Group A Streptococcus (GAS) Carbohydrate as an Immunogen for Protection against GAS Infection

Hemant Sabharwal,1 Francis Michon,4 Daniel Nelson,1 Wenling Dong,4 Kathleen Fuchs,2 Roberto Carreño Manjarrez,6 Arun Sarkar,4 Catherine Ulitz,7 Ann Viteri-Jackson,1 Romeo S. Rodriguez Suarez,7 Milan Blake,5 and John B. Zabriskie1,2

1Research Division, Hospital for Special Surgery, and Laboratories of 2Clinical Microbiology and Immunology and 3Microbial Pathogenesis and Immunology, Rockefeller University, New York, New York; 4BioVeris Corporation, Gaithersburg, and 5Division of Bacterial, Parasitic and Allergenic Products, Laboratory of Bacterial Polysaccharides, Food and Drug Administration, Rockville, Maryland; 6Department of Internal Medicine, Hospital Infantil De Mexico, and 7National Center for the Health of Infants and Adolescents, Secretary of Health, Mexico City, Mexico

Previous studies have shown that human serum containing anti–group A streptococcus carbohydrate (GAS CHO) antibodies were opsonic for different M protein–carrying serotypes. To investigate the role that anti–GAS CHO antibodies play in passive and active protection, mice were immunized subcutaneously or intranasally with GAS CHO conjugated to tetanus toxoid, and mortality and oral colonization were monitored after challenge with live GAS. Compared with control mice, immunized mice were significantly protected against systemic or nasal challenge with GAS. Furthermore, studies of serum samples and throat cultures from Mexican children revealed an inverse relationship between high serum titers of anti–GAS CHO antibodies and the presence of GAS in the throat. Anti–GAS CHO antibodies were also tested for cross-reactivity with human tissues and cytoskeletal proteins. No cross-reactivity was observed in either assay. The present study demonstrates that GAS CHO is both immunogenic and protective against GAS infections.

Previous work from our laboratory has shown that serum samples from healthy children contain antibodies to group A streptococcus carbohydrate (GAS CHO) and that the titers of these antibodies increase with increasing age. These antibodies were also shown to be opsonic for several M protein–carrying (M+) serotypes of GAS in an in vitro phagocytic assay. In addition, the specificity of these antibodies for GAS CHO was clearly demonstrated, because removal of them by absorption with N-acetyl glucosamine coupled to Sepharose beads resulted in the loss of the phagocytic properties of the serum [1]. Further studies revealed that GAS CHO conjugated to tetanus toxoid (TT) induced high serum titers of anti–GAS CHO antibodies in rabbits. These antibodies were also able to promote phagocytosis in an in vitro assay [1]. An important observation in these studies was that the addition of fresh complement devoid of anti–GAS CHO antibodies was required for the phagocytic system to be effective.

Although these studies clearly demonstrated the role that anti–GAS CHO antibodies play in promoting phagocytosis, several questions remain unanswered. First, are these antibodies protective against challenge with GAS, either in passive protection experiments or in active immunization experiments in a mouse model? Second, does immunization of mice with GAS CHO protect against oral colonization of mice by GAS? Third, is there a correlation between the presence or absence of GAS in the throats of humans and the titers of anti–GAS CHO antibodies in their serum samples? Finally, when it is used as an immunogen, does GAS CHO induce antibodies that cross-react with human tissues? The present study clearly demonstrates that the answer to
the first 3 questions is affirmative and that no cross-reactivity with human tissues occurs.

SUBJECTS, MATERIALS, AND METHODS

Bacterial strains. All streptococcal strains were obtained from the Rockefeller University Collection. The following strains were used: S43/46 (serotype M6), D58/93/7 (serotype M3), and S23 (serotype M14). All strains were passaged repeatedly in mice to increase the virulence of each strain as much as possible [2, 3]. Animal experimental guidelines set forth by the Rockefeller University animal facility were followed in the conduct of all animal studies.

GAS CHO conjugate vaccine. The parent GAS strain was isolated by D. Colebrook (Queen Charlotte Hospital, London, United Kingdom) from a patient with puerperal sepsis and was sent to R. C. Lancefield (Rockefeller University, New York, New York) in 1937. The strain, originally called GAS serotype 3, was passaged several times in mice and was finally isolated as strain D58X, an M protein–negative mutant. GAS CHO was taken from the cell wall and purified using the sodium nitrite extraction method [4]. Briefly, after a 20-L fermentation, the culture was harvested by tangential flow filtration (TFF) with a 0.1-μm hollow-fiber cartridge filter. The filtration retentate was centrifuged at 3000 g, the cell pellet was resuspended in 500 mL of saline, and the solution was distilled with distilled water to a volume of 1.5 L. 4 N sodium nitrite (150 mL) was slowly added, and then 150 mL of glacial acetic acid was slowly added while the mixture was stirred. The mixture was stirred for 1 h, diluted with distilled water to a final volume of 7.5 L, and thoroughly mixed. The cell lysate suspension was clarified by TFF with a 0.1-μm hollow-fiber cartridge filter, and the permeate was processed for polysaccharide purification. The molecular weight of GAS CHO was 10 kDa, and the material was free of proteins and nucleic acids (F.M., unpublished data). GAS CHO was conjugated to TT (Statens Serum Institute) in a ratio of 3:1 (wt:wt) by reductive amination [5] with solid sodium cyanoborohydride (Sigma Aldrich). A 0.2-mmol/L phosphate buffer (pH 8.0) was used for the coupling reaction. After incubation at 37°C for 3 days, the conjugate (GAS CHO–TT) was purified from the reaction mixture by TFF with a 100-kDa cutoff membrane. The final conjugate contained ~3 polysaccharide chains/mole of TT.

Intraperitoneal (ip) challenge assay. The GAS strain to be tested was grown as described by Salvadori et al. [1]. The culture was then appropriately diluted to deliver a sufficient number of GAS to cause 100% mortality in control mice in 72 h. Live GAS in a volume of 200 μL was administered to mice by ip injection. Samples of the same dilutions in 20-μL and 100-μL increments were placed in petri dishes to which 10 mL of brain-heart infusion agar (2.4%) containing 0.5% defibrinated sheep’s blood was added. The plates were incubated at 37°C overnight, and the number of colonies was counted the next day.

Intranasal (inl) immunization assay. The method of immunization was the same as that used in the inl challenge experiments of Bessen and Fischetti [3]. Briefly, every other day for 1 week, mice were given inl immunizations of 20 µL of GAS CHO (27 µg/20 µL) mixed with 30 µg of free cholera toxin B subunit (CTB). The mice were allowed to recover for 3 weeks, after which time serum samples were obtained for measurement of titers of anti–GAS CHO antibodies. Three days before challenge, another dose of GAS CHO and CTB was administered to mice. Control mice received CTB in saline on the same schedule.

Oral challenge assay. Mice were given streptomycin water (5 g/L; Difco) 24 h before challenge, to reduce the normal oral bacterial flora. GAS (serotype M3; streptomycin resistant) were grown overnight in Todd-Hewitt (TH) broth with streptomycin (200 µg/mL), pelleted, suspended 1:3 in TH broth, and allowed to grow at 37°C for 2 h or until the OD₆₀₀ was 0.73. A total of 30 µL (10 µL orally and 10 µL to each nostril) of live GAS was administered through a micropipette to control mice (n = 32) and mice immunized with GAS CHO (n = 30). For the next 3 consecutive 24-h intervals, mice were orally swabbed with calcium alginate fiber–tipped ultrafine swabs (Fisher), and the swabs were streaked on streptomyacin-treated blood agar plates for the enumeration of colony-forming units. The presence of a single β-hemolytic colony on the plate was considered to be indicative of a positive culture.

Passive immunization. Rabbits were immunized subcutaneously (sc) in 4 separate sites with 10 µg of GAS CHO–TT in complete Freund’s adjuvant. The second dose of GAS CHO–TT was given in incomplete Freund’s adjuvant 3 weeks later. Blood samples were collected 2 weeks later, and the titers of anti–GAS CHO antibodies were assayed. Rabbits were given 2 booster injections of the same dose of GAS CHO in Freund’s incomplete adjuvant. In the mouse challenge model, on the day of the challenge, mice were given ip injections of 0.3 mL of rabbit anti–GAS CHO antibodies (usually diluted 1:5 or 1:10) or normal rabbit serum (NRS) either 1 h or 24 h before the injection of GAS.

Active immunization. Mice were injected sc with GAS CHO–TT at a concentration of 5 µg/dose and alum (total volume, 200 µL). The second dose was given 3 weeks later, and the procedure was repeated twice more, for a total of 4 doses. Ten days after the last dose, blood samples for the measurement of anti–GAS CHO antibodies were obtained by retro-orbital bleeding. Control mice were injected in the same manner with alum and TT alone. All mice were given ip injections of GAS, as described above.

Human population studies. A total of 306 Mexican chil-
Children were enrolled into the study. Written, informed consent (in Spanish) to draw a small sample of blood from each child was obtained from either the parent or the guardian. The serum was separated and stored at −70°C until use. A throat swab sample was obtained from each child as well. All children appeared to be healthy at the time of testing, and none complained of sore throat or fever, according to a questionnaire filled out by the parent or guardian. Approval for the study protocol was obtained from the institutional review boards of Rockefeller University and the Ministry of Health, Mexico. Children whose throat cultures tested positive for GAS were asked to return to the clinic and were treated with penicillin.

**Throat cultures.** At the time of the clinic visit, swabs were used to obtain a sample from the throat of each child, and the samples were transported to the Hospital Infantil de Mexico, where they were streaked on blood agar plates and incubated at 37°C overnight. On the following day, colonies suspected of being streptococcal were isolated and then were grown in blood broth at 37°C overnight. On the following day, the organisms were centrifuged at 875 g for 10 min and then were washed, and the CHO was extracted by boiling the organisms for 10 min at pH 2.5. The supernatant was neutralized to pH 7.0 with 1 mmol/L NaOH and was grouped with antisera prepared against the common streptococcal groups (Diagnostic Reagents Oxoid; Unipath).

**Serum ELISAs.** ELISAs were performed as described elsewhere [6] but with the following modification. To avoid measurement of the TT antibodies commonly found in human serum, GAS CHO was conjugated to human serum albumin before use in the ELISA. The conjugated material was then plated in a 96-well plate at a concentration of 0.1 μg of carbohydrate/well in PBS (pH 7.4). Starting serum dilutions were 1:1000, and samples were serially diluted until the OD_{450} was 0.5. Only titters of IgG antibodies to GAS CHO were measured in the serum samples from children. All tests were performed in duplicate.

**Immunohistochemical analysis of human tissues.** Within 12 h after death, pieces (1 × 1 cm) of the appropriate human tissues were obtained, embedded in Tissue-Tek (Electron Microscopy Sciences), snap frozen in a mixture of alcohol and dry ice (−80°C), and stored at −70°C until use. Each tissue sample was cut into 4-μm sections on a cryostat microtome. The sections were placed on glass slides and allowed to dry in a desiccator overnight. On the day of use, the sections were treated with acetone for 1 min and then were washed twice in PBS. The excess fluid was removed with tissue wipes, and 10–20 μL of the appropriate primary antibody (diluted 1:5 in PBS) was added. The slides were incubated at room temperature in a moist, dark chamber for 30 min. After 1 wash in 100 μL of PBS, the antigen-specific fluorescein isothiocyanate (FITC)–labeled antibody (diluted 1:20) was added, and the sections were incubated for another 30 min as before. After washing, 20–30 μL of 1:1 PBS:glycerol solution was placed on each section, and a cover slip was applied. FITC fluorescence was read on a Nikon Optiphot microscope using UV light and a 495-nm filter. Either secondary antibody alone or NRS was used as a negative control. Positive controls were rabbit antisera specific for tissue proteins.

### Table 1. Passive protection tests in mice after intraperitoneal challenge with live group A streptococcus (GAS).

<table>
<thead>
<tr>
<th>GAS strain, serum</th>
<th>Colonies, range</th>
<th>Mice, no. that survived/ no. injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotype M6 strain S43/46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>200–500</td>
<td>3/26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit serum with anti–GAS CHO antibodies</td>
<td>200–500</td>
<td>16/26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serotype M3 strain D58/93/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>1.7 × 10^7–4.6 × 10^8</td>
<td>3/15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit serum with anti–GAS CHO antibodies</td>
<td>1.7 × 10^7–4.6 × 10^8</td>
<td>13/15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**NOTE.** GAS CHO, GAS carbohydrate.

<sup>a</sup> P<.01, Fisher’s exact test.
<sup>b</sup> P<.01, Fisher’s exact test.

### Table 2. Active immunization studies in mice after intraperitoneal challenge with different M protein–carrying group A streptococcus (GAS).

<table>
<thead>
<tr>
<th>GAS strain, antigen</th>
<th>Inoculum range, cfu</th>
<th>Mice, no. that survived/ no. injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotype M6 strain S43/46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT with alum</td>
<td>3 × 10^6–7 × 10^8</td>
<td>2/15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAS CHO–TT with alum</td>
<td>3 × 10^6–7 × 10^8</td>
<td>11/15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serotype M14 strain S23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT with alum</td>
<td>3 × 10^6–3.6 × 10^8</td>
<td>5/22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAS CHO–TT with alum</td>
<td>3 × 10^6–3.6 × 10^8</td>
<td>18/22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**NOTE.** GAS CHO–TT, GAS carbohydrate conjugated to tetanus toxoid.

<sup>a</sup> P<.003, Fisher’s exact test.
<sup>b</sup> P<.001, Fisher’s exact test.
RESULTS

Passive protection tests. Table 1 shows that serum obtained from rabbits immunized with GAS CHO–TT was capable of conferring significant protection against live strains from 2 different GAS M+ serotypes in a lethal murine challenge model. As can be seen from the data, the number of organisms needed to kill ∼80% of the mice given only NRS differed markedly, depending on the serotype of the strain used. This occurred in spite of repeated ip passage of the strains in mice, and it demonstrates the difficulty and variability in adapting human GAS to mice.

Active protection tests. Mice were actively immunized with GAS CHO–TT sc with an average of 4 doses. The average of the serum titers, as measured with an ELISA, ranged from a dilution of 10^1 to 10^4 anti–GAS CHO antibodies. Strains from 2 different M+ serotypes were used in these experiments, and the number of organisms needed to kill 82% (average) of the control mice is shown in table 2. As can be seen from the data, immunized mice had significantly lower mortality, irrespective of the serotype of the strain used for the challenge, than control mice.

Inl protection studies. Mice are normally not susceptible to inl colonization by GAS in nature. However, under optimal experimental conditions with 6-week-old mice, colonization rates can be 60%–70% [7]. We utilized this model and were able to show a stable colonization rate in control mice for 3 days (day 1, 50%; day 2, 53%; day 3, 50% [n = 32]). Our lower colonization rate can most likely be attributed to the advanced age of our mice (13–14 weeks), which were age-matched to immunized mice [3]. In contrast, immunized mice (n = 30) had colonization rates of 30% on days 1 and 2 and 20% on day 3 (P < .1, Fisher’s exact test for all days). Moreover, of the mice that were colonized in each group, immunized mice had a significantly lower number of colony-forming units than did control mice (P < .05, analysis of variance) (figure 1).

Human studies. Throat swab samples were obtained from 306 Mexican children, and 61 throat cultures (20%) tested positive for GAS. A smaller percentage of throat cultures tested positive for group B (6%), C (2%), F (5%), or G (9%) streptococcus. A total of 173 throat cultures (57%) tested negative for all streptococcal groups. Table 3 shows the distribution of the streptococcal groups obtained from the throat cultures. The children varied in age from 5 to 14 years. All age groups were colonized with equal percentages of each streptococcal group.

To determine whether higher serum titers of anti–GAS CHO antibodies correlated with GAS-negative throat cultures, anti–GAS CHO antibodies were measured in children with GAS-positive throat cultures and then were compared with those in children with GAS-negative throat cultures. Figure 2 shows that the serum titers were twice as high in children with GAS-negative throat cultures than in children with GAS-positive throat cultures (P < .003).

Figure 3 demonstrates that serum titers of anti–streptolysin O (ASO) antibodies were significantly higher in children with GAS-positive throat cultures than in children with GAS-negative throat cultures (P < .001). These findings also suggest that children with GAS-negative throat cultures had lower serum ASO antibody titers secondary to the elimination of colonization of the throat by GAS. A similar trend was seen when serum titers for anti–DNAse B antibodies were compared (data not shown), but the difference was not statistically significant (P = .3).

Cross-reactive antibody assays. Because of the numerous studies [8–10] demonstrating the ability of GAS and their antigens to induce antibodies that are cross-reactive with mammalian tissues, we performed both an ELISA and an immunofluorescence assay with rabbit anti–GAS CHO antibodies. Using a number of cytoskeletal proteins known to be present in mammalian tissues, we performed an ELISA to determine whether anti–GAS CHO antibodies bound to these proteins in

<table>
<thead>
<tr>
<th>Streptococcal group</th>
<th>Positive throat culture, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>61 (20)</td>
</tr>
<tr>
<td>B</td>
<td>18 (6)</td>
</tr>
<tr>
<td>C</td>
<td>7 (2)</td>
</tr>
<tr>
<td>F</td>
<td>14 (5)</td>
</tr>
<tr>
<td>G</td>
<td>26 (9)</td>
</tr>
<tr>
<td>Not groupable</td>
<td>7 (2)</td>
</tr>
<tr>
<td>None</td>
<td>173 (57)</td>
</tr>
</tbody>
</table>
Figure 2. Anti–group A streptococcus carbohydrate (GAS CHO) serum antibody titers in children with positive or negative throat cultures for GAS. Note the significantly higher serum anti–GAS CHO antibody titers in children with GAS-positive throat cultures ($P < .003$).

There was no significant difference between the binding of anti–GAS CHO antibodies and NRS to all of the cytoskeletal proteins tested (data not shown).

We used an indirect immunofluorescence assay to determine whether GAS CHO induced cross-reactive antibodies to human tissues. Heart, brain, kidney, and liver tissues, many of which are known to be cross-reactive with streptococcal antigens [8], were tested. As is seen in figure 4, rabbit anti–GAS CHO antibodies, at the same titers as the positive control antibodies specific for the particular tissue proteins, did not cross-react with any of the human tissues tested. Of particular note was the observation that the rabbit anti–GAS CHO antibodies did not stain human heart tissue (figure 4, middle panels). This is important, because the major pathological damage seen in rheumatic fever occurs in the heart tissue and heart valves.

**DISCUSSION**

Although many investigators have had difficulty in demonstrating the presence of precipitating antibodies to GAS CHO in human serum, beginning in 1965 a series of experiments [11–15] using direct and indirect agglutinating techniques clearly demonstrated that antibodies to GAS CHO were present in human serum. The direct agglutinating antibodies were primarily raised against the GAS variant moiety, whereas the indirect agglutinating antibodies were raised against the N-acetyl glucosamine moiety. Furthermore, Todome et al. [13] noted that these antibodies were primarily of the IgG2a class.

Lancefield and Todd [2] were unable to demonstrate, however, that anti–GAS antibodies were protective against challenge in mice. Other investigators have confirmed these findings, but they all used the in vitro phagocytosis test with heparin. It is now known that heparin blocks complement components, especially C1q [14–16]. Furthermore, when we added heparin to the phagocytic assays used by Salvadori et al. [1], the phagocytic abilities of these antibodies were significantly decreased. Although these studies suggest that M protein opsonization could occur by Fc receptors on the neutrophil, a recent study by Nilsson et al. [17] indicated that the complement component C3b, but not the Fc receptor, plays an important role in the killing of GAS. In the present study, removal of anti–GAS CHO antibodies by affinity absorption resulted in a significant decrease in the phagocytic abilities of human serum.

On the basis of these observations, we addressed whether the serum titers of these anti–GAS CHO antibodies increased with increasing age, whether these antibodies were phagocytic for several different M+ serotypes, and whether the phagocytosis caused by the serum was related specifically to anti–GAS CHO antibodies. The answer to all 3 questions was affirmative [1].

Although previous studies demonstrated the opsonic properties of anti–GAS CHO antibodies, the present study addressed 4 new questions: (1) Did anti–GAS CHO antibodies passively protect mice against a lethal challenge with live strains from different GAS M+ serotypes? (2) Did active immunization protect against ip or inl challenge? (3) Did the serum titers of anti–GAS CHO antibodies correlate with the presence or absence of GAS-positive or GAS-negative throat cultures from healthy children? (4) Did anti–GAS CHO antibodies cross-react with human tissues?

Concerning the first 2 questions, we have clearly shown that anti–GAS CHO antibodies both passively and actively protect mice against challenge with live GAS. As we noted, this protection extended to strains from several M+ serotypes. Thus, the protection was broad-based and encompassed >1 M+ serotype. Of interest was that there were marked differences between the numbers of colonies needed to kill control mice even...
Figure 4. Indirect immunofluorescence studies. Human tissue sections were stained with rabbit serum containing either anti–streptococcal group A carbohydrate (GAS CHO) antibodies or antigen-specific fluorescein isothiocyanate–labeled antibodies. Top panels, Human brain tissue sections stained with hematoxylin-eosin (HE; A), secondary antibody (B), anti-proteoglycan antibodies (C), or anti–GAS CHO antibodies (D). Note the specific staining in brain tissue stained with anti-proteoglycan antibodies (C), compared with the nonspecific staining in brain tissue stained with anti–GAS CHO antibodies (D). Middle panels, Human heart tissue sections stained with HE (A), secondary antibody (B), anti-myosin antibodies (C), anti-tropomyosin antibodies (D), anti-streptococcal serotype M6 antibodies (E), or anti–GAS CHO antibodies (F). Note the specific staining in heart tissue stained with either anti-myosin antibodies (C) or anti-tropomyosin antibodies (D), compared with the nonspecific staining in heart tissue stained with anti–GAS CHO antibodies (F). Bottom panels, Human kidney tissue sections stained with HE (A), secondary antibody (B), anti-proteoglycan antibodies (C), or anti–GAS CHO antibodies (D). Note the specific staining in kidney tissue stained with anti-proteoglycan antibodies (C), compared with the nonspecific staining in kidney tissue stained with anti–GAS CHO antibodies (D).

when the same strain (serotype M6 strain S43/46) was used. This difference in protection occurred in spite of extensive passage of the strains in mice. This finding again points to the difficulties inherent in using the mouse model of infection to study human GAS. Whatever may be the correct explanation for it, in both active and passive immunization studies, the protection conferred was significant.

With respect to whether high titers of anti–GAS CHO antibodies in human serum decreases colonization of the throat by GAS, we have shown a definite correlation between increased serum titers of anti–GAS CHO antibodies in children and decreased colonization of the throat by GAS. Furthermore, the correlation between decreased serum titers of other anti-streptococcal antibodies (ASO and anti–DNAse B) and decreased colonization of the throat by GAS lends further support to our belief that higher serum titers of anti–GAS CHO antibodies do result in decreased colonization of the throat by GAS.

These human studies were further strengthened by our inl
immunization studies in mice. After inl immunization with GAS CHO and CTB, there was a significant difference in the number of colony-forming units isolated from the throats of immunized mice than that in control mice.

We also addressed whether immunization with GAS CHO induces cross-reactive antibodies to human tissues, as was suggested by Shikhman et al. [18, 19] and Kirvan et al. [20]. As is seen in figure 4, in immunofluorescence studies with various human tissues known to be involved in nonsuppurative sequelae of GAS infections, we did not observe any binding of anti–GAS CHO antibodies to any of the tissues studied. In our ELISAs, we obtained similar results when we used a number of cytoskeletal proteins, including keratin (data not shown). These findings are in contrast to those of Shikhman et al. [18, 19], who found that a particular keratin peptide cross-reacted with anti–GAS CHO antibodies. The results of both of our assays are also in contrast to those of a study by Kirvan et al. [20] in which “humanized” monoclonal antibodies cross-reacted with N-acetyl glucosamine and certain ganglioside moieties in the brain. The discrepancy between the results of our study and those of Cunningham et al. may be related to the type of antibodies (monoclonal vs. polyclonal) used. It should also be noted that high serum titers of anti–GAS CHO antibodies were found in many healthy children who had no evidence of neurological dysfunction or active cardiac disease.

In summary, the results of the present study and those of our previous studies strongly suggest that GAS CHO is a potent immunogen and can induce antibodies in both humans and animals. The titers of these antibodies naturally increase with increasing age, and the antibodies are protective in children against colonization by GAS. Additionally, they are opsonic and can induce passive and active protection in mice. Finally, the titers of these antibodies in the serum correlate quite well with the presence or absence of GAS in throat cultures. Taken together, these results suggest that GAS CHO can be used as an effective immunogen in the prevention of streptococcal infections.

References