

ELISA to evaluate plasma anti-asparaginase IgG concentrations in patients with acute lymphoblastic leukemia

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Received 10 January 2000; received in revised form 13 March 2000; accepted 17 March 2000

Abstract

The development of antibodies to asparaginase may attenuate the pharmacologic effect of asparaginase treatment, may be associated with hypersensitivity reactions, and may necessitate switching to a different commercial asparaginase preparation for current or future therapy. Thus, development of an ELISA for measurement of anti-asparaginase antibody levels is important in the clinical setting. An anti-asparaginase antibody reference was established by screening 65 plasma samples from six patients with acute lymphoblastic leukemia (ALL) who had recently developed a hypersensitivity reaction to *Escherichia coli* or *Erwinia chrysanthemi* asparaginase therapy. Twenty-one plasma samples were selected for the anti-asparaginase antibody reference pool. Five micrograms per milliliter of commercial *E. coli* and *Erwinia* asparaginase and 10 µg/ml of *E. coli* asparaginase conjugated with polyethylene glycol (PEG asparaginase) were found to be optimal as coating antigen concentrations. Anti-asparaginase antibody concentrations were determined using a commercial polyclonal goat anti-human IgG horseradish peroxidase conjugate. The antibody reference curves were linear in a range of absorbance from 0.1 to 1.5 O.D. units for dilutions from 1:1600 to 1:51,200. Inter-assay coefficients of variation were 9.04, 14.7 and 13.0%, and intra-assay coefficients of variation were 1.44, 4.43 and 3.28% for antibodies against *E. coli*, *Erwinia*, and PEG L-asparaginase, respectively. The cut-off for positivity in plasma was determined as mean+2 S.D. of the optical density values for plasma from untreated healthy volunteers. Measurement of specific IgG by this ELISA allows for the evaluation of plasma anti-asparaginase antibody concentrations in patients receiving one or more of the multiple commercial L-asparaginase preparations. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: ELISA; L-Asparaginase; Anti-asparaginase antibodies; Acute lymphoblastic leukemia

Abbreviations: ALL, acute lymphoblastic leukemia; O.D., optical density; OPD, o-phenylenediamine dihydrochloride; NHP, normal human plasma; NHS, normal human serum

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1. Introduction

L-Asparaginase is an enzyme that catalyses the hydrolysis of L-asparagine to aspartic acid and ammonia. The antileukemic effect of asparaginase is postulated to result from the rapid and complete

depletion of the circulating pool of L-asparagine, as tumor cells require asparagine for optimal growth (Asselin et al., 1989, 1991; Capizzi et al., 1971). Due to the specificity of the mechanism of activity, asparaginase has been widely used to treat acute lymphoblastic leukemia (ALL) and lymphoma for over 25 years (Evans et al., 1982). Two primary commercial sources of asparaginase are derived from *Escherichia coli* and *Erwinia chrysanthemia* (Evans et al., 1982). The third commercial preparation is a modified *E. coli* asparaginase with two polyethylene glycol (PEG) chains (Asselin et al., 1993). Asparaginase hypersensitivity reactions ranging in severity from mild allergic reactions to anaphylactic shock have been frequently observed (Park et al., 1981; Yoshimoto et al., 1986; Billett et al., 1992; Woo et al., 2000). The reported frequency of anti-asparaginase antibodies is highly variable, and antibodies have been reported in up to 79% of adults and 70% of children following intravenous or intramuscular administration of *E. coli* asparaginase (Oettgen et al., 1970a). Although drug hypersensitivity has most commonly been related to IgE, antibodies to asparaginase have been characterized as IgG (Cheung et al., 1986). Development of anti-asparaginase antibodies may hinder the anti-leukemic effect by shortening its half life (Asselin et al., 1993), interfering with circulating enzyme activity (Peterson et al., 1971), or lowering enzyme activity by deposition of antigen–antibody complex in the reticuloendothelial system (Killander et al., 1976). Hypersensitivity reactions usually require discontinuation of *E. coli* asparaginase, and substitution with either the *Erwinia* preparation or with PEG–asparaginase.

Hypersensitivity reactions to asparaginase in leukemia patients have been characterized by clinical symptoms (Dellinger and Miale, 1976; Steuber et al., 1978; Evans et al., 1982; Billett et al., 1992), and have been correlated with non-specific antibody determination by immunodiffusion (Oettgen et al., 1970b), with passive hemagglutination assay (Peterson et al., 1971), and with radioimmunosorbent assay (RIA) (Dohlwitz et al., 1970; Killander et al., 1976; Cheung et al., 1986). The ELISA assay offers a number of advantages over RIA. Reagents used in the ELISA assay are relatively inexpensive and easy to handle. The peroxidase-labeled reagents are stable

and easily stored for long periods of time without loss of activity. Multiwell microtiter plates, automated plate readers, and multiwell washers allow large numbers of samples to be assayed quickly and easily. Therefore, we describe here an ELISA to specifically measure asparaginase-induced IgG levels in plasma samples from ALL patients. The assay has been standardized and an anti-asparaginase antibody reference pool has been established from the plasma of ALL patients who recently had allergic reactions. Negative controls were obtained from untreated healthy volunteers. Commercial preparations of *E. coli*, *Erwinia*, and PEG asparaginase were used for evaluation of anti-asparaginase antibody concentrations in patients receiving multiple asparaginase preparations.

2. Materials and methods

2.1. Anti-asparaginase antibody reference pool

An initial ELISA was achieved by using one plasma sample from an ALL patient who recently had an allergic reaction. The antibody level of this sample had an O.D. value of 1.0 at a 1:10,000 dilution. Using this plasma sample as a positive control, and normal human plasma as a negative control, we then tested 65 samples from six ALL patients who received *E. coli*, *Erwinia* or both preparations, and of whom one received PEG asparaginase. Subsequently, 21 samples (from six patients) with O.D. values greater than 1.0 at a 1:3200 dilution were selected for establishing an anti-asparaginase antibody reference pool. After combining these 21 samples, the antibody reference was re-analyzed and then aliquoted and stored at -70°C . Normal human plasma (NHP) samples were obtained from young individuals (students at the University of Tennessee, Memphis) who had never received asparaginase. Informed consent was obtained from patients according to Institutional Review Board guidelines and from students according to University of Tennessee Review Board guidelines.

2.2. Asparaginase antigens

Using this anti-asparaginase antibody reference

pool, the optimal concentrations of *E. coli* (Elspar, Merck & Co., Inc.), *Erwinia* (Erwinia, MRA/CAMR, Salisbury, UK) and PEG (Oncaspar, Enzon, Inc.) commercial asparaginase preparations were standardized as distinct antigens. The ELISA was performed with these three antigens using separate well-coating concentrations ranging from 0.3125 to 40 µg/ml in the same plate. The antibody reference plasma was diluted 1:3200 and aliquoted to each well. NHP at 1:3200 (v/v) was used as a negative control. To ensure that the antigen completely saturated the plate, the optimal concentrations of the three coating antigens were chosen as the second concentration above that defining the plateau absorbance value.

2.3. Measurement of anti-asparaginase IgG antibodies by ELISA

Microplates (Nunc-Immuno plate, Thomas Scientific Product, cat. no. 62409-50) were coated with 100 µl of *E. coli*, *Erwinia* or PEG asparaginase commercial preparations diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.5 (Sigma cat. no. S7795/S6297; Sigma, St. Louis, MO, USA) and incubated overnight at 2–8°C. The wells were drained without washing and blocked for at least 90 min at room temperature with 300 µl/well of a solution of 0.1 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% bovine serum albumin (BSA; Sigma cat. no. A7888) and 0.05% Tween-20 (Sigma cat. no. P7949). The plates were then washed three times with 0.05% Tween-20 in saline. For each asparaginase preparation, 100 µl of two-fold serial dilutions of the antibody reference, NHP, or plasma samples with 0.05% Tween-20 in PBS were added, then incubated in appropriate wells for 1 h at room temperature. After incubation, the plates were again washed as described above. A polyclonal goat anti-human IgG horseradish peroxidase conjugate (specific for human IgG, but not for IgA, IgM, Bence Jones Kappa and Lambda myeloma proteins, Sigma cat. no. A6029) was freshly diluted to 1:10,000 (v/v). A 100-µl volume of diluted conjugate was added to each well and incubated for 1 h at room temperature. The wells were drained, washed with Tween-20–saline four times, and incubated with 100 µl of a freshly made substrate solution containing 20

mg of *o*-phenylenediamine dihydrochloride (OPD; Sigma cat. no. P1526) in 50 ml of 0.1 M citrate buffer, pH 6.0, and 167 µl of 3% H₂O₂ for 30 min at room temperature in the dark. After exactly 30 min, a 100-µl aliquot of 1.0 M phosphoric acid solution was added to stop the reaction. Anti-asparaginase antibody concentrations were measured at 490 nm for the enzymatic product (subtracting the absorbance at 650 nm for non-specific absorbance) using a microplate reader (Model: THERMO max, Molecular Devices). The cut-offs for positivity in plasma were determined as the mean plus two standard deviations of the O.D. values determined for plasma from healthy volunteers. Assays were performed in duplicate.

2.4. Statistical analysis

The data are expressed as the mean ± S.D., unless otherwise indicated. The statistical analysis was performed using an unpaired Student's *t*-test.

3. Results

3.1. Validation of reagents

3.1.1. Positive antibody reference pool

Sixty-five plasma samples from six ALL patients who had allergic reactions to *E. coli* asparaginase were collected for an initial test. Plates were coated with 5 µg/ml of *E. coli* asparaginase and incubated with four two-fold dilutions of 1:400, 1:800, 1:1600, and 1:3200 for the analysis of each sample. The samples whose titration curves showed evidence of saturating the ELISA at a dilution of 1:3200 (i.e., O.D. > 1.0) were selected to establish an anti-asparaginase antibody reference pool. The antibody reference pool was established from 21 samples and continues to exhibit very high titers against *E. coli* asparaginase as well as *Erwinia*- and PEG asparaginase. This antibody reference was used to establish the linearity of standard curves, and, for the controls, to evaluate intra-assay and inter-assay variation in the analysis of ALL plasma samples.

3.1.2. Coating antigens

E. coli, *Erwinia*, and PEG asparaginase were

coated on microtiter plates with concentrations ranging from 0.3125 to 40 $\mu\text{g/ml}$. The antibody reference pool and NHP at a dilution of 1:3200 (v/v) were used. The antibodies appeared to bind to *E. coli*, *Erwinia*, and PEG asparaginase at all antigen concentrations examined. The absorbance approached an apparent plateau for both *E. coli* and *Erwinia* asparaginase at a concentration of 1.25 $\mu\text{g/ml}$, and for PEG asparaginase at a concentration of 2.50 $\mu\text{g/ml}$ (Fig. 1). However, there was no significant antibody binding with NHP. The optimal concentrations were determined as the second concentration higher than the dilution that yielded the absorbance value of the plateau. The concentrations were defined as 5 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$ for *E. coli*, *Erwinia*, and PEG asparaginase, respectively. These coating antigen concentrations were

used for validation of the assay and for analysis of patient samples.

3.1.3. Reactivity of anti-asparaginase antibody in plasma

Plates were coated with 5 $\mu\text{g/ml}$ of *E. coli* asparaginase antigen. Twenty-two plasma samples (NHP) from normal volunteers who had never received asparaginase, and 22 plasma samples from ALL patients (subset from Woo et al., 1998) at the end of induction of asparaginase administration (no hypersensitivity reactions) and 22 plasma samples from the same patients at the end of reinduction of asparaginase administration (all had hypersensitivity reactions) were incubated in the plates at a 1:3200 dilution. Fig. 2 indicates that the O.D. readings from

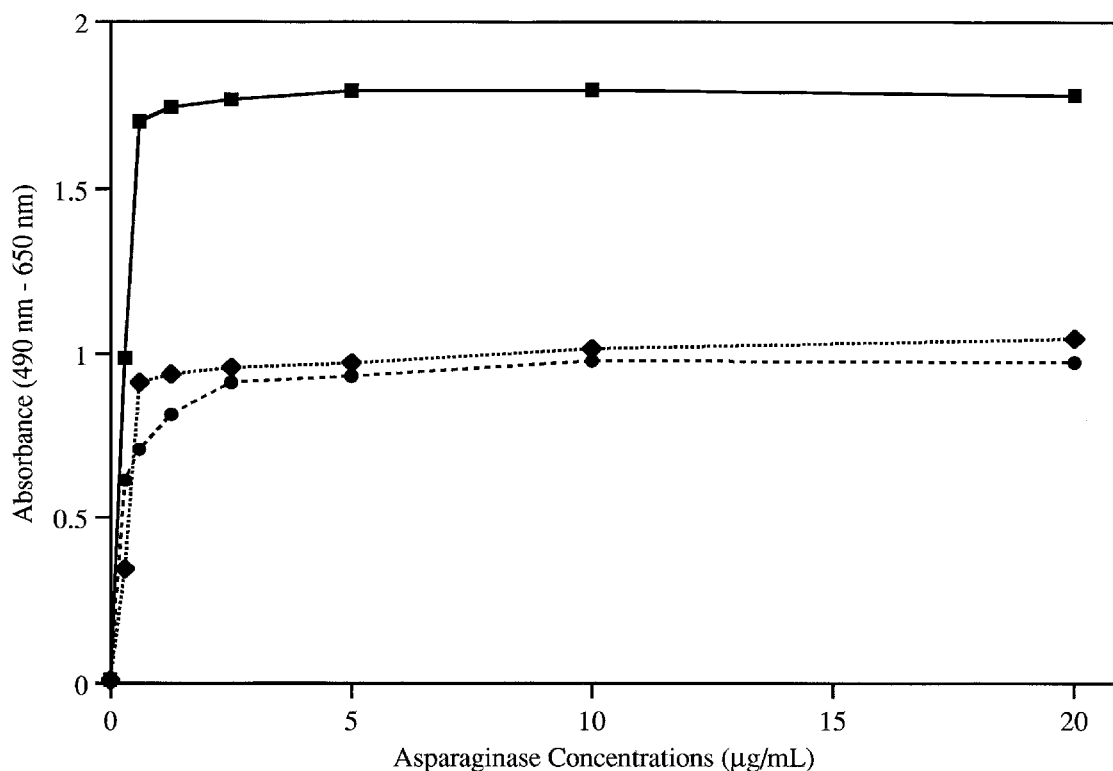


Fig. 1. Determination of the optimal concentration curve for *E. coli* (■), *Erwinia* (◆), and PEG (●) asparaginase antigens using a 1:3200 dilution of the anti-asparaginase antibody reference pool. The data for antigens at 40–80 $\mu\text{g/ml}$ are not shown.

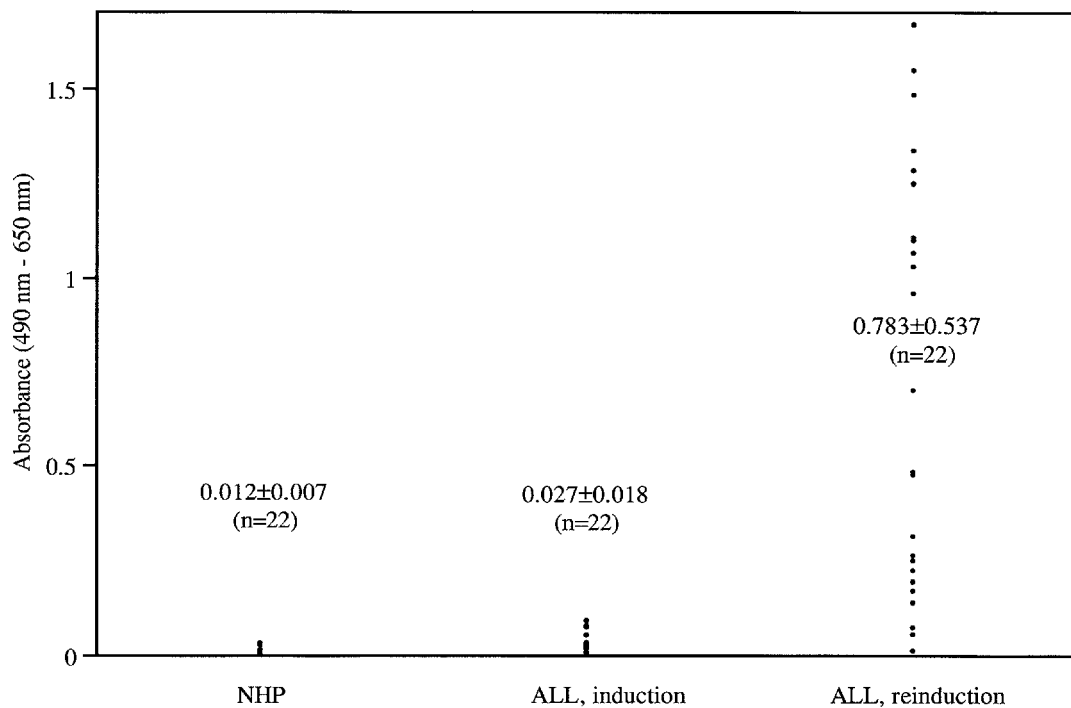


Fig. 2. Reactivity of anti-asparaginase antibodies in plasma of normal volunteers (NHP) compared to ALL patients at the end of induction of asparaginase administration (no hypersensitivity reaction), and to the same ALL patients at the end of reinduction of asparaginase administration (all had hypersensitivity reaction). The inset values are expressed as the mean $\pm$ S.D. (Number of samples) from that group.

NHP (0.012 ± 0.007) were different from those from plasma samples of ALL patients at the end of induction (0.027 ± 0.018 , $P < 0.001$). However, O.D. readings were much higher in plasma samples of ALL patients at the end of reinduction (0.783 ± 0.537 , $P < 0.001$ vs. NHP, $P < 0.001$ vs. ALL at the end of induction).

3.1.4. Reactivity of peroxidase-conjugated anti-human IgG

Three antigens coated in plates were incubated with the antibody reference pool at a 1:3200 dilution. Plates were then incubated with peroxidase-conjugated goat anti-human IgG polyclonal antibody at dilutions from 1:2500 to 1:20,000. Fig. 3 indicates that the reactivity of the conjugate is approximately linear across these dilutions. The dilution 1:10,000 was used for all subsequent assays.

3.2. Assessment of efficiency

3.2.1. Linearity of an anti-asparaginase antibody reference pool

The antibody reference pool and NHP samples were tested for linearity in two separate experiments on different days. Fig. 4 shows that the O.D. reading is approximately linear between 0.1 and 1.5 with antibody reference dilutions from 1:800 to 1:51,200 ($y = 0.292x - 0.229$, $r^2 = 0.998$ in experiment 1; $y = 0.278x - 0.216$, $r^2 = 0.997$ in experiment 2).

3.2.2. Reproducibility

To assess the intra- and inter-assay variability, independent repeats of the antibody reference pool were examined by ELISA. The mean, standard deviation, and coefficient of variation were determined (Table 1). The coefficients of variation were 1.44, 4.43, and 3.28% in intra-assay variability and

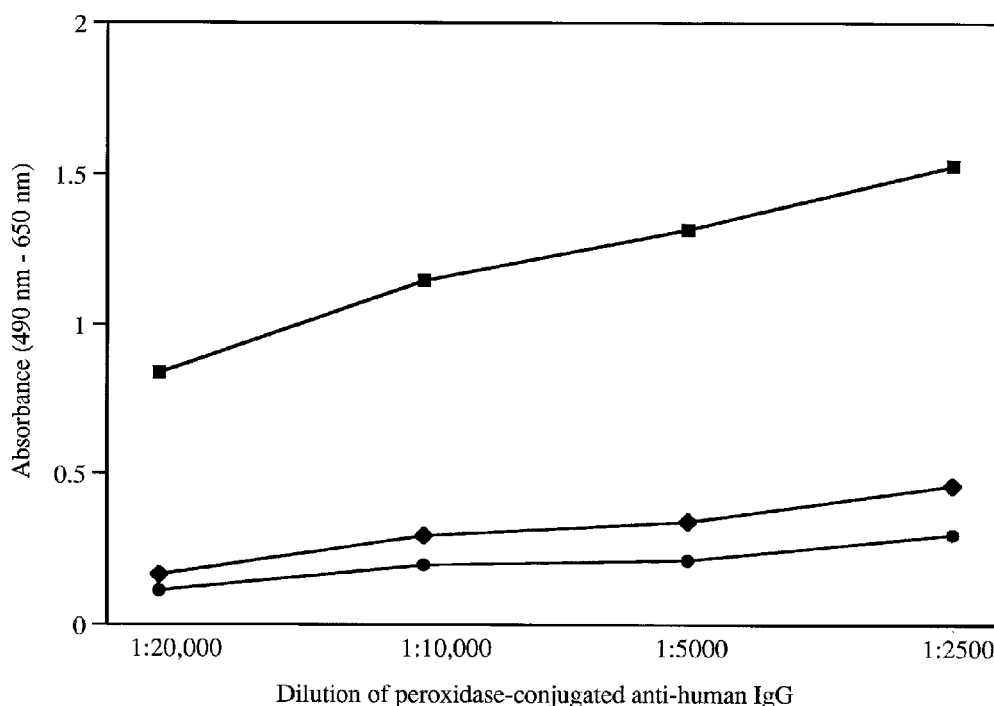


Fig. 3. Reactivity of peroxidase-conjugated goat anti-human IgG polyclonal antibody using a 1:3200 dilution of the positive reference pool. *E. coli* (■), *Erwinia* (◆), and PEG (●) asparaginase antigens.

9.04, 14.7, and 13.0% in inter-assay variability, respectively, for *E. coli*, *Erwinia*, and PEG asparaginase at a 1:3200 dilution of the antibody reference pool.

3.2.3. Absorption of nonspecific antibody

The cut-off value for positivity was established based on samples from 20 healthy volunteers. The plasma samples were analyzed at dilutions of 1:50, 1:400, 1:800, 1:1600, and 1:3200 (Fig. 5). The cut-off values for describing positivity of anti-asparaginase antibodies were chosen as the mean value plus 2 S.D. Based on these data, these values can be defined as greater than 0.108 at a 1:400 dilution or greater than 0.017 at a 1:3200 dilution.

4. Discussion

The development of an ELISA for determining anti-asparaginase IgG antibodies was limited by the

source of anti-asparaginase-positive antibodies, which could only be obtained from patients who had an allergic reaction to asparaginase or from asparaginase-immunized animals. We chose to establish a positive antibody reference pool by examining anti-asparaginase-specific IgG levels in ALL patient plasma samples. Since the plasma samples were collected from ALL patients who received two or three asparaginase preparations, antibodies in the reference pool appeared to react with *E. coli* asparaginase as well as *Erwinia* and PEG asparaginase. The antibody reference pool provides a reasonable amount of positive antibody reference with a constant high antibody titer against three commercial asparaginase preparations and can conceivably be used for long-term studies.

Due to the possible use of multiple asparaginase preparations in ALL patients, we have used the anti-asparaginase antibody reference plasma to standardize the concentrations for coating three commercial asparaginase antigens in an ELISA. This ap-

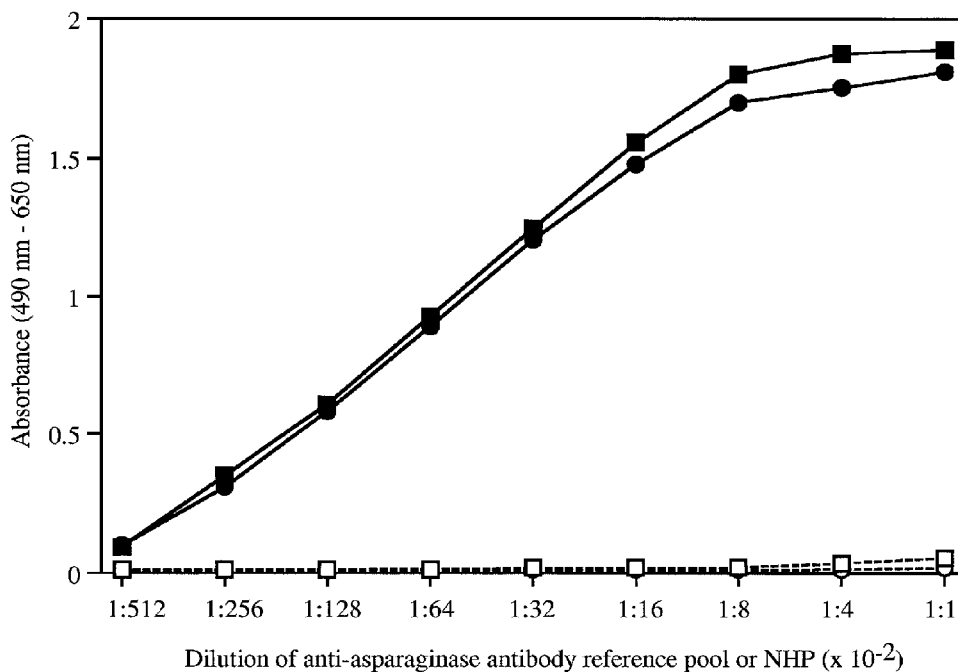


Fig. 4. ELISA showing the linearity of the antibody reference (Ab^+) between absorbance values of 0.1 and 1.5 at 490 nm at dilutions ranging from 1:800 to 1:51,200 in two experiments with the negative control (NHP). Ab^+ (■), NHP (□) in experiment 1; Ab^+ (●), NHP (○) in experiment 2.

proach provides for the measurement of anti-asparaginase IgG antibodies against three antigen preparations in a single microtiter plate.

In order to accurately determine the anti-as-

paraginase antibody concentration, sample dilutions were carefully chosen based on the linearity of the antibody reference pool as well as on the background absorbance of untreated healthy volunteers. The antibody reference pool was linear starting at a 1:800 dilution. The background absorbance of healthy volunteers showed no significant difference at dilutions of 1:400 and greater. Therefore, the dilutions of 1:400, 1:800, 1:1600, and 1:3200 have been used for sample analysis. The cut-off calculation for determination of the positivity for anti-asparaginase IgG in plasma was based on the mean of the absorbance found for healthy controls plus two S.D., similar to Belec et al., 1995 and Artenstein et al., 1997.

In the process of developing this ELISA, reducing the plate's background reading was deemed critical. To determine optimal blocking conditions for the assay, various protocols using BSA and the non-ionic detergent Tween-20, or a combination of the two plus normal human serum (NHS) were assessed. The inclusion of NHS in the blocking and antibody

Table 1
Reproducibility of ELISA

Antigens	Cut-off index ^a (mean±S.D.)	Coefficient of variation (%)
Intra-assay (n=8)		
<i>E. coli</i>	1.339±0.019	1.44
<i>Erwinia</i>	0.274±0.012	4.43
PEG	0.332±0.011	3.28
Inter-assay (n=10)		
<i>E. coli</i>	1.311±0.119	9.04
<i>Erwinia</i>	0.214±0.031	14.7
PEG	0.313±0.041	13.0

^a O.D. readings at a 1:3200 dilution in anti-asparaginase antibody reference pool.

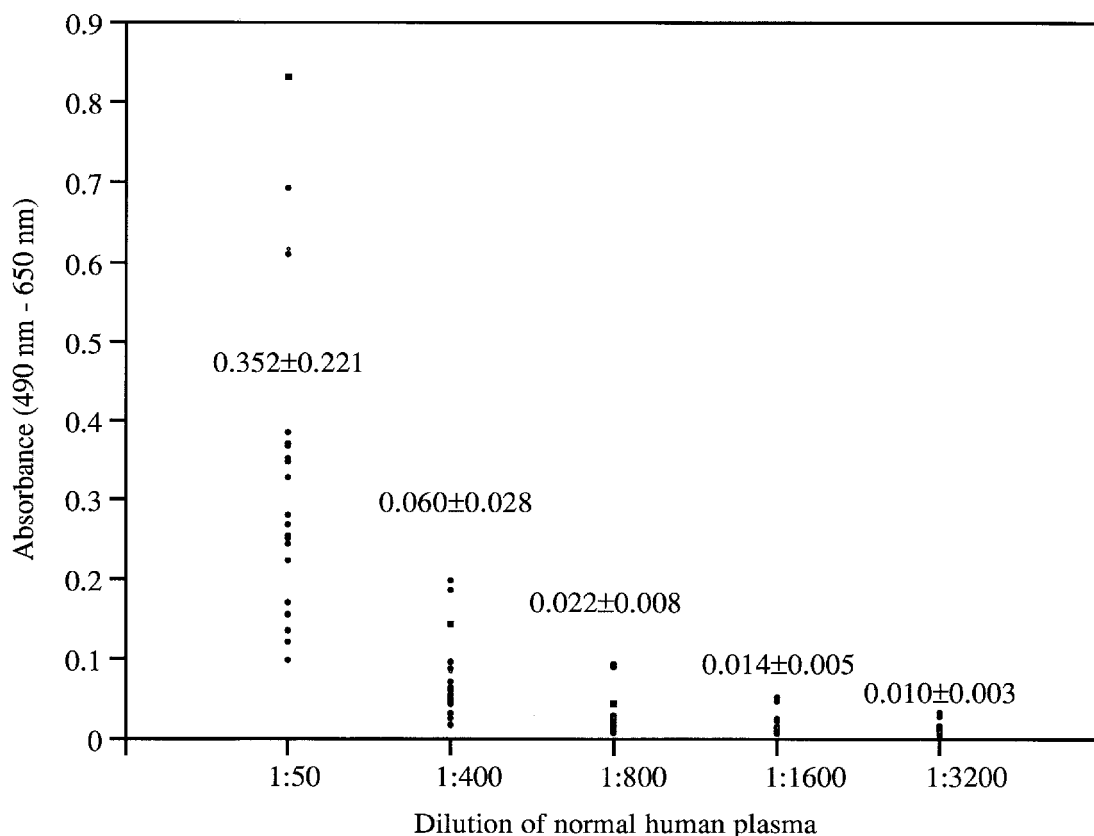


Fig. 5. Absorption of non-specific antibodies in normal human plasma. The inset values are expressed as the mean $\pm$ S.D. ($n=22$).

diluting buffers resulted in higher background (data not shown).

During assay development, we found that the inter-assay variations were largely influenced by two factors: the preparation of the substrate solution and the incubation period of the substrate solution. We had less inter-assay variation (%C.V.<15%) when we first dissolved OPD in citrate buffer in the dark for 10 min and then quickly added H_2O_2 to the solution before finally adding the substrate solution to the plates.

While developing and validating this assay for plasma samples, we had the opportunity to compare plasma versus serum samples collected at the same time from the same patient. For the limited samples available, plasma and serum samples gave similar absorbance values (data not shown). We suggest that this ELISA may be used for either plasma or serum samples.

This assay has been used to evaluate anti-*E. coli* asparaginase IgG levels in the plasma samples of ALL patients (Woo et al., 1998). That study indicated that patients with hypersensitivity reactions had higher antibody levels than similarly treated patients who did not have reactions. Therefore, antibody analysis may be of clinical value in future treatment.

Acknowledgements

This study was supported by a grant from Rhône-Poulenc Rorer Pharmaceuticals, Inc., a grant from the state of Tennessee Center of Excellence in Pediatric Pharmacokinetics and Therapeutics, the National Institutes of Health, Bethesda, MD, Cancer Center CORE grant no. CA-21765, CA-51001, and American Lebanese Syrian Associated Charities (ALSAC).

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