NOTES

Relative Avidities of Human Immunoglobulin G Antibodies for Streptococcal Pyrogenic Exotoxins A and B

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Group A streptococci (GAS) are a common cause of a wide variety of mild human infections, including pharyngitis, scarlet fever, and erysipelas. In addition, GAS are sometimes involved in life-threatening diseases like streptococcal toxic shock-like syndrome (TSS) and necrotizing fasciitis. Since the mid-1980s, outbreaks of severe GAS infections have been reported in various parts of the world (1, 14). Despite prompt antibiotic treatment, the mortality rate in patients with these syndromes remains high (30 to 80%) (1, 14). Predisposing conditions such as advanced age, diabetes, malignancy, and immunosuppression have been recognized but, remarkably, a substantial proportion of patients consists of previously healthy, young adults (1, 14). Of the extracellular GAS products, streptococcal superantigens, including streptococcal pyrogenic exotoxin A (SPE-A) and SPE-B, have been implicated in the pathogenesis of streptococcal TSS and necrotizing fasciitis (1, 14). Properties of these pyrogenic exotoxins include pyrogenicity, the ability to induce vast lymphocyte proliferation, and the enhancement of host susceptibility to endotoxin shock. Superantigens are microbial proteins capable of activating a large proportion of lymphocytes, thereby causing an excessive release of proinflammatory cytokines (5, 6).

We have previously demonstrated that patients with invasive GAS disease are relatively deficient in antibodies against SPE-A and SPE-B compared with healthy individuals (7, 8). In addition, anti-SPE-A antibodies were associated with a favorable outcome of TSS (8). In vivo and in vitro studies have suggested that intravenous immunoglobulin (IVIG) preparations would be beneficial as adjunctive treatment for patients with severe GAS infections (10, 11). The mechanism by which IVIG may improve clinical outcome in the setting of acute infectious diseases has not been totally elucidated. Nevertheless, in vitro studies have revealed that IVIG has neutralizing activity against a large variety of superantigens released by clinical GAS isolates. Evidence has been presented that the superantigen-counteracting effect of IVIG is conferred to patients following treatment (10). However, sera that contain exotoxin-specific antibodies do not always inhibit exotoxin-induced proliferation (9). In addition, several studies have shown variation between individuals and even between different IVIG preparations with regard to the levels and neutralizing capacities of SPE-specific antibodies (9, 10). The present study was designed to investigate the average functional capacities of polyclonal immunoglobulin G (IgG) antibodies to bind SPE-A and SPE-B, comparing sera from patients with fatal GAS infections with sera from healthy individuals and with IVIG preparations. The affinity values obtained represent the overall binding properties of a heterogeneous population of antigen-specific serum antibodies as measured by competitive enzyme-linked immunosorbent assay (ELISA) and are therefore described as relative avidities.

Sera. In the period from 1994 to 1998, serum samples from six Dutch patients (mean age, 52 years; age range, 30 to 74 years) who died of streptococcal TSS and from eight healthy individuals (mean age, 41 years; age range, 25 to 61 years) were taken as sources of polyclonal anti-SPE-A (α-SPE-A) and α-SPE-B antibodies. Cases of TSS were defined by previously published criteria (16). From one patient, there was no more serum available for the estimation of the relative avidity for SPE-B. Informed consent was obtained from the patients or their relatives and from all controls. Six of the controls were donors at a blood bank (Stichting Rode Kruis Bloedbank, Utrecht, The Netherlands), and two were coworkers in our own department. We also studied α-SPE-A and α-SPE-B antibodies in six batches of IVIG preparations (Sandoglobulin; Sandoz, Basel, Switzerland) each dissolved to a concentration of 3 mg/ml. Sera were stored at −70°C until use.

Antigens. SPE-A was purified from GAS strain NY-5 by ultrafiltration, ethanol precipitation, and anion-exchange chromatography as described by Mascini et al. (6). SPE-B, isolated from GAS strain MGAS 1719 as described by Kapur et al. (4), was kindly provided by J. M. Musser (Houston, Tex.). SPE-A and SPE-B were more than 99% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis combined with silver staining (6).

Detection of α-SPE-A and α-SPE-B antibodies. α-SPE-A and α-SPE-B antibody levels were determined by exotoxin-specific ELISAs (6). Unless otherwise mentioned, all incuba-
tion steps were performed at 37°C. Phosphate-buffered saline supplemented with 0.1% Tween 20 was used in the washing procedures. ELISA plates (96-well, flat bottom microtiter; Costar 2595, Cambridge, Mass.) were coated for 1 h with 100 μl of SPE-A or SPE-B (0.5 μg/ml of saline). Blocking (1 h) was performed with 0.1% gelatin. Afterwards, the plates were incubated with serum, serially diluted in washing buffer (dilutions ranged from 1:30 to 1:10,000), for 1 h. The plates were then subjected to incubation with peroxidase-labeled sheep anti-human IgG antibodies (ICN Biochemicals, Amsterdam, The Netherlands). A tetramethylbenzidine (2% [wt/vol])–hydrogen peroxide (0.02% [wt/vol]) mixture was used as chromogenic substrate. Optical densities in the wells were determined by using competitive ELISAs for SPE-A and SPE-B. Each exotoxin was serially diluted (10⁻⁴ to 10⁻⁷ g/liter) in uncoated, 96-well, flat-bottom microtiter plates (Greiner GmbH, Frickenhausen, Germany) and mixed 1:1 with a fixed concentration of the serum to be tested. The fixed serum concentrations we used in the avidity tests were deduced from the ELISA-obtained titers shown in Fig. 1: concentrations from the linear part of the ELISA curve corresponding to 80% of the maximum values obtained by ELISA were used. This means that different concentrations were used for individual serum samples or immunoglobulin preparations, depending on the antibody titers of the samples to be tested. The exotoxin-antibody mixtures were incubated at 37°C for 1 h (incubation times of up to 24 h longer yielded results similar to those obtained with the 1-h incubation). Separately, 96-well, flat-bottom microtiter ELISA plates (Costar 3590, Cambridge, Mass.) were coated with either SPE-A or SPE-B and blocked as described above. Aliquots (100 μl) of the exotoxin-antibody mixtures were pipetted into the coated plates and incubated for 1 h; the concentration of free antibody was then determined by competitive ELISA. A regular ELISA was performed with the same plate used for the competitive ELISA in order to establish the absorbance value corresponding to half the antibody concentration used in the competitive ELISA. Next, the plates were incubated with peroxidase-labeled sheep anti-human IgG and the ELISAs were developed as described above. Optical densities in the wells were determined with an ELISA microplate reader (Bio-Rad) operating at 450 nm. Since the antibody concentration that yields half saturation of the coated antigen corresponds to the reciprocal of the relative avidity, the Kₐ values estimated in this way could vary

served, although the patients showed a larger proportion of low (<10 U/ml; log = 1) α-SPE-A levels than healthy individuals (χ² = 5.091; P < 0.025).

Relative avidity. Relative avidities (Kᵢ's) of human serum antibodies for SPE-A and SPE-B were estimated based on the following formulas and assumptions. If the equation [Ag] + [Ab] = k₂k₁/ [AgAb], where [Ag], [Ab], and [AgAb] represent the concentrations of antigen, antibody, and antigen-antibody complexes, respectively, and k₁ and k₂ represent the association and dissociation velocities, respectively, is considered, then the avidity at equilibrium can be described by the following:

\[ K_a = [Ag] \cdot [Ab]/[AgAb] = k_2/k_1 = 1/K_d \] (1)

where Kₐ is the association constant. Subsequently, another parameter, the fraction of bound antibody (r), can be defined by the equation

\[ r = [AgAb]/[Ab_{tot}] = [AgAb]/([AgAb] + [Ab]) = \frac{1}{1 + [Ab]/[AgAb]} \] (2)

where Ab_{tot} is the total amount of bound and free antibodies. Substitution of equation 1 in equation 2 results in

\[ r = 1/(1 + K_a/[Ag]) = [Ag]/([Ag] + K_a) \] (3)

This means that when half of the antibody-binding sites are occupied, then [Ag]/([Ag] + Kₐ) = 0.5, which means that Kₐ = [Ag].

In practice, Kᵢ's of human serum IgG for SPE-A and SPE-B were determined by using competitive ELISAs for SPE-A and SPE-B. Each exotoxin was serially diluted (10⁻⁴ to 10⁻⁷ g/liter) in uncoated, 96-well, flat-bottom microtiter plates (Greiner GmbH, Frickenhausen, Germany) and mixed 1:1 with a fixed concentration of the serum to be tested. The fixed serum antibody levels were expressed in arbitrary units (percentage of the titer of the simultaneously tested positive controls). AU, arbitrary units.

FIG. 1. Individual titers of polyclonal IgG antibodies against SPE-A (A) and SPE-B (B) in sera from patients with fatal GAS disease, in sera from controls, and in IVIG batches. No significant differences in antiexotoxin titers between patients and controls were observed, although patients showed a larger proportion of low (<10 U/ml; log = 1) α-SPE-A levels than healthy individuals (χ² = 5.091; P < 0.025).
somewhat with the coating concentrations used and polyclonal antibodies may have a broad array of $K_d$'s, the term relative avidity rather than $K_d$ value is used in this paper. Polyclonal antibodies derived from different sources, sera from patients with fatal streptococcal TSS, sera from healthy controls, and different batches of IVIG preparations, were tested.

There was a marked variation in the relative avidities estimated for both exotoxins between individual antibody sources (Fig. 3). Values for SPE-A varied from $4 \times 10^{-7}$ to $1 \times 10^{-10}$ M and values for SPE-B varied from $3 \times 10^{-9}$ to $1 \times 10^{-11}$ M. Compared with IVIG batches, sera from control individuals showed slightly high, but not significantly different, values for SPE-A ($7 \times 10^{-10}$ M and $1 \times 10^{-9}$ M, respectively) and SPE-B ($2 \times 10^{-11}$ M and $4 \times 10^{-11}$ M, respectively), as determined by Mann-Whitney analysis. We observed that relative avidities of antibodies for SPE-B were higher in sera from patients than in sera from controls, with regard to SPE-A, there were no significant differences between results for the separate serum sources. Table 1 shows the comparison between geometric mean $K_d$ values for SPE-A and SPE-B.

In this study, we addressed the question of whether the relative avidities of sera from patients with serious GAS disease could be related to the clinical outcome of disease. Despite the small number of serum samples tested, we perceived that the average affinities of polyclonal antibodies for both exotoxins tended to be substantially lower in sera from patients than in sera from controls; with regard to SPE-B, significant differences between patients and controls were found. In fact, mean relative avidities for exotoxins A and B were about 10-fold lower in the sera of patients than in sera of controls, which implies that about 10 times as many antibodies are required to obtain a similar binding of exotoxin in patient sera. Our data are in line with those of others who found an association, in Scandinavian patients, between low capacities of sera to neutralize exotoxin-induced T-cell proliferation and serious GAS infections (9). Interestingly, those authors reported that the capacity of human SPE-A- and SPE-B-specific antibodies to neutralize the mitogenic activity of the corresponding proteins was highly variable. We also noted a considerable variation in relative avidity values, not only within the groups of patients and controls but also between different IVIG batches. Remarkably, the range of average affinity values for SPE-B within control sera and immunoglobulin preparations appeared relatively small, ranging from $1 \times 10^{-11}$ to $5 \times 10^{-11}$ M. Otherwise, the relative avidities of $\alpha$-SPE-A antibodies within these two groups showed an impressive variation, with values between $3 \times 10^{-10}$ M and $3 \times 10^{-10}$ M. The fact

FIG. 2. Principles of competitive ELISA used for the determination of the relative avidity of polyclonal antibodies for SPE-A. (A) Regular ELISA to determine the serum concentration (x) used in the competitive ELISA and to establish the absorbance value corresponding to half the antibody concentration (1/2x). (B) Competitive ELISA. The relative avidity is deduced at the point where the concentration of exotoxin equals the value obtained by regular ELISA (K). OD450, optical density at 450 nm.

FIG. 3. Individual avidity values of IgG antibodies for SPE-A (A) and SPE-B (B) in sera from patients with fatal GAS disease, in sera from controls, and in IVIG batches. Avidities of antibodies for SPE-A and SPE-B were higher in sera from controls and in IVIG preparations than in sera from patients ($P = 0.112$ and $P = 0.014$, respectively).
that sera from controls showed slightly higher relative avidity values than immunoglobulin preparations might be explained by the procedures involved in the manufacturing of immunoglobulin preparations. The fairly high relative avidity values presented here are in line with a similar value (1.8 × 10⁻¹⁰ M) for a mouse monoclonal IgG2a antibody against SPE-A (15).

The relative avidities of antibodies for SPE-B were significantly higher than those for SPE-A in all three groups. In line with this, neutralization of SPE-A activity has been reported to require serum concentrations higher than those required by other streptococcal superantigens (10). The antiexotoxin titers as well as the capacity of the antibodies to bind SPE-A and SPE-B present in IVIG preparations are likely to determine the neutralizing activity against streptococcal products and, thereby, whether or not a particular batch is beneficial for patients with severe GAS disease. These data stress the importance of screening IVIG pools prior to use in the clinical setting.

We developed a solid-phase assay with immobilized antigen to determine the concentration of the free (not associated with exotoxin) antibody. We first incubated the antibodies and the antigen in homogeneous solution long enough to reach equilibrium, and only then was the concentration of free antibodies estimated by ELISA. Indirect competitive ELISA methods offer many advantages as long as they are used under conditions in which equilibrium is reached and not subsequently perturbed by the immunoassay (2, 3). First, only one of the components (antigen or antibody) needs to be purified (2). Second, problems concerning steric hindrance, antibody valence, and antigen density and mobility on the surface, which affect the position of equilibrium on a surface, are not encountered (2). Third, we are dealing with unmodified molecules, since no labeling of either the antigen or the antibody is required. In support of our method, a significant correlation between competitive enzyme immunoassays and methods established by others (as long as avidity values were read from the linear part of the saturation curve obtained at high saturation of the antibody by the antigen) has been reported (2, 3, 13). One disadvantage of our approach is that it may be difficult to estimate relative avidities for sera with low antibody levels. Moreover, in the case of high-avidity values requiring low concentrations of exotoxin in the incubation mixture, a shift of the equilibrium in the fluid phase may be induced by the relatively high SPE concentration in the coating, thus yielding an underestimation of the avidity values (3). Otherwise, problems with competitive ELISA are related to the restricted sensitivity of the solid-phase assay; the limiting affinity level of antibodies in most test systems appears to be 10⁻¹⁵ M, but obviously the majority of antibodies in enzyme immunoassays have lower avidity constants (12). Furthermore, we cannot provide information as to the heterogeneity of antibody affinities in a polyclonal response, since this was beyond our goal of comparing operational affinities.

In conclusion, the relative avidities of antibodies for both toxins were considerably lower in sera from patients with fatal TSS than in sera from controls or in IVIG batches, in addition to the levels of antibody being lower in the serum. It might be argued that patients who develop severe GAS disease have a deficiency in high-avidity antibodies against SPE-A and SPE-B, which is probably best described by the product of titer and relative avidity. The variation between different sera and immunoglobulin preparations in SPE-binding capacity is likely to reflect the variation in SPE neutralization of these samples.

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REFERENCES


