Human intravenous immunoglobulin for experimental streptococcal toxic shock: bacterial clearance and modulation of inflammation

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Objectives: Polyclonal human intravenous immunoglobulin (IVIG) has been advocated as an adjunct to therapy in severe invasive streptococcal toxic shock because of its ability to neutralize superantigen toxins. The aim of this study was to assess IVIG therapeutic efficacy in an experimental model of streptococcal toxic shock.

Methods: To confirm the in vitro activity of IVIG against the Streptococcus pyogenes strain used in the study, IVIG was tested for superantigen neutralizing and bacterial opsonizing activity prior to in vivo studies. To evaluate the in vivo effects of IVIG in terms of microbiological outcome and disease severity in a superantigen-sensitive transgenic model of streptococcal shock, HLA-DQ transgenic mice were treated with IVIG either at the time of infection or after infection with S. pyogenes. Antibiotics were included in some studies.

Results: The IVIG preparation neutralized superantigenicity of S. pyogenes in vitro and enhanced bacterial killing in a whole blood assay. When given to mice at the time of S. pyogenes infection, IVIG neutralized circulating superantigens and reduced systemic inflammatory response. Remarkably, IVIG-enhanced systemic clearance of bacteria and enhanced neutrophil infiltrate into the infected tissues. However, when used in combination with penicillin and clindamycin in a delayed treatment setting, IVIG did not confer additional therapeutic benefit, in terms of inflammatory response, bacterial clearance or survival.

Conclusions: IVIG monotherapy can confer benefit in experimental streptococcal shock, but extension of these findings to the clinical situation will require further evaluation.

Keywords: Streptococcus pyogenes, polyclonal human intravenous immunoglobulin, septic shock, superantigen

Introduction

Trials of polyclonal human intravenous immunoglobulin (IVIG) as an adjunct to treatment of severe sepsis have failed to demonstrate a reduction in mortality. Indeed, a recent meta-analysis concluded that IVIG should only be used in the context of randomized clinical trials in sepsis. Nonetheless, high dose (0.5–1.0 g/kg/day) IVIG has been advocated as a useful adjunct in the specific setting of invasive group A streptococcal disease and the streptococcal toxic shock syndrome (STSS).

Invasive group A streptococcal infection is associated with a mortality of 30–60%, particularly when associated with STSS. Although Streptococcus pyogenes has good in vitro susceptibility to antibiotics such as penicillin, deaths occur even in those appropriately treated. Mortality from STSS can be reduced by early recognition and medical treatment, prompt surgical debridement and inclusion of clindamycin in the drug regimen. Evidence for these strategies stems from a mixture of retrospective case–control studies, animal data and in vitro data. A recent European randomized controlled trial of IVIG in invasive streptococcal disease was terminated early because of slow patient recruitment. The small numbers included in the study, coupled with a mortality of 30% in the placebo group precluded any definitive conclusions regarding the efficacy of IVIG in STSS, although there were some indications of potential benefit. In an earlier comparative observational study, Kaul et al. suggested

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that IVIG may be of benefit in STSS, though analysis of the data may have been confounded by the use of historical controls who were less likely to have received clindamycin or surgical intervention than IVIG-treated patients.

Mouse models of invasive streptococcal disease are well-established and have been used previously to study experimental approaches to therapy, such as the efficacy of clindamycin in treatment,


reported that IVIG therapy started 8 h after onset of infection provided no therapeutic benefit (either alone or in combination with antibiotics) in terms of bacterial clearance from thigh muscle in *S. pyogenes*-infected C57BL/6 mice. This study did not examine systemic inflammatory response, and also did not report the *in vitro* efficacy of IVIG against the *S. pyogenes* isolate used in the studies, in relation to either opsonizing efficiency or neutralization of toxins, both of which may be key beneficial properties of IVIG in STSS. IVIG is specifically proposed to inhibit harmful host pro-inflammatory responses to streptococcal superantigens which underlie STSS, but can also augment opsonization of bacteria and theoretically may assist bacterial clearance. C57BL/6 mice are resistant to the pro-inflammatory effects of streptococcal superantigens such as streptococcal pyrogenic exotoxin A (SPEA) and streptococcal mitogenic exotoxin Z (SMEZ), compared with ‘humanized’ transgenic mice which express human HLA class II molecules. It is therefore possible that any anti-inflammatory effect of IVIG would be masked in the C57BL/6 strain.

In this experimental study, we evaluated whether IVIG could provide any benefit against STSS in terms of bacterial clearance and severity of disease (evaluated by IL-6 levels, superantigen levels, organ histopathology and mortality) using HLA-DQ transgenic mice which are sensitive to streptococcal superantigens and which demonstrate clear pathological responses during sepsis that underlie STSS, but can also augment opsonization of bacteria and theoretically may assist bacterial clearance. C57BL/6 mice are resistant to the pro-inflammatory effects of streptococcal superantigens such as streptococcal pyrogenic exotoxin A (SPEA) and streptococcal mitogenic exotoxin Z (SMEZ), compared with ‘humanized’ transgenic mice which express human HLA class II molecules. It is therefore possible that any anti-inflammatory effect of IVIG would be masked in the C57BL/6 strain.

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IVIG in streptococcal shock

**Results**

**Human IVIG can neutralize superantigens produced by strain H305**

Significant depression of *S. pyogenes*-induced mitogenesis was seen with 0.5 mg/mL IVIG in the bioassay for superantigens (Figure 2a), confirming that the preparation could neutralize the combination of superantigens produced by this strain. Inhibition of *S. pyogenes*-induced mitogenesis was more marked than inhibition of phytohaemagglutinin (PHA)-induced mitogenesis (Figure 2b). Goat polyclonal IgG had no inhibitory effect on H305-induced mitogenesis, even when used at 5 mg/mL (not shown).

**IVIG can assist killing of *S. pyogenes* H305 in human and murine whole blood**

Although humans are exposed to group A streptococci throughout life, opsonizing M-type-specific antibody is not always present in blood samples drawn from healthy donors. Strain H305 grew 100-fold in non-immune human blood. Growth was inhibited when cultures were co-incubated with 2.5 mg/mL IVIG and was reduced by 90% when 5 mg/mL IVIG was used. (Figure 2c).

In-bred mice have no prior exposure to group A streptococci and are therefore non-immune, permitting growth of *S. pyogenes* in blood. IVIG 5 mg/mL reduced growth of H305 by only 40% in murine blood (Figure 2d) and further inhibition could not be achieved, even by increasing IVIG concentrations to 10 mg/mL (not shown). However, when blood from mice pre-treated with G-CSF was used, to improve neutrophil numbers and expression of Fc receptors (FcR)-γ, growth of H305 in murine blood was inhibited by IVIG to a greater extent compared with controls (Figure 2d). Hence, human IVIG can assist the *in vitro* clearance of H305 from murine blood, though activity in the whole blood assay may be limited by the lesser neutrophil number.

**IVIG given at time of infection can reduce IL-6 in superantigen-sensitive mice and can reduce bacterial spread**

IVIG treatment given at the start of infection ($t = 0$ h) reduced 24 h IL-6 levels in *S. pyogenes*-infected mice compared with albumin-treated controls (Figure 3a). Intriguingly, systemic spread of bacteria was also reduced by IVIG treatment, as demonstrated by significant reductions in spleen (Figure 3b) and blood colony counts (Figure 3c).

**IVIG given at time of infection can improve neutrophil recruitment to site of infection**

To further investigate the mechanism of IVIG-enhanced bacterial clearance, histopathological examination of muscle from IVIG and albumin-treated *S. pyogenes*-infected HLA-DQ mice was undertaken. This suggested a marked difference in recruitment of neutrophils to the site of infection (Figure 4a–d). Semi-quantitative analysis showed that IVIG-treated mice had more neutrophils within muscle than albumin-treated mice (Figure 4e).
IVIG given at time of infection neutralizes circulating bioactive superantigens

To study the mechanism of IVIG-induced reduction in IL-6, we investigated the extent to which IVIG treatment had reduced circulating superantigens in infected mice using an ex vivo bioassay. IVIG administration significantly reduced the amount of circulating bioactive superantigen by 60% in H305-infected mice when blood was drawn 24 h after onset of infection (Figure 5). Nonetheless, superantigenic effects were still detectable in the serum, hence the dose of IVIG was doubled to 2 g/kg on day 1, followed by 1 g/kg for subsequent studies where treatment was delayed.

Delayed treatment using IVIG with antibiotics does not enhance bacterial clearance from muscle

The effect of delaying treatment to 24 h and using antibiotics at the same time as IVIG was then investigated. Preliminary studies had...
shown that penicillin and clindamycin treatment led to sterilization of blood and spleen cultures, hence it was necessary to collect thigh muscle in order to determine whether bacterial clearance was affected by IVIG therapy. Muscle was collected 72 h after start of infection (48 h after start of treatment). Surprisingly, group A streptococci were easily cultured from muscle, despite exposure to antibiotics which had cleared bacteria from blood and spleen. Mice receiving albumin in conjunction with antibiotics had a similar level of bacterial growth in thigh muscle (median, 4.7 × 10^7 cfu/g tissue; range, 0.9–17 × 10^7 cfu/g) to mice receiving IVIG with antibiotics (median, 5.3 × 10^7 cfu/g tissue; range, 2.2–7.7 × 10^7 cfu/g). Hence, when combined with antibiotics, IVIG did not enhance bacterial clearance from muscle.

We considered the possibility that viable streptococci may persist in thigh muscle due to poor penetration of antibiotics into necrotic tissue or internalization of streptococci into host cells. However, muscle tissue antibiotic activity, as measured by inhibition zone diameter, was easily detectable and indistinguishable between infected and non-infected mice (21.3 ± 1.5 mm from neat non-infected muscle extract compared with 22.3 ± 2.8 mm from infected muscle extract; further dilution did not reveal any divergence between the study groups and activity was detected in samples diluted 1:8). Antibiotic activity could also be easily detected in serum from infected mice, even when diluted 4-fold (not shown). Histopathological examination showed that all visible bacteria were extracellular.

Delayed treatment using IVIG with antibiotics does not affect severity of infection or survival

In conjunction with antibiotic treatment, albumin-treated (control) S. pyogenes-infected mice still had significantly elevated mitogenic activity in serum 24 h after onset of treatment (25 541.2 ± 5589 cpm, n = 9). However, in conjunction with antibiotic treatment, IVIG therapy reduced mitogenicity of infected mouse serum (9872 ± 6691 cpm, n = 7) to levels seen in normal, uninfected mouse serum (8025 ± 1517 cpm, n = 7). Despite this, in conjunction with antibiotic treatment, IVIG therapy did not affect production of IL-6, a recognized marker of disease severity, when compared with albumin-treated controls 24 h after start of treatment (48 h after onset of infection). The experiment was conducted using both low (10^5 cfu) and high (3 × 10^8 cfu) inocula to achieve mild (no mortality) and moderate (40% mortality) severity of infection (Figure 6). Furthermore, IVIG treatment did not affect IL-6 levels measured at two other time points after infection in antibiotic-treated mice.
inter-species differences in FcR, though human IgG is known to ine blood may be limited by lower neutrophil counts and possible study. We hypothesized that may in part explain the lack of protection seen in the earlier extent, murine blood. The lesser activity of IVIG in murine blood thermore, provided opsonizing activity in human and, to a lesser activity against the superantigens produced by S. pyogenes evidence.1,22 This study represents the only in-depth analysis for streptococcal shock despite the lack of definitive clinical therapeutic advantage in an antibiotic-treated model. S. pyogenes sensitive mice and an IVIG preparation with known activity ment is commenced at the onset of infection. Using superantigen-severity in experimental streptococcal shock provided that treat clearance, neutralize circulating superantigen, and reduce illness This study provides evidence that IVIG can assist in bacterial infection. Quantification of neutrophils by myeloperoxidase in leg tissue. We speculate that IVIG may neu additional stimulation to murine neutrophils, facilitating the inter- and the cytokine burst that accompanies infection may provide additional stimulation to murine neutrophils, facilitating the inter-activity with human IVIG. IVIG had no direct antibacterial activity (data not shown).

Discussion

This study provides evidence that IVIG can assist in bacterial clearance, neutralize circulating superantigen, and reduce illness severity in experimental streptococcal shock provided that treatment is commenced at the onset of infection. Using superantigen-sensitive mice and an IVIG preparation with known activity against S. pyogenes, delayed IVIG therapy did not confer overall therapeutic advantage in an antibiotic-treated model.

IVIG is widely considered an effective adjunctive treatment for streptococcal shock despite the lack of definitive clinical evidence.1,22 This study represents the only in-depth analysis of the efficacy and mechanism of IVIG action in experimental STSS, using an HLA-DQ transgenic model of sepsis which is highly responsive to superantigens. In contrast to an earlier study,14 the IVIG used here had demonstrable neutralizing activity against the superantigens produced by S. pyogenes and, furthermore, provided opsonizing activity in human and, to a lesser extent, murine blood. The lesser activity of IVIG in murine blood may in part explain the lack of protection seen in the earlier study. We hypothesized that in vitro opsonizing activity in murine blood may be limited by lower neutrophil counts and possible inter-species differences in FcR, though human IgG is known to successfully interact with rodent FcR.23 The in vitro opsonizing activity of IVIG in mouse blood improved following G-CSF therapy, which is known to increase neutrophil counts and FcR expression,23 confirming that the activity of human IVIG in mouse blood is limited by quantitative differences in neutrophil or FcR number rather than inter-species incompatibility. Surprisingly, IVIG administered to infected mice at 0 h markedly reduced the systemic bacterial load and systemic inflammatory response in mice infected with S. pyogenes. G-CSF and interferon-γ are significantly raised in HLA-DQ8 transgenic mice compared with wild-type controls during S. pyogenes infection (S. Sriskandan, M. Ferguson and L. Faulkner, unpublished data), and the cytokine burst that accompanies infection may provide additional stimulation to murine neutrophils, facilitating the interaction with human IVIG. IVIG had no direct antibacterial activity (data not shown).

Intriguingly, histopathology showed that IVIG treatment was associated with a greater influx of neutrophils to the site of infection. Quantification of neutrophils by myeloperoxidase assay was not feasible due to high background levels of myeloperoxidase in leg tissue. We speculate that IVIG may neutralize a range of S. pyogenes virulence factors which impede neutrophil recruitment and activation, such as the IL-8 cleaving cell envelope proteinase SpyCEP,24,25 thereby improving the overall host response to infection. Importantly, this study is the first to demonstrate any benefit of polyspecific human
immunoglobulin on systemic bacterial clearance in experimental S. pyogenes sepsis. IVIG administered at time 0 h also reduced serum IL-6. IL-6 is known to be a reliable marker of disease severity in this model and high levels are associated with early mortality.19 Other cytokines are difficult to detect except immediately prior to death and could not be assayed in this model using standard ELISA due to interference from the high prevailing concentrations of IVIG. IVIG-induced reduction in disease severity may relate directly to enhanced bacterial clearance or to neutralization of bacterial toxins known to cause inflammation, such as superantigens. An earlier clinical study showed that IVIG present in patient plasma has the potential to neutralize exogenous superantigens ex vivo.16 We now show for the first time that IVIG directly neutralizes systemic superantigen activity produced during experimental sepsis. Although the serum bioassay for superantigens may reflect the presence of circulating pro-mitogenic cytokines in addition to superantigens, we have previously shown that the mitogenic effects of serum from patients with STSS can be obliterated specifically with anti-superantigen antibodies, suggesting that the contribution of cytokines is small.26

Having established that IVIG pre-treatment can reduce bacterial load, superantigen activity, and illness severity in experimental S. pyogenes sepsis, the effects of delayed therapy were then evaluated in antibiotic-treated mice, to assess the likely benefits of IVIG in a model which resembled the clinical setting. The delayed dual antibiotic-treated model represents a significant advance in modelling, as it reproduced the illness severity commonly encountered in clinical practice, with a mortality of 60–70%. IVIG did not reduce severity in the antibiotic-treated model, using IL-6 as a severity marker. As an alternative to IL-6, plasma lactate was also measured in groups receiving delayed treatment with antibiotics and IVIG or albumin, though no differences were seen between treatment groups (data not shown). Delayed antibiotic therapy cleared bacteria from the blood and spleen, and therefore microbiological outcome was measured by bacterial quantification from infected muscle. IVIG treatment did not reduce bacterial load in muscle in antibiotic-treated mice, however. We were surprised that viable S. pyogenes persisted in muscle despite bactericidal levels of antibiotic in both serum and muscle extract. We speculate that S. pyogenes can acquire an antimicrobial-resistant or biofilm phenotype during invasive infection in vivo if not actively growing, leading to relative resistance to antimicrobials.27 Careful histopathological examination showed that extracellular bacteria dominated, though we cannot exclude the possibility that small foci of intracellular bacteria might act as a source for waves of replication, or that the combination of antibiotics used led to a bacteriostatic effect in muscle rather than bactericidal effect. Consistent with bacteriology and IL-6 data, mortality was unaffected by IVIG treatment in the antibiotic-treated setting. It is possible that a beneficial effect of IVIG might be revealed in settings where treatment, such as antibiotic dose, is suboptimal. Antibiotic doses used in this study were based on recommendations in the European Strep-Ig study protocol and UK formularies.

Using IVIG with defined anti-S. pyogenes activity and a superantigen-sensitive humanized murine model of invasive streptococcal disease, we have demonstrated that human IVIG has therapeutic effect when administered at the time of infection. Despite these findings, IVIG did not confer detectable additional protection when used in conjunction with standard antibiotic therapy in the delayed treatment setting. Notwithstanding the difficulties inherent in conducting clinical studies in STSS, additional clinical trial data will be required before one can confidently state that IVIG is indicated in therapy for STSS.

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