Treatment of collagen induced arthritis in DBA/1 mice with L-asparaginase

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Abstract

Objective

To evaluate the safety and efficacy of L-asparaginase as an immunosuppressive agent in a mouse model of rheumatoid arthritis.

Methods

Male DBA/1 mice with collagen-induced arthritis (CIA) were treated at different intervals with various doses of native and pegylated L-asparaginase from E. coli. The mice were observed for 4 weeks during which time arthritis was scored. Outcome parameters included effect on severity and progression of established arthritis as well as prevention of disease. In addition, X-rays from the affected joints were obtained for comparison.

Results

Both native L-asparaginase at a dose of 50 IU/injection intraperitoneally three days a week and pegylated asparaginase (PEG-L-asparaginase) at a dose of 25 IU/injection twice a week, significantly reduced the mean arthritic score (MAS) in mice with established arthritis ($p < 0.001$ for PEG-L-asparaginase). When native L-asparaginase was administered before the onset of arthritis (days 14-post immunization) the number of mice developing arthritis as well as the number of arthritic paws and the severity of arthritis in the treatment group were significantly decreased ($p < 0.0001$). Significant differences were found in the X-ray evaluation between treated and control mice. None of the animals died due to drug related events or showed signs of asparaginase induced toxicity.

Conclusion

Our data provide the first direct evidence that L-asparaginase is a potent antiarthritic agent and may represent an effective second line agent for future treatment studies in juvenile and adult rheumatoid arthritis.

Key words

CIA, L-asparaginase, treatment.

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Introduction

The rediscovery of cancer drugs, such as methotrexate, for the treatment of autoimmune diseases has led to renewed interest in other chemotherapeutic agents that might be effective as immunosuppressants and potent anti-inflammatory agents. L-asparaginase is used as an adjunct to treat acute lymphoblastic and non-lymphoblastic leukemia and is incorporated in the treatment of high-risk refractory acute lymphatic leukemia (ALL) and recurrent ALL in children and adults (1-4).

Enzymes depleting asparagine or glutamine are known for their immunosuppressive potential and have been studied in cancer patients in the past (5-7). Most asparaginases catalyze the deamidation of L-asparagine and L-glutamine to aspartic acid and glutamate respectively. The asparaginase isolated from *E. coli* has high substrate affinity for both asparagine and glutamine and has been shown to deplete plasma levels of both of these amino acids in humans (1, 4). The immunosuppressive effects of L-asparaginase have been attributed to its glutaminase property and are restricted to the lymphoid system, suggesting a potential role for the treatment of T cell regulated autoimmune diseases (8, 9). L-asparaginase also suppresses the humoral and cell mediated immunological response to T cell dependent immunogens on sheep red blood cells (SRBCs) and markedly reduces splenic immunoglobulin producing B-cells. As a consequence, a significant decrease in the size and reactivity of the germinal centers in the spleen can be observed (10).

Unlike other anti-tumor agent (cyclophosphamide, etc.), asparaginases are not mutagenic and are not associated with secondary malignancies (1). At the same time, L-asparaginase is not myelosuppressive, hence patients treated with asparaginase are not at high risk for sepsis or for severe life threatening infections (2).

The asparaginases in current clinical use are immunogenic proteins derived from *Escherichia coli* (*E.coli*) or *Erwinia carotovora*. *E. coli* possesses two asparaginase enzymes, one constitutive and another induced by the anaerobic state, the latter being responsible for the tumor inhibitory effect (11).

Immune responses against these bacterial antigens result in allergic reactions and are the major side effect of L-asparaginase therapy. For this reason modified enzyme derivatives have been developed by covalently attaching immobilized enzymes to polyethylene glycol (PEG). These pegylated enzymes (e.g. PEG-L-asparaginase) are less immunogenic and have a prolonged half-life (12-16).

Collagen induced arthritis (CIA) is an experimental animal model of arthritis with immunologic, pathologic and genetic features resembling rheumatoid arthritis in humans (17-19). Susceptibility to CIA is linked to HLA class II and requires the presence of T cells expressing V beta chains of their T cell receptor (20, 21). CIA in mice has been used to test a variety of different antiarthritic drugs. However, very few of these conventional and clinically successful agents have been shown to treat established CIA, and only prevented or delayed the onset of arthritis in these animals. In addition treatment protocols involving newer therapeutic compounds, such as biologic response modifiers (anti-CD4 antibodies, IL1 receptor antagonist, and monoclonal antibodies against TNF) or thalidomide are characterized by brief observation periods and little or no effect on established arthritis (22-28).

Due to its T cell immunosuppressive properties, we hypothesized that *E. coli* asparaginase might be a useful agent in the prevention and/or the treatment of established CIA. Herein, we show evidence that native and pegylated asparaginase from *E. coli* have potent antiarthritic activity in CIA.

Materials and methods

Animals and reagents

Male DBA/1 LacJ mice, 4 - 5 weeks of age, were obtained from Jackson Laboratory (Bel Harbor, MA) and quarantined for 3 weeks prior to the experiments. The study was approved by the Animal Care Committee (ACC) of Childrens Hospital Los Angeles.

Arthritis was induced by injection of 100 g of bovine collagen type II

emulsified 1:1 in complete Freund's adjuvant (CFA) (Difco Detroit, ME) at the base of the tail. On day 21 the mice were given a booster injection of another 100 g of bovine collagen type II in complete Freund's adjuvant at the base of the tail. The mice were weighed weekly and their overall health status was assessed three times a week after the initial injection.

Fore and hind paws were assessed for arthritis using an established scoring system (0 = normal, 1 = erythema, 2 = erythema and minor edema, 3 = pronounced edema, 4 = rigidity) (21). Each limb was graded separately, giving a maximal possible score of 16 per mouse. Our results are presented as a mean arthritic score (MAS) for each treatment and control group. The MAS was calculated by dividing the sum of the severity scores by the number of mice per group.

L-asparaginase preparations

L-asparaginase derived from *Escherichia coli* in the form of L-asparagine amidohydrolase, type ECII, was obtained from Merck Sharp and Dohme, West Point, PA. PEG-asparaginase was kindly provided by Enzon, Inc. PEG-L-asparaginase is produced by covalently conjugating units of monomethoxypolyethyleneglycol (PEG), molecular weight 5000 KD, to the native *E. coli* L-asparaginase. The enzyme activity for both asparaginase preparations was determined by the amount of ammonia produced upon hydrolysis of L-asparagine (Nessler's test) (10). Activity was expressed in International Units (IU) which is the amount of enzyme catalyzing the formation of one mol ammonia per minute under the conditions of the assay. In all assays a standard curve with ammonium sulfate was prepared.

Mice are resistant to the toxicity of L-asparaginase up to 2000 IU/kg. Due to its high molecular weight (138,000 KD) little L-asparaginase enters the extravascular space and peak extravascular levels are usually around 10% of the blood levels. Therefore, the half life in mice is approximately 2.6 hours, while in humans *E. coli* asparaginase has a half life between 11 and 22.6 hours for regular L-asparaginase and about 7 days for the PEG-L- asparaginase (12).

Therapeutic protocol

In all therapeutic protocols, mice were randomized from a pool of animals with established arthritis in order to match the severity of their arthritis as closely as possible. All experiments were repeated at least 2 times in order to validate the results.

1. Classic CIA

E. coli *asparaginase (L-asparaginase).* Forty-eight days post immunization mice with arthritis were treated with 50 IU of L-asparaginase intraperitoneally (IP) 3 times per week for a total of 4 weeks. Controls received saline injections. Twelve mice per group were studied. All limbs were evaluated and the presence of arthritis on each paw was graded three times per week. An average MAS for each group was obtained weekly. After 4 weeks mice were sacrificed for histopathological studies.

E. coli PEG-asparaginase (PEG-L-asparaginase). Forty-eight days post immunization mice with arthritis were treated with 25 IU of PEG-L-asparaginase IP 2 times per week (due to the longer half-life of this agent). The mice were treated for a total of 3 weeks and compared to controls. Fifteen mice per group were studied. The presence of arthritis in each paw was graded 3 times per week and an average MAS for each group was obtained weekly.

2. Accelerated CIA

LPS induced CIA produces histopathologic changes in the murine arthritis model that are comparable to the classic CIA model (24). Twenty mice without macroscopic evidence of arthritis on Day 54 post immunization were injected with a single IP dose of 40 g of LPS. This resulted in severe arthritis in all animals within 3 days and they were randomly divided into two groups. In the treatment group animals received IP injection of 50 IU of L-asparaginase 3 times weekly, while control animals received saline IP injections. The animals were followed for 4 weeks, the presence of arthritis in each paw was graded 3 times per week, and an average MAS for each group was obtained weekly.

Preventive protocol

In order to study the effect of L-asparaginase on preventing arthritis 50% of the DBA/1 mice received IP doses of 50 IU of L-asparaginase three times weekly 14 days post immunization while controls were immunized and received saline injections. Forty-five animals per group were studied. The incidence of arthritis, as well as the number of affected paws and the MAS after day 28 post immunization in the treated versus the controls, was compared. The mice were examined three times a week for 4 weeks and an average MAS for each group was obtained weekly.

Data analysis

The distribution of mean arthritic score (MAS) was examined as a univariate variable, by titrate, and by titrate over time. Regression lines were fitted for each titrate and compared with the control. Regression lines were tested for parallel slopes. Analysis of variance modeling time effects were run. Equality of slope hypotheses were tested using analysis of covariance models. Slopes were unequal during treatment and indifferent post-treatment at the = 0.05 level of significance. Therefore, non-parametric regression models were fitted over treatment periods. Repeated measures of analysis of variance were performed on the *E. coli* asparaginase data. Treatment effects, time effects, and interaction effects were plotted and tested for significance.

Assessment of radiographs

For radiographic analysis paws from mice with established arthritis, which had been treated with either saline or asparaginase, were removed and placed in the ventral position on Kodak X–OMAT MA films and exposed with 28 kV, 125 mAmps at a distance of 45 cm from the x-ray device.

The radiographic evaluation of treated and untreated animals was assessed by a blinded radiologist. Individual films were read 3 times in random order by the same radiologist in order to control for intra-observer variations. A modified Sharp Score was applied where 0 = no radiographic changes, 1 = equivocal

findings, 2 = beginning erosions and joint space narrowing, 3 = advanced erosions and joint space narrowing, 4 = severe erosions and loss of joint space.

Results

The course of collagen-induced arthritis in DBA/1 mice is presented in Figure 1. The first symptoms of arthritis usually occurred around 27 days post immunization. The incidence reached a peak on Day 46 and declined thereafter (data not shown). The total incidence of arthritis after 48 days was approximately 50%.

Therapeutic protocol

1. Effect of asparaginase on classic CIA

We first assessed the effect of *E. coli* asparaginase on established CIA. Figure 2 is a representation of several experiments and demonstrates the inhibitory effect of *E. coli* L-asparaginase on CIA. Twelve mice per group were studied.

The MAS in the L-asparaginase treatment group was 6.75 compared to 5.95 in the saline controls at the beginning of treatment. Over a 4 week treatment period with 3 weekly doses of 50 IU of native L-asparaginase the MAS decreased in the treated group while remaining consistently elevated in the saline controls. The main difference was observed at weeks 2 and 3 with a significant difference in the MAS scores ($p = 0.0047$). The MAS in the treated group at week 3 was also statistically significant when compared to baseline, whereas there was no difference in the saline controls. In the fourth week of treatment the MAS in the treatment group slightly increased but was still below the baseline value.

To determine if the observed anti-arthritic effect was related to antigenic competition due to the amount of injected protein, or truly due to the enzymatic activity of L-asparaginase, native *E. coli* L-asparaginase was inactivated by heating in a boiling water bath. This procedure was repeated 5 times to yield inactive enzyme as verified by a Nessler's test. Inactivated L-asparaginase had no significant anti-arthritic effect and the MAS in this group of mice was not significantly different when compared to controls (data not shown).

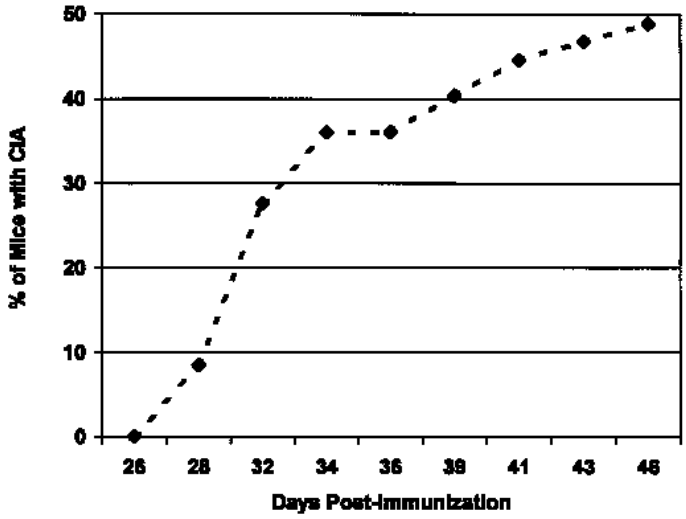

Fig. 1. Time course of arthritis development in DBA/1 mice. The first signs of arthritis usually developed around day 26 post immunization and approximately 50% of the animals had developed arthritis around day 46.

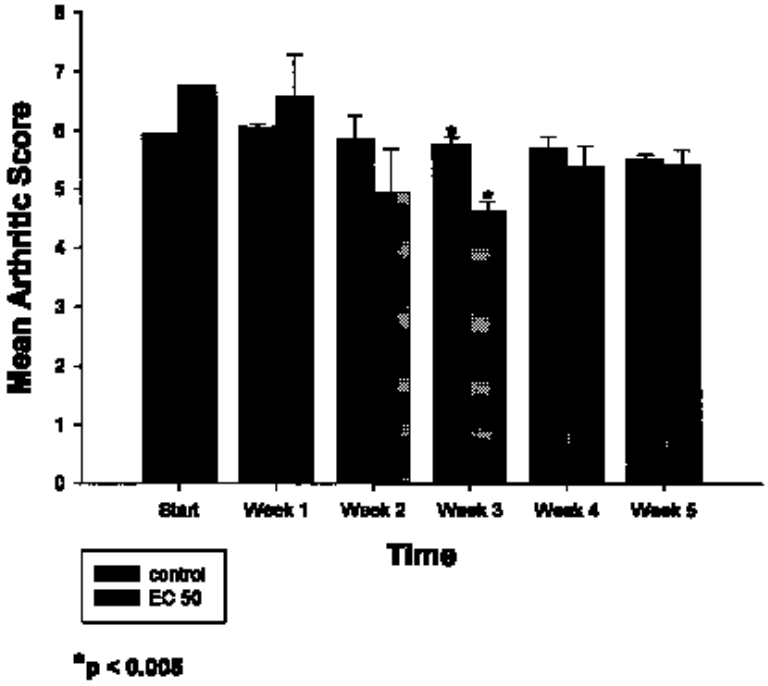

Fig. 2. Inhibitory effect of native *E. coli* L-asparaginase on established CIA. Mice were injected IP with 50 IU of *E.coli*. L-asparaginase three times a week.

In order to determine the optimal treatment dose we simultaneously tested 5, 10, 25 and 50 IU per injection of L-asparaginase. Fifteen mice per group were studied. The results are presented in Figure 3.

A statistically significant difference was again observed after two and three weeks with 50 IU ($p < 0.005$) and during the third week with 25 IU of L-asparaginase ($p < 0.05$). The other lower doses failed to demonstrate any anti-arthritic effect.

We therefore conclude that L-asparaginase has a dose dependent anti-arthritic effect.

2. Effect of PEG-L-asparaginase on CIA

Next we determined the effect of a dif-

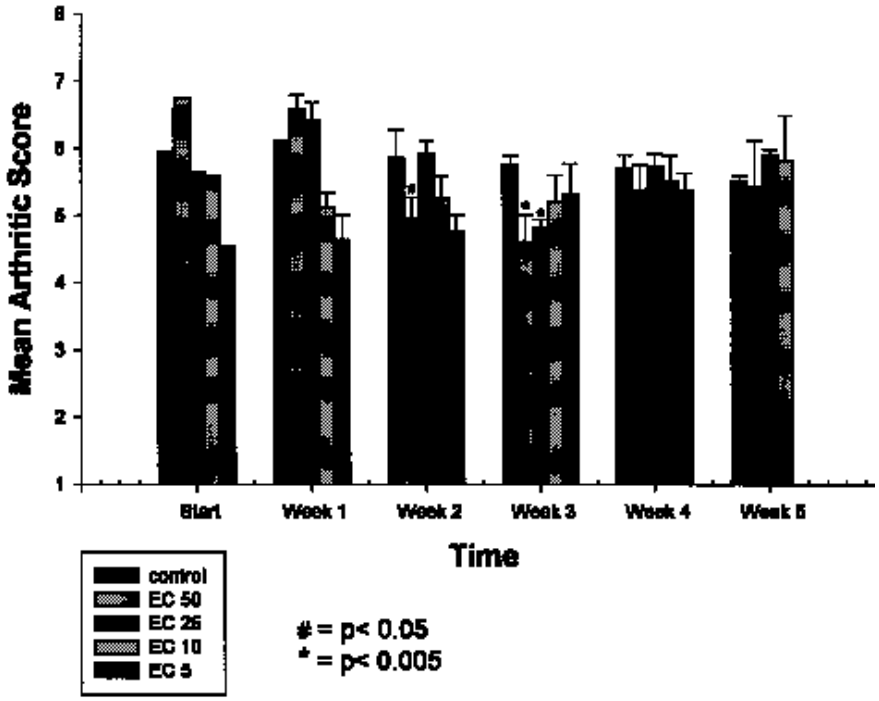

Fig. 3. Titration of native *E. coli* L-asparaginase in established CIA. Doses of 5,10, 25 and 50 IU per injection 3 times a week are compared. A statistically significant difference was observed after two and three weeks with 50 IU ($p < 0.05$ at week 2, $p < 0.005$ at week 3) and during the third week with 25 IU of L-asparaginase ($p < 0.005$).

ferent formulation of *E. coli* asparaginase, PEG-L-asparaginase, by treating mice with 25 IU IP twice a week. Fifteen mice per group were studied and randomized as described above. The data are presented in Figure 4.

The baseline MAS at the beginning of treatment (48 days post immunization), was 5.2 for the study group vs 5.8 for the control group. The scores for PEG-L-asparaginase treated vs control mice over the next 5 consecutive weeks were 4.1 vs 7.1, 3.5 vs 6.1, 3.8 vs 8.1, 3.8 vs 7.7 and 6.1 vs 8.5. All scores were statistically significant by ANOVA ($p < 0.04$). Applying the more powerful regression analysis addressing only the 3 weeks of treatment, we found that these statistical differences became even more pronounced ($p < 0.001$).

After 3 weeks treatment was discontinued and the mice were followed clinically for signs of disease flare. During the next 5 weeks the average MAS in the PEG-L-asparaginase treated mice remained unchanged until the ninth week, after which there was a slow increase. Scores in the control group increased slightly during this observation period. From these data we conclude that PEG asparaginase has potent antiarthritic activity in this animal model.

3. Effect of asparaginase on accelerated CIA.

The inhibitory effect of *E. coli* L-asparaginase on LPS accelerated arthritic mice is shown in Figure 5. The MAS at the beginning of treatment (Day 61 post immunization) was 7.45 in the treatment group and 6.9 in the controls. After receiving 50 IU of *E.coli*. L-asparaginase three times a week, paw swelling and redness decreased. The MAS of the treatment group dropped significantly to 5.5 during weeks 3 and 4 after the onset of treatment ($p < 0.015$), and then increased during week

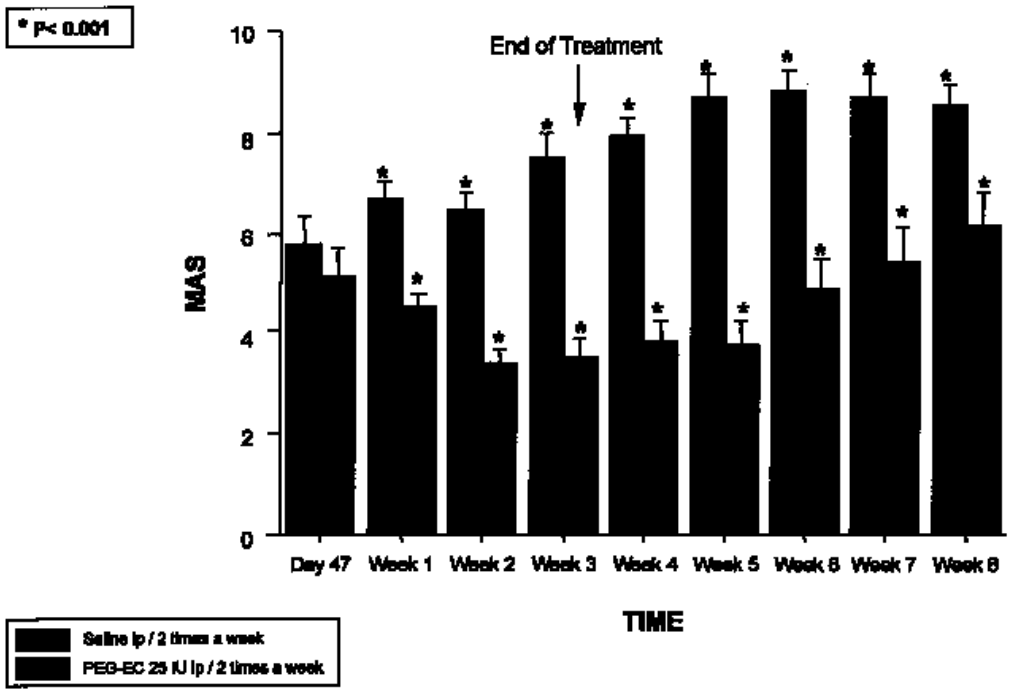

Fig. 4. Inhibitory effect of PEG - L-asparaginase on established CIA. Mice were treated with 25 IU IP twice a week.

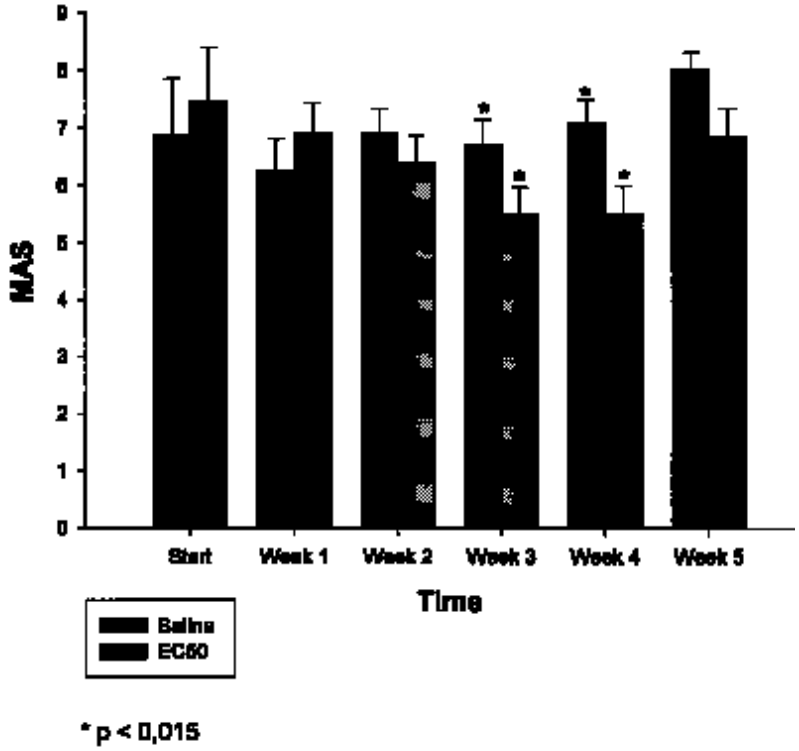

Fig. 5. Inhibitory effect of native *E. coli* L-asparaginase on LPS accelerated arthritis. Mice were injected IP with 50 IU of *E.coli* L-asparaginase three times a week.

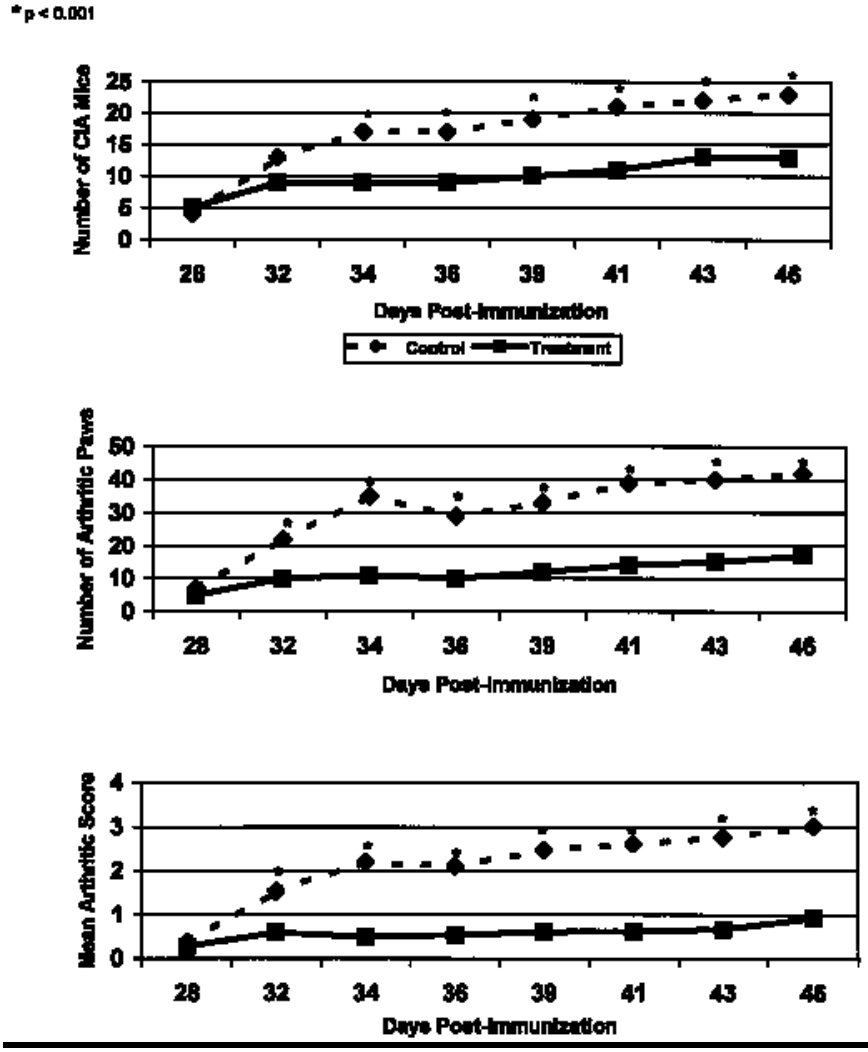

Fig. 6. Preventive effect of native *E. coli*. L-asparaginase in CIA onset. 50% of the mice received IP doses of 50 IU of L-asparaginase three times weekly 14 days post immunization prior to the onset of arthritis. Controls were immunized and boosted, but not pretreated.

Table I. Radiologic evaluation of L-asparaginase in DBA/1 mice.

	No. of affected joints	Severity score
Controls		
Mouse 1	6	10
Mouse 2	5	9
Mouse 3	6	9
Average	5.6	9.3
Saline		
Mouse 1	10	19
Mouse 2	9	10
Mouse 3	8	17
Average	9	15.3
L-Asparaginase		
Mouse 1	7	11
Mouse 2	7	11
Mouse 3	8	13
Average	7.3	11.6

Controls = DBA/1 mice without arthritis
Saline = DBA/1 mice with arthritis treated with saline injections
L-asparaginase = DBA/1 mice with arthritis treated with l-asparaginase
Scoring system:all 4 extremities were evaluated. 0= normal, 1= equivocal 2=joint space narrowing and beginning erosions, 3= joint space narrowing and advanced erosions, 4= severe erosions and joint fusion.

5. The MAS in the control group fluctuated slightly between 6.9 at treatment start and 7.1 at the end of week 5, but remained high overall during the observation period. Hence, these results demonstrate that asparaginase has significant antiarthritic activity in this most treatment resistant model for autoimmune arthritis.

Preventive protocol

The data are presented in Figure 6. In both treated and control groups the onset of arthritis occurred around day 28 despite L-asparaginase given three times weekly at days 14 and 21 post immunization. However, the number of mice developing arthritis in the treatment group was significantly reduced. After 46 days 23 mice (49%) in the control group and only 13 mice (30%) in the treated group had developed arthritis ($p < 0.0001$). During the same time period arthritis developed in 42 paws in the control animals, but in only 17 paws in the treated animals ($p < 0.0001$). Furthermore, the MAS indicating the severity of arthritis was significantly decreased to 0.9 in the treatment group vs 3.0 in the untreated group ($p < 0.0001$). We therefore conclude that asparaginase has an effect on the early induction stages of CIA.

Assessment of radiographs

Radiographs of 3 groups with 2 mice and 8 joints each were compared: non-immunized mice without arthritis (controls), immunized mice with arthritis that received saline injections (saline controls), and immunized mice with arthritis treated with L-asparaginase.

Five joints with an average Sharp score of 9.3 were read as abnormal in the control mice, suggesting that subclinical arthritis may be present in these genetically predisposed but clinically unaffected mice. In contrast the average number of affected joints in the saline controls was 9 with a mean Sharp score of 15.3. In the L-asparaginase treated mice these numbers were 7 and 11.6, respectively (Table I). A one-way analysis of variance test on the number of affected joints and severity of arthritis was performed. Orthogonal contrasts were used to make pair-wise comparisons of the controls, saline, and L-asparaginase treated mice. P-values for the pair-wise comparisons were based on the student t-distribution.

Statistically significant differences were found for both the number of radiographically affected joints and the Sharp score between the control group and saline treated mice ($p < 0.001$). However, when comparing saline treated and L-asparaginase treated mice a statistically significant difference was only found for the number of affected joints but not for the severity of the arthritis ($p < 0.05$).

Discussion

Although the depletion of non-essential amino acids such as asparagine and/or glutamine may account for the unique therapeutic effect of L-asparaginase in cancer treatment, it does not necessarily explain the potent action of L-asparaginase in autoimmune disease. While malignant cells are dependent on an exogenous source of asparagine due to rapid cell turnover, non-malignant cells are able to synthesize asparagine and should consequently be less affected by the enzymatic depletion with L-asparaginase.

L-asparaginase has multiple immunosuppressive and anti-inflammatory effects, which are partially attributed to its activity as a glutaminase (8-10). It has been shown that T cell mediated B cell responses are more susceptible to suppression by L-asparaginase than T cell independent B cell responses, such as LPS induced B cell proliferation (29). In addition, L-asparaginase has a direct effect on lymphocytes, as demonstrated by inhibition of the blastogenic response to PHA stimulated PBLs *in vitro* (5). Its effect on the lymphoid system suggests a potential role for the treatment of autoimmune diseases related to abnormal T cell responses. Although the pathogenesis of rheumatoid arthritis is multifactorial, T cells play an important role in the induction phase, followed by activation of macrophages and synovial fibroblasts and excessive cytokine production. Furthermore L-asparaginase suppresses the production of antibodies to SRBC, graft vs host reactions, allograft rejection and delayed hypersensitivity reactions (5, 30-32).

L-asparaginase could also have anti-inflammatory properties. A possible anti-inflammatory mechanism of L-asparaginase is the intracellular accumulation of aspartic acid, which could interrupt the cellular biosynthetic feedback and interfere with the energy producing mechanism of the cell, causing cytotoxicity. An overall inhibition of protein synthesis, however, seems unlikely. Moreover asparaginase induces apoptosis in lymphoid leukemia cells and this could also occur in normal lymphoid cells. The elucidation of possible mechanisms of action of asparaginase on autoimmunity will require further investigation.

Our observation that L-asparaginase and its pegylated variants effectively treat established arthritis and dramatically prevent the onset and reduce the severity of arthritis in CIA mice suggests that this agent might be a potent agent for treatment of autoimmune diseases, such as rheumatoid arthritis.

When compared to published data using other agents to treat established CIA, L-asparaginase may be superior to drugs such as cyclosporine A or TNF fusion proteins since these drugs have very little impact on established CIA (23-28). In CIA, L-asparaginase appears to be as effective but less toxic than cyclophosphamide.

It remains to be determined in clinical trials, whether asparaginase treatment will have long-lasting effects on arthritis perhaps by deleting reactive clones of lymphoid cells, which may result in prolonged remission. We therefore propose that clinical trials of PEG-L-asparaginase be carried out in adults and children with rheumatoid arthritis.

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