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Peptides That Mimic the Group B Streptococcal Type III Capsular Polysaccharide Antigen

Seth H. Pincus,* Michael J. Smith,* Harold J. Jennings,† James B. Burritt,* and Pati M. Glee*

Microbial polysaccharides are notably poor immunogens. We have developed an alternate route for the production of Abs to important carbohydrate epitopes. mAb S9, a protective mAb against the type III capsular polysaccharide of group B streptococci (GBS), was used to select epitope analogues from a peptide display phage library. Depending upon desorption conditions, two populations of phage were identified with displayed sequences of WENWMMGNA and FDTGAFDPDWPA. ELISA results demonstrated that these phage bound to S9 and no other Abs. Phage blocked the binding of S9 to type III GBS, but did not block binding by another anti-GBS mAb. Phage displaying the latter peptide sequence showed greater inhibition. Ab S9 and other monoclonal and polyclonal anti-GBS type III antisera bound the synthetic peptide FDTGAFDPDWPA. The binding of S9 to GBS was inhibited by the free peptide with an IC_{50} of 30 μg/ml. The binding of polyclonal anti-GBS antibodies to peptide could be blocked by intact GBS as well as purified capsular polysaccharide. The peptide was conjugated to three different carriers and was used to immunize mice. All mice produced a significant antibody response to GBS and to the purified capsular polysaccharide following a single immunization. These data demonstrate that a peptide mimetic of the GBS capsular polysaccharide is both antigenic and immunogenic. The incorporation of such peptides into vaccine preparations may enhance the efficacy of vaccines in inducing Ab responses to important carbohydrate epitopes. The Journal of Immunology. 1998, 160: 293–298.

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roup B streptococci (GBS) are a major cause of neonatal sepsis and meningitis (1). Neonatal infection occurs as a consequence of maternal colonization with GBS and perinatal acquisition of the bacteria. When mothers lack protective anti-GBS Abs, neonates are at risk of infection (2–4). Efforts to prevent this disease include maternal immunization to induce protective Ab. Initial vaccines, containing only GBS carbohydrates, were marginally effective in inducing Ab (5). Conjugation of the carbohydrate to a protein carrier, tetanus toxoid, produced a much more effective vaccine (6). However, it is not yet established whether these conjugates induce optimal levels of Ab, or whether more effective immunogens can be constructed.

The GBS carbohydrates are one of many examples of microbial polysaccharides that are notably poor immunogens. Consisting of repeating carbohydrate subunits, microbial polysaccharides often induce only low levels of Ab despite repeated immunization. They frequently function as T-independent Ags, primarily provoking IgM Abs and little immunologic memory. Methods have been developed to circumvent this problem, including conjugation of the polysaccharide to protein carriers (6, 7). Here we demonstrate the feasibility of an alternative approach, the use of peptides that mimic the structure of microbial polysaccharides (8).

Phage display libraries allow the screening of large numbers of phage expressing different fusion proteins on their surface. Libraries have been constructed that express peptides with random sequences of amino acids (9, 10). These have been used in epitope or ligand mapping studies to define peptides that bind to a given mAb or receptor molecule. Such libraries have been used to identify peptides that bind to carbohydrate-binding molecules such as lectins (11) and Abs (12, 13). Here we have used a phage display library to identify peptides that bind to mAbs specific for GBS carbohydrate Ags. One of these Abs, directed against the type III capsular polysaccharide, has demonstrated in vivo protective efficacy (14–16). The peptides specifically block binding of anti-GBS Abs to GBS and elicited an anti-GBS Ab response in mice. This approach may have general utility to produce immunogens for other micro-organisms with immunodominant polysaccharide Ags.

Materials and Methods

Abs, bacteria, phage library, and peptide

The murine mAbs used in this study are listed in Table I. Abs S7, S9, and S10 are IgM Abs against GBS (15). B6.1 is a protective IgM Ab directed against a β-1,2-linked trimannose epitope of Candida albicans (17), and 924 is an irrelevant IgG1 anti-HIV gp120 (18). Abs 1A6, 2B1, and 2A6 were produced by immunization with tetanus toxoid-type III capsular polysaccharide conjugate (H. J. Jennings, manuscript in preparation). Polyclonal anti-type III GBS antisera was obtained by repeated infection of BALB/c mice with 10^8 live GBS strain 1.2 as described previously (19). Mice were bled 18 days following the first infection (primary bleed) and 1 wk following the second and third infections. Rabbit antiserum against M13 bacteriophage was made in our laboratories. Alkaline phosphatase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Zymed Laboratories (South San Francisco, CA). GBS type III, strain 1.2, were described previously (20). Capsular polysaccharide was extracted from GBS strain 1.2 using a modification of the protocol of Lancefield (21). GBS were washed twice in water and boiled in HCl, pH 2.0, for 10 min, and the GBS was pelleted out. The HCl extract was neutralized with Tris base, chilled, and precipitated with 80% ethanol. The ethanol extract was then treated with DNase (5 μg/ml), RNase (1 μg/ml), and proteinase K.
Selection of phage

mAbs S7, S9, and S10 were separately immobilized on cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) at 3 mg of Ab/ml of beads. Phage (4 × 10^12 plaque-forming units (pfu)) were preadsorbed five times on beads containing immobilized Ab to remove any phage that would bind to all IgM Abs. The preadsorbed library was divided into two aliquots for affinity selection with either Ab S7 or S9. The phage, diluted in Tris-buffered saline, 1% BSA, and 1% Tween-20, were incubated overnight with 2.5 ml of immobilized Ab. The beads were washed extensively with Tris-buffered saline/Tween (15 batchwise elutions of 15 ml each and on a column with 75 ml) and eluted with 0.1 M glycine, pH 2.2. Following an additional wash, the beads were further eluted with 0.5 M NH4OH, pH 11. The eluted phage were neutralized to pH 7 immediately, and those phage eluted with high or low pH were maintained as distinct pools. The titer of phage in the last wash and that in each eluate were determined. The eluted phage were then amplified in Escherichia coli strain K91 to a titer of 10^12 pfu and reapplied to the column. The same incubation and washing procedures were used, and bound phage eluted with either glycine or NH4OH, depending upon which pool of phage was used. Each aliquot of phage was subjected to three such rounds of selection. The third round eluate had >10^6 pfu. Phage were tested for binding to the selecting Ab, but not to irrelevant IgMs, using immunoblots of plaques. Phage with the desired reactivity were cloned, amplified to high titer, and purified by precipitation with 2.5% polyethylene glycol (8000 m.w./w.)0.5 M NaCl.

ELISA

ELISA was used to measure the binding of phage to Ab, Ab to peptide, and Ab to GBS or capsular polysaccharide. Protein or peptide Ags were coated onto microtiter wells (Immulon 2, Dynatech, McLean, VA) at 5 to 10 μg/ml. GBS were coated onto microtiter wells using polyl-t-lysine and glutaraldehyde as described previously (15, 19). Capsular polysaccharide was coated directly onto microtiter wells. Plates were blocked with 1% BSA or 1% OVA and used within 1 wk. Primary Abs were incubated in microtiter wells at 4°C for 18 h. The plates were washed and incubated with alkaline phosphatase-conjugated anti-Ig for 6 h, followed by washing and addition of colorimetric substrate p-nitrophenyl phosphate (Sigma Chemical). A_405 was determined using a microplate reader (EL-320, BioTek Instruments, Winoski, VT). Binding of phage was measured by incubation of phage in coated microtiter wells, washing, addition of rabbit anti-phage antisera, and detection of rabbit Ig with alkaline phosphatase-conjugated anti-rabbit Ig.

Immunization of mice

Peptide was conjugated to maleimide-derivitized BSA, OVA, and keyhole limpet hemocyanin (KLH; all purchased from Pierce Chemical Co., Rockford, IL). The efficacy of conjugation was demonstrated by reactivity of the conjugate, but not by that of the unconjugated maleimide derivative, with Ab S9. Three groups of two mice each were immunized s.c. with a single 50-μg dose of each conjugate in CFA (Difco, Detroit, MI). Mice were bled on the day of immunization and on day 35.

Results

Selection of phage

A phage display library expressing a random nine-amino acid sequence was selected for binding to one of two different anti-GBS mAbs: S9, a protective mAb that binds to the type III capsular polysaccharide, and S7, which is specific for the group B carbohydrate (15). The latter was used primarily as a specificity control. Within each selection, two separate desorption protocols were used to identify two populations of phage: 0.1 M glycine, pH 2.2, or 0.5 M NH4OH, pH 11. Phage with binding specificity for the selecting Ab were identified by immunoblots of plaques. Forty clones were selected, 10 from each elution condition and selecting Ab, and amplified to a titer of 10^13 to 10^14 pfu/ml.

We have sequenced the DNA encoding the displayed peptide from the two different pools of S9-selected phage. Within each pool, the sequence of each clone was identical, but two very different sequences were seen depending upon the eluting pH. The 9AA displayed sequence for the glycine (pH 2.2)-eluted phage was WENWMMGNA. The sequence displayed by the NH4 OH-eluted phage had >10^6 pfu. Phage were tested for binding to the selecting Ab, but not to irrelevant IgMs, using immunoblots of plaques. Phage with the desired reactivity were cloned, amplified to high titer, and purified by precipitation with 2.5% polyethylene glycol (8000 m.w./w.)0.5 M NaCl.

Specificity of Ab binding to displayed peptides

To show the specificity of phage binding, ELISA plates were coated with Abs. The immobilized Abs were incubated with representative phage clones from each selection (10^10 pfu/well), and binding was measured. The results are shown in Table II. The parental phage (M13KbSt) bound to no Ab. Phage selected with Ab S7 or S9 bound only to the selecting Ab. Phage that were first absorbed on Ab S10, before the selection on S7 or S9, bound to all IgM Abs, indicating that within the library there is a population of phage that bind to all IgMs.

Binding of Abs to the synthetic peptide FDTGAFDPDWPAC was also demonstrated by ELISA. Figure 1 shows binding of the
Abs to peptide. Figure 1A shows binding of the mAbs S7 and S9. Binding to the peptide was seen only with Ab S9. Figure 1B shows that two of three other IgG anti-GBS type III mAbs bind to the peptide. Figure 1C shows that the sera of mice infected with type III GBS bind to the peptide; binding to the peptide parallels the total Ab response to type III GBS (19). These data demonstrate that anti-GBS Abs other than the selecting Ab also recognize the peptide sequence.

Phage and peptides block the binding of Ab to GBS

The demonstration of specific recognition of the peptide sequence by the selecting Ab is a good indication that the phage bind to the variable regions. However, to demonstrate that the displayed sequence actually resembles the carbohydrate epitope of GBS, we must show blocking of Ab binding to GBS Ags. To perform these experiments, we used an ELISA in which Ab and inhibitor (phage or peptide) were premixed, incubated for 1 h, and then plated onto the microtiter plates with GBS. Inhibition of the Ab’s binding to GBS indicated that the phage or peptide was successfully competing with the GBS for Ab binding.

In Table III, intact phage were used to inhibit the binding of Abs S7 and S9 to GBS. The concentrations of S7 used were slightly higher than those of S9 because there are fewer antigenic determinants recognized by S7 on the surface of GBS (15). The concentrations of Ab used for inhibition are in the middle third of the linear portion of the binding curve. Ab B6.1 was used to indicate the level of background binding of IgM to GBS. The S9-selected phage inhibited the binding of S9, but not that of S7, to GBS, while the S7-selected phage inhibited the binding of only S7. The parental phage did not produce significant inhibition of either S7 or S9. In some cases, the inhibition of Ab binding was virtually complete. The inhibition of S9 induced by the phage eluted at high pH (S9–24 and S9–26) was considerably greater than that seen with the phage eluted at low pH. To confirm that the phage eluted at high pH were better inhibitors, a titration of phage was performed (Table IV). The data indicate that the phage eluted at high pH were approximately 5 times more efficient at inhibiting Ab S9 than the low pH phage; equivalent inhibition was seen with $2 \times 10^{10}$ pfu of S9–24 or S9–26 as with $10^{11}$ pfu of S9–11 or S9–16. The greater inhibition may be a reflection of the amino acid sequence of the displayed peptide or of its greater length. The increased inhibition is an indication that the peptide displayed by phage clones S9–24 and S9–26 binds to Ab S9 with a higher avidity than that of the other displayed peptides.

### Table III. Inhibition by phage of Ab binding to GBS

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>S9 (µg/ml)</th>
<th>S7 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No phage</td>
<td>2.03</td>
<td>0.63</td>
</tr>
<tr>
<td>M13KBst</td>
<td>1.97</td>
<td>0.51</td>
</tr>
<tr>
<td>S9-11</td>
<td>1.47</td>
<td>0.16</td>
</tr>
<tr>
<td>S9-16</td>
<td>0.89</td>
<td>0.14</td>
</tr>
<tr>
<td>S9-24</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td>S9-26</td>
<td>0.21</td>
<td>0.14</td>
</tr>
<tr>
<td>S7-A</td>
<td>1.96</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Abs S7 or S9 were diluted to the concentrations shown and mixed with $10^{11}$ pfu of the indicated phage. Following a 1-h incubation, the phage and Ab were transferred to GBS-coated microtiter wells, where they were incubated overnight. The plates were washed, and Ab binding was detected with alkaline phosphatase-conjugated anti-mouse IgM and substrate. The values are $A_{405}$ and are the mean of duplicate samples. Background binding of irrelevant IgM Ab B6.1 is 0.14.

### Table IV. Titration of phage-mediated inhibition of Ab binding to GBS

<table>
<thead>
<tr>
<th>Phage Number</th>
<th>Ab Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>M13KBst</td>
<td>1.97</td>
</tr>
<tr>
<td>S9–11</td>
<td>1.47</td>
</tr>
<tr>
<td>S9–24</td>
<td>1.66</td>
</tr>
<tr>
<td>S9–26</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Ab S9 (0.1 µg/ml) was mixed with the indicated number (pfu) of phage, incubated for 1 h, transferred to GBS-coated microtiter wells, and incubated overnight. The plates were washed, and Ab binding was detected with alkaline phosphatase-conjugated anti-mouse IgM and substrate. The values are $A_{405}$ and are the mean of duplicate samples.

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**FIGURE 1.** Binding of Abs to synthetic peptide FDTGAFDPDWPAC. Microtiter wells were coated with peptide at 10 µg/ml and then blocked with 1% BSA. Test Abs were added to the wells, incubated, and washed. Ab binding was detected with alkaline phosphatase-conjugated anti-Ig and then substrate. The values are $A_{405}$ and are the mean of duplicate or triplicate samples. The binding of mAbs S7 and S9 is shown in A. B shows binding of three IgG anti-GBS type III and one irrelevant mAbs, and C shows the sera from mice infected with 10^8 live GBS type III (primary, secondary, and tertiary refer to the number of times the mice were infected). The binding of all Abs to BSA was <0.1.
Because inhibition of Ab binding seen with intact phage may have resulted from steric inhibition due to the large size of a filamentous phage, the inhibition experiments were repeated with the synthetic peptide FDTGAFDPDWPAC. Those results are shown in Figure 2A. The binding of S9 to GBS was inhibited by free peptide with an IC₅₀ of approximately 30 µg/ml. There was no inhibition of the binding of Ab S7.

Binding of anti-GBS Ab to peptide is inhibited by capsular polysaccharide

To demonstrate that anti-GBS polyclonal Abs that bind to the peptide are specific for the type III capsular polysaccharide, we used both intact GBS and extracted capsular polysaccharide to inhibit binding to peptide (Fig. 2B). Two different dilutions of sera from mice infected with GBS were premixed with either an equal volume of GBS (OD = 0.9) or dilutions of the capsular polysaccharide. The GBS produced complete inhibition of binding to peptide. Although the inhibition by the capsular polysaccharide was not quite complete, the increasing inhibition with greater concentrations of polysaccharide or lesser amounts of serum suggests that maximal inhibition had not been obtained.

Mice immunized with peptide make anti-GBS Ab

Mice were immunized with peptide conjugated to KLH, BSA, or OVA. All mice made peptide-specific Ab (not shown) as well as Ab to GBS and to purified capsular polysaccharide (Fig. 3). The results demonstrate a high background of binding to GBS in the prebleed sera, perhaps as a result of binding to bacterial Fc receptors. As a comparison, the anti-GBS Ab response of mice that were infected with live GBS was also measured. A single immunization with peptide-protein conjugate induced a greater anti-GBS Ab response than seen following infection with 10⁶ GBS and a response comparable to that seen following a second infection.

Discussion

Group B streptococcus, or Streptococcus agalactiae, causes invasive infections of newborns, pregnant women, and adults with underlying medical conditions. Although the bacteria are sensitive to antibiotics such as penicillin, case fatality rates are estimated to be 5 to 20% in neonates and 15 to 32% in adults (1). Infection is most commonly seen as bacteremia, meningitis, and pneumonia. Survivors of neonatal infection may suffer permanent neurologic sequelae as a result of meningitis. Prevention of GBS infections in both adults and neonates is a major public health priority (1). The development of maternal vaccines is considered a leading approach to the prevention of neonatal GBS disease (23). Passive administration of Ab has defined protective epitopes of GBS (15, 16, 24–27), responses to which are critical for an effective vaccine.

In this communication, we have used mAbs directed against GBS polysaccharide Ags to select phage from a peptide display library. These phage display a peptide sequence that mimics the carbohydrate epitope. The selected phage and peptides derived from the displayed sequence of the phage are bound by the selecting Ab, and this binding is highly specific; S9 binds to S9-selected sequences and not to S7-selected sequences, while S7 only binds to S7-selected sequences. Monoclonal and polyclonal anti-type III Abs, which were not used in the phage selection, also bind to the peptide. Antigenic resemblance between the peptide and carbohydrate epitope was demonstrated by peptide-specific inhibition of Ab binding to GBS and inhibition of Ab recognition of the peptide by GBS and capsular polysaccharide. Immunization with the peptide conjugates elicited anti-GBS Ab, showing that the peptide also mimics the carbohydrate immunologically.
Because of the obvious public health ramifications, considerable effort has been devoted to the development of GBS vaccines. Attempts to develop a GBS vaccine have focused on using the capsular polysaccharide. An initial vaccine consisting of GBS type III capsular polysaccharide underwent both animal and human testing (5). Although the vaccine elicited Abs in only 63% of pregnant women immunized, the passive transfer of anti-GBS Abs to neonates was shown, thus demonstrating the feasibility of maternal immunization. To improve the immunogenicity of capsular polysaccharide, it was conjugated to the carrier protein, tetanus toxoid (28). Subsequent clinical trials of this vaccine have demonstrated an Ab response in approximately 90% of recipients (6). These studies indicate that while progress is being made toward the development of a GBS vaccine, the final goal has not yet been achieved because significant protective efficacy in humans has not been shown. On the basis of studies described here, the incorporation of carbohydrate mimetic peptides may enhance the immunogenicity of anti-GBS vaccines.

Although it is not intrinsically obvious that peptides can mimic nonpeptide structures, there are naturally occurring compounds that do so. The protein tendamistat binds to the enzyme α-amylase, with the tripeptide WRY occupying the carbohydrate binding site of the enzyme. Using phage display libraries, other peptides that mimic carbohydrate structures have been demonstrated. Con A, a lectin that interacts with oligosaccharides with terminal α-linked mannose or glucose residues, specifically selected YPY-bearing peptides from a peptide display phage library (11). Phage and synthetic peptides with the motif blocked the binding of the lectin to its carbohydrate ligand. Other investigators have used this approach with Abs recognizing carbohydrate epitopes. Ab B3 binds to the Leα carbohydrate Ag on the surface of tumor cells. A peptide with the sequence PWLY was identified that blocked the binding of Ab to tumor cells and to purified carbohydrate (13).

The two S9 binding sequences are considerably different: WEN WMMGNA and FDTGAFDPDWPA. However, there are similarities in motif. In each case there are aromatic, acidic, and hydrophobic residues. The presence of aromatic residues is characteristically seen in peptides mimicking carbohydrates. The presence of acidic residues probably reflects the sialic acid in the carbohydrate epitope. However, the molecular basis underlying the antigenic mimicry of the carbohydrate structure by the peptides is not known.

A number of investigators have used anti-idiotypic Abs to elicit an immune response to carbohydrate Ags of Streptococcus pneumonia (29), Pseudomonas aeruginosa (30), E. coli (31), and group A streptococci (32). Using the resemblance between anti-Id and Ag to identify a carbohydrate mimetic peptide, Westerink et al. (8) were able to elicit protective immunity to group C meningococci. Interestingly, 5 of 12 AA residues within this peptide were aromatic. Thus, there are good data to suggest that proteins and peptides can mimic polysaccharide Ags.

Recent data by Harris et al. (12), using peptides selected from phage libraries with a panel of Abs to group A streptococcal cell wall polysaccharide, has been interpreted to suggest that the binding of peptides occurs by a different mechanism than binding of carbohydrate, and that this is neither antigenic nor immunologic mimicry. We have addressed this issue and found that two of three IgG mAbs to type III GBS also bind to this peptide (Fig. 1b). Our interpretation of these data is that while each of the mAbs binds to the same polysaccharide structure, some may recognize different aspects of that structure. This interpretation was considered by Harris et al. but was discarded in favor of the above interpretation (12). We have also demonstrated that polyclonal anti-GBS Abs bind well to the peptide mimetic. Thus, our data contradict those of Harris (12).

In this manuscript we have demonstrated that the peptide FDT GAFDPDWPA resembles the protective epitope of the type III GBS capsular polysaccharide both antigenically and immunologically. Further studies will be required to determine whether the approach outlined in this manuscript is broadly applicable to other microbial polysaccharides.

Acknowledgments

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References


