MAJOR ARTICLE

Differences in Potency of Intravenous Polyspecific Immunoglobulin G against Streptococcal and Staphylococcal Superantigens: Implications for Therapy of Toxic Shock Syndrome

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Administration of intravenous polyspecific immunoglobulin G (IVIG) has been proposed as adjunctive therapy for toxic shock syndrome caused by *Streptococcus pyogenes* or *Staphylococcus aureus*. We investigated whether superantigen-containing culture supernatants prepared from streptococcal isolates ($n = 21$) and staphylococcal isolates ($n = 20$) from cases of severe sepsis were inhibited to an equal extent by IVIG in proliferation experiments that used human peripheral blood mononuclear cells. All 3 IVIG preparations tested were highly efficient in neutralizing the superantigens, and most supernatants were completely inhibited at concentrations ranging from 0.05 to 2.5 mg IVIG/mL. An important finding was that culture supernatants from *S. pyogenes* isolates were consistently inhibited to a greater extent than those of *S. aureus* isolates ($P < .01$). The findings demonstrate that staphylococcal superantigens are not inhibited as efficiently as streptococcal superantigens by IVIG, and, hence, a higher dose of IVIG may be required for therapy of staphylococcal toxic shock syndrome in order to achieve protective titers and clinical efficacy.

Staphyloccocus aureus and *Streptococcus pyogenes* are pathogens that can cause a variety of diseases, ranging from mild superficial skin infections to more severe and life-threatening conditions, such as septicemia and endocarditis, as well as toxic shock syndrome (TSS). There are 2 subsets of TSS caused by *S. aureus:* menstrual and nonmenstrual. The mortality for both subtypes ranges from 3% to 5%, whereas streptococcal TSS is usually associated with a much higher fatality rate of 30%–80% [1].

S. aureus and *S. pyogenes* produce and release exotoxins with superantigenic activities. These superantigens are defined by their ability to bypass the normal rules of antigen presentation by binding (without prior cellular processing) to the outside of the antigen-binding cleft of MHC class II molecules of antigen-presenting cells and to specific variable regions of the β-chain of the T cell receptor [2]. This binding results in activation of up to 40% of the naive T cell population, which in turn leads to a massive release of proinflammatory cytokines. Superantigens have been recognized as central mediators of the systemic effects of TSS, mainly by virtue of their potent induction of proinflammatory responses [1, 3]. Most staphylococcal and streptococcal strains express several different superantigens, and at least 24 staphylococcal and 12 distinct streptococcal superantigens are known today [1, 3–5].

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A crucial role of humoral immunity against superantigens in the protection against invasive streptococcal and staphylococcal infections has been demonstrated. In healthy adults, antibodies against TSS toxin 1 (TSST-1) can regularly be detected by ELISA [6, 7], and an antibody response against TSST-1 can usually be demonstrated following septicemia with a TSST-1–producing *S. aureus* strain [8, 9]. Patients with low antibody levels or with a deficient antibody response against the staphylococcal superantigens seem to be at risk for TSS or recurrence of TSS [6, 10–13]. Several studies have shown that patients with invasive *S. pyogenes* infections have significantly lower levels of both opsonic anti-M antibodies and neutralizing antisuperantigen antibodies, compared with controls [14–17].

On the basis of these findings, administration of intravenous polyspecific IgG (IVIG) was proposed as efficient adjunctive therapy for TSS, and in vitro studies showed that IVIG preparations contained opsonic and neutralizing antibodies directed against both staphylococcal and streptococcal superantigens [18–26]. The clinical efficacy of IVIG as adjunctive therapy for streptococcal TSS has been reported in 2 trials: 1 observational cohort study [27] and 1 multicenter, placebo-controlled trial [28]. Both reported a reduction in mortality following IVIG therapy. The clinical efficacy of IVIG therapy in staphylococcal TSS is not as well documented; it has been noted in only 2 case reports [29, 30].

In our recently performed, placebo-controlled trial of IVIG therapy for streptococcal TSS [28], a patient with staphylococcal TSS was enrolled in the study as having a presumed case of streptococcal TSS. In vitro studies revealed that the toxins from the *S. aureus* culture supernatant were not as efficiently neutralized by IVIG or by plasma obtained from the infected patient after IVIG therapy (hereafter, "post-IVIG plasma"), whereas the *S. pyogenes* culture supernatants were completely inhibited by post-IVIG plasma and by the IVIG [28]. These results led us to further investigate whether there is a difference in the ability of IVIG to neutralize streptococcal and staphylococcal superantigens produced by clinical isolates.

METHODS

Clinical isolates. Clinical isolates of *S. pyogenes* ($n = 20$) and *S. aureus* ($n = 21$) from cultures of blood from patients with severe sepsis or TSS were included in the study (tables 1 and 2). Sepsis and septic shock were defined according to established criteria [31]. Isolates were selected in order to obtain a strain collection representing a variety of streptococcal sero-

Table 1. Serotypes and superantigen genotypes for *Streptococcus pyogenes* isolates.

Isolate	Patient diagnosis	Serotype		Superantigen genotype									Total no. of SAG genes
		M-Type	T-Type	*speA*	*speB*	*speC*	*speF*	*speG*	*speH*	*speJ*	*ssa*	*smeZ/2*	
0201	STSS, NF	M1	T1	+	+	−	+	+	−	+	−	+	6
0202	STSS	M1	T1	+	+	−	+	+	−	+	−	+	6
0203	STSS, NF	M12	T12	−	+	+	+	+	+	−	−	+	6
0502	STSS, erysipelas	M1	T1	+	+	+	+	+	−	+	−	+	7
0503	STSS	M22	NT	+	+	−	+	+	+	−	+	+	7
0801	STSS	M1	T1	+	+	−	+	+	+	+	−	+	7
0803	STSS, NF	M3	NT	+	+	−	+	+	+	−	+	+	7
0804	STSS	M1	T1	+	+	−	+	+	−	+	−	+	6
0805	STSS, NF	M1	T1	+	+	−	+	+	−	+	−	+	6
0901	STSS	M1	T1	+	+	+	+	+	−	+	−	+	7
1002	STSS	M3	T3	+	+	−	+	+	+	−	+	+	7
1201	STSS, NF	M1	T1	+	+	+	+	+	+	+	−	+	8
1202	STSS	M4	T4	−	+	+	+	−	−	−	+	+	5
1701	STSS, NF	M12	T12	−	+	+	+	+	+	−	−	+	6
1801	STSS, NF	M93	NT	−	+	−	+	+	−	+	−	+	5
5442	STSS, NF	M1	T1	+	+	−	+	+	−	+	−	+	6
5444	STSS, NF	M1	T1	+	+	−	+	+	−	+	−	+	6
5448	STSS	M1	T1	+	+	+	+	+	−	+	−	+	7
8096	STSS, NF	M1	T1	+	+	+	+	+	−	+	−	+	7
8157	STSS, NF	M1	T1	+	+	−	+	+	−	+	−	+	6

NOTE. NF, necrotizing fasciitis; NT, not typable; SAG, superantigen; STSS, streptococcal toxic shock syndrome. Genes designated *spe* code for streptococcal pyrogenic exotoxin, the gene *sme/Z* codes for streptococcal mitogenic exotoxin, and the gene *ssa* codes for streptococcal superantigen.

Table 2. Superantigen expression in *Staphylococcus aureus* isolates.

Patient	Diagnosis	Detection limit, by superantigen[a]							Total no. of SAGs expressed
		SEA	SEB	SEC	SED	TSST-1	ETA	ETB	
1	Sepsis, wound infection	0	0	128	0	0	0	0	1
2	Sepsis, pyelitis, osteitis of the foot	0	0	0	0	32	0	0	1
4	Sepsis, endocarditis, meningitis	0	0	16	0	0	0	0	1
5	Septic shock	0	0	32	0	0	0	0	1
7	Sepsis, soft tissue infection	0	>128	0	0	0	0	0	1
8	Sepsis, prosthetic joint infection	128	0	0	0	0	0	0	1
9	Sepsis, septic arthritis	0	0	>128	0	0	0	0	1
10	Sepsis, septic arthritis	0	0	0	0	64	0	0	1
11	Sepsis, coxitis	128	0	0	128	0	0	0	2
12	Sepsis, pneumonia	0	0	>128	0	0	0	0	1
13	Sepsis, vertebral osteomyelitis	0	0	128	128	0	0	0	2
14	Sepsis, meningitis	0	0	>128	0	0	0	0	1
15	Sepsis, septic arthritis	16	0	0	0	>64	0	0	2
17	Sepsis, soft tissue infection	64	0	0	0	0	0	0	1
21	Sepsis, endocarditis, coxitis	16	0	0	0	64	0	0	2
24	Sepsis, osteomylitis	32	0	0	0	>64	0	0	2
25	Sepsis	>128	0	0	128	0	0	0	2
31	Septic shock, endocarditis	64	0	0	128	0	0	0	2
32	Sepsis, osteomyelitis	0	0	0	>128	0	0	0	1
33	Sepsis, wound infection	32	0	0	0	64	0	0	2

NOTE. ETA-B, exfoliative toxins A and B; SAG, superantigen; SEA–D, staphylococcal enterotoxins A–D; TSST-1, toxic shock syndrome toxin 1.

[a] Values indicate the detection limit of each superantigen in a series of $\log_2$ dilutions.

types as well as varying superantigen profiles. All staphylococcal isolates and the majority of the streptococcal isolates were obtained in Sweden [9, 28]. The study also included 5 isolates of *S. pyogenes* serotype M1T1 obtained in Canada (kindly provided by Donald E. Low, Mount Sinai Hospital, Toronto, Canada).

Superantigen characterization. Genomic DNA from the *S. pyogenes* isolates was prepared as described elsewhere [32]. Multiplex PCR amplifications using primer pairs specific for each superantigen gene were performed to detect the gene encoding the streptococcal superantigen *(ssa)*, the genes encoding the streptoccocal pyrogenic exotoxins *(speA, speB, speC, speF, speG, speH,* and *speJ)*, and the gene encoding streptoccocal mitogenic exotoxin Z *(smeZ/2)*, as described in detail elsewhere [33].

Production of staphylococcal enterotoxins A, B, C, D, and TSST-1 by the *S. aureus* isolates was assessed in culture supernatants by reverse passive latex agglutination (RPLA) with use of the commercial kits SET-RPLA and TST-RPLA (Oxoid) according to the manufacturer's instructions. The *S. aureus* isolates were also tested for expression of exfoliative toxins A and B by real-time PCR assays using the LightCycler system with SYBR-Green-I (Roche Diagnostics). The primers used were based on sequences described elsewhere [34].

M-serotyping and T-serotyping of* S. pyogenes *isolates. M-types were determined by direct sequencing of the *emm* gene, essentially as described elsewhere [35], but using the same DNA preparation as was used for the superantigen characterization, and with smaller reaction volumes. The sequences obtained were compared with the nucleotide sequences encoding the N-terminal hypervariable portion of streptococcal M proteins, which are available on the Internet at the Centers for Disease Control and Prevention Web site (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). T-typing was performed by conventional agglutination methods at the Swedish Institute for Infectious Disease Control, Solna, Sweden, and at the National Reference Center for the Streptococcus, Edmonton, Alberta, Canada.

Preparation of bacterial culture supernatant. The *S. pyogenes* and *S. aureus* isolates were cultured overnight at 37°C in 15 mL Todd-Hewitt broth (Difco) supplemented with 1.5% yeast extract (Difco). Supernatants were separated from the bacteria by centrifugation, and proteins in the culture supernatants were precipitated in ethanol overnight, as previously described in detail [22]. Cell-free culture supernatants were stored at −20°C until used. In parallel, viable bacterial counts (in colony-forming units per milliliter) were determined for

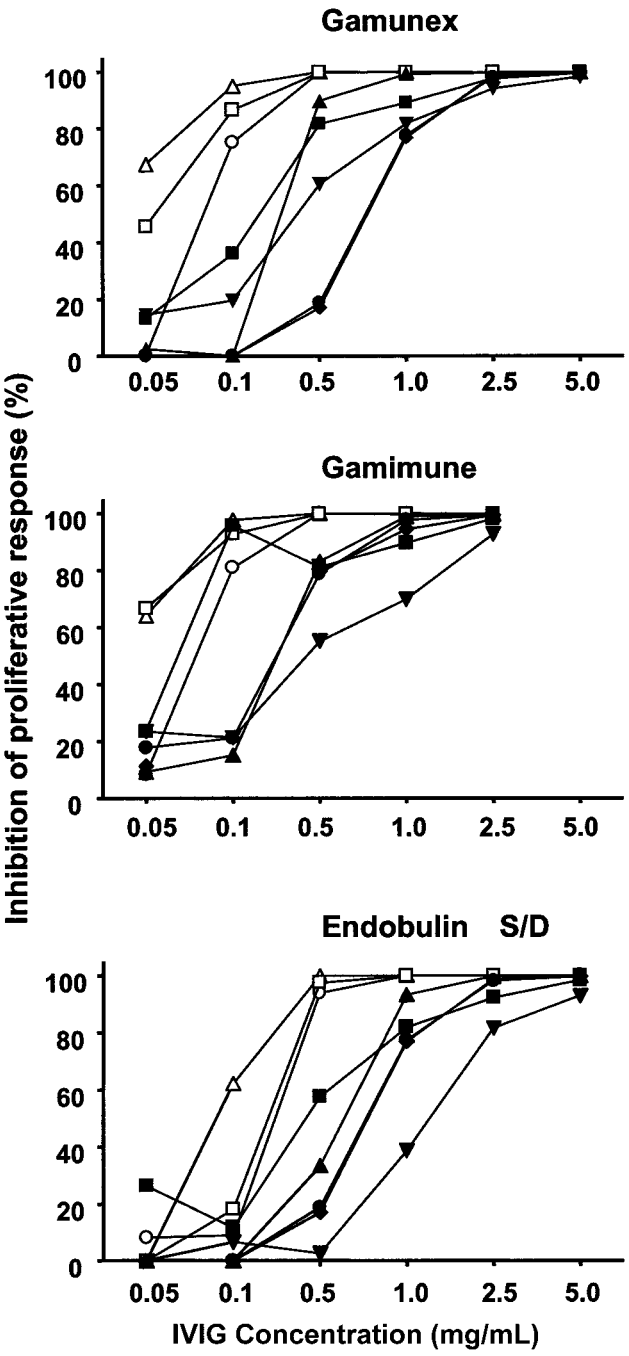

Figure 1. Inhibition of proliferative responses in human peripheral blood mononuclear cells from healthy individuals that were stimulated with culture supernatants prepared from *Staphylococcus aureus* clinical isolates ($n = 5$; isolates 9, 11, 13, 11, and 31) (table 2) and *Streptococcus pyogenes* clinical isolates ($n = 3$; isolates 1201, 1701, and 1801) (table 1) of different serotypes and with different superantigen profiles, in the presence of varying concentrations of 3 different intravenous polyspecific IgG preparations. Proliferative responses were assessed after 72 h of stimulation. Each symbol represents 1 isolate. Percentages are relative to inhibition without adjunctive IVIG. *Open symbols,* culture supernatants from streptococcal isolates; *filled symbols,* culture supernatants from staphylococcal isolates.

each isolate by serial dilution and plating on blood-agar plates. Dose-response experiments were performed to determine the optimal concentration of cell-free culture supernatants.

Neutralization assays. Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor by Ficoll-Hypaque gradient centrifugation. The PBMCs were cultured in RPMI 1640 medium (concentration, 1×10^6 cells/mL) supplemented with 25 mmol/L HEPES, 4 mmol/L L-glutamine, 100 U/mL penicillin-streptomycin, and 5% fetal calf serum. The cells were stimulated with the cell-free bacterial culture supernatants in the presence of different concentrations of IVIG, as previously described [22]. After 72 h, the cells were pulsed for 6 h with 1 μCi per well of [^{3}H]-thymidine (specific activity, 6.7 Ci/mmol; ICN Biomedical). Phytohemagglutinin-L (Sigma) was used as a positive control at a concentration of 1 μg/mL. All samples were assayed in triplicate.

Three different IVIG preparations were tested for neutralizing activity, including 5 batches each of Gamimune N and Gamunex (both at concentrations of 10%; Bayer) and 1 batch of Endobulin S/D (Baxter Medical).

Statistical analysis. Statistically significant differences between groups were assessed by Student's t test, or by ANOVA followed by the Tukey post hoc test. $P < .05$ was considered significant.

RESULTS

The *S. pyogenes* isolates used in this study were predominantly of serotype M1T1 (65% of isolates), but this study also included isolates of other serotypes (i.e., M3T3, M4T4, M12T12) and isolates that were not typable with respect to their T type but that had varying M-types, including M3, M22, and M93 (table 1). *Spe* genotyping revealed that all streptococcal isolates harboured genes encoding for 5–8 different superantigens (table 1). As expected, all isolates harbored the chromosomally encoded *speB, speF, speG* (in all except 1 isolate), and *smeZ* genes, whereas the presence of other superantigen genes varied among the serotypes. The *S. aureus* isolates produced at least 1 superantigen each—SEA-D and/or TSST-1—but none of the isolates tested positive for exfoliative toxins A or B (table 2). Culture supernatants prepared from *S. aureus* and *S. pyogenes* isolates all induced very potent proliferative responses, and dose-response experiments showed that a dilution corresponding to 5×10^7 cfu/mL resulted in an optimal response, which typically ranged from 30,000 to 50,000 cpm (data not shown). No correlation between a specific superantigen profile and magnitude of proliferative response could be noted (data not shown).

The 3 different IVIG preparations caused a dose-dependent inhibition of the proliferative response associated both with staphylococcal culture supernatants ($n = 5$) and with strep-

tococcal culture supernatants ($n = 3$), and 100% inhibition was achieved at 2.5–5.0 mg IVIG/mL (figure 1). An important finding was dectection of a distinct difference in inhibitory activity against streptococcal culture supernatants and against staphylococcal culture supernatants; IVIG (concentrations ranging from 0.05 to 2.5 mg/mL) consistently inhibited streptococcal supernatant–induced proliferation to a greater extent than it inhibited staphylococcal supernatant–induced proliferation.

This difference was further confirmed in the extended analysis using 20 *S. pyogenes* and 20 *S. aureus* culture supernatants. Inhibition of proliferation induced by *S. pyogenes* supernatants was significantly different than the effects on *S. aureus* supernatant–induced proliferation for all IVIG preparations tested ($P < .01$) (figure 2). All lots of Gamunex and Gamimune exerted a greater inhibition of *S. pyogenes* and *S. aureus* exotoxins (99% and 93% inhibition, respectively) than did Endobulin S/D (93% and 82% inhibition, respectively) ($P < .0001$) (figure 2*B*). A significant difference in *S. aureus* exotoxin neutralization was found between 2 of the Gamunex batches ($P < .05$) (figure 2*A*), whereas no interbatch variation could be detected for Gamimune. There was no particular isolate or superantigen that could be identified as being more or less neutralized by the IVIG (data not shown).

DISCUSSION

Administration of IVIG has been proposed as adjunctive immunotherapy for superantigen-mediated TSS. Despite the lack of large, controlled, multicenter trials, which is a consequence of the low incidence of these diseases, there are strong cross-

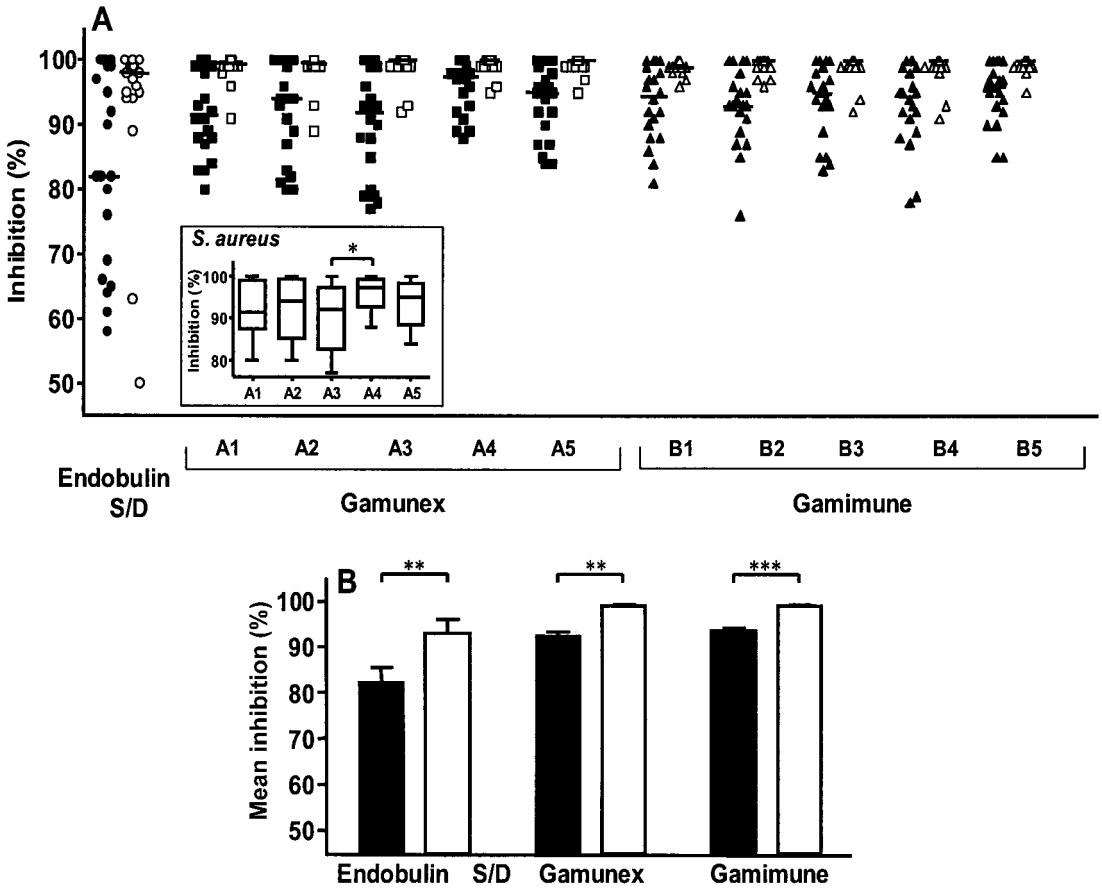

Figure 2. Inhibition of streptococcal supernatant–induced and staphylococcal supernatant–induced proliferation of peripheral blood mononuclear cells (PBMCs) by intravenous polyspecific IgG (IVIG). Human PBMCs from healthy individuals were stimulated with culture supernatants prepared from *Staphylococcus aureus* clinical isolates *(filled symbols)* ($n = 20$) and *Streptococcus pyogenes* clinical isolates *(open symbols)* ($n = 20$) in the presence of 3 preparations of IVIG: 5 batches each of Gamunex *(rectangles)* and Gamimune *(triangles)*, and 1 batch of Endobulin S/D *(circles)*, at concentrations 0.5 mg/mL. Proliferative responses were assessed after 72 h of stimulation. *A,* The individual data for each isolate. *Horizontal lines,* median values of inhibition by the different batches of IVIG. *Insert, S. aureus*–induced proliferation inhibited by Gamunex. *Horizontal line,* median; *bars,* 25th–75th percentiles; *whiskers,* range. *B,* The mean of percentages (± SDs) of inhibition of the streptococcal supernatants *(filled bars)* and staphylococcal supernatants *(open bars)*. Variations in values for different IVIG preparations and different batches were analyzed by ANOVA followed by the Tukey post hoc test. Significant differences are indicated in the figure: $*P = .044$; $**P < .001$; $***P < .0001$.

validated clinical data [27, 28] that, together with powerful functional data, provide strong support for the use of IVIG to treat superantigen-mediated TSS. In vitro studies have demonstrated the presence of antibodies against defined streptococcal and staphylococcal superantigens in IVIG [18–21]. Here we show for the first time (to our knowledge) that culture supernatants prepared from clinical *S. aureus* isolates, which contain a mixture of superantigens and other exotoxins, are functionally inhibited by IVIG. We have chosen to conduct the studies using culture supernatants from clinical isolates because this more closely mimics the clinical situation with potential synergistic or additive activities between superantigens and other secreted factors. As in the previous studies that reported IVIG-mediated broad-spectrum inhibition of streptococcal superantigens (reviewed in [36]), in this study IVIG seemed to exhibit a broad polyspecificity against staphylococcal superantigens; all 20 staphylococcal isolates we tested were equally inhibited, regardless of their superantigen expression profile.

In our recent placebo-controlled trial of the use of IVIG for streptococcal TSS, in vitro studies revealed that the superantigen-containing culture supernatant of the *S. aureus* clinical TSS isolate was less efficiently inhibited by IVIG than were the streptococcal superantigens tested [28]. This observation, made on the basis of only 1 isolate, was indeed confirmed in this study, in which we demonstrated significant differences between the neutralization of *S. aureus* and *S. pyogenes* superantigens by IVIG. The reason for this difference in inhibitory activity is unknown, but is likely due to differences in the quality and/or quantity of antibodies directed to streptococcal or staphylococcal superantigens. Previous studies have shown that the antibody titers measured by ELISA or immunoblot analysis do not always correlate with the neutralizing activity [16, 17, 23, 24, 26]. Differential neutralizing activity of IVIG has previously been reported for defined streptococcal superantigens, with the superantigen speA being less efficiently neutralized, compared with other streptococcal superantigens, despite equal antibody levels. Hence, several studies have demonstrated that the titer of the antibodies does not determine the functional activity of the antibody, but the reason for this discrepancy between titer and functional activity is, as yet, unknown.

Previous studies have demonstrated that different IVIG preparations and different batches of the same preparation may have varying levels of neutralizing activity against *S. pyogenes* superantigens [24, 26]. In this study, all lots of Gamunex and Gamimune showed superior neutralizing activity against streptococcal and staphylococcal superantigens, compared with Endobulin S/D. Interbatch variation could also be demonstrated, because 2 lots of Gamunex differed in their neutralizing activity against *S. aureus* exotoxins. This was the only significant difference found within and between the different Gamunex and Gamimune lots. Because we tested only 1 lot of Endobulin S/D, the observed difference in the neutralizating activity of this preparation in comparison with Gamunex or Gamimune remains to be validated.

The difference in the degree of IVIG-induced neutralizing activity against staphylococcal and against streptococcal culture supernatants was relatively small, ranging from 6% to 11% at physiological concentrations of IVIG. However, we believe that even this small difference in neutralizing activity may have an impact on clinical efficacy, because for the patient with staphylococcal TSS in the IVIG trial [28], post-IVIG plasma had only 41% neutralizing activity, compared with 98% activity for post-IVIG plasma from patients with streptococcal TSS. This was in concordance with in vitro data that showed reduced IVIG neutralizing activity against the staphylococcal isolate—65%, compared with 99% activity against the streptococcal isolates [28]. Furthermore, a correlation between the in vitro neutralizing activity of the IVIG preparation and the neutralizing activity of plasma obtained after therapy has previously been reported for patients with streptococcal TSS [24]. Hence, taken together, the data suggest that even slight variations in superantigen-neutralizing activity may be clinically important, and that a higher dose of IVIG may be needed to obtain protective antibody levels in the treatment of staphylococcal TSS. It is important to note that our in vitro data demonstrate that 100% inhibition of *S. aureus* superantigens can be obtained with higher doses of IVIG.

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