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Alteration of the circulating life and antigenic properties of bovine adenosine deaminase in mice by attachment of polyethylene glycol

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SUMMARY

Polyethylene glycol was attached covalently to adenosine deaminase (ADA) using cyanuric chloride as the coupling agent. The modified adenosine deaminase (PEG-ADA) appears to lose its immunogenicity in mice following multiple intravenous injections. PEG-ADA does not react with antibodies raised against native ADA. The circulating half-life ($T_{\frac{1}{2}}$) of PEG-ADA was increased to 28 hr. The lack of detectable antibody formation and long circulating life may make PEG-ADA suitable for treating human ADA deficiency.

INTRODUCTION

An X-linked and autosomal recessive form of SCID has been described which may be associated with deficiency of adenosine deaminase (ADA) (Meuwissen, Pollara & Pickering, 1975). Frozen irradiated erythrocytes have been used to treat patients with ADA deficiency (Polmar *et al.*, 1976) because they are a source of ADA. Following transfusion, lymphocyte ATP levels drop and proliferative capacity increases. Despite the partial success of this therapy, it has been anticipated that long-term transfusions may lead to haemosiderosis, hepatitis and the development of antibodies against minor erythrocyte antigenic determinants (Polmar *et al.*, 1976).

Similarly, the injection of non-human enzymes would be highly limited due to adverse immunological sequelae. We have shown that the attachment of polyethylene glycol (PEG) can reduce the antigenicity of soluble proteins (Abuchowski *et al.*, 1977).

We describe the preparation, immunogenicity and circulating life in mice of PEG covalently attached to ADA.

EXPERIMENTAL PROCEDURES

Materials. Adenosine deaminase from calf intestine was obtained from Boehringer Mannheim Biochemicals. Adenosine was purchased from Sigma Chemical Co. and methoxypolyethylene glycol of 5,000 daltons from Union Carbide. BALB/c mice were used.

Covalent attachment of PEG to ADA. ADA was dialysed with 0·05 M, pH 7·4 potassium

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phosphate buffer. 'Activated PEG' was prepared by the method of Abuchowski *et al.* (1977). Cyanuric chloride (4·6 g) was dissolved in 300 ml of anhydrous benzene containing 10 g of anhydrous sodium carbonate. PEG (25 g) was added to the mixture and stirred for 3 days at room temperature. The solution was filtered and 300 ml of petroleum ether added slowly. The precipitate was dried in a vacuum desiccator. Enzyme in 0·05 M, pH 7·4 phosphate buffer was mixed with an equal amount of 0·1 M sodium tetraborate, pH 9·2. To this solution, activated PEG in a five-fold molar excess was added. The mixture was stirred for 1 hr and unreacted PEG removed using an Amicon ultrafilter with an XM-50 membrane. The protein concentration of ADA and PEG-ADA was determined by the biuret method using albumin for comparison. The percentage of PEG attachment was determined by the method of Habeeb (1966). The resultant preparation had 60% of ADA-free amino groups attached to PEG-ADA; activity was 28% of the unmodified enzyme.

Enzyme assay. Native ADA and PEG-ADA were measured by the method of Bergmeyer (1974). The decrease in absorbency/minute (Δ A/min) was measured at 265 nm as adenosine was converted to inosine. Activity of adenosine deaminase, i.e. Δ A/min, was linear over a wide concentration (1×10^{-6} to 1×10^{-4} mg/ml).

Mice were injected i.p. with 250 μg ADA every week for 4 weeks in Freund's incomplete adjuvant. Two weeks later the animals were bled from the orbit, sera were pooled and stored at −20°C. Gel diffusion slides in 1·0% agarose in PBS, pH 7·3, with 1·0% sodium azide, were incubated overnight at 25°C, and stained with Coomassie blue.

Two groups of mice were injected in the tail vein with 0·05 mg of PEG-ADA, or 0·05 mg of native ADA. Blood was obtained from four mice in each group at specified times. Blood was centrifuged; 10 μl of serum was added to 960 μl of 0·05 M, pH 7·4 PBS and 30 μl of 1·4 mM adenosine in 0·05 M, pH 7·4 PBS and assayed for ADA as described.

The circulating life of ADA and PEG-ADA was determined following i.p. injection in two groups of three mice. Each mouse received 0·05 mg of enzyme.

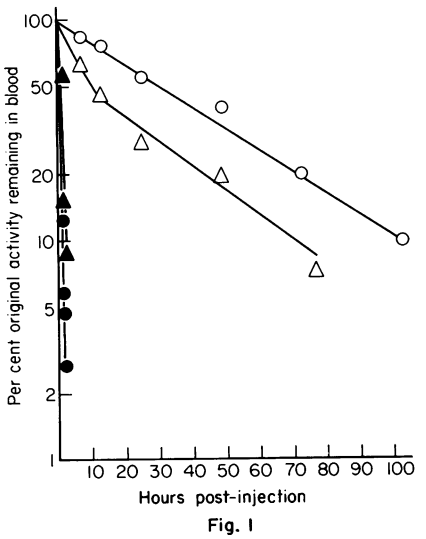

Fig. 1

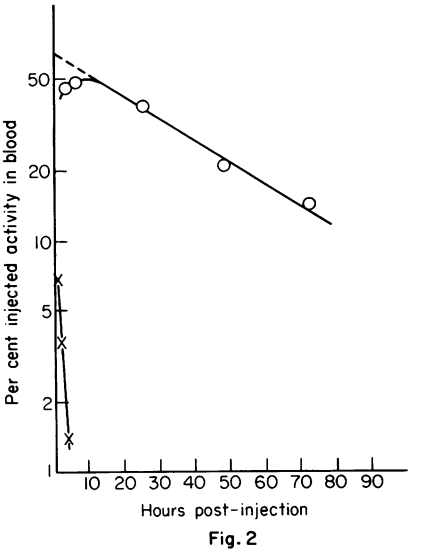

Fig. 2

Fig. 1. Blood circulating life of i.v. injected native ADA and PEG-ADA. (▵——▵) PEG-ADA (first injection), (●——●) native ADA (first injection), (○——○) PEG-ADA (13th injection), (▲——▲) native ADA (13th injection).

Fig. 2. Blood circulating life of i.p. injected native ADA and PEG-ADA. (○——○) PEG-ADA, (×——×) native ADA. The ordinate (% injected activity) is a shortened notation representing the percentage of the theoretical activity (Δ A_{265}/min) that would be expected in the serum. The endogenous ADA levels were subtracted prior to calculating the percentages.

RESULTS

The kinetics of serum ADA activity in mice following the initial and in mice following 13 injections of both native and PEG-ADA is shown in Fig. 1. Following the first injection, native ADA was virtually gone after 2 hr; at 2 hr, only 2·7% of 0-hr activity remained. Calculated $T_{\frac{1}{2}}$ for native ADA was 30 min. In mice given 13 injections of native ADA, the 0-hr activity was only 42% of the 0-hr level seen in mice given a single injection; the $T_{\frac{1}{2}}$ was less than 30 min. The reduction in 0-hr enzyme level seen in mice given 13 injections suggests early removal of activity by pre-existing antibodies.

PEG-ADA had a greatly prolonged circulating life in mice given both single injections and 13 injections. Approximately 15% PEG-ADA activity was present in the serum 100 hr after a single injection; the $T_{\frac{1}{2}}$ for the first single injection was 28 hr, for mice given 13 injections it was 30 hr. Following the 13th injection, the 0-hr activity was 95% of the 0-hr level seen in mice given a single injection. No toxicity was seen in the PEG-ADA-injected group of mice.

Serum ADA and PEG-ADA activity following a single i.p. injection is shown in Fig. 2. The $T_{\frac{1}{2}}$ of native ADA injected i.p. was 40 min, the $T_{\frac{1}{2}}$ for i.p. PEG-ADA was 32 hr.

Immunodiffusion studies. Sera harvested from animals injected with ADA or PEG-ADA were set up as immunodiffusion plates to test for precipitating activity. Serum raised in mice injected with native ADA reacted strongly with ADA but not with PEG-ADA indicating that the antigenic sites on ADA had been masked by the PEG. Serum from PEG-ADA-injected mice did not react with ADA or PEG-ADA suggesting that PEG-ADA does not elicit a significant antibody response. The fact that an antiserum to PEG-ADA could not be raised using immunodiffusion techniques for detection suggests that *in vivo* breakdown of PEG-ADA did not produce moieties capable of stimulating antibodies to native ADA.

DISCUSSION

In the present communication we describe the preparation, circulating life and immunogenicity of ADA attached to PEG. Methoxypolyethylene glycols have the general structure $CH_3(OCH_2CH_2)_NOH$, are non-immunogenic, uncharged and water-soluble. These characteristics make PEGs ideal polymers to render enzymes of potentially great use in clinical medicine. PEG enzymes are removed slowly from the circulating by macrophage pinocytosis. No free PEG is detectable in our enzyme preparations so that PEG toxicity has not been seen nor is anticipated. Our success in preparing a long-lived, non-immunogenic ADA in mice compares favourably with a PEG-asparaginase we have evaluated in humans (Abuchowski, Davis & Davis, 1981).

PEG-ADA exhibited a highly extended circulating life when injected i.v. in mice. The circulating life was unchanged after 13 injections. ADA and PEG-ADA were both detected in the blood stream following i.p. injection. Once in the blood stream, PEG-ADA behaved as in the i.v. injected group. When injected i.p., taking into account dilution, amount of blood removed for assay, dilution prior to assay, optimal predicted blood activity and 28% activity as compared to native ADA, the activity of PEG-ADA at 6 hr following i.p. injection (48%, Fig. 2) was close to the theoretical maximal amount; the level of native ADA at 1 hr was only 6·9% of theoretical calculated level. This suggests that PEG-ADA can move freely across biological membranes without loss of activity.

Polmar *et al.* (1976) suggested that ADA contained in frozen irradiated erythrocytes following administration to SCID patients did not leave the erythrocytes to move to areas where needed. This problem would hopefully be overcome because of the free mobility of PEG-ADA. In addition, transfusions have the risks of hepatitis, haemochromatosis and immunological reactions to red cell antigens. If PEG-ADA retains its non-immunogenicity in humans, it will serve as an effective mode of therapy in ADA-deficient SCID.

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