaminase (ADA) deficiency was the first of the immunodeficiency diseases for which the underlying biochemical defect was discovered (1). This unexpected finding revealed the crucial importance of ADA for the development and function of the immune system. Subsequent analysis indicated that ADA deficiency accounted for approximately 20% of the incidence of combined immunodeficiency disease in humans (2). However, the causal link between purine metabolic disturbances associated with ADA deficiency and the resulting immunodeficiency has not been determined. In recent years, ADA deficiency has received added interest because it has served as a testing ground for the development of novel therapies, especially enzyme therapy (3) and gene therapy (4,5).

ADA deficiency is the most severe of the immunodeficiencies in humans (6). Without intervention, affected individuals die from overwhelming opportunistic infections within the first few months of life (2,7). The immunodeficiency is the most studied feature of ADA deficiency, however, other abnormalities and disturbances are seen. Additional features include hepatocellular damage (8), pulmonary insufficiency (2), neurological disorders (2), renal problems and bony abnormalities (9). Although these conditions are often thought to be secondary to the immunodeficiency, some investigators have suggested that they may be a primary consequence of ADA deficiency and not secondarily related to infections stemming from the immunodeficiency (8,10).

In attempting to understand the multitude of abnormalities associated with ADA deficiency, most attention has focused on the substrates of the ADA catalyzed reaction, adenosine and 2’-deoxyadenosine. Each nucleoside is known to be elevated in ADA-deficient patients (11-13), and may cause developmental or physiological disturbances in a number of
ways. Adenosine functions as an intercellular signaling molecule by engaging G-protein-coupled receptors on the surface of target cells (14). Mammalian cells possess four different adenosine receptors, A1, A2a, A2b, and A3, that function in a variety of physiological circumstances. In this regard, adenosine is involved in T cell receptor signaling (15), heart rate (16), blood pressure (17), renal function (18), inflammatory responses (19,20) and neurotransmission (21). ADA plays a critical role in controlling the concentration of adenosine, thereby affecting many areas of intercellular signaling. Thus, in the absence of ADA, the uncontrolled elevations of adenosine could unleash a variety of adenosine signaling cascades with detrimental effects on numerous areas of physiology. The other ADA substrate, 2’-deoxyadenosine, is known to be cytotoxic by interference with a number of critical metabolic pathways. High levels of 2’-deoxyadenosine may lead to the interference of transmethylation reactions (22), reduction of deoxynucleotide synthesis (2), or activation of the apoptotic caspase cascade (23). We have previously shown that some tissues in mice, especially the thymus and spleen, produce high levels of 2’-deoxyadenosine (24,25), making them especially sensitive to metabolic consequences of 2’-deoxyadenosine-induced metabolic cytotoxicity. We have also shown that the murine fetal liver is sensitive to 2’-deoxyadenosine elevations (26). Thus, ADA deficiency may provoke a variety of consequences, either through metabolic disturbances caused by elevated 2’-deoxyadenosine, or cell signaling disturbances caused by elevated adenosine.

We have recently used a two-stage genetic engineering strategy to produce a line of ADA-deficient mice (25). These mice develop a combined immunodeficiency that is associated with profound disturbances in purine metabolism. Elevations in ADA substrates were greatest in the thymus and spleen (25), suggesting the metabolic disturbances in these major immune organs play a role in the ensuing immunodeficiency. In addition to immunodeficiency, ADA-deficient
mice develop severe abnormalities including rib cage alterations, renal abnormalities, and pulmonary insufficiency (25). The mice fail to thrive and die from respiratory insufficiency by three weeks of age (25,27).

ADA-deficient mice serve as a useful model for elucidating the biochemical basis of the various phenotypic abnormalities associated with the loss of ADA function. In considering the complexity of the phenotypes associated with ADA deficiency in humans and mice, it is likely that some features may be attributed to alterations in adenosine signaling, whereas others are due to 2’-deoxyadenosine-induced metabolic disturbances. Here we report the use of ADA enzyme therapy to regulate the level of metabolic disturbances associated with ADA deficiency and show that relatively low levels of ADA enzyme therapy correct the pulmonary abnormalities. Much higher doses of enzyme therapy are required to alleviate the immunodeficiency, a finding relevant to the use of enzyme therapy to treat ADA deficiency in humans. Available evidence suggests that the pulmonary insufficiency in ADA-deficient mice results from abnormalities in adenosine signaling (27), whereas the immunodeficiency stems largely from 2’-deoxyadenosine-induced metabolic cytotoxicity. Thus, ADA enzyme therapy serves as a convenient experimental strategy to regulate the metabolic consequences of ADA deficiency and differentially correct abnormalities associated with ADA deficiency.
EXPERIMENTAL PROCEDURES

Transgenic mice- Mice heterozygous for the null Ada allele (m1/+) and carrying a placenta-specific ADA minigene necessary for prenatal rescue (Tg) were intercrossed (25). Genotypes were determined by Southern blot analysis of genomic DNA obtained from tails at weaning (25). Wild type (+/+) and heterozygous (m1/+) mice with or without the ADA minigene (Tg) were designated as “ADA+ controls”, whereas homozygous mutant mice carrying the ADA minigene (Tg-m1/m1) were designated ADA-deficient (25). All mice were housed in cages equipped with microisolator lids and maintained under strict containment protocols.

PEG-ADA enzyme therapy- Polyethylene glycol-modified-ADA (PEG-ADA, Adagen®), was kindly provided by Enzon Inc. (Piscataway, NJ). A “low-“ and “high-dose” protocol was utilized. For the “low-dose” protocol, mice were injected intramuscularly with dosages of PEG-ADA designed to deliver 100 to 500 Units (1 Unit is defined as the amount of enzyme necessary to convert 1 μmole adenosine to inosine per min at 25°C) of PEG-ADA per kg body weight. Injections were started on postnatal day three and were given every seven days for six weeks. For the ‘high-dose” protocol, mice were injected intraperitoneally with dosages of PEG-ADA designed to deliver 1000-5000 Units of PEG-ADA per kg body weight. Injections were started on postnatal day 10 and were given every three to four days.

Zymogram analysis and determination of PEG-ADA trough values- Whole blood was collected from the tail vein or from the heart at the time of euthanasia. One volume of homogenizing buffer (10 mM Tris pH 8.0, 1 mM Na₂EDTA and 1 mM β-mercaptoethanol) was added and the samples were lysed by several rounds of freeze-thawing. Tissues were dissected, rinsed in cold
PBS and sonicated in three volumes of homogenizing buffer. Samples were then centrifuged at 14,000 x g at 4°C and protein concentration determined in the supernatants (Bio-Rad). Samples were run on thin agarose gels (Innovative Chemistry) in the cold, and were then overlaid with enzyme substrate and colorimetric detection cocktail (24). For determination of ADA trough values, heparinized blood samples were collected from the tail vein of mice just prior to the next injection of PEG-ADA, and plasma isolated by low-speed centrifugation. ADA specific activity in plasma was determined using established protocols (28).

**Histological analysis**- Animals were sacrificed and the lungs infused with 0.5 ml of fixative (4% paraformaldehyde in PBS) prior to fixation overnight at 4°C. Fixed lung tissues were rinsed in PBS, dehydrated, and embedded in paraffin according to standard techniques. Sections (5 µm) were stained with hematoxylin and eosin (H&E) using a Rapid Chrome staining kit (Shandon Lipshaw).

**Flow Cytometry of Leukocyte Populations**- The following monoclonal antibodies used for flow cytometric analysis were obtained from PharMingen, Inc. as fluorescein isothiocyanate (FITC) or phycoerthrin (PE) conjugates: anti CD3-ε, clone 145-2C11; anti-CD4, clone RM4-5; anti-CD8a, clone 53-6.7; anti-CD45R/B220, clone RA3-6B2; clone M1/70; anti-TCRβ chain, clone H57-597; anti-pan-NK cells, clone DX5; anti-IgM, clone R6-60.2; Fc control anti-CD16/CD32 (FcγIII/IIR), clone 2.4G2. Phenotyping of leukocyte populations in thymuses and spleens was conducted by direct two-color analysis on an EPIC Profile Analyzer (PharMingen, Inc.). Thymuses and spleens were excised from control and ADA-deficient age-matched littermates or mice treated with PEG-ADA and processed for flow cytometry (24). Two-color flow cytometry
analysis was conducted on $1 \times 10^6$ viable leukocytes, and data were obtained on two-color dot plot profiles.

**Analysis of adenine nucleosides and nucleotides and determination of AdoHcy hydrolase enzymatic activity** - Tissues were removed from mice and quickly frozen in liquid nitrogen. Nucleosides and nucleotides were extracted and analyzed by HPLC according to established procedures (24,29). AdoHcy hydrolase enzymatic activity was determined in freshly prepared tissue extracts according to established procedures (24,30).
RESULTS

Stability and distribution of PEG-ADA in mice- Before attempting to use PEG-ADA to treat ADA-deficient mice, it was necessary to conduct experiments to determine adequate PEG-ADA treatment protocols. ADA+ control mice were treated with PEG-ADA in order to monitor the consistency of drug delivery. Mice were given an intramuscular injection of PEG-ADA (2.5 to 10 µl, depending on age) once weekly for six weeks. This dose was designed to deliver between 100 and 500 Units of PEG-ADA per kg body weight. PEG-ADA levels were estimated semiquantitatively in whole blood two days following the last injection and were found to be similar in all samples (Fig. 1A), suggesting that the treatment protocol was capable of consistently delivering enzyme to the circulation of mice. To determine the stability of PEG-ADA in the circulation, ADA+ control mice were treated with PEG-ADA as described above, and blood PEG-ADA levels were estimated at various time points following the last treatment (Fig. 1B). At two days following the last injection, PEG-ADA levels were approximately three times greater than endogenous ADA. By four days following the last injection, blood levels had dropped, and were equivalent to endogenous ADA levels. At eight days post-PEG-ADA, only trace amounts were seen, and by 16 days post-treatment PEG-ADA was not detectable. From these results it was estimated that the half-life of PEG-ADA in the murine circulatory system was three to four days. Trough values of PEG-ADA activity achieved with this protocol are shown in Table 1. This regimen was referred to as a “low-dose” treatment protocol, in comparison with the higher dose regimen used in later studies (see below).

PEG-ADA enzyme therapy rescues ADA-deficient mice- ADA-deficient mice die by three weeks of age in association with respiratory distress, lymphopenia and severe metabolic disturbances
To determine if PEG-ADA enzyme therapy could rescue ADA-deficient mice, three-day-old pups were treated intramuscularly with PEG-ADA according to the “low-dose” regimen. At three weeks of age, blood was collected and analyzed for endogenous ADA or PEG-ADA (Fig. 2A). As expected, blood collected from an untreated ADA-deficient animal (Fig. 2A, lane 2) did not exhibit ADA or PEG-ADA enzyme activity. This animal was smaller than control littermates, exhibited severe respiratory distress and died late on day 21. In contrast, blood collected from a treated ADA-deficient animal showed only PEG-ADA enzyme activity (Fig. 2A, lane 4). Growth of this animal was indistinguishable from that of ADA+ control littermates and it survived more than six months on this treatment protocol with no sign of morbidity. This result demonstrated that a “low-dose” of PEG-ADA resulted in circulating PEG-ADA activity sufficient to prevent lethality in ADA-deficient mice.

**PEG-ADA is found only in the circulatory system of ADA-deficient mice**—PEG-ADA does not enter erythrocytes or blood lymphocytes of human ADA-deficient patients, however, the distribution of PEG-ADA in other tissues is not known (2,28). ADA-deficient mice provided the opportunity to examine the levels of PEG-ADA present in various non-hematopoietic tissues. Shown in Fig. 2B is the analysis of PEG-ADA activity in extracts from various tissues taken from an ADA-deficient animal two days following its last PEG-ADA injection (“low dose” regimen). Whereas PEG-ADA was detectable in the blood of this animal, it was not detected in any of the tissues examined, including the thymus, spleen, liver, kidney and lung. These data suggested that most if not all of the PEG-ADA in ADA-deficient mice was found in the circulatory system, as has been assumed to be the case in humans (2,28).
“Low-dose” PEG-ADA treatments prevent lung inflammation and damage—ADA-deficient mice succumb to severe respiratory distress by three weeks of age (25,27). Lungs of these animals show defects in alveogenesis and severe lung inflammation and damage is present (Fig. 3B) (27). The ability of the “low dose” PEG-ADA regimen to rescue ADA-deficient mice from lethality (Fig. 2A) was associated with significant reduction in the severe lung inflammation and damage seen in untreated ADA-deficient mice (Fig. 3). These findings suggested that “low-dose” PEG-ADA provided adequate metabolic protection to prevent the pulmonary phenotype and allowed for the survival of these animals.

High doses of PEG-ADA are required to establish ADA trough values in ADA-deficient mice similar to those seen in ADA-deficient humans— The initial treatment for seriously ill, newly diagnosed human infants with ADA-deficient SCID, is a twice weekly injection of 30 Units/kg PEG-ADA (31,32). This treatment protocol typically leads to trough plasma ADA activity levels that range from approximately 50-120 µmol/hr/ml (2). Based on body weight, the “low-dose” treatment protocol used in ADA-deficient mice was considerably higher than this initial dosage used in ADA-deficient humans. However, the mean trough plasma ADA activity in mice treated with this regimen was considerably lower than the trough levels observed in ADA-deficient humans (Table 1, (2)). Therefore, we also treated mice with 10-fold greater amounts of PEG-ADA. This “high-dose” protocol was sufficient to maintain trough plasma PEG-ADA activity at levels equivalent to that observed with a 60 Unit/kg/week regimen in ADA-deficient humans (Table 1, (2)). These data demonstrated that much higher doses of PEG-ADA, on a per kg body weight basis, are required in the mouse to establish plasma PEG-ADA levels that are therapeutically effective in humans.
High doses of PEG-ADA overcome the block in thymocyte development seen in ADA-deficient mice. ADA deficiency in humans (2) and mice (25) is associated with pronounced lymphopenia. Treatment of ADA-deficient humans with PEG-ADA results in varying degrees of restoration of mature lymphoid cells (2,31). To begin to assess the impact of PEG-ADA enzyme therapy on lymphoid development in ADA-deficient mice, total lymphoid counts were examined in the thymuses of ADA+ control and ADA-deficient mice given either a “low-“ or “high-dose” of PEG-ADA (Fig. 4A and B). Lymphoid counts in ADA-deficient thymuses were greatly reduced on postnatal day 17 (Fig. 4A). Low doses of PEG-ADA did not have a significant impact on the lymphopenia seen in the thymus (Fig. 4B), however, the “high-dose” treatment allowed for an improvement in thymocyte counts to near control values (Fig. 4B).

Immature thymocytes are organized into two major groups based on the cell surface expression of CD4 and CD8 (33). The most immature thymocytes express neither CD4 or CD8 and are termed double negative (DN), while more mature thymocytes express both CD4 and CD8 and are termed double positive (DP). Flow cytometry using antibodies to specific markers of thymocyte differentiation were used to determine the effect of ADA enzyme therapy on thymocyte differentiation. The percentage of DP cells was diminished from 80% in ADA+ control thymuses to 20% in ADA-deficient thymuses on postnatal day 17 (Fig. 4C). In addition, there was an increase in DN cells in ADA-deficient thymuses, verifying a potential block in thymocyte differentiation before the DP stage. The distribution of DP and DN cells in ADA-deficient thymuses improved slightly following “low-dose” PEG-ADA treatment, while a “high-dose” treatment improved the distribution of cells in ADA-deficient thymuses to near control
values (Fig. 4D). These data suggested that high doses of PEG-ADA are needed to see an improvement in thymocyte differentiation in ADA-deficient mice.

**Improvement of peripheral T, B and NK cell lymphopenia in ADA-deficient mice treated with PEG-ADA-** ADA deficiency in humans leads to a combined immunodeficiency that is characterized by a pronounced T, B and NK cell lymphopenia (6). PEG-ADA enzyme therapy has been shown to be effective in improving the status of these cell types (2,31,34). ADA-deficient mice are known to exhibit T and B cell lymphopenia (25), however, the status of NK cells in these mice was not known. Antibodies against cell surface markers specific for mature T, B and NK cells were used to monitor these cell populations in ADA-deficient mice and ADA-deficient mice treated with PEG-ADA. Similar to what is seen in ADA-deficient humans, all three lymphoid cell populations were greatly diminished in ADA-deficient mice on postnatal day 17 (Fig. 5B). There was a slight improvement in the absolute numbers of these cells in the spleens of ADA-deficient mice treated with a “low-dose” of PEG-ADA, and an even greater improvement in ADA-deficient mice treated with a “high-dose” regimen (Fig. 5C). These results suggested that the immune phenotype seen in ADA-deficient mice resembled that seen in ADA-deficient humans. Furthermore, a “high-dose” of ADA enzyme therapy was capable of providing a substantial improvement in T, B and NK cells numbers.

**The improvement of metabolic disturbances in tissues of ADA-deficient mice varies with the amount of PEG-ADA given-** Elevated levels of the ADA substrates adenosine and 2’-deoxyadenosine in plasma and urine, resulting in markedly elevated pools of dATP and reduced AdoHcy hydrolase activity in red blood cells, are the biochemical hallmarks of ADA deficiency
PEG-ADA enzyme therapy has been shown to correct these metabolic disturbances in erythrocytes of ADA-deficient humans (2,3,31). However, it has not been possible to quantitate the levels of substrate elevations in their tissues. The generation of ADA-deficient mice has made assessment of tissue substrate elevations possible, and it has been shown that both adenosine and 2’-deoxyadenosine are highly elevated in the circulation and in tissues of ADA-deficient mice (25,35). To determine what effect PEG-ADA enzyme therapy has on ADA substrates in tissues, adenine nucleosides were extracted from tissues of ADA-deficient mice treated with PEG-ADA, and were quantitated by reversed-phase HPLC (Fig. 6). Treatment of ADA-deficient mice with a “low-dose” regimen of PEG-ADA prevented elevations of adenosine and 2’-deoxyadenosine in the serum and lung, but not in the thymus and spleen (Fig. 6). In contrast, high doses of PEG-ADA were able to markedly improve the metabolic disturbances found in the serum and lung as well as the thymus and spleen (Fig. 6). These results demonstrated that PEG-ADA provided varying degrees of metabolic protection in the whole animal.

AdoHcy hydrolase enzyme activity and elevations in dATP vary in tissues of ADA-deficient mice following PEG-ADA enzyme therapy— Decreased AdoHcy hydrolase enzymatic activity and the accumulation of dATP in RBCs are commonly noted features in ADA-deficient patients (2,36,37). In humans, PEG-ADA enzyme therapy readily prevents the inhibition of AdoHcy hydrolase and dATP accumulation in RBCs (2,3,31). However, as with the assessment of substrate elevations, it has not been possible to examine the effects of PEG-ADA enzyme therapy on AdoHcy hydrolase and dATP in other tissues. To address this, we determined the levels of AdoHcy hydrolase enzyme activity and dATP levels in various tissues of ADA-
deficient mice following PEG-ADA treatment. Previous examination of AdoHcy hydrolase enzymatic activity in ADA-deficient mice demonstrated that the greatest decrease in activity was seen in RBCs and in the thymus and spleen (25,35). In ADA-deficient mice treated with a “low-dose” regimen of PEG-ADA, a substantial decrease in AdoHcy hydrolase activity was still noted in the thymus and spleen, and to a lesser extent in RBCs and the lung (Fig. 7A). A “high-dose” regimen restored AdoHcy hydrolase activity in RBCs and lung and provided partial improvement in the thymus and spleen (Fig. 7A). The levels of dATP that accumulate in RBCs of ADA-deficient mice are seven- to eight-fold higher than those measured in the thymus (25,35). In contrast, the levels of dATP that accumulated in the RBCs of ADA-deficient mice treated with a “low-dose” regimen of PEG-ADA were similar to those measured in other tissues from the same animals (Fig. 7B). Elevations in dATP were noted in all tissues following a “low-dose” regimen, with the greatest relative accumulation occurring in the thymus (Fig. 7B). This suggested that a “low-dose” of PEG-ADA was capable of preventing much of the accumulation of dATP in erythrocytes and lungs, but not dATP accumulation in other tissues. Treatment of ADA-deficient mice with a “high-dose” of PEG-ADA completely prevented the accumulation of dATP in both erythrocytes and tissues (Fig. 7B). Improvements in these metabolic endpoints correlated with the degree of immune reconstitution, adding support to the hypothesis that metabolic abnormalities are mechanistically involved in the immune phenotypes associated with ADA deficiency.
DISCUSSION

ADA deficiency in humans is most often associated with a severe combined immunodeficiency (2,6). However, additional non-lymphoid abnormalities have been noted, including bone, kidney and adrenal abnormalities (9), liver damage (8) and problems associated with the nervous system (10). Pulmonary insufficiency in ADA-deficient patients is similar to pulmonary insufficiency seen in patients with other forms of SCID. It is not present at birth, and is most commonly found in association with known pathogens. The lung injury in ADA-deficient mice is present early in life and is not associated with infection (27). In the current study, PEG-ADA enzyme therapy was used to study the impact of tissue metabolic disturbances on the pulmonary and immune phenotypes seen in ADA-deficient mice (25,27). ADA-deficient mice were initially given a “low-dose” of PEG-ADA that resulted in circulating enzyme activities below plasma PEG-ADA levels achieved in patients with ADA-deficient SCID (2,31,32). Nonetheless, this "low-dose" of PEG-ADA was capable of rescuing ADA-deficient mice from lethality due to pulmonary injury. This “low-dose” treatment corrected systemic and lung accumulations of adenosine and 2’-deoxyadenosine, but did not prevent elevations of adenosine and 2’-deoxyadenosine in the thymus and spleen. Consequently, defects in lymphoid development persisted. A PEG-ADA treatment protocol designed to maintain circulating PEG-ADA activity at concentrations that are in the effective range seen in humans (31,32), resulted in substantial improvements in immune status as well as a major reduction in the degree of substrate elevations in the thymus and spleen. These findings suggest that the pulmonary and immune phenotypes seen in ADA-deficient mice are separable and are mediated by the different degrees of metabolic disturbances associated with ADA deficiency.
The pulmonary phenotype seen in ADA-deficient mice is characterized by a developmental defect in alveogenesis that results in enlarged alveolar airways (27). In addition, these animals develop severe lung inflammation and damage that kills these animals by three weeks of age. Lung damage and inflammation was associated with the elevation of adenosine and to a lesser extent, 2'-deoxyadenosine in the lungs (25). We show that maintaining ADA-deficient mice on a “low-dose” of PEG-ADA resulted in normal lung development, and lung inflammation and damage was not seen. This was associated with prevention of adenosine and 2'-deoxyadenosine elevations in the lungs, suggesting ADA substrates are mediating the abnormal lung development and inflammation. Both adenosine (14) and 2'-deoxyadenosine (2) have potent physiological effects on cells. Adenosine elicits its actions on cells by engaging G-protein-coupled receptors on the cell surface (14). This signaling pathway plays important roles in many aspects of lung inflammation and damage (20), and adenosine elevations in the lung could influence normal adenosine signaling during lung development, and may promote inflammation and damage in ADA-deficient lungs. Efforts to identify the adenosine receptor subtypes expressed in normal and ADA-deficient lungs, and the use of genetic and pharmacological approaches to study their function will help us to understand the impact of adenosine signaling in this model. 2'-Deoxyadenosine elevation has been implicated to lead to the disruption of cell growth and development (38,39) and influence apoptosis (23). Although 2'-deoxyadenosine elevations in ADA-deficient lungs were relatively low, the potential impact of 2'-deoxyadenosine cytotoxicity on the pulmonary phenotype cannot be ruled out at this time.

A major finding of this study was the observation that the pulmonary and immune phenotypes could be separated. This was made possible by the finding that a “low-dose” of PEG-ADA was sufficient to normalize adenosine and 2'-deoxyadenosine levels in the circulation
and in the well vascularized lung, but was not sufficient to prevent substrate elevations in the thymus and spleen. Elevated thymic nucleosides are likely due to high levels of adenosine and 2'-deoxyadenosine generated in the thymus due to the large amount of apoptosis naturally occurring in this organ as a part of thymocyte development (24,25). This idea is supported by the observation that enzyme treatments that maintained high PEG-ADA trough values were needed to achieve metabolic and phenotypic improvement in the thymus and spleen. The benefits of PEG-ADA enzyme therapy in humans have focused predominantly on the immune system; however, some studies show a rapid improvement of non-immune phenotypes following PEG-ADA treatment or red cell transfusion (8,10). Pulmonary insufficiency is common in ADA-deficient patients, and these insufficiencies are most often attributed to bacterial or viral pneumonia that arises from a compromised immune system. However, in many cases of interstitial pneumonia an organism cannot be isolated (2). Our observations in ADA-deficient mice suggest that it is possible that the metabolic disturbances may directly contribute to the pulmonary insufficiency occurring in ADA-deficient patients.

ADA deficiency in humans results in a severe combined immunodeficiency characterized by a depletion of T, B and NK cells (6). All three of these cell populations were reduced in ADA-deficient mice, making them a valuable model for studying mechanisms governing the immunodeficiency seen as a result of ADA deficiency. In this model we were able to conduct experiments not permissible in humans, including the removal of lymphoid organs for the examination of specific lymphoid cells, and the investigation of metabolic disturbances in various tissues. Results showed an apparent block in T cell development at the transition from DN to DP stage in ADA-deficient thymuses, which was in agreement with previously observed findings (24,25). High doses of PEG-ADA were required to see an improvement in lymphocyte
differentiation and an increase in the number of mature T, B and NK cells. T cells have been demonstrated to be sensitive *in vitro* to the metabolic consequences of ADA deficiency (reviewed in (2)). Toxicity has been demonstrated for adenosine (40), however; more notable are the effects of 2'-deoxyadenosine cytotoxicity on lymphoid cells (2,30,41). Less is known about the impact of ADA substrate accumulation on B and NK cells. However, results presented here, and results in ADA-deficient patients during PEG-ADA therapy and after its discontinuation (8,34), suggest that B and NK cells are directly impacted by ADA substrate accumulation. More research into the mechanisms of substrate actions on B and NK cells are needed to improve our understanding of the immunodeficiency associated with ADA-deficiency.

These studies lay the groundwork for the use of ADA-deficient mice to advance the treatment of ADA deficiency in humans. Rescuing ADA-deficient mice from lethality using PEG-ADA enzyme therapy will make possible the examination of bone marrow transplantation and gene therapy interventions in these animals. Furthermore, this model will be useful in the analysis of the metabolic and immunologic consequences associated with the cessation of PEG-ADA enzyme therapy. This information will be important for fully understanding the potential benefits of ADA gene therapy, which until now has been performed in conjunction with PEG-ADA therapy. In addition to therapeutic advances, the ability to manipulate substrate accumulations in ADA-deficient mice using various doses of ADA enzyme therapy will allow for the examination of the impact of these substrates or their metabolites on the immune system and other phenotypes associated with ADA deficiency. In particular, the use of PEG-ADA in ADA-deficient mice provides a means to manipulate the levels of adenosine in tissues and cells *in vivo*. This may provide an opportunity to study a vast array of physiological systems that are influenced by adenosine signaling.
FOOTNOTES

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1The abbreviations used are: ADA, adenosine deaminase; AdoHcy, S-adenosylhomocysteine; dATP, deoxyadenosine triphosphate; RBCs, red blood cells; PEG-ADA, polyethylene glycol-ADA; NK cells, natural killer cells; DN, double negative; DP, double positive; HPLC, high performance liquid chromatography.

2Linda F. Thompson et al., unpublished observations

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FIG. 1. **Equal delivery and half-life determination of PEG-ADA.** ADA+ control mice were treated with a “low-dose” regimen of PEG-ADA for six weeks. Panel A Levels of endogenous ADA (mADA) and PEG-ADA were examined by zymogram analysis. Hemoglobin (Hg) was used as an internal control for equal loading of samples. The genotypes of the mice were determined by Southern blot analysis and are shown above each sample. Panel B Blood was isolated at 2, 4, 8 and 16 days following the last “low-dose” PEG-ADA treatment.

FIG. 2. **Zymogram analysis demonstrating the rescue of ADA-deficient mice, and the tissue distribution of PEG-ADA.** Panel A Zymogram analysis of blood from untreated mice (lanes 1 and 2) or mice treated (lanes 3-5) with a “low-dose” of PEG-ADA. Panel B Zymogram analysis of equal amounts of protein from ADA+ control blood or blood and various tissues from an ADA-deficient mouse two days after a “low-dose” regimen of PEG-ADA. Purine nucleoside phosphorylase (PNP) was used as a positive control. Protein concentrations for ADA were 5 µg per lane, while protein concentrations for PNP were 1 µg per lane.

FIG. 3. **Prevention of the pulmonary phenotype in ADA-deficient mice treated with a “low-dose” of PEG-ADA.** Lungs were collected from ADA+ control mice and processed for histological analysis. Panel A H&E stained lung section from an 18-day old ADA+ control mouse. Panel B H&E stained section from an 18-day old ADA-deficient mouse demonstrating severe alveolar abnormalities (*), inflammation (arrow), and thickening of pulmonary blood vessel walls (bv). H&E section through 18-day old (panel C) or six week old (panel D) ADA-deficient mouse maintained on a “low-dose” regimen of PEG-ADA. Notice that the pulmonary...
abnormalities described in B are prevented in ADA-deficient mice at both stages. The scale bar in each panel is equal to 50 µm. Day 18 analysis was repeated four times, and six week analysis was conducted twice, all with similar results.

FIG. 4. Lymphoid cell counts and thymocyte distributions. Panel A Lymphoid cell counts from ADA+ control (open bar, n = 6) and ADA-deficient (black bar, n = 6) thymuses at postnatal day 17. Panel B Cell counts from thymuses of 7-8 week old ADA+ control mice (open bar, n = 5), six week old ADA-deficient mice two days after a “low-dose” (shaded bar, n = 6) or “high-dose” (black bar, n = 3) regimen of PEG-ADA. All mean cell counts are given in millions ± S.E. and percentages represent percent of control values. Double positive (DP) and double negative (DN) cells were determined using anti-CD4 and anti-CD8. Panel C Relative percentage of DN and DP thymocytes found in the thymus of 17-day old ADA+ control (open bar) and ADA-deficient (black bar) mice. Panel D Relative percentage of DN and DP thymocytes found in the thymus of 7-8-week old ADA+ control mice (open bar) and ADA-deficient mice two days after a “low-dose” (shaded bar) and “high-dose” (black bar) regimen of PEG-ADA. Values are presented as mean percentages ± S.E., and the n for each sample is the same as earlier in this legend.

FIG. 5. Analysis of T, B and NK cell populations. Splenocytes were collected and subjected to flow cytometric analysis. Specific antibodies to cell surface markers were used to identify T cells (anti-CD3 and anti-TCRβ), B cells (anti-CD45R and anti-IgM) and NK cells (anti DX5). Total cells counts for each were determined in spleens from 17-day old ADA+ control (panel A, n = 6) and ADA-deficient (panel B, n = 6) mice, and from 7-8-week old ADA+ control mice and
ADA-deficient mice treated with a “low-dose” (n = 6) or “high-dose” (n = 3) regimen of PEG-ADA (panel C). Values are presented as mean cell counts ± S.E. and percentages represent percent of control values.

FIG. 6. Metabolic disturbances in tissues. Adenine nucleosides were extracted from various tissues of 7-8-week old ADA+ control mice (open bars) or ADA-deficient mice two days following a “low-dose” (shaded bar) or “high-dose” (black bar) regimen of PEG-ADA, and quantitated using HPLC. Panel A Adenosine levels. Panel B 2’-Deoxyadenosine levels. Values are given as mean nmoles per mg protein ± S.E., n = 3 for each. nd, not detectable at a minimal detection value of < 0.001 nmoles/mg protein.

FIG. 7. AdoHcy hydrolase enzymatic activity and dATP accumulation. Panel A AdoHcy hydrolase enzymatic activity in various tissues collected from 7-8-week old ADA+ control mice (open bars) and ADA-deficient mice two days following a “low-dose” (shaded bars) or “high-dose” (black bars) regimen of PEG-ADA. Mean AdoHcy hydrolase specific activities are given as nmoles of adenosine converted to AdoHcy per min per mg protein ± S.E., n = 5 for each. Panel B dATP was quantitated in various tissues of 7-8-week old ADA+ control mice (open bars) and ADA-deficient mice two days following a “low-dose” (shaded bars) and “high-dose” (black bars) regimen of PEG-ADA. Mean dATP values are presented as nmoles/mg protein ± S.E., n = 4 for each.
Table 1. Plasma ADA Activity*

<table>
<thead>
<tr>
<th></th>
<th>µmol/hr/ml</th>
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<tr>
<td>control</td>
<td>1.03 ± 0.09</td>
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<tr>
<td>ADA-deficient Low PEG-ADA</td>
<td>13.40 ± 8.63</td>
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<tr>
<td>ADA-deficient High PEG-ADA</td>
<td>154.20 ± 67.90</td>
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</table>

* Mice were treated with either a “low-dose” or “high-dose” regimen of PEG-ADA as described in the methods and. Plasma was collected just prior to the last PEG-ADA injection to determine trough levels of PEG-ADA. Mean plasma ADA specific activity are presented as µmol/hr/ml ± S.E., n = 3 for each condition.
Blackburn, et al. Figure 1

A

mADA-  Hg-  PEGADA-

B

mADA-  Hg-  PEGADA-

days post PEGADA-  2  4  8  16
Figure 2

A

<table>
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<tr>
<th></th>
<th>Tg-m1/+</th>
<th>Tg-m1/m1</th>
<th>Tg-+/+</th>
<th>Tg-m1/m1 PEGADA</th>
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</table>

1 2 3 4 5 6

B

PEGADA blood(ADA+) blood(ADA-) thymus(ADA-) spleen(ADA-) liver(ADA-) kidney(ADA-) lung(ADA-)

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<tr>
<th></th>
<th>mADA-</th>
<th>Hg-</th>
<th>PEGADA-</th>
<th>PNP-</th>
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A

**ADENOSINE**
(nmoles/mg protein)

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<th>thymus</th>
<th>spleen</th>
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B

**2'-DEOXYADENOSINE**
(nmoles/mg protein)

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</tbody>
</table>
Blackburn, et al. Figure 7

A

ADOHCY HYDROLASE ACTIVITY (nmoles/min/mg protein)

RBC thymus spleen lung

B

dATP (nmoles/mg protein)

RBC thymus spleen lung

nd
The use of enzyme therapy to regulate the metabolic and phenotypic consequences of adenosine deaminase deficiency in mice: Differential impact on pulmonary and immunologic abnormalities


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