Protection from Group B Streptococcal Infection in Neonatal Mice by Maternal Immunization with Recombinant Sip Protein

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Group B streptococcus (GBS) is the leading cause of life-threatening bacterial infections in newborns (28). Vaccination of pregnant women or women of reproductive age is often reported as being the best long-term preventive measure that could be developed against GBS infection (10, 21, 28). Already, several GBS capsular polysaccharide (CPS) types have been tested as vaccines in human adult volunteers (1, 3, 7). They were shown to be safe, but their immunogenicity was disappointing (3, 7). These CPS, when coupled to carrier proteins such as tetanus toxoid, were shown to be more immunogenic in preclinical animal assays (15, 20, 24, 25, 30–32) and human clinical trials (5, 6, 13), and as expected, the protection was shown to be type specific (3, 12, 13). Based on the current information on serotype distribution, a conjugate vaccine would have to include types Ia, Ib, II, III, and V to prevent the majority of disease in North America. However, the formulation would have to be modified to be effective in other parts of the world, such as Japan, where other serotypes, such as VI and VIII, are more prevalent (14).

Noncapsular surface antigens are being investigated as potential vaccine candidates or carrier proteins (11, 29). One of these noncapsular antigens, the Sip protein, was found to be highly conserved and produced by every GBS strain examined to date, which included representative isolates of all serotypes (8). It was also established that Sip-specific antibodies recognized their epitopes at the cell surfaces of different GBS strains (26). More importantly, the immune response to purified recombinant Sip protein (rSip) efficiently protected adult mice against experimental infection with GBS strains representing serotypes Ia/c, Ib, II/R, III, V, and VI (8). It was also found that humans exposed to GBS developed a specific antibody response against the Sip protein. Indeed, this protein was identified after the immunoscreening of a GBS genomic library with a pool of normal human adult sera (8). Although these studies suggested that these Sip-specific antibodies could play a role in the prevention of GBS infection, it is essential to clearly establish a direct link between the presence of Sip-specific antibodies and protection of neonates. In this study, we verified that the rSip protein could protect against GBS infection by using the mouse neonate model, which simulates human disease (22, 27).

MATERIALS AND METHODS

GBS strains. The GBS strain C88890 (Ia/c) was isolated from the cerebrospinal fluid of a child with meningitis and was obtained from the Children’s Hospital of Eastern Ontario (Ottawa, Ontario, Canada). The strains NCS 251 (II/e) and NCS 535 (V) were, respectively, isolated from joint fluid and blood samples from adult patients. Strain NCS 437 (III/R) was isolated from the blood of an infant. The latter three strains were generously provided by the National Centre for Streptococcus, Provincial Laboratory of Public Health for Northern Alberta (Edmonton, Alberta, Canada). The GBS strain ATCC 12401 (Ib) was obtained from the American Type Culture Collection (Manassas, Va.). The GBS strains were grown overnight on tryptic soy agar plates containing 5% (vol/vol) sheep blood (Qualab Laboratories, Montreal, Quebec, Canada) or in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) at 37°C in the presence of 8% CO2. Strains were stored at −80°C in brain heart infusion broth (Difco) containing 20% (vol/vol) glycerol (Sigma Chemical Co., St. Louis, Mo.).

Production and purification of recombinant Sip protein. The purified recombinant plasmid pURV32 (8) was used to transform Escherichia coli strain BLR (Novagen, Inc., Madison, Wis.) by electroporation with the Gene Pulser II apparatus (Bio-Rad Laboratories, Hercules, Calif.) following the manufacturer’s recommendations. This recombinant strain was inoculated in Luria-Bertani broth (Gibco-BRL, Gaithersburg, Md.) containing 40 μg of kanamycin per ml (Sigma), and incubated at 34°C for approximately 3 h with agitation (optical density at 600 nm [OD600] = 0.6), after which time, the temperature was increased to 39°C for 4 to 5 h in order to induce the production of the recombinant protein. After the induction period, the bacterial cells were removed from the culture media by centrifugation at 12,000 × g for 30 min at 4°C. The supernatant was then filtered onto a 0.22-μm-pore-diameter membrane and concentrated with an ultrafiltration apparatus and a Diaflo ultrafiltration membrane YM 10 (Amicon, Inc., Beverly, Mass.). The concentrated supernatant was submitted to 50% (wt/vol) ammonium sulfate precipitation, and the precipitated proteins were collected by centrifugation and suspended in 50 mM Tris-HCl buffer (pH 8.5). The rSip protein was purified by two successive chromatographic steps: first by hydrophobic interaction chromatography with Sepharose HP (Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada) and then by anionic-exchange
Immunization and protection model. A 2.5-kg (body weight) New Zealand White female rabbit (Charles River Laboratories, St-Constant, Quebec, Canada) was immunized subcutaneously (s.c.) three times at 3-week intervals on several sites on the back with 100 μg of rSip protein in the presence of Freund's complete and incomplete adjuvant (Gibeo-BRL). Serum samples were collected before the first immunization and again 2 weeks after the last immunization. The antibodies present in the serum samples were partially purified by ammonium sulfate precipitation followed by extensive dialysis against phosphate-buffered saline (PBS).

Female CD-1 mice (Charles River Laboratories), 5 to 7 weeks old, were injected s.c. three times at 3-week intervals with either 20 μg of purified rSip protein in 0.1 ml of PBS mixed with 20 μg of QuilA adjuvant (Cedarlane Laboratories, Hornby, Ontario, Canada) or 20 μg of QuilA in PBS as a negative control. Serum samples from each mouse were taken before each immunization and the day the challenge was performed. At the end of the immunization period, the mice were mated. For passive immunization, pregnant mice were injected intravenously (i.v.) with 500 μl of partially purified rabbit anti-Sip serum or the corresponding preimmune serum on day 16 of gestation. The mouse neonatal model of infection was adapted from that of Rodewald et al. (27). The bacterial challenges were performed between 9 to 14 weeks after the third immunization. The lethal dose, which varied between $4 \times 10^5$ and $8 \times 10^6$ CFU, depending on the GBS strain, was administered to the pups s.c. between 24 and 48 h after birth. To enhance virulence, GBS strains were passed by intraperitoneal (i.p.) injection several times in female CD-1 mice as described by Lancefield et al. (16), and early-exponential-phase stock cultures were frozen at −80°C in brain heart infusion broth containing 20% glycerol. The GBS strains C388/90 (Ia/c), ATCC 12401 (Ib), NCS 251 (II/c), NCS 437 (III/R), and NCS 535 (V). Mortality was recorded daily for the next 7 days. Statistical significance was estimated by Fisher's exact test.

Analysis of the mouse sera. Determination of Sip-specific antibody titers in the sera collected from dams and selected pups in the litters were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μl of carbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ [pH 9.6]) containing purified rSip protein at a concentration of 1 μg/ml was added to each well of a flat-bottom microtitration plate (Falcon 3415; Becton Dickinson, Franklin Lakes, N.J.) and incubated overnight at room temperature. The plate was washed three times with PBS containing 0.05% (vol/vol) Tween 20. The mouse sera were serially diluted in PBS-Tween buffer, and 100 μl of each dilution was added to the appropriate well. The plate was incubated for 90 min at room temperature and then washed three times with PBS-Tween buffer. One hundred microliters of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted in PBS-Tween buffer was added to each well, and the plate was incubated for 60 min at room temperature. The plates were washed three times, 100 μl of tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories) was added, and this mixture was then incubated for 10 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M phosphoric acid. The OD$_{450}$ was read with a SpectraMax 340 (Molecular Devices Corporation, Sunnyvale, Calif.) microplate reader. A pool of high responder mouse antisera was assigned a Sip-specific arbitrary antibody unit (AU) value of 1,000 AU/ml and was used as the reference standard. For each serum, the optical values were transformed into AU per milliliter by using a reference standard curve calculated from the values obtained for the reference serum. A modified ELISA was used to evaluate the transfer of rabbit antibodies from the dams to their litters. In this assay, a goat alkaline phosphatase-labeled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Mississauga, Ontario, Canada) and $p$-nitrophenylphosphate disodium substrate (Sigma) were used instead of the previously described reagents. The serum dilution for which an absorbance reading of 0.1 ($A = 405/630 \text{ nm}$) was recorded after background subtraction was considered the titer of this serum. Immunoblots with purified recombinant protein were performed as described previously (8). The Sip-specific monoclonal antibody (MAb) 5A12, which was used as a positive control, was described previously (8).

<table>
<thead>
<tr>
<th>Strain used for challenge (serotype)</th>
<th>Group</th>
<th>No. of mothers</th>
<th>No. of neonates surviving GBS challenge (total %)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C388/90 (Ia/c)</td>
<td>Sip</td>
<td>7</td>
<td>72/73 (98)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4</td>
<td>2/45 (4)</td>
</tr>
<tr>
<td>ATCC 12401 (Ib)</td>
<td>Sip</td>
<td>2</td>
<td>21/28 (75)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2</td>
<td>0/23 (0)</td>
</tr>
<tr>
<td>NCS 251 (II/c)</td>
<td>Sip</td>
<td>4</td>
<td>42/44 (95)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3</td>
<td>0/34 (0)</td>
</tr>
<tr>
<td>NCS 437 (III/R)</td>
<td>Sip</td>
<td>6</td>
<td>48/61 (78)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4</td>
<td>1/41 (2)</td>
</tr>
<tr>
<td>NCS 535 (V)</td>
<td>Sip</td>
<td>3</td>
<td>26/27 (96)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2</td>
<td>3/24 (12)</td>
</tr>
</tbody>
</table>

* The number of survivors was evaluated for 7 days after challenge. The pups were challenged s.c. with 50 μl of a GBS culture. The bacterial challenge doses were adjusted to $4 \times 10^5$, $8 \times 10^5$, $6 \times 10^5$, $6 \times 10^4$, and $8 \times 10^4$ CFU, respectively, for GBS strains C388/90 (Ia/c), ATCC 12401 (Ib), NCS 251 (Ia/c), NCS 437 (III/R), and NCS 535 (V). The numbers of survivors in the Sip-immunized groups were shown to be statistically different from the numbers of survivors in the control groups at $P < 0.001$ by using Fisher’s exact test.

RESULTS

Passive immunization. The potential of Sip-specific antibodies to protect neonates against GBS infection was evaluated by passive administration of semipurified rabbit antibodies. After three injections with purified rSip protein, the reciprocal Sip-specific antibody titer of the rabbit antiserum was determined to be $5 \times 10^5$. A total of three pregnant mice on day 16 of gestation were injected i.v. with 500 μl of partially purified rabbit antibodies. Two control pregnant mice received the same volume of either (i) partially purified antibodies collected from the same rabbit before its immunization or (ii) PBS. The pups were challenged s.c. between 24 and 48 h after birth with a lethal dose of $4 \times 10^5$ CFU from the serotype Ia/c GBS strain C388/90. The survival data, representing monitoring of the number of survivors for 7 days after challenge, are presented as follows. None of the 19 pups from the control groups treated with rabbit preimmune serum (0 of 6 pups; $n = 1$ dam) or PBS (0 of 13 pups; $n = 1$ dam) survived the challenge, compared to 24 out of 25 pups (96%) whose dams ($n = 3$) had received the semipurified rabbit antibodies ($P < 0.001$ by Fisher’s exact test against both control groups). Immunoblots confirmed the presence of rabbit Sip-specific antibodies in the sera of the pups collected 7 days after the GBS challenge (data not shown).

Active immunization. To evaluate the ability of Sip protein to protect neonates against GBS infection, female CD-1 mice were immunized s.c. with 20 μg of purified rSip protein with QuilA adjuvant. Female control mice were injected with PBS mixed with QuilA adjuvant. At the end of the immunization period, these mice were mated, and their pups were challenged with one of five serologically distinct GBS strains (Table 1). The strains used to challenge the pups were C388/90, ATCC 12401, NCS 251, NCS 437, and NCS 535, which were found to be of serotypes Ia/c, Ib, II/c, III/R, and V, respectively. A range of 75 to 98% of the pups born from immunized dams survived the GBS challenges, compared to 0 to 12% for the control
groups. In all cases, the number of surviving pups in the immunized groups was shown, by Fisher’s exact test, to be significantly different ($P < 0.001$) from the number of survivors recorded in the control groups. The number of survivors for each litter is presented in Table 2. When pooled together, the protection data indicated that 209 of the 233 (90%) pups born from control dams (lanes 1 to 7) or control dams (lanes 8 to 11) (B), and sera collected 7 days after challenge from surviving pups born from Sip-immunized dams (lanes 1 to 7) (C). The reactivity of the Sip-specific MAb 5A12 with the purified recombinant Sip is presented in lanes 6, 12, and 8 of panels A, B, and C, respectively.

**DISCUSSION**

The importance of maternal antibody transfer for the protection of newborns human was clearly established (2, 4). Indeed, Baker et al. (2) demonstrated the existence of a correlation between maternal antibody deficiency at delivery and susceptibility to neonatal infection. These findings suggested that vaccination of pregnant women could become a very efficient prophylactic strategy to prevent GBS infection in neonates. Indeed, this approach could stimulate placental transfer of GBS-specific antibodies from the mother to the fetus, thus considerably increasing the level of protective antibodies present at the time of delivery (7).

Models to study transfer of IgG from the pregnant mother to the fetus and neonatal protection have been developed in the mouse (27), rat (9, 18), and primate (22). For this study, we selected the mouse neonatal model, since it is very well suited for testing in offspring the efficacy of antibodies acquired transplacentally from actively vaccinated dams (17). Indeed, in the absence of a mature immune system, protection in newborn pups can only be achieved via the acquisition of protective maternal antibodies. Furthermore, this model was recently used to evaluate the potency of different GBS conjugated CPS vaccines used for clinical phase 1 and 2 trials (19).

The neonate model was first designed to evaluate the functional activity of immune sera passively administered to pregnant dams on neonatal pup survival (23, 27). Indeed, Rodewald et al. (27) first reported that administration of a rabbit serum raised against GBS type III CPS vaccine passively protected 100% of the offspring. In this study, passive administration of Sip-specific rabbit antibodies to pregnant dams protected 96% of the pups against a lethal GBS challenge. The presence of circulating rabbit Sip-specific antibodies in the sera collected from these pups was confirmed by immunoblots (data not shown). As expected, no pup born from a dam that had received partially purified preimmune serum survived the GBS
challenge. These results clearly demonstrate that Sip-specific antibodies produced in another animal species can efficiently cross the placenta to enter the fetal blood circulation and then confer protection to the newborn pups against GBS infection. Additional experiments should be conducted in order to determine the minimal amount of Sip-specific antibodies needed to confer the observed protection.

As previously presented, the Sip protein can be found at the surface of every GBS strains tested (26). Thus, maternal immunization with this protein could provide protective immunity that would not be restricted to a particular group of serologically related strains, as is the case for conjugated CPS vaccines (3, 12, 13). To demonstrate this, female CD-1 mice were immunized with purified rSip protein and mated. The newborn pups were then challenged with one of the serotype Ia/c, Ib, II, III, or V GBS strains (Table 1). A range of 75 to 98% of the pups born from immunized dams with the rSip protein survived the challenge with these five serologically different GBS strains. In an effort to understand the variation in the level of protection, we tried to establish a relationship between the number of survivors per litter and a variety of factors, such as the number of pups per litter, the mean body weight of the pups, the number of weeks between the last immunization and the bacterial challenge, and the number of hours between the time of delivery and challenge. Unfortunately, no correlation could be established. A higher number of survivors was recorded when the pups were challenged with serotypes Ia/c (98%) and V (96%). The lowest levels of protection were observed when pups were challenged with serotypes Ib (75%) and III (78%). ELISA (Table 2) and immunoblots (Fig. 1) indicated that maternal Sip-specific antibodies had crossed the placenta and were found in the sera from the pups. Detectable levels of Sip-specific antibodies were still present in the sera collected from pups 64 days after the challenge. These results suggest that maternal Sip-specific antibodies generated after vaccination could be present long enough in the blood circulation of newborns to confer protection against early- and late-onset GBS diseases. As was clearly established for conjugated CPS vaccine through clinical trials (5, 13), the levels of Sip-specific antibodies needed to confer protection still have to be determined.

In conclusion, the data presented in this report confirm that Sip-specific antibodies can cross the placenta in order to confer protection to the newborn pups against GBS diseases. Further studies are required to determine the particular surface epitopes that could play a role in antibody-mediated protective immunity.

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REFERENCES

tive immunity and is expressed by most strains causing invasive infections. J. Exp. Med. 177:1593–1603.

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