Immunogenicity of Native or Pegylated *E. coli* and *Erwinia* Asparaginases Assessed by ELISA and Surface Plasmon Resonance (SPR-Biacore) Assays of IgG Antibodies (Ab) in Sera from Patients with Acute Lymphoblastic Leukemia (ALL)

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Abstract. *Background: Therapeutic uses of asparaginases (ASNase) have been shown to elicit immune responses resulting in the development of potentially life-threatening human anti-bacterial antibodies (Ab). A robust screening enzyme-linked immunosorbent assay (ELISA) to detect binding Ab(+) against ASNase has been developed and validated for therapeutic monitoring to support clinical trials. Recently, a protein chip bioassay (Biacore) was developed for the Ab of these proteins. These methods were compared. Materials and Methods: A Biacore T-100 analyzer using a protein bioassay and an ELISA assay were used to determine the IgG immmuboglobulin Ab against ASNase in sera from 84 acute lymphoblastic leukemia (ALL) patients plus 6 controls (n=121 samples). These samples were characterized for anti-ASNase Ab neutralizing activity. Human E. coli ASNase, pegaspargase and Erwinase proteins were covalently coupled to the carboxy-methylated dextran matrix of a CM5 sensor chip (surface plasmon resonance, SPR). In the course of a nested experimental design, a wide range of human sera from patients who had obvious clinical allergic reactions after either native or pegaspargase treatments were tested. The data were fitted by a parametric logistic equation (±95% confidence interval, CI), which ranged from <3.0% to <14%. Results: The specificity of Ab(+) was evaluated using "spiked" human IgG antibodies. Both assays provide near excellent linearity and sensitivity of response (<0.8 to <500 ratio and 1-3000 resonance units [RU]) of anti-ASNase Ab in human sera with low variance. The bioassay method was ten times more sensitive than the ELISA Ab assay. The lowest limit of quantification of Ab(+) ratio for the SPR assay was 0.6 whereas the upper limit of quantification was 3,000 RU. The SPR assay results were in excellent accord with both the Ab(–) and the Ab(+). Ab(–) by the ELISA method (<1.003 ratio) was related to a mean RU value of 8.1. Despite the narrow range of ambiguity around the 1.1 Ab(+) ratio values, the majority of the specimens (93.2%) were determined to be Ab(+) by either ELISA or SPR determination. Conclusion: The vast majority (81/84 = 96.4%) of the IgG Ab(+) were neutralizing. The SPR Ab determination technique is reliable, accurate and more sensitive than the ELISA method.*

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Key Words: Immunogenicity, asparaginases, antibody, IgG immunoglobulin, ALL.

Protein therapeutics can elicit an immune response after multiple administrations (1-3). When antigen-binding antibody (Ab) is developed, the subsequent immune reaction may cause unexpected adverse effects, including Ab-inactivation of the antigen, resulting in severe allergic reactions. In this case, discontinuation of treatment is suggested, which may limit the long-term event-free survival (EFS) of patients. Therefore, it is essential that Ab assays of therapeutic proteins be applied during clinical treatment assessments. Asparaginases (ASNase) are bacterial proteins that are used in the treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma (2-8). Many clinical studies have reported a wide range of human anti-ASNase Ab(+) formation, all of which have been assayed by an antibody-capture enzyme-linked immunosorbent (ELISA) assay (2, 3-10).

The presence of Ab in patients with hypersensitivity reactions to ASNase formulations leads to a more rapid clearance of the enzymatic activity and hence in the half-life of native or pegylated (PEG) *Escherichia coli* preparations (3, 9, 10). The Ab(+) in a subpopulation of ALL patients has been correlated with inferior outcome (3). The clinical

allergic symptoms in these patients ranged from localized skin rash and swelling to respiratory difficulties and/or anaphylaxis. Neutralizing anti-PEG-ASNase Abs have also been seen in 85% of allergic patients, although some patients with an apparent clinical reaction were found to have no Ab and substantial ASNase activity (11). Once an immune response has been detected, a non-cross reacting ASNase (Erwinase) can be administered (2, 3). Therefore, monitoring ASNase activity and Ab may be useful for guiding ASNase therapy in ALL.

In patients who developed hypersensitivity post-ASNase treatments, an inverse relationship between high Ab(+) levels and low ASNase activity due to neutralization of the antigen-drug has been found (2, 3). Clearly, the immune response is very heterogeneous, thus assessment methods must be able to detect different Ab isotypes (IgG, IgM *etc*.), their affinities and specificities. A limited number of publications comparing ELISA with other assays for Ab to protein-drugs for the determination of their immunogenicity have been reported (12). However, a simple, reliable, accurate and rapid method is needed to determine the Ab presence in large numbers of patients. Surface plasmon resonance (SPR) (Biacore) has emerged as a biosensor assay that allows for the sensitive detection of both low and high affinity Ab (13). This biosensor Ab assay has been developed to detect Ab(+) binding to immobilized ASNase protein testing Ab(–) and Ab(+) sera for the presence of neutralizing IgG or IgE Ab(+) (14). A single amino acid (tryptophan) can be the cause for a diagnostically relevant peptide from a major allergen/antigen of *Aspergillus fumigatus*, a mechanism determined by kinetic studies with higher binding to its Ab (SPR) (15). The SPR and ELISA Ab detection methods were compared in sera specimens from patients and in volunteers in terms of sensitivity, specificity and reproducibility.

Materials and Methods

Equipment and reagents. The following instruments and reagents were available from GE Lifesciences Uppsala, Sweden. A Biacore T100 processing unit; Biacore CM5 Sensor Chip (part number BR-1006-68); 1x Biacore HBS-EP+ (part number BR-1008-26) (0.1 M HEPES, 1.5 M NaCl, 30 mM EDTA and 0.5% v/v surfactant P20, pH 7), and Amine Coupling Kit 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (part number BR-1000-50), *N*-hydroxysuccinimide (NHS) and 1.0 methanolamine-HCl, pH 8.5.

Biacore antibody assay: creating a sensor surface. Using Biacore's CM5 sensor chips and following standard amine chemistry protocols, *Erwinia* ASNase was diluted to 150 mg/ml in 10 mM sodium acetate (pH 4.5) and immobilized to a level of 4,000 resonance units (RU) into flow cell 2. *E. coli* ASNase was also diluted to 150 mg/ml in 10 mM sodium acetate (pH=4.0) and immobilized to a level of 2,000 RU into flow-cell 4. Flow cells 1 and 3 were activated and blocked and assigned as "blank" controls. RU is a measure of a 0.0001 degree-shift in refractive index. It is approximately equivalent to 1 pg/mm^2 in dextran. The RU for each serum specimen, both control and experimental samples, were obtained over 20 hours after multiple cycles of analysis. The reproducibility of these values was superimposable from assay to assay.

Excellent linearity between $\log_{10}$ antibody ratio (ELISA assay) *vs*. the $\log_{10}$ Ab(+) at dilution of 1:1,000 of the original serum specimen has previously been determined (10). The ELISA Ab(+) assay was determined in triplicate in n=3 separate experiments of the same stock Ab(+), but with separate dilutions, or total n=9 determinations.

Binding analysis. The Ab assay was performed using Biacore's HBS-EP+ running buffer of the protein-embedded chips. The serum was diluted 1:10 with running buffer and was injected over all four sensor surfaces (SPR) for 60 seconds at 30 ml/minute, followed by a 60 second injection of anti-isotype specific antibody. The complex was regenerated back to immobilized protein-drug (ASNase) using 10 mM glycine, pH 2.0. Biacore software can be constrained to comply with the Food and Drug Administration's (FDA) regulation 21 CFR - part 11.

Patients and treatment. Between May 2004 and November 2006, patients with newly diagnosed ALL and HR features receiving institutional ALL chemotherapy treatments, which included *E. coli* native ASNase or pegaspargase, after the appropriate institutional board review (IRB) and the individual informed consents had been obtained. Serum specimens were evaluated on a compassionate basis from patients (84 pediatric patients, n=115 samples) who developed clinical allergy to native ASNase or pegaspargase treatment, plus 6 sera from volunteers (11). When Ab(+) was found, the patients were treated with alternative chemotherapy after discontinuation of ASNase treatments. The majority of the patients who reacted with obvious clinical allergy post-ASNase treatment were male >10 years of age (HR ALL).

ASNase formulations. All the preparations of ASNase were given by intramuscular (IM) injection. The doses were 6,000 IU/m^2 for each dose of native *E. coli*-ASNase (ELSPAR, Merck and Co. Inc. West Point, PA, USA) and 2.5 IU/m^2 for pegaspargase (2, 3, 9-11).

Clinical sample testing. An enzymatic activity assay and ELISA for the quantification of Ab have been developed previously with quality controls for *E. coli*, pegaspargase, or *Erwinia* ASNase activity, anti-*E. coli*, anti-pegaspargase, and/or anti-*Erwinia* ASNase Ab in human sera (3, 9, 10). The linearity of the calibration lines, the lower limit of quantification (LLOQ), the inter-and intra- batch accuracy and precision of these assays were excellent (3, 9, 10).

Statistical analyses. Population parametric analyses were used to determine the critical statistical values in a similar manner as the one reported previously (3, 9, 10).

Results

Response and function of linearity of ELISA and Biacore Ab(+) assays. The sera from six individuals who were Ab(–) by ELISA had very low RU values (average= 8.1±6.6 RU). There was a wide range between low Ab(–) and high titer

anti-*E. coli* ASNase Ab(+) repeated with high reproducibility in 120 cycles (20 hours). One patient who had the highest Ab(+) by ELISA assay (290 ratio) had a value of 2,750 RU. Thus, the SPR Ab assay could distinguish between an Ab(+) from Ab(–) sera at very low RU values and it was effective over a wide range: (means: 8.1-2800 RU). The specificity of Ab(+) was evaluated by "spiking" human IgG Ab and observing the RU response. All neutralizing Ab(+) were IgG. Moreover, the association and dissociation rate constants of Ab(+) to specific ASNase antigen in each flow-cell was measured.

An excellent relationship existed between the $\log_{10}$ RU by SPR assay *vs*. the $\log_{10}$ Ab ratio obtained from the ELISA assay. All but one set of data fell within the 95% CI of the linear relationship between ELISA and SPR (Biacore).

Only 3 out of the 84 patients (3.6%) who were Ab(+) by ELISA were found not to be anti-ASNase IgG Ab(+) by the SPR method, as shown in Figure 1. The cut-off point of Ab(+) or the LLOQ below which all the values were considered as Ab(–) was then calculated as 8.1 RU and 1.003 ratio over (–) control for the Biacore and ELISA assays, respectively (Figure 1). The Ab(–) values were statistically significantly different from the Ab(+) values at $p>0.05$. All Ab(–) samples remained Ab(–) in 120 cycles by SPR methods, as they had by repeated ELISA determinations.

Comparison of the ELISA and SPR for assessing clinical immunogenicity of IgG antibodies (Ab). The mean (±SEM) Ab ratio in the Ab(–) patient samples was 1.003±0.69 by ELISA compared with 8.1±6.6 RU (mean±SEM) from the SPR method. In contrast, the Ab(+) sera specimens had a mean of 4.8±1.1by the ELISA assay as compared to 255.7±85.3 RU for the SPR method ($p=0.04$). Eighteen out of the 21 (85.71%) samples which were determined to be Ab(–) by ELISA method (<1.1 ratio) were well below an RU value of 8.1. Figure 1 shows the comparison of the Ab(+) ratio as determined by ELISA assay and RU units as determined by the SPR method in the Ab(–) ($n_1=21$) and Ab(+) ($n_2=63$) patients (N=115 + 6 Ab(–) total samples). In 18 out of 21 patients who were initially Ab(–) by ELISA and SPR, upon repeated sampling in approximately 4 weeks time became Ab(+). Despite the narrow range of ambiguity around the 1.1 Ab(+) ratio values, the vast majority of the specimens (93.2%) were determined to be Ab(+) by either ELISA or SPR determination. These sets of data (square symbols) are illustrated in Figure 1 as being above the 8.1 RU line for the SPR method, demonstrating a positive Ab(+), and to the right of 1.003 Ab ratio, as depicted by the ellipsis and indicating an Ab(+) value by the ELISA method.

Only one of the very high Ab(+) ratio specimens to both native and pegaspargase cross-reacted with Erwinase. The lack of cross-reactivity between *E. coli* and *Erwinia* ASNase formulations was reassuring.

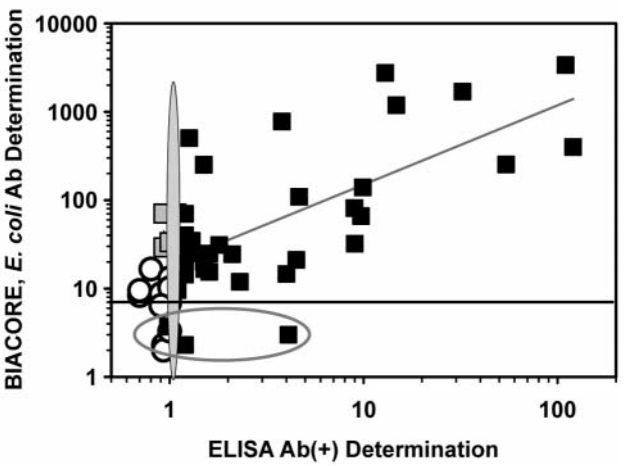

Figure 1. *Comparison of the ELISA and Biacore protein-antibody interaction methods of Ab(+) (squares) and Ab(–) (circles) sera for the native anti-E. coli asparaginase antibody. Three serum samples which were determined to be Ab(+) by the ELISA assay appeared to be Ab(–) by the Biacore anti-E. coli ASNase assay.*

Discussion

Anti-leukemic therapy protocols require the use of asparaginases. Since these bacterial proteins induce immune responses, monitoring the anti-asparaginase Ab and its antigen-neutralizing activity may serve as a predictor of poor prognosis (3). Currently there are accepted ELISA methods for the evaluation of anti-ASNase Ab in patients (3, 6-11). However, the uncertainty of below the LLOQ (BLLOQ) Ab by ELISA alone in patients with obvious clinical allergy symptoms needs to be clarified. To this end, an SPR assay with high sensitivity, specificity, and reproducibility of the RU response has been developed. A wide range of IgG Ab(+) sensitivity in RU values from 1 to <3,000 RU was achieved by the SPR assay, although the majority of the Ab(+) serum specimens were in the 20 to <300 RU range (Figure 1). The results demonstrated that the SPR assay could detect weak affinity Ab(+) by sensitivity to an approximately 10-fold lower concentration level, at which the Ab could be washed away in the ELISA assay. The difference in performance between these assays is related to the real-time analysis of the SPR method occurring over 300 seconds, compared with the ELISA, which is an end-point analysis (24 h). Furthermore, the SPR method determined the subtype of Ab (IgG) and whether or not the Ab was neutralizing, which were not detected by the ELISA assay.

During cross-validation, using the same Ab(+) and Ab(–) serum controls (blinded samples), the SPR assay was shown to be more sensitive in RU values than the Ab(+) ratio over negative control from the ELISA assay. This evidence provided the external validation for each method, with the

SPR being more sensitive and able to identify clearly the isotype of the Ab(+) formed.

The IgG sub-type of Ab(+) is suggested to be the cause of the post-ASNase administrations, allergic clinical reactions in patients. This isoform of Ab(+) was determined by the SPR method. This type of Ab(+) against ASNase is regarded as critical for patient safety and efficacious treatment, possibly requiring intervention, *i.e.* treatment with *Erwinia* ASNase. The vast majority of the IgG Ab(+) were neutralizing suggesting that the Ab(+) negated the pharmacodynamic (PD) efficacy against ALL (3, 9, 10).

Finally, due to the very low "noise" of the Biacore T-100 unit, the range between LLOQ and BLLOQ RU signals was very wide so that the probability of false-negative results was diminished. Thus, it is proposed that the SPR assay detects Ab(+) in samples faster, with accuracy and with more specificity against ASNase than the ELISA method. This very sensitive and specific SPR Ab detection method is now available for use in determining immunogenicity to biological oncology products. Clinical trials with pegaspargase in childhood and adult ALL are pending.

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Received July 18, 2008
Revised December 3, 2008
Accepted December 8, 2008