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Evidence for the Presence of Streptococcal-Superantigen-Neutralizing Antibodies in Normal Polyspecific Immunoglobulin G

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Recently we demonstrated that normal polyspecific immunoglobulin given intravenously (IVIG) and plasma samples from patients treated with IVIG neutralize the mitogenic and cytokine-inducing activities of group A streptococcal (GAS) superantigens. Here we investigated whether this neutralizing activity is mediated by antibodies to these superantigens. IVIG and plasma samples collected from a patient with GAS necrotizing fasciitis post-IVIG infusions markedly inhibited the mitogenic activity elicited by the streptococcal pyrogenic exotoxins SpeB and SpeC, as well as by GAS culture supernatant. Immunoblot analysis showed marked increases in the levels of antibodies to SpeC and proteins in the GAS culture supernatant in post-IVIG over those of pre-IVIG plasma samples. Removal of antisuperantigen antibodies in IVIG by adsorption to SpeC- and GAS culture supernatant-coupled Sepharose markedly reduced the neutralizing ability of IVIG against respective stimuli. The neutralizing activity was totally recovered in the eluted antibodies. By contrast, although pre- and post-IVIG plasma samples contained antibodies to SpeA, these antibodies did not block the activity of this superantigen. Nonspecific immunomodulatory activity of IVIG was ruled out because neither the IVIG nor the affinity-purified antibodies significantly inhibited the response to the polyclonal T-cell mitogen phytohemagglutinin A. These data provide direct evidence that the neutralizing activity in IVIG, and in patient plasma samples following IVIG treatment is mediated by antibodies to superantigens and indicate that the quality rather than the quantity of these antibodies may be more clinically relevant.

Superantigens have been implicated in the pathogenesis of staphylococcal and streptococcal toxic shock syndrome (3, 4, 11, 13). Superantigens are microbial proteins capable of activating a large proportion of the immune cells, thereby causing an excessive release of inflammatory cytokines (3, 4, 11, 13). This activity is attributed to the fact that superantigens are bifunctional molecules that interact with relatively invariable regions of the T-cell receptor and with major histocompatibility complex class II molecules on specialized cells including B cells and monocytes (3, 4, 11, 13).

Several studies have suggested that normal polyspecific immunoglobulin given intravenously (IVIG) may be effective in treatment of acute infectious diseases, such as staphylococcal toxic shock (TSS) and severe invasive group A streptococcal (GAS) infections, including streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF) (1, 2, 7). In a recent case control study, mortality was dramatically reduced in STSS patients treated with IVIG (2).

The mechanism by which IVIG may improve clinical outcome in the setting of acute infectious diseases has not been totally elucidated. However, IVIG has been shown to have a profound immunomodulatory effect on the activity of staphylococcal and streptococcal superantigens, as evident by a strong inhibition of the T-cell proliferation and cytokine production induced by these proteins (8, 12, 14). Recently, our group reported that the inhibitory activity of IVIG was directed against a large variety of streptococcal superantigens and that this broad specificity and neutralizing activity were transferred to the plasma of patients with severe invasive GAS infections following IVIG treatment (8). The previous studies, however, did not examine whether this inhibitory activity was mediated by antibodies to superantigens or by other nonspecific immunomodulatory agents in the IVIG. This study provides evidence that the inhibitory activity present in IVIG, and in the plasma of IVIG-treated patients, is directly attributed to antibodies against streptococcal superantigens and that these antibodies are transferred to the patient's plasma following IVIG administration.

Clinical data and collection of samples from the patient. A 20-year-old male suffered an abrasion to his left knee, resulting in a GAS cellulitis. Despite treatment with penicillin and clindamycin, cellulitis progressed rapidly. He was transferred to Mount Sinai Hospital in Toronto, Canada, where he received a total of 2 g of IVIG per kg of body weight in two doses (Cutter Biologicals, Toronto, Canada). At surgery he was found to have septic arthritis and NF, with the latter diagnosis confirmed by histopathology. Plasma samples were drawn from the patient on the day of admission (pre-IVIG) and immediately after the first and second IVIG infusions (post-IVIG1 and post-IVIG2, respectively). A plasma sample was also collected during the convalescent phase of infection (8 weeks after the onset of infection). The bacterial isolate from the site of infection was identified as *Streptococcus pyogenes* at the National Center for Streptococcus Research (Edmonton, Canada). The isolate and patient plasma samples were promptly frozen and stored at −80°C until processed. The presence of

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TABLE 1. Toxin-neutralizing activity in plasma samples drawn from a patient during the acute and convalescent phases of infection[a]

Sampling time[b]	% Inhibition of toxin mitogenicity by patient plasma[c]						
	SpeA		SpeB		SpeC		GAS SUP (1% plasma)
	1%	5%	1%	5%	1%	5%	
Pre-IVIG	0	0	25	56	0	0	0
Post-IVIG1	0	0	89	99	42	100	100
Post-IVIG2	0	3	100	96	91	100	ND[d]
Convalescence	0	0	94	100	29	83	0

[a] PBMCs from a healthy individual were cultured in the presence of 1 and 5% patient plasma samples, and the proliferative responses induced by 10 ng of SpeA or SpeC per ml, 50 ng of SpeB per ml, and 1:100 dilution of the supernatant from the patient's clinical GAS isolate were compared with that obtained in the presence of the same concentration (1 or 5%) of FBS. All samples were assayed in triplicate. The data are presented as percent inhibition of toxin mitogenicity, as calculated by the equation given in the text.

[b] The post-IVIG1 and post-IVIG2 plasma samples were drawn after the first and second IVIG infusions, respectively. The convalescent-phase plasma sample was drawn 8 weeks after the onset of infection.

[c] SpeA, SpeB, and SpeC are streptococcal pyrogenic exotoxins. GAS SUP is the supernatant from the culture of the patient's clinical isolate grown overnight.

[d] ND, not determined.

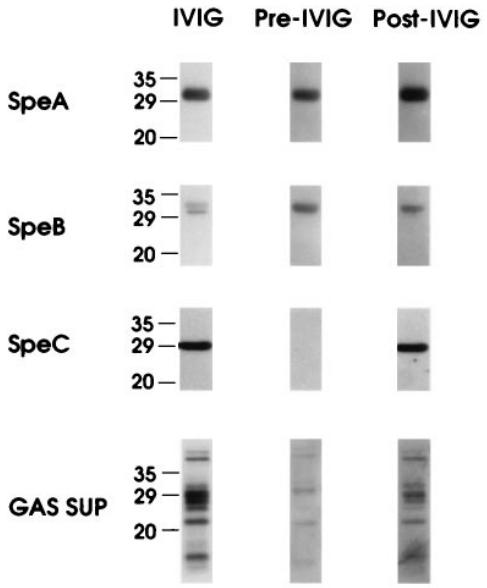

FIG. 1. Detection of antisuperantigen antibodies present in patient plasma samples and in IVIG by Western blot analysis. SpeA, SpeB, and SpeC (1 μg each) and GAS culture supernatant (120 μl) (GAS SUP) were separated on SDS–12% polyacrylamide gels and blotted onto nitrocellulose membranes. The blots were incubated with 5% pre- or post-IVIG patient plasma samples or 200 μg of pure IVIG per ml and then with a secondary antibody and finally with the luminol-chemiluminescence reagents (Amersham ECL). Shown are the autoradiograms obtained following incubation with IVIG and pre- and post-IVIG plasma. The molecular masses of the standards are shown to the left of the autoradiograms and are given in kilodaltons.

the genes encoding the streptococcal pyrogenic exotoxins SpeA, SpeC, and SpeF and streptococcal superantigen (SSA), was determined by PCR as previously described (6, 9, 15).

Preparation of bacterial culture supernatant. The GAS isolate was cultured overnight in 10 ml of chemically defined medium (JHR Biosciences, Lenexa, Kans.), and the culture supernatant was collected and ethanol precipitated as previously described (8). The precipitate was dissolved in 1 ml of distilled H_2O and dialyzed for 24 h against distilled H_2O, filter sterilized, and stored at −20°C. The supernatant was tested at different dilutions for its ability to induce T-cell proliferation, and the optimal concentration was then used in further experiments.

PBMC preparation and proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from a healthy individual by Ficoll-Hypaque gradient centrifugation. A total of 1.5×10^6 cells per ml were cultured in RPMI 1640 medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 4 mM L-glutamine, and 100 U of penicillin and streptomycin per ml (complete RPMI medium). The cells were stimulated with the optimal concentration (predetermined from dose-response curves) of SpeA, SpeB, and SpeC (Toxin Technology, Sarasota, Fla.) in the presence of the same concentration (ranging from 1 to 5%) of either heat-inactivated patient plasma or fetal bovine serum (FBS). Proliferation was determined after 3 days of incubation at 37°C, 5% CO_2, and 95% humidity, as previously described (8). All samples were assayed in triplicate, and the data are presented as mean cpm of [^{3}H]thymidine uptake ± standard deviation or as percent inhibition of toxin mitogenicity, as calculated by the following equation (8, 10): $\{1 - [(cpm_{plasma + stimulus} - cpm_{plasma})/(cpm_{FBS + stimulus} - cpm_{FBS})]\} \times 100$.

SDS-PAGE and Western blotting (immunoblotting). Protein samples, 1 μg of SpeA, SpeB, or SpeC, or 120 μl of lyophilized culture supernatant, were dissolved and boiled for 5 min in loading buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 5% glycerol) for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% total acrylamide, 2.7% bisacrylamide). Proteins were electroblotted onto nitrocellulose membranes, and the blots were blocked overnight with 6% nonfat dry milk in Tris-buffered saline (50 mM Tris (pH 7.5), 150 mM NaCl). The blots were then incubated with primary antibody (200 μg of IVIG per ml or 5% pre- or post-IVIG patient plasma diluted in 0.1% nonfat dry milk in Tris-buffered saline), washed, and then incubated with secondary antibody (anti-human immunoglobulin conjugated to horseradish peroxidase) (Fisher Biotech, Pittsburgh, Pa.). Binding was detected by the luminol-chemiluminescence reagents (Amersham ECL). The processed blots were exposed to X-ray films, and the autoradiograms were analyzed.

Adsorption of antibodies to superantigens in IVIG. The dialyzed GAS culture supernatant was lyophilized and resuspended in 0.5 ml of coupling buffer (0.5 M $NaHCO_3$, 0.5 M NaCl; pH 8.3). The culture supernatant and 0.5 mg of SpeC (resuspended in 0.5 ml of coupling buffer) were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech, Sollentuna, Sweden) by rotating for 2 h at 25°C. The remaining active groups on Sepharose were blocked with 0.2 M glycine, pH 4.0.

IVIG (5 mg/ml) was added to the toxin-coupled Sepharose, agitated for 24 h at 4°C, and then centrifuged, and the supernatants were saved for further analyses. Sequential elution of the bound antibodies was achieved by adding 3 volumes of pH 7.0 elution buffer (50 mM NaH_2PO_4, 0.15 M NaCl; pH 7.0), pH 5.5 elution buffer (50 mM sodium citrate, 0.15 M NaCl; pH 5.5), pH 4.3 elution buffer (0.5 M sodium acetate, 0.15 M NaCl; pH 4.3), and pH 2.3 elution buffer (0.5 M glycine, 0.15 M NaCl; pH 2.3). After each addition, the mixtures were centrifuged and the supernatants from each elution step were pooled, dialyzed against several changes of distilled H_2O overnight, and lyophilized. The lyophilized antibodies were resuspended in complete RPMI medium and added to the cultures at a 1:20 final dilution.

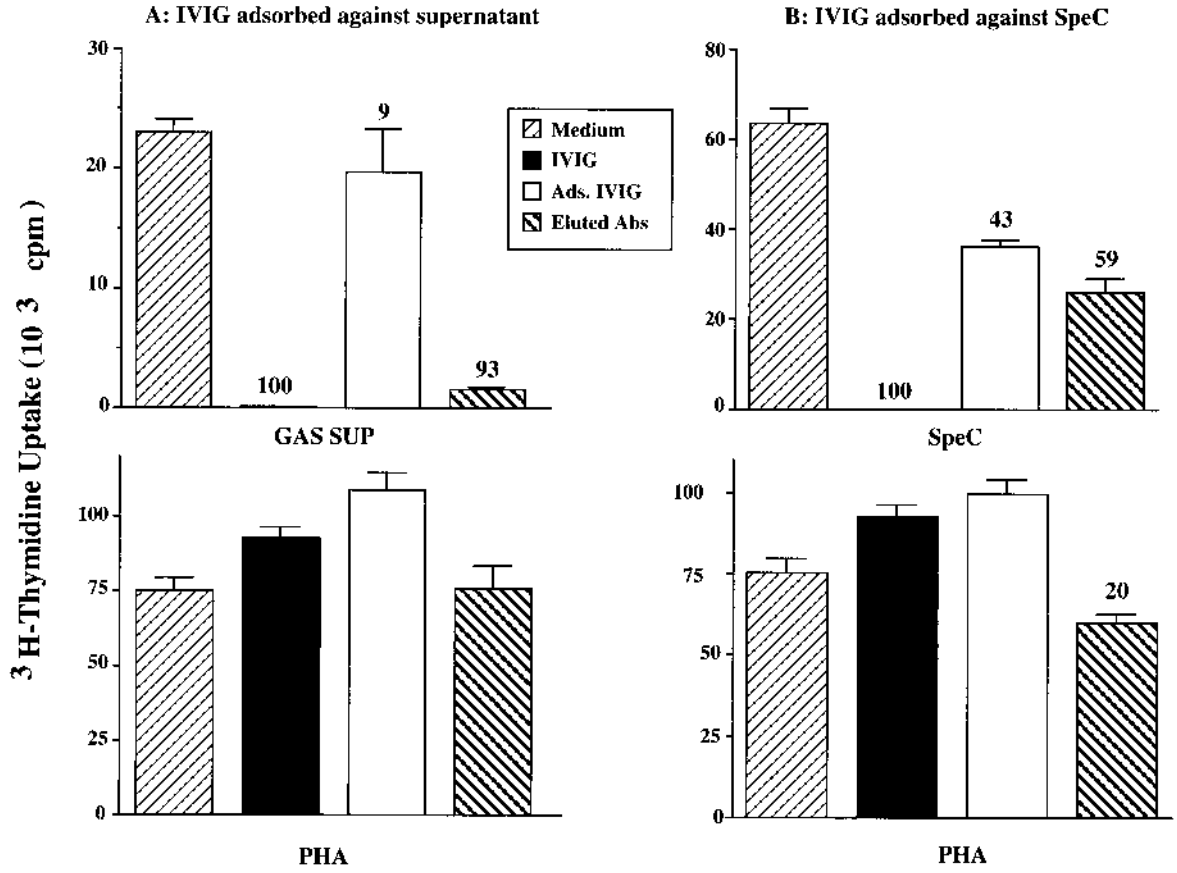

FIG. 2. Neutralizing activity of IVIG is attributed to antisuperantigen antibodies. IVIG was adsorbed to GAS culture supernatant- and SpeC-coupled Sepharose, and the bound antibodies were eluted by sequentially decreasing the pH. The adsorbed IVIG (Ads. IVIG) and the eluted antibodies (Eluted Abs) were tested for neutralizing activity against GAS culture supernatant or SpeC and PHA. PBMC (1.5×10^6 cells per well) from a healthy individual were stimulated with a 1/200 dilution of GAS culture supernatants, 1 ng of SpeC per ml, and 1 μg of PHA per ml in the presence of 0.25 mg of IVIG per ml, 0.25 mg of adsorbed IVIG per ml, or eluted antibodies (diluted 1:20). The data are presented as mean cpm of [^{3}H]thymidine uptake ± standard deviation (SD) of triplicate samples. (A) Results obtained with IVIG adsorbed to GAS culture supernatant-coupled Sepharose; (B) results obtained with IVIG adsorbed to SpeC-coupled Sepharose. The numbers above the bars indicate percent inhibition of mitogenicity compared with the value obtained for medium alone. The mean cpm values ± SDs for the different cultures were as follows: unstimulated cells in cultures with medium alone, 4,393 ± 112; cultures with IVIG, 339 ± 33; cultures with GAS SUP-adsorbed IVIG, 831 ± 87; cultures with eluted GAS SUP antibodies, 2,934 ± 197; cultures with SpeC-adsorbed IVIG, 688 ± 281.

Superantigenic activity in GAS culture supernatant. The GAS isolate studied was serotyped M3/T3 and harbored the genes encoding SpeF and SSA but lacked those encoding SpeA and SpeC (data not shown). Since certain GAS strains produce superantigens that have not yet been characterized (15), both purified Spe proteins and the concentrated GAS culture supernatant were used as sources of superantigens. The GAS culture supernatant induced a brisk proliferative response even at a 1:200 dilution (data not shown).

Patient plasma and IVIG contain antisuperantigen antibodies. IVIG (0.25 and 0.5 mg/ml) and post-IVIG patient plasma samples completely neutralized the mitogenic activity of the GAS culture supernatant (Table 1). The plasma sample drawn from this patient before IVIG administration had no neutralizing activity against SpeA, SpeC, or the GAS culture supernatant and had only low inhibitory activity against SpeB. With the exception of SpeA, the neutralizing activity against purified superantigens and GAS culture supernatant was markedly increased in plasma samples drawn post-IVIG administration (Table 1). The increase in neutralizing activity noted in the post-IVIG plasma samples against SpeC and culture supernatant was only temporary, since the inhibitory activity was lower in the plasma sample drawn during the convalescent phase of infection. By contrast, the anti-SpeB activity remained high in the convalescent-phase sample. The inhibition of exotoxin mitogenic activity was not due to nonspecific toxicity, since the phytohemagglutinin A (PHA)-induced proliferation was inhibited only 18 and 24% by plasma samples drawn post-IVIG and during the convalescent phase, respectively (data not shown).

Next, we investigated whether the neutralizing activity in IVIG and post-IVIG plasma samples was mediated by antibodies directed against superantigens. Immunoblots revealed that IVIG contained antibodies to SpeA, SpeB, and SpeC and reacted with eight visible protein bands in the GAS culture supernatant (Fig. 1). The pre-IVIG plasma sample contained antibodies to SpeA and SpeB but not to SpeC. Low reactivity of this plasma sample with GAS culture supernatant was observed (Fig. 1). By contrast, post-IVIG plasma samples reacted strongly with all purified superantigens tested, as well as with the GAS culture supernatant. Interestingly, the pattern of reactivity with the latter was identical to that observed with pure IVIG (Fig. 1).

We then examined whether these antibodies are directly responsible for blocking the mitogenic activity of streptococcal superantigens. Antisuperantigen antibodies in IVIG were adsorbed to SpeC- and GAS culture supernatant-coupled Sepha-

rose. Adsorption of IVIG to culture supernatant and SpeC resulted in loss of neutralizing activity against these stimuli (Fig. 2). The removal of inhibitory activity was most pronounced following adsorption to GAS culture supernatant, where the inhibition was reduced from 100 to 9%. The inhibitory activity of the adsorbed IVIG against SpeC was reduced from 100% to 43%. Most importantly, the neutralizing activity was totally recovered in the eluted antibodies (93 and 59%, respectively). Nonspecific toxicity of the eluted antibodies could be ruled out, since PHA-induced proliferation was only marginally affected by the addition of the eluted antibodies (Fig. 2).

Despite prompt antibiotic treatment, the mortality rate in patients with severe invasive GAS infections, such as STSS and NF, remains high. In a recent report from Canada, the mortality rates for patients with STSS and NF were 81 and 45%, respectively (5). In vitro and in vivo studies have suggested that the use of IVIG as adjunctive treatment for patients with severe GAS infections is clinically beneficial (2, 7, 8, 12, 14). Our previous in vitro studies showed that IVIG could inhibit both defined streptococcal superantigens and superantigenic activity present in cultures of clinical GAS isolates (8). Most importantly, we reported that the inhibitory activity of IVIG was conferred to patients following treatment (8). In this study we examined whether the inhibition was mediated by antibodies directed to superantigens or by some other immunomodulatory effect.

Consistent with our previous study (8), the neutralizing activity in plasma against superantigens present in culture supernatant and SpeC increased markedly following IVIG treatment. The increase in neutralizing activity correlated well with the results of immunoblotting showing a marked increase in antibodies to SpeC and proteins in the GAS culture supernatant in post- compared with pre-IVIG plasma. This correlation between increased inhibitory activity and enhanced immunoreactivity against SpeC and GAS culture supernatant was not found with SpeA and SpeB. Although anti-SpeA antibodies were present in comparable levels in pre- and post-IVIG plasma samples, these antibodies apparently lacked neutralizing activity. Recently, we showed that the majority of patients lack SpeA-neutralizing activity and that IVIG has much lower levels of inhibitory activity against this superantigen than SpeB and SpeC (8). The reason for low titers of SpeA-neutralizing antibodies is not clear, however, our studies suggest that these anti-SpeA antibodies are present but they are not capable of blocking the activity of SpeA. It is quite possible that IVIG preparations from other sources (i.e., different geographical areas) may show higher titers of SpeA-neutralizing antibodies. The data underscore the importance of screening different IVIG pools prior to use in clinical settings. The levels of anti-SpeB antibodies were also similar in pre- and post-IVIG plasma samples; however, a marked increase in the SpeB-neutralizing activity was noted after IVIG treatment. Furthermore, the pattern of reactivity of post-IVIG plasma and IVIG in the immunoblots of the culture supernatant was identical. These data suggest that the quality rather than quantity of antibodies to superantigens may be more clinically related. The mode of binding of the antibody, i.e., the region of the superantigen to which the antibody is directed against, might determine whether neutralization of the superantigen is achieved. This is in concordance with a previous study which reported that Spe-specific enzyme-linked immunosorbent assay antibody titers in plasma samples from patients with various GAS infections did not always correlate with neutralizing activity of the plasma (10).

Direct evidence that the neutralizing activity of IVIG is mediated by antisuperantigen antibodies was obtained from experiments where IVIG adsorbed against SpeC- and GAS culture supernatant-coupled Sepharose had a markedly reduced inhibitory activity against the respective stimulus. Furthermore, the neutralizing activity was recovered in the eluted antibodies. The total loss of inhibition against the culture supernatant following adsorption further emphasizes the broad specificity of IVIG against streptococcal superantigens and provides a rationale for its use in treatment of diseases associated with these toxins.

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