

Immunological and Pharmacological Characterization of Poly-DL-Alanyl-modified *Erwinia carotovora* L-Asparaginase¹

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ABSTRACT

The covalent attachment of poly-DL-alanine peptides to lysyl residues on the surface of *Erwinia carotovora* L-asparaginase has produced a modified enzyme which is much less immunogenic in mice and demonstrates 100-fold longer plasma half-life in the rhesus monkey. Immunogenic responses towards both the immunoglobulin G (IgG) and immunoglobulin E (IgE) antibody subclasses were evaluated in C57BL × DBA/2 F₁ mice exposed to 250 rads of whole-body irradiation 4 hr prior to immunization with 5-diazo-4-oxynorvaline-inactivated native and modified L-asparaginase in complete Freund's adjuvant. Under these immunologically stressful conditions, the native enzyme evoked an IgE and IgG response which could be further amplified by a secondary immunization, whereas the modified enzyme evoked no IgE or IgG response even after a tertiary immunization. In experiments mimicking an intensive therapeutic schedule, whereby two groups of mice were given weekly injections of 5 to 10 units of either native or modified asparaginase for up to 14 weeks, neither enzyme form evoked a significant IgE response, and only the mice given injections of the native enzyme produced an IgG response. In a preliminary patient study, skin testing of a child who had shown an allergic reaction to the native enzyme resulted in a negative response after an intradermal injection of the modified enzyme, whereas a wheal and flare reaction was observed to both the native enzyme and a histamine control. All of these results suggest that the modified enzyme should show a definite reduction in immunological reactions associated with L-asparaginase treatment of childhood leukemia.

INTRODUCTION

Hypersensitivity reactions, ranging in severity from mild allergic reactions to anaphylactic shock, occur in up to 25% of acute lymphocytic leukemia patients treated with L-asparaginase (8, 12). In addition, anti-asparaginase IgG antibodies may develop, which greatly accelerate enzyme clearance and consequently limit therapeutic effectiveness (5, 8, 12). These complications may be minimized by (a) administering short courses only during remission induction or consolidation, (b) using combinations of chemotherapeutic agents many of which are immunosuppressive, and (c) switching to a non-cross-reacting enzyme when allergic reactions do occur. Nevertheless, allergic reactions frequently require the discontinuance of asparaginase therapy in patients who might otherwise continue

to benefit from its use. Therefore, the modification of asparaginase to decrease its immunogenicity can not only decrease the allergic reactions associated with the use of the enzyme but also broaden the ways and expand the time over which the enzyme can be given effectively.

A previous report from our laboratory has documented the improvement in therapeutic, immunological, and clearance properties associated with the attachment of poly-DL-alanyl peptides to the L-asparaginases obtained from *Escherichia coli* and *Erwinia carotovora* (14). This work is a continuation of those studies and demonstrates a pronounced decrease in the immunogenicity of the poly-DL-alanyl-modified *Erwinia* enzyme in eliciting both IgG and IgE antibody responses and 100-fold prolongation of the plasma clearance in a rhesus monkey compared to the native enzyme.

MATERIALS AND METHODS

Materials. L-Asparaginase from *E. carotovora* was obtained from the Microbiological Research Establishment, Salisbury, England. DL-Alanine-N-carboxyanhydride was prepared from N-carbobenzoxy-DL-alanine (Sigma Chemical Co., St. Louis, Mo.) in dry diethyl ether by the PCl₅ procedure (6) (yield, 85%; m.p., 33–38°). DONV³ was a gift of Dr. Robert Handschumacher, Yale University. FA adjuvant was obtained from Grand Island Biological Co., Grand Island, N. Y. Young adult male C57BL × DBA/2 F₁ (hereafter known as BD2 F₁ mice were obtained from The Jackson Laboratory, Bar Harbor, Me., and young adult female CD rats came from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Preparation of Pyrogen-free Poly-DL-alanyl Asparaginase. To the contents of a vial (10 mg) of *Erwinia* asparaginase (Lot MRE17) were added 10 ml of 50% dimethyl sulfoxide in 0.05 M NaHCO₃, pH 8.6, at –20°. To this material were added 150 mg of DL-alanine-N-carboxyanhydride and 1 drop of antifoam B (Sigma). The reaction mixture was stirred for 1 hr at 4° and then diluted with 20 ml of water. The diluted reaction mixture was then applied to an autoclaved 20-ml Bio-Rad hydroxylapatite column equilibrated with 0.01 M potassium phosphate buffer, pH 7.2. The column was washed with 20 ml of the 0.01 M potassium phosphate buffer and eluted with 10-ml aliquots of 0.3 M potassium phosphate. Fractions 2 and 3 demonstrated enzyme activity and were collected. All reagents, buffers, and glassware were sterile and pyrogen free except for the DL-alanine-N-carboxyanhydride, antifoam B, and hydroxylapatite.

Eight preparations of modified asparaginase prepared in this manner had 31 ± 7% (S.D.) of the original specific activity. None would form precipitates in Ouchterlony immunodiffusion assays with anti-native asparaginase sera. All had 10-fold longer plasma half-lives in mice. All had 1000 ± 400 more alanine residues per mol than did the native enzyme. Also, all contained less than 0.1 ng pyrogen per ml, as measured by the Worthington *Limulus* Pyrostat Assay. For assay

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³ The abbreviations used are: DONV, 5-diazo-4-oxonorvaline; CFA, complete Freund's adjuvant; PCA, passive cutaneous anaphylaxis; RID, reversed single radial immunodiffusion; i.d., intradermal.

conditions, see Ref. 14 except for pyrogen assays, which were performed as described in the accompanying Worthington literature.

Immunization Conditions. Optimal immunization conditions were obtained by examining the effects of dosage, adjuvant, and irradiation on the IgE response in BD2 F₁ mice to an i.p. injection of DONV-inactivated asparaginase. DONV inactivation was performed by incubating 0.5 mg enzyme per ml with 2 mM DONV in 0.05 M KH₂PO₄ buffer, pH 6.8, for 1 hr. Immunization conditions included: 10, 1, or 0.1 mg DONV-inactivated enzyme per kg adsorbed on 1 mg aluminum hydroxide gel with 6×10^9 *Bordetella pertussis* cells or emulsified with an equal volume of CFA and administered to groups of mice either left unirradiated or exposed to 250 rads of whole-body irradiation from a Gammacell 40 ¹³⁷Cs source 4 hr prior to immunization. Maximal IgE titers determined by PCA were observed in mice given injections of 10 mg of inactivated native asparaginase per kg in CFA with the prior exposure to 250 rads of whole-body irradiation.

Immunosuppression was investigated by first eliciting an IgE response with 10 mg of inactivated native enzyme per kg in CFA after prior exposure to irradiation. After elicitation of the IgE response, groups of 5 mice were treated with 1 mg of either the native or modified enzyme per kg emulsified in CFA or 20 mg of either the native or modified enzyme per kg without adjuvant (repeated 3 days later). All 4 groups of mice were challenged at a later date with 1 mg native enzyme per kg with adjuvant. The mice were bled from the retroorbital plexus at weekly intervals and assayed for IgE titer by PCA and IgG levels by RID (see below).

Immunotolerance was investigated by immunizing groups of 5 mice by the optimal conditions described above with either the native or the modified enzyme followed by reimmunization 5 weeks later with 1 mg of native enzyme per kg with adjuvant. Mice were bled weekly and assayed for IgE and IgG levels.

Immunoassays. The IgE titer of a serum was measured by PCA in rats. Injections (i.d.) of 0.1 ml of sera in 2-fold dilutions were given 48 hr prior to an i.v. injection of 125 µg of native asparaginase in 1 ml of 0.5% Evans blue dye. Rats were killed after 30 min, and the diameter of the extravasated dye was measured on the underside of the skin. Results were recorded both as the reciprocal of the highest dilution of antiserum giving a positive reaction and as the area of the diffused dye. When sera from mice immunized with the modified enzyme were used, i.v. challenge was with the modified and the native enzyme.

Anti-asparaginase IgG antibodies in serum were measured by RID and competitive radioimmunoassay. Asparaginase (62.5 µg/ml) was incorporated into 1.5% agarose gel, and 5-ml aliquots were dispensed in immunodiffusion plates. Wells were cut, and 5 µl of undiluted sera were introduced. After 14 to 16 hr at room temperature, the diameter of the single precipitin ring was recorded. The addition of the modified asparaginase to the gel in place of the native enzyme gave comparable results when sera from mice immunized with the modified enzyme were used. Antisera elicited to the native enzyme do not cross-react with the modified enzyme, but antisera induced to the modified enzyme will cross-react with the native enzyme (14).

A solid-phase competitive radioimmunoassay was performed with radiolabeled native asparaginase prepared as described in Ref. 14. Dynatech Immulon microtiter plates were coated with a 1:1000-diluted high-titer anti-asparaginase serum in 0.1 M glycine:0.1% sodium azide buffer, pH 9.5. After the plates were washed, 0.1 ml of 1% bovine serum albumin in 5 mM EDTA was added to each well followed by dilutions of the unknown sera and 10,000 cpm of labeled antigen. After overnight incubation at 4°, the plates were washed, and 0.1 ml of 1% sodium lauryl sulfate was added to each well. The contents of the wells were transferred to vials and counted in a Beckman Model 4000 γ-counter. Results were expressed as the percentage of inhibition of the cpm bound in the absence of added competing sera. With 625-fold diluted sera, a RID diameter of 1.5 mm shows 10% inhibition of the cpm bound, 2.0 mm shows 30% inhibition, 2.5 mm shows 60% inhibition, and 3.0 mm shows 80% inhibition. In a related assay in which the antigen rather than the antisera was used to compete for the

labeled enzyme binding, 8 ng of the native enzyme would inhibit the binding of 50% of the cpm in this assay whereas 3200 ng of the modified enzyme were required for 50% inhibition.

RESULTS

IgG and IgE Responses to Poly-DL-alanyl Asparaginase. In evaluating the immunological response to L-asparaginase, one should be cognizant of the fact that the enzyme exhibits immunosuppressive activity, which is probably due to the glutamine requirements of a properly functioning immune system (3). To overcome this difficulty, the enzyme was inactivated with the site-specific reagent DONV. This reagent forms a 1:1 covalent complex at the active site of the enzyme (7). Unlike the *E. coli* asparaginase, the *Erwinia* enzyme does not degrade DONV at an appreciable rate. Therefore, 50% dimethyl sulfoxide, which was necessary to retard the decomposition of DONV by the *E. coli* enzyme (7), was not necessary during inactivation of the *Erwinia* enzyme.

Unlike other allergens (e.g., ragweed, ovalbumin, etc.), it was difficult to evoke a substantial IgE response to inactivated asparaginase as measured by PCA in rats. After evaluating various immunization schedules, doses, and adjuvants (see "Materials and Methods"), we found that, by the use of 250 rads of whole-body irradiation 4 to 5 hr prior to immunization with 10 mg antigen per kg in CFA, a maximal IgE response could be observed. Whole-body irradiation has been shown to preferentially destroy IgE suppressor T-cells (13). With the use of this primary immunization procedure and 1 mg antigen per kg in CFA at 4 and 10 weeks as secondary and tertiary immunizations, the results in Chart 1 were observed. These results demonstrate a total lack of IgE and IgG production in response to the modified enzyme as contrasted to the substantial responses to the native enzyme. Immunization under the above conditions without prior irradiation has produced an IgG response to the modified enzyme but at a reduced level when compared with the native enzyme. We have not found conditions which will evoke an IgE response to the modified enzyme.

Immunological Response to Therapeutic Levels of Native and Modified Asparaginase. Two groups of 10 BD2 F₁ mice were given weekly injections of a standard dose for the treatment of experimental murine leukemia of 5 to 10 IU of either native or modified asparaginase for 14 weeks. When blood samples were monitored for the presence of IgE and IgE anti-asparaginase antibodies, the results in Chart 2 were observed. In the absence of adjuvant and prior inactivation, neither enzyme produced a substantial IgE response, and only the native enzyme produced an IgG response.

Combined Native and Modified Enzyme Immunization. Recent results from other laboratories on the immunological effects of the attachment of polyethylene glycol and D-glutamyl-D-lysyl peptides to allergens have suggested that both the production of specific immunological tolerance and suppression of ongoing sensitivity can be achieved by the administration of the modified allergens (9, 10, 15). Consequently, we have examined desensitization schedules (see "Materials and Methods") involving the native and modified enzyme with and without adjuvant. Such treatments have not reduced significantly the IgE and IgG response to the native enzyme. However, an intervening injection of the modified enzyme without adjuvant resulted in titers 6 to 25% of control following a booster

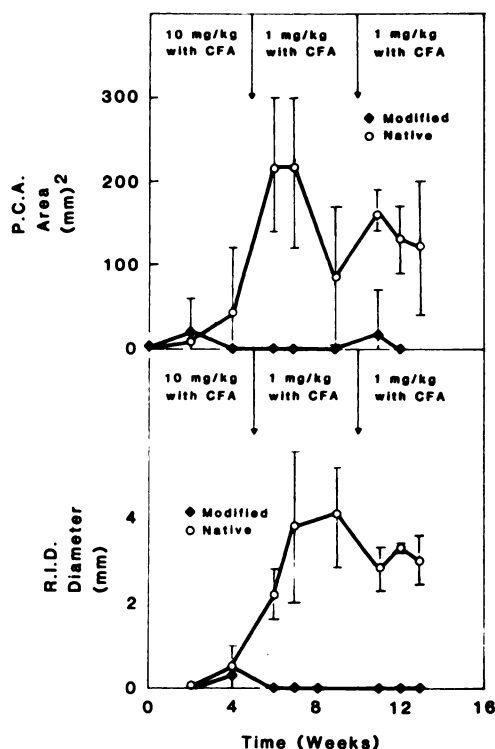


Chart 1. IgE (top) and IgG (bottom) responses of mice immunized with native or poly-DL-alanyl *Erwinia* L-asparaginase. Groups of 5 mice were immunized with 10 mg of DONV-inactivated native or -modified L-asparaginase per kg in CFA 4 hr after 250 rads of whole-body irradiation. Booster injections of 1 mg DONV-inactivated native or -modified enzyme per kg in CFA were given at 5 and 10 weeks. Mice were bled weekly, the serum IgE content was measured by PCA, and IgG content was measured by RID (see "Materials and Methods"). Radioimmunoassay confirmed the lack of an IgG response to the modified enzyme and high levels of IgG to the native enzyme.

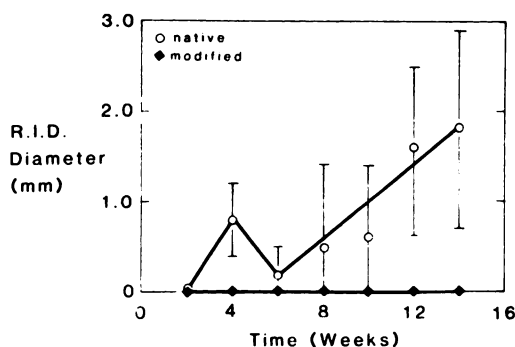


Chart 2. Immunological responses to therapeutic levels of native or poly-DL-alanyl-modified *Erwinia* L-asparaginase. Two groups of 10 mice were given weekly injections of 5 to 10 IU (1.6 mg/kg) of native or 5 to 10 IU (5 mg/kg) of modified asparaginase without adjuvant for 14 weeks. Mice were bled every 2 weeks, and the serum IgE and IgG content was measured by PCA and RID (see "Materials and Methods"). Neither group evoked an IgE response, and all the mice given injections of the native enzyme showed an IgG response.

dose of the native enzyme. Treatment with the modified enzyme prior to immunization with the native enzyme also did not reduce the IgE or IgG titers to the native enzyme. These results do not necessarily suggest that immunological tolerance or suppression cannot be produced by our modified preparations but only that such tolerance or suppression may be schedule and/or dose dependent and consequently difficult to extrapolate to the clinical setting.

Patient Skin Testing. Two patients who had reacted to *E.*

coli asparaginase and therefore had been switched to the *Erwinia* enzyme subsequently developed signs of hypersensitivity (light rash or hives and tightness in the chest). Serum from neither patient inhibited our radioimmunobinding assay, suggesting a lack of circulating IgG antibodies in these patients. Injections (i.d.) of 0.05 ml of 0.5 mg sterile pyrogen-free native or modified enzyme per kg produced a wheal and flare reaction to the native enzyme in one patient, while neither patient reacted to the modified enzyme, and both patients responded to a control of an i.d. injection of histamine diphosphate (0.3 mg/ml). These results suggest that one of these patients was sensitive to the *Erwinia* enzyme, but these antibodies would not cross-react with the modified preparation.

Plasma Clearance Rates in Rhesus Monkey. Under the assumption that primates are better models for the pharmacokinetics of drug distribution and clearance in humans, we investigated the plasma clearance kinetics of the native and polymer-modified enzyme in the rhesus monkey. Chart 3 shows these results. The observed volume of distribution for the native and modified asparaginases was approximately equal to the blood volume (8% of the body weight). The plasma half-life of the modified enzyme ($t_{1/2} = 254$ hr) was over 100 times longer than that of the native enzyme ($t_{1/2} = 1.6$ hr). The half-life determination of the native enzyme was performed both before and after the injection of the modified enzyme. In both cases, a 2-hr half-life was observed, indicating that no circulating antibodies were present which might alter the observed enzyme clearance.

This extended plasma half-life was much greater than the 10-fold longer half-life of the modified enzyme observed in mice and rats (14). However, the short half-life of the native enzyme in the monkey questions how well the monkey model represents the human system where a half-life of 7 to 13 hr has been reported for the native *Erwinia* enzyme (11). Blood counts, liver function tests, and amylase levels were within the normal range at 1, 2, and 3 weeks following the injection of the modified enzyme. Liver function tests consisted of measurements of serum levels of glutamic-oxaloacetic transaminase, lactate dehydrogenase, alkaline phosphatase, and bilirubin.

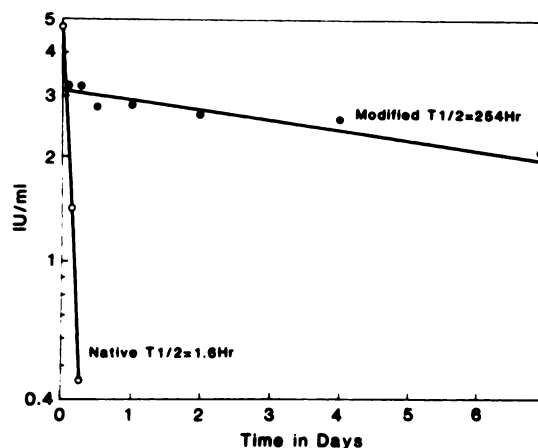


Chart 3. Plasma clearance of native or poly-DL-alanyl-modified *Erwinia* L-asparaginase in the rhesus monkey. One thousand units of sterile pyrogen-free native asparaginase were injected i.v. into an anesthetized (10 mg ketamine per kg) 7.7-kg rhesus monkey. Blood samples were collected from the anesthetized monkey, and asparaginase activity was measured by the coupled enzyme assay of Cooney et al. (2). One week later, the same monkey was given injections of 600 units of sterile pyrogen-free-modified asparaginase, and blood samples were assayed for asparaginase content.

DISCUSSION

Two overlapping but distinct immunological problems are associated with the clinical use of L-asparaginase. They consist of: (a) the development of rapid enzyme clearance and concomitant decrease in therapeutic activity thought to result from the clearance of IgG or IgM antibody:enzyme complexes (8, 12); and (b) the expression of serious hypersensitivity reactions thought to be triggered by IgE:enzyme complexes (8, 12). These hypersensitivity reactions are, in fact, the most undesirable side effects in asparaginase therapy and occur in up to 25% of the patients (8). Patients have demonstrated rapid enzyme clearance with or without hypersensitivity reactions; therefore, neither is predictive of the other (4, 8). Consequently, the immunological response of both the IgE and IgG antibody subclasses to a modified enzyme is of clinical importance. The total lack of IgE and IgG responses to the modified asparaginase under very immunologically stressful conditions (Chart 1) and therapeutic conditions (Chart 2) suggests that clinical trials with these forms should be undertaken. Similarly, the lack of patient skin test cross-reactivity suggests that the modified enzyme may be of value in treating patients who have become sensitized to the native enzyme. The extent to which the 10-fold longer plasma half-life of the modified enzyme in mice and its immunosuppressive activity affect the immunological responses under therapeutic conditions is not known.

Any proposed clinical trial should also take into consideration the much longer plasma half-life of the modified enzyme. The 100-fold increase in plasma life span observed in the rhesus monkey is much longer than the 10-fold increase in half-life observed in mice, which resulted in a more extended depletion in plasma asparagine and improved therapeutic response (14). If such increases in plasma half-life are also observed in humans, it will more than compensate for the loss in activity (60%) brought about by the modification procedure, since pharmacological activity is a composite of both drug concentration and duration of action. The longer half-life of the modified enzyme may also affect its toxicity, but asparaginase toxicity has been shown not to be a strict function of dosage in the 10- to 200-IU/kg/day range (1).

It is interesting to speculate about the mechanism responsible for both the decrease in immunogenicity and prolonged plasma half-life. It is generally accepted in modern immunology that an immunological response to an antigen requires cooperation between helper T-cells, suppressor T-cells, and the antibody producing B-cells. An excess of suppressor cells turns off an antibody response, while an excess of helper cells stimulates the response. What is perhaps less widely recognized is that macrophages need to process and present the antigen to the helper cells in order to stimulate their action. A parallel processing and presentation to suppressor cells does not seem to be necessary. Consequently, the attachment of polymers to the surface of an antigen may interrupt the mac-

rophage processing or presentation and prevent helper cell activation but not suppressor cell activation (15). Similarly, a lack of macrophage engulfment may prolong the plasma life span of the antigen. Therefore, the observed lack of immunogenicity and extended plasma half-lives of the modified asparaginases may be related to the same process. The resistance to proteolytic attack of these modified enzymes (14) may also be part of this process.

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