Decreased Protective Efficacy of Reduced and Alkylated Human Immune Serum Globulin in Experimental Infection with Haemophilus influenzae Type b

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Conventionally prepared immune serum globulin frequently produces severe side effects when administered intravenously. A modified preparation in which 4 to 5 interchain disulfide bonds have been reduced and alkylated has been made for intravenous use. However, reduction and alkylation may affect Fc-mediated functions of immunoglobulin G, particularly its ability to fix complement by the classical pathway. To determine whether reduction and alkylation alters the protective activity of immune serum globulin in vivo we compared it with two less harshly prepared globulins (pH 4 treated or ultrafiltered) in an infant rat model of Haemophilus influenzae b infection. Antibody binding to the capsular and noncapsular components of H. influenzae b and in vitro bactericidal activity were similar in the globulin preparations. Infant rats were treated with various doses of globulins adjusted to provide identical concentrations of capsular antibodies as measured by the Farr radioactive antigen binding assay. At high doses of anticapsular antibody (>1,500 ng per pup), all preparations protected well. At marginal doses (750 ng per pup), however, rats given reduced and alkylated globulin had a significantly greater incidence of bacteremia (P < 0.05), meningoitis (P < 0.01), and death (P < 0.05) and a higher magnitude of bacteremia (P < 0.02) than rats who received pH4-treated or ultrafiltered globulins. These differences were not due to differences in capsular antibody concentrations achieved in the serum. The 50% protective serum concentrations of capsular antibody in this model were 200 to 300 ng/ml for reduced and alkylated globulin and 100 to 200 ng/ml for acid-treated globulin. Absorption of the globulins with purified H. influenzae b capsule reduced in vitro bactericidal activity and rat protective activity. However, the magnitude of bacteremia was lower in rats receiving absorbed pH 4-treated globulin than in those receiving absorbed reduced and alkylated globulin (P < 0.05). We conclude that reduced and alkylated immunoglobulin G provides significantly less protective activity against H. influenzae b infection in this model than globulins not so modified, and we suggest that the altered Fc function of the immunoglobulin G, such as the decreased ability to fix complement by the classical pathway or decreased Fc-mediated opsonization, may be responsible for this impairment.

Human immune serum globulin (ISG) has been found to be useful in reducing infections in patients with humoral immune deficiencies (4, 15, 31). The dose of ISG that can be administered has been limited by the necessity of intramuscular administration due to serious systemic side effects that occurred with intravenous use (4, 5). Although the mechanism of these effects is not fully understood, spontaneous activation of complement by immunoglobulin G aggregates formed during the plasma fractionation process may be responsible (5, 23).

An intravenous globulin that avoids these problems would permit larger and less frequent doses of ISG, as well as provide ISG therapy to patients previously unable to receive it (7). Ideally, an intravenous globulin should have normal antigen binding, normal Fc-mediated function, such as fixation of complement and opsonization, and absence of systemic side effects. A number of ISGs suitable for intravenous administration have recently been developed. One preparation, modified by reduction and alkylation, has been available commercially in the United States for several years. This process uses controlled methods to selectively reduce an average of four to five disulfide bonds on each ISG molecule (27, 28), yielding an ISG that has decreased anticomplementary activity, normal antigen binding, normal staphylococcal protein A binding and intact bactericidal activity (6, 9, 12, 23, 25). However, reduction and alkylation decreases the Fc ability to bind to the first component of complement (6, 21, 35). Complement activation does occur, however, primarily through the alternative pathway and with altered kinetics and decreased cooperativity (6, 21, 36). In addition, Fc-mediated opsonization has been found to be abnormal (25, 32). The decreased functional activity of reduced and alkylated ISG has thus far not been demonstrated to decrease protective activity against bacterial infection in vivo (9, 26). Recently, more gently prepared “native” immunoglobulins, such as ultrafiltered ISG (UF-ISG) and pH4-treated ISG (pH 4-ISG), have become available. UF-ISG, an essentially unmodified ISG that has not been exposed to excessive denaturation during the fractionation process, has minimal amounts of immunoglobulin G aggregates. pH 4-ISG is briefly treated with low pH and trace pepsin to minimize aggregate formation. Both these ISGs have intact interheavy chain disulfide bonds, exhibit apparently normal Fc function, including C1Q binding, have normal in vitro antibody function (23, 28), and represent alternatives to reduced and alkylated ISG. We undertook this study to determine whether reduced and alkylated ISG exhibits decreased protective activity in vivo compared with

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these more gently prepared globulins. We chose an infant rat model of Haemophilus influenzae type b (Hib) infection to demonstrate protective differences, since this organism is a major pathogen in patients with antibody deficiencies who are likely to receive therapeutic ISG (11, 15). In addition, it has previously been shown that human ISG containing antibodies to the capsular polysaccharide of Hib (polyribosylribitol phosphate [PRP]) can prevent infection in this model (2, 17). Furthermore, this model provides multiple outcomes (bacteremia, meningitis, death) as well as quantitative outcome (magnitude of bacteremia), thus facilitating the evaluation of subtle but important differences in prophylactic regimens (17, 20, 29).

**MATERIALS AND METHODS**

**Intravenous immune serum globulins.** Three preparations were used for comparison. All are fractionated from plasma by modifications of the cold ethanol procedure of Cohn et al. and Oncley et al. (8, 19). Reduced and alkylated ISG (RA-ISG) was obtained commercially (Cutter Biologic Laboratories, Inc., Berkeley, Calif.) and is supplied as a 5% protein solution stabilized with 10% maltose. This ISG is prepared from Cohn fraction II that has been reduced with dithiothreitol and alkylated with iodoacetamide (12, 27). pH 4-ISG was obtained from, Sandoz Inc., Basle, Switzerland, in lyophilized form. The preparation is prepared by brief treatment of Cohn fraction II with pH 4 in the presence of trace amounts of pepsin and is stabilized with 10% sucrose. The ISG was reconstituted as a 5% protein solution with sterile saline. UF-ISG was obtained from Massachusetts Public Health Biologic Laboratories, Boston, in lyophilized form. This preparation is made by a modified Cohn fractionation technique, using diafiltration to minimize exposure to high alcohol concentrations and prevent excessive denaturation. The globulin is stabilized with 5% sucrose and 1% albumin and was reconstituted with sterile saline to a 5% protein solution.

**Bacteriological methods.** A streptomycin-resistant mutant of Hib strain Eagan (E-1) was used in most experiments. The parent strain was stored in skim milk at −70°C, and fresh subcultures were used for each experiment. Inocula for animal challenge were prepared by growing Hib to mid-log phase (5 × 10^8 CFU/ml) in 10 ml of brain heart infusion (BHI) broth supplemented with NAD and hemin. The suspension was centrifuged at 12,000 × g for 10 min at 4°C, washed twice in phosphate-buffered saline (PBS), resuspended in PBS to a concentration of 5 × 10^10 CFU/ml and stored on ice until use. Hib for bactericidal assay was prepared in the same manner but was grown to a final concentration of 5 × 10^10 CFU/ml.

Cultures of blood and spinal fluid of rats were performed on supplemented BHI agar containing 50 µg of streptomycin and 5 U of bacitracin per ml to inhibit growth of contaminants. Plates were incubated overnight at 37°C in 5% CO₂ atmosphere, and colonies were counted the next morning.

**Bactericidal assay.** The bactericidal assay was performed with Hib strain Eagan (E-1) which was grown and washed as noted. Globulin samples were serially diluted, and 25-µl samples were added to each well of a sterile plastic tray. Hib (200 µl) at a concentration of 5 × 10^10 CFU/ml was added to 800 µl of a solution of PBS containing 0.5 mM MgSO₄, 0.15 mM CaCl₂, 5% horse serum albumin (immunoglobulin free; Sigma Chemical Co., St. Louis, Mo.), and 25% colostrum-deprived calf serum as the complement source (22). A 25-µl sample of this mixture was then added to each well containing various concentrations of ISG. The plastic tray was incubated on a shaker for 60 min at 37°C and 800 µl of supplemented BHI agar (kept liquid at 45°C) was added to each well. Plates were incubated overnight at 37°C in 5% CO₂, and colonies were counted the next day. The globulin dilution showing ≥99% reduction in CFU was used as the endpoint.

**Antibody assays.** Antibody to the Hib capsule was measured in globulins and animal sera by a Farr-type radioactive antigen-binding assay (3), using tritiated PRP supplied by Porter Anderson, University of Rochester, Rochester, N.Y. A standard serum containing 40 µg of capsular antibody per ml was used for quantitation (22).

Antibody to noncapsular Hib components was assayed by using an enzyme-linked immunosorbent assay to Hib noncapsular surface antigens. A Hib (strain Eagan) capsule-deficient mutant (supplied by Robert S. Daum, Tulane Medical Center, New Orleans, La.) was grown for 18 h in 200 ml of supplemented BHI broth. The bacteria were spun at 12,000 × g for 10 min at 4°C, and the pellet was washed twice in PBS and then resuspended in 1 M Tris buffer with 2.5 mM EDTA. Absorbance was determined on a spectrophotometer (Stasar II; Gilford Instruments, Oberlin, Ohio), and the bacteria were then sonicated on ice (Sonicator cell disrupter, Ultrasonics, Plainview, N.Y.) until a >50% decrease in absorbance was observed. The disrupted Hib cells were repelleted at 12,000 × g, the pellet was discarded, and the supernatant was saved and resuspended in 30,000 × g for 20 min at 4°C. This new pellet was suspended in PBS, the protein concentration was assayed by the method of Lowry (16), and the solution was diluted to 1 µg/ml of protein concentration. Latex particle agglutination showed less than 1 ng of residual PRP per ml (33). Wells of plastic microtiter plates (Linbro, Titertek; Flow Laboratories, McLean, Va.) were coated with 100 µl of the concentration (1 µg/ml) of protein and washed with PBS-Tween 20, and various dilutions of globulins then added. After 24 h of incubation at 4°C, plates were again washed, and gamma chain-specific goat anti-human IgG (Atlantic Antibody, Scarborough, Maine) conjugated with alkaline phosphatase was added. After 2.5 h of incubation at 37°C, plates were washed again, and 110 µl of phosphatase developer was added. Optical density was read on an enzyme-linked immunosorbent assay reader (Biotek Instruments Inc., Burlington, Vt.). The endpoint titer was defined as the dilution giving an optical density at 450 nm of 0.300.

**Absorption of ISG with PRP.** Purified PRP (lot 764; Merck & Merck Sharp & Dohme, West Point, Pa.) was added to each ISG at a concentration five times the weight of the capsular antibody in the ISG. After incubation at 4°C for 5 days, the mixture was centrifuged at 12,000 × g for 30 min. The supernatant was dialyzed against 0.02 M Na₂HPO₄, pH 8.0, and applied to a DEAE column. The effluent was monitored for absorption at 280 nm, and the protein-containing fractions were pooled and concentrated with a Minicon B-15 concentrator (Amicon Corp., Danvers, Mass.) to a concentration of 4%. The final anti-PRP concentration was 1 µg/ml, approximately 5% of that present in the parent ISGs. No residual PRP remained in the ISGs as determined by a latex particle agglutination assay (33). This material was diluted 1/2 before administration to infant rats.

**Animal model.** Outbred, pathogen-free, Sprague-Dawley albino rats were obtained from Camm Research, Wayne, N.J. The rats were 25-30 days old. A single dose of capsular antibody by Farr assay. Pregnant female rats were allowed to deliver in our laboratories. On day 2 of life,
pups were randomized among mothers so that no mother had less than 8 or more than 11 pups and to minimize effects of genetic variation among litters.

Infant rats, 5 days old, were given ISG of various dilutions in a volume of 0.15 ml intraperitoneally (i.p.). Controls received 0.15 ml of 0.9 M glycine-buffered saline (pH 8.0). After 18-h, 30 to 50 µl of blood was obtained by tail vein puncture for assay of antcapsular antibody levels. Hib (5 × 10^3 CFU) in a volume of 0.1 ml was then given i.p. with a 1-cc tuberculin syringe and a 27-gauge 1/2-in (ca. 12.7 mm) needle. Certain animals were also given ISG but not challenged with Hib. Antibody levels in these uninfected animals were then monitored on day 1 and 3 after globulin administration.

Blood bacterial density was monitored 1, 2, and 4 days post-Hib inoculation. The distal tail vein of unanesthetized pups was cleansed with isopropyl alcohol, dried, and punctured with a sterile lancet.Volumes (1 and 10 µl) of blood were spread on opposite halves of petri plates containing supplemented BHI agar, permitting quantitation of 10⁴ to 10³ CFU/ml of blood.

Cerebrospinal fluid cultures were obtained 2 days after Hib inoculation. Pups were held with head flexed, the occipital area was cleansed with isopropyl alcohol, and puncture of the cisterna magna was performed with a 27-gauge needle. Approximately 10 µl of cerebrospinal fluid was cultured on supplemented BHI agar.

Statistical methods. Data analysis were performed on the PROPHET System, a national computer system sponsored by the National Institutes of Health. Comparison of means was performed by a paired t-test for parametric data and by the Mann-Whitney test for nonparametric data (two tailed). The Fisher exact test was used to determine significance of differences in incidence of bacteremia, meningitis, and mortality among animal groups and for differences in minimum protective antibody levels.

RESULTS

In vitro characterization of immune serum globulins. All globulins were diluted to 5% protein concentrations for assay. Antcapsular antibody concentrations were similar in each preparation ranging from 20,000 to 30,000 ng/ml as measured by Farr assay. Antibody titers to H. influenzae noncapsular surface antigens measured by enzyme-linked immunosorbent assay were also similar in RA-ISG and pH 4-ISG (Table 1).

Two lots of RA-ISG and pH 4-ISG had cidal titers of 1.8 to 1:16. One lot of UF-ISG had a cidal titer of 1:16 to 1:32. Antibody absorption with purified PRP removed 95% of the anti-PRP antibody and resulted in a fourfold reduction in bactericidal titer of pH 4-ISG and RA-ISG (Table 1), suggesting that anti-PRP antibody provided most of the bactericidal activity.

Animal protection. Animals given saline and challenged with Hib sustained a high incidence of bacteremia and meningitis, a high cumulative death rate, and a high magnitude of blood bacterial density (Table 2). Doses of pH4-ISG or RA-ISG containing large amounts of anti-PRP antibody protected infant rats from Hib infection; a globulin dose containing 3,000 ng of anti-PRP antibody given before Hib challenge prevented bacteremia, meningitis, and death when compared to saline injected controls (Table 2). One rat receiving RA-ISG at this dose developed low-grade transient bacteremia.

Lower doses of antibody revealed differences in protective efficacy between RA-, pH 4-, and UF-ISGs. Rats receiving RA-ISG containing 1,500 ng of anti-PRP antibody developed a higher incidence of bacteremia (40 versus 0%; P < 0.05) than rats receiving the same dose of pH 4-ISG. Similarly, at a dose of 750 ng of anti-PRP antibody, RA-ISG recipients, despite similar serum antibody levels, had significantly higher incidences of bacteremia, meningitis, and death as well as a higher magnitude of bacteremia than did pH 4-ISG recipients (Table 2). The differences in protection afforded by the globulins persisted for the 5-day duration of the experiment (Fig. 1). To ensure that the difference was not confined to single lots of RA-ISG and pH 4-ISG, two lots of each were examined and compared. Similar differences in protection were found (data not shown), and these data are therefore combined in Table 2. Finally, a globulin prepared by a third method, ultrafiltration, showed similar efficacy as the pH 4-ISG.

To determine whether the protective activities of RA- and pH 4-ISGs might differ due to different activities of antibody to noncapsular determinants, animals were injected with PRP-absorbed ISGs containing primarily antismsomatic antibody. Animals receiving either absorbed RA-ISG or absorbed pH 4-ISG had a lower magnitude of bacteremia than did saline-injected controls. Since these animals had no detectable antcapsular antibody in their serum, the moderate protection observed was most likely due to antibodies to noncapsular components of Hib. Animals receiving absorbed pH 4-ISG, however, had a significantly lower mag-

<table>
<thead>
<tr>
<th>Immunoglobulin prepnt*</th>
<th>Anti-PRP antibody (ng/ml)</th>
<th>Antibody titer to Hib noncapsular antigens*</th>
<th>Bactericidal titer</th>
<th>Bactericidal titer after PRP absorption</th>
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<tbody>
<tr>
<td>pH 4-ISG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lot A</td>
<td>20,000</td>
<td>1/750</td>
<td>1/8</td>
<td>1:1/2</td>
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<tr>
<td>Lot B</td>
<td>25,000</td>
<td>ND</td>
<td>1/16</td>
<td>ND</td>
</tr>
<tr>
<td>UF-ISG</td>
<td>30,000</td>
<td>ND</td>
<td>1/16-1/32</td>
<td>ND</td>
</tr>
<tr>
<td>RA-ISG</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lot A</td>
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<td>1/600</td>
<td>1/8</td>
<td>1:1/2</td>
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<tr>
<td>Lot B</td>
<td>21,000</td>
<td>ND</td>
<td>1/8</td>
<td>ND</td>
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</tbody>
</table>

* All ISGs were diluted to 5% protein concentration.

* Titer giving an optical density measured at 405 nm of 0.3 by enzyme-linked immunosorbent assay.

* Residual anti-PRP antibody was 1,000 ng/ml in each preparation.

* ND, Not done.
TABLE 2. Comparison of protective efficacy of pH 4-ISG, UF-ISG, and RA-ISG in infant rats given Hib i.p.

<table>
<thead>
<tr>
<th>Immunoglobulin prep (dose of anti-PRP antibody [ng])</th>
<th>No. of pups</th>
<th>Day 1 mean serum anti-PRP antibody concn (ng/ml)</th>
<th>Incidence (%) of:</th>
<th>Day 2 geometric mean bacteremia (CFU/ml of blood)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Bacteremia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Meningitis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Death&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saline (0)</td>
<td>16</td>
<td>&lt;25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pH 4-ISG (3,000)</td>
<td>6</td>
<td>771</td>
<td>0</td>
<td>0</td>
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<tr>
<td>RA-ISG (3,000)</td>
<td>6</td>
<td>804</td>
<td>17</td>
<td>0</td>
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<tr>
<td>pH 4-ISG (1,500)</td>
<td>11</td>
<td>342</td>
<td>0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>RA-ISG (1,500)</td>
<td>10</td>
<td>366</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>pH 4-ISG (750)</td>
<td>22</td>
<td>215&lt;sup&gt;e&lt;/sup&gt;</td>
<td>23&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>RA-ISG (750)</td>
<td>21</td>
<td>156</td>
<td>86</td>
<td>67</td>
</tr>
<tr>
<td>UF-ISG (750)</td>
<td>6</td>
<td>191</td>
<td>33</td>
<td>0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 4-ISG (375)</td>
<td>9</td>
<td>118</td>
<td>89</td>
<td>56</td>
</tr>
<tr>
<td>RA-ISG (375)</td>
<td>9</td>
<td>97</td>
<td>100</td>
<td>89</td>
</tr>
</tbody>
</table>

<sup>a</sup> Day 2.
<sup>b</sup> Day 3.
<sup>c</sup> Day 5.
<sup>d</sup> Cumulative.
<sup>e</sup> P < 0.05 as compared to RA-ISG-treated rats receiving the same dose of anti-PRP antibody by Fisher's exact test.
<sup>f</sup> P > 0.05 as compared to day 1 mean anti-PRP antibody levels in RA-ISG-treated rats receiving the same dose of anti-PRP antibody.
<sup>g</sup> P < 0.05 as compared to RA-ISG-treated rats receiving the same dose of anti-PRP antibody by Fisher's exact test.

The relationship between day 1 serum anti-PRP levels and bacteremia 24 h after Hib challenge is shown in Fig. 3. Of rats receiving pH 4-ISG, 54% were protected from Hib infection when initial serum anti-PRP antibody levels were between 100 and 200 ng/ml. This contrasted with 66% protection in RA recipients with the same initial antibody levels (P < 0.01). The initial anti-PRP antibody level that protected 50% of pups receiving reduced and alkylated ISG from infection was approximately 100 ng/ml higher than native globulin recipients.

DISCUSSION

RA-ISG afforded less protection from bacteremia, meningitis, and death than rats given absorbed RA-ISG (Table 3).

Serum antcapsular antibody levels. Serum anti-PRP antibody levels were measured in each rat pup to exclude the possibility that differences in protection were due to differences in the absorption and pharmacology of each ISG. Mean serum anti-PRP antibody levels were similar in each dosage group (Table 2) 24 h after i.p. injection. Uninfected control animals given pH 4-ISG or RA-ISG containing 1,500 ng of anti-PRP antibody also maintained similar antibody levels over a 3-day period (Fig. 2).

The difference in efficacy was not due to differences in anti-PRP or noncapsular antibody content in the globulins, nor was it due to differences in antcapsular antibody levels in the infant rats which were similar in each dosage group. Decreased protective efficacy of RA-ISG was especially apparent at doses delivering borderline protective quantities of anti-PRP antibody.

The initial serum anti-PRP antibody level in rats predicted protection (Fig. 3). The protective level of antibody in rats receiving native ISG was similar to that predicted for humans (22). The serum concentration of anti-PRP antibody required for protection of infant rats challenged i.p. with Hib (100 to 200 ng/ml) was slightly higher than the reported previously in an intranasal rat model of Hib infection (2). This difference is probably due to the larger number of organisms entering the circulation after i.p. injections than after intranasal administration (17, 18, 29). The protective level of anti-PRP antibody in rats receiving RA-ISG was twofold higher, suggesting decreased function of the antibody in vivo.

The differences in protective effect of the ISGs were not due to differences in pharmacokinetics in the infant rats. Uninfected pups had similar anti-PRP antibody levels over a 3-day period after being given either RA-ISG or pH 4-ISG i.p.
The differences in protective effect were also not due to different concentrations of antibody to noncapsular surface antigens of Hib. Enzyme-linked immunosorbent assay measurements of antibody titers confirmed that concentrations of somatic antibody were similar in RA-ISG and pH 4-ISG. Use of the PRP-absorbed ISGs in vivo showed some protective activity. Again, however, absorbed RA-ISG was significantly less protective than absorbed pH 4-ISG, suggesting decreased functional activity of antibody to noncapsular components of Hib in vivo.

The mechanism of the decreased efficacy of RA-ISG in protection of infant rats from Hib infection is unclear. Previous studies, as well as the current study, have documented normal in vitro cidal activity and antigen binding of RA-ISG. However, it seems clear that reduction and alkylation alters Fc function so that RA-ISG activates complement primarily through the alternative pathway. Function of the classical pathway, however, may be important to optimal host defense against bacterial infection. Bactericidal activity and opsonization by serum is more rapid when the classical pathway is intact (24). An in vivo correlate of this finding exists in C4-deficient guinea pigs, which, when challenged with pneumococci, exhibit more rapid reappearance of bacteraemia than normal animals and reduced protective activity of type specific antibody (14). Although humans genetically deficient in early complement components are not always subject to infections, some C2-deficient humans have been found to be more susceptible to bacterial infections, including Hib, despite normal alternative pathway function (1, 35).

The differences in efficacy of RA-ISG and less harshly treated ISGs were especially pronounced when low anti-capsular antibody levels were present in infant rats. Others have shown that low levels of specific antibody to type III group B streptococcus utilizes the classical pathway, whereas

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### TABLE 3. Effect of PRP absorption on protective efficacy of pH 4-ISG and RA-ISG

<table>
<thead>
<tr>
<th>Immuno-globulin prepn</th>
<th>No. of pups</th>
<th>Total anti-PRP antibody dose (ng)</th>
<th>Day 1 mean serum anti-PRP antibody concn (ng/ml)</th>
<th>Incidence (%) of Bacteremia*</th>
<th>Meningitis*</th>
<th>Death*</th>
<th>Day 2 geometric mean bacterial (CFU/ml blood)</th>
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<tr>
<td>pH 4-ISG</td>
<td>5</td>
<td>75</td>
<td>&lt;25</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>6.9 × 10^2/</td>
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<tr>
<td>RA-ISG</td>
<td>6</td>
<td>75</td>
<td>&lt;25</td>
<td>100</td>
<td>100</td>
<td>17</td>
<td>2.6 × 10^4/</td>
</tr>
<tr>
<td>Saline</td>
<td>16</td>
<td>0</td>
<td>&lt;25</td>
<td>100</td>
<td>100</td>
<td>81</td>
<td>3.7 × 10^2/</td>
</tr>
</tbody>
</table>

* Day 2.
* Day 3.
Cumulative.
* P < 0.01 as compared to RA-ISG- and saline-treated rats.
* P < 0.05 as compared to saline-treated rats.

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**FIG. 2.** Mean serum anti-PRP antibody levels ± standard error of the mean in uninfected rat pups after administration of either pH 4-ISG or RA-ISG containing 1,500 ng of anti-PRP antibody (n = 5 in pH 4-ISG group and n = 6 in RA-ISG group).

**FIG. 3.** Initial serum anti-PRP antibody levels in bacteremic and nonbacteremic rats who received either pH 4-ISG or RA-ISG before i.p. Hib challenge.
higher antibody levels recruit the alternative pathway (10). Similar findings have recently been described for anti-PRP antibody (30). Low levels of reduced and alkylated capsular antibody may thus be insufficient to activate adequate quantities of complement via the classical pathway for optimal promotion of bacterial lysis and opsonophagocytosis. Previous studies have demonstrated that antibodies to noncapsular Hib components may also be protective in infant rats (13). Our PRP-absorbed preparations containing anti-somatic antibodies were mildly protective at the concentrations given. PRP-absorbed RA-ISG, however, exhibited decreased protective efficacy compared to PRP-absorbed pH 4-ISG. The decreased protective efficacy of the PRP-absorbed RA-ISG may also be due to impaired complement activation by the noncapsular antibody in the preparation. Noncapsular antibody has recently been found to be unable to facilitate complement-mediated bactericidal activity via the alternative pathway (30). Reduction and alkylation may further reduce complement activation by these antibodies by decreasing C1q binding necessary for classical pathway activation. Other Fc-mediated functions of reduced and alkylated globulin, however, are also abnormal. Although opsonic activity of RA-ISG appears to be adequate in the presence of complement (25), this ISG has decreased opsonic activity for a variety of organisms when no complement is present (32). Since opsonization and subsequent clearance of Hib by the liver and spleen plays a major role in removal of Hib from the blood (34), alterations in Fc-receptor function might also result in decreased in vivo efficacy of RA-ISG. Whether due to poor classical pathway activation, altered opsonic ability, or other Fc-mediated function, intravenous RA-ISG exhibits decreased protective efficacy compared to two less harshly prepared ISGs in infant rats challenged with a common pediatric pathogen, Hib. Equivalent protection of infant rats required almost twofold more RA-ISG than native ISG as judged by rates of bacteremia, meningitis, and death as well as minimum protective antibody levels. If such a relationship holds true in human recipients, approximately twice as much RA-ISG as native ISG would be required to be administered to achieve protection from Hib infection. Further studies will be necessary to determine which functional defects of RA-ISG are responsible for decreased protective efficacy in vivo and whether protection against other bacterial pathogens is also impaired.

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LITERATURE CITED


