PROTECTION OF MICE FROM EXPERIMENTAL INFECTION WITH TYPE III GROUP B STREPTOCOCCUS USING MONOCLONAL ANTIBODIES

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It is widely believed that antibodies to the group B streptococcal (GBS) type-specific polysaccharides are of major importance in human host defenses against GBS infection. All four of the type-specific polysaccharides elicit antibodies against at least two forms of the antigens: a complete antigen that contains N-acetylneuraminic acid (sialic acid) at the nonreducing ends of the side chains and an incomplete or nonsialated antigen (1-4). This was first demonstrated for the type II polysaccharide by Lancefield and Freimer (1) when they showed that rabbit antisera directed against either the complete or incomplete antigen would protect mice from a lethal challenge of live type II organisms. They were unable to carry out studies with type III GBS using similar passive protection tests because these organisms were not by themselves pathogenic for mice.

The question of antibody specificity in protection against type III GBS infections remains controversial. There are conflicting reports regarding the protective properties of human and rabbit antibodies directed against the type III antigens and against the capsular polysaccharide of the type 14 pneumococcus, which cross-reacts with the incomplete GBS type III antigen (5). Although it is possible to select for certain specificities in anti-GBS serum by specific absorptions with purified antigens, as was done by Lancefield and Freimer (1), the polyclonal nature of antiserum makes it difficult to clearly determine the immunoprotective effects of various specificities and different isotypes of the antibodies in question.

Harris et al. (6) recently reported that mice could be protected against GBS types Ia, Ib, Ic, or II by antibody-producing tumors formed 2 wk after subcapsular injection of hybridoma cells. The protection was serotype specific. Similar experiments could not be carried out using type III GBS because this serotype was not virulent in the mouse model.

This report describes the production of mouse hybridoma antibodies of different isotypes directed against both sialated and nonsialated forms of the GBS type III polysaccharide. The protective properties of antibodies were examined under conditions where type III GBS were lethal for mice.

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Materials and Methods

Bacterial Strains. Type III GBS, strain M732, was grown in Todd-Hewitt broth to log phase, harvested by centrifugation, and washed in PBS. Two vaccines were prepared, one by heat-killing at 80°C for 15 min, the other by treating with 2 N acetic acid at 80°C for 1 h to remove sialic acid residues. Live organisms for protection tests were suspended in Todd-Hewitt broth containing 15% glycerol (vol/vol), and 1-ml aliquots were frozen at -70°C until used.

Hybridoma Antibodies. BALB/c female mice were immunized intraperitoneally with 0.2 ml of a suspension of streptococcal cells (A590nm = 0.6). To obtain hybridomas against the complete type III antigen, heat-killed log-phase cells were used; for hybridomas against the incomplete type III antigen, acid-treated cells were used. A secondary immunization was performed (0.1 ml intravenously and 0.2 ml intraperitoneally) 2 wk later. 4 d after boosting, the spleen cells were fused with Ag 8.653 cells (7) in the presence of 35% PEG 6000, 20% fetal calf serum, 1 × 10^{-4} M hypoxanthine, 4 × 10^{-7} M aminopterin, and 1.6 × 10^{-6} M thymidine (HAT medium). 2 wk after fusing, the supernates from those wells containing growing cells were tested for antibody to group B type III polysaccharides using the enzyme-linked immunosorbent assay (ELISA) described below. Clones producing monoclonal antibodies of interest were obtained by limiting dilution on mouse peritoneal exudate feeders. Isotypes of the clones were determined by double diffusion in 1% agarose in phosphate-buffered saline (PBS) of the cell culture supernates (concentrated 20-50-fold by (NH₄)₂SO₄ precipitation) against isotype-specific anti-mouse immunoglobulin (Litton Bionetics Inc., Kensington, MD). Larger quantities of the monoclonal antibodies were obtained from the ascitic fluid of mice in which the hybridoma had been grown intraperitoneally. The BALB/c female mice used for this purpose were injected with 0.5 ml of tetramethylpentadecane (Aldrich Chemical Co., Milwaukee, WI) at least 2 wk before the injection of hybridoma cells.

Screening Hybridomas. An ELISA system was used to screen hybridoma culture supernates for the presence of antibody, essentially as previously described (8). Nonsialated type III antigen was prepared by acid extraction and ethanol fractionation (1). Complete type III antigen was prepared from cells sonicated in cold 5% trichloroacetic acid and purified on a wheat germ agglutinin affinity column (B. M. Gray, H. C. Dillon, Jr., and D. G. Pritchard, manuscript in preparation). Polystyrene autoanalyzer cups (2 ml) were coated with the appropriate antigen coupled to poly-t-lysine (8). After washing out unabsorbed antigen, hybridoma supernates (0.1 ml) were added to the cups, diluted with 1 ml PBS containing 0.05% Brij-35 (Aldrich Chemical Co.), and incubated for 3 h at room temperature. Rabbit antibody against both heavy and light chains of mouse immunoglobulin (Litton Bionetics Inc., Kensington, MD), and incubated for 3 h at room temperature. Rabbit antibody against both heavy and light chains of mouse immunoglobulins, conjugated to horseradish peroxidase (Cappel Laboratories Inc., West Chester, PA), was added and incubated for 2 h. After washing out excess conjugate, O-phenylenediamine/H₂O₂ substrate was added, color was allowed to develop for 30 min, and was read at 420 nm with a P 1000 colorimeter (Brinkmann Instruments, Inc., Westbury, NY) (8).

Mouse Protection Test. Male C3H/HeN mice, 13-wk-old and ~30 g, were obtained from the National Cancer Institute. The mucin model of Fleming (9, 10) was used in all experiments. A 10% (wt/vol) suspension of type II hog gastric mucin (Sigma Chemical Co.) in Dulbecco's balanced salt solution was prepared and autoclaved. Test mixtures were prepared by mixing 1 ml of the bacterial dilution, containing 1,560 colony-forming units (cfu) (8 × LD₉₀), 1 ml of diluted (1:5) ascitic fluid or Dulbecco's balanced salt solution for the controls, and 2 ml of mucin. Each mouse was injected intraperitoneally with a total of 0.5 ml of the mixture. The LD₉₀ was estimated by the method of Reed and Muench (11) and corresponded to 170 cfu.

Results

Table I lists the hybridoma antibodies, their specificity, and isotypes. The hybridomas obtained after immunization with acid-treated GBS III (fusion 1)
TABLE I
Hybridoma Antibodies to Type III GBS

| Clone No.* | Specificity* | Isotype | Clone No.* | Specificity* | Isotype |
|------------|-------------|---------|------------|-------------|---------|
| Fusion 1 (acid-treated cells) | Fusion 2 (log-phase cells) |
| SA19C1     | III (NS)    | IgM     | SS15C1     | III (CPL)   | IgA     |
| SA19C3     | III (NS)    | IgM     | SS15C2     | III (CPL)   | IgA     |
| SA23C3     | III (NS)    | IgM     | SS8C3      | III (CPL)   | IgG2a   |
| SB1C1      | III (NS)    | IgM     | SV18C1     | III (CPL)   | IgM     |
| SB1C2      | III (NS)    | IgM     | SV18C2     | III (CPL)   | IgM     |
| SB2C2      | III (NS)    | IgM     | SV18C3     | III (CPL)   | IgM     |
| SB3C1      | III (NS)    | IgM     | SV20C1     | III (CPL)   | IgM     |
| SB3C2      | III (NS)    | IgM     | SW8C2      | III (NS)    | IgG2a   |
| SB3C3      | III (NS)    | IgM     | SW8C3      | III (NS)    | IgG2a   |
| SD16C2     | III (NS)    | IgG3    | SZ3C1      | III (CPL)   | IgM     |
| SE11C1     | III (NS)    | IgM     | SZ18C1     | III (CPL)   | IgM     |
| SE11C2     | III (NS)    | IgM     |           |             |         |
| SE11C3     | III (NS)    | IgM     |           |             |         |
| SF2C2      | III (NS)    | IgM     |           |             |         |
| SF23C2     | III (NS)    | IgG3    |           |             |         |
| SJ9C1      | III (NS)    | IgM     |           |             |         |
| SJ9C2      | III (NS)    | IgM     |           |             |         |
| SJ9C3      | III (NS)    | IgM     |           |             |         |

* The first two letters and number of the hybridoma code numbers designate the original hybridoma; C followed by a number indicates the clone.

NS, nonsialated; CPL, complete.

produced monoclonal antibodies directed exclusively against the nonsialated type III antigen. Most of these yielded IgM antibodies, but two clones produced IgG3 (SD16C2 and SF23C2). The hybridoma antibodies obtained after immunization with whole heat-killed organisms were predominantly against the complete type III antigen and included clones producing IgM, IgG2a, and IgA antibodies. One hybridoma (SW8) from this fusion, however, produced IgG2a antibody against the nonsialated antigen. The exclusive specificity of each monoclonal antibody for either the complete or incomplete antigen is illustrated in Fig. 1.

Preliminary experiments indicated that the C3H/HeN strain of mice was well-suited for studies on the protective effect of anti-GBS antibodies. Whereas mucin was required for a fatal infection, a relatively small inoculum produced consistent results. It was determined that the LD₉₀ for C3H/HeN mice was ~170 cfu of the type III strain M732. Results obtained with the six monoclonal antibodies selected for use in the mouse protection studies are listed in Table II. Control mice, which did not receive antibody, were not protected from the challenge and all died. Most mice receiving monoclonal antibodies to the nonsialated
Figure 1. Immunodiffusion reactions in 1% agarose gels between the purified type III GBS polysaccharides (0.3 mg/ml) and the monoclonal antibodies (ascitic fluids) used in the mouse protection tests. (A) the complete (sialated) type III polysaccharide; (B) the nonsialated type III polysaccharide. The ascitic fluids in the outer well are as follows: well 1, SV18C2 (IgM); well 2, SE11C2 (IgM); well 3, SS8C3 (IgG2a); well 4, SW8C3 (IgG2a); well 5, SS15C2 (IgA); well 6, SF23C2 (IgG3).

Table II
Protective Effect of Monoclonal Antibodies in Mice Challenged with Strain M732 GBS

| Monoclonal antibody | Specificity | Isotype | µg injected* | No. died/No. challenged |
|---------------------|-------------|---------|--------------|------------------------|
| SE11C2              | III (nonsialated) | IgM     | ND           | 5/6                    |
| SW8C3               | III (nonsialated) | IgG2a   | 200 µg       | 6/6                    |
| SF23C2              | III (nonsialated) | IgG3    | 105 µg       | 5/6                    |
| SV18C2              | III (complete)   | IgM     | 198 µg       | 0/12                   |
| SS8C5               | III (complete)   | IgG2a   | ND           | 0/6                    |
| SS15C2              | III (complete)   | IgA     | 60 µg        | 0/5                    |
| Control*            | **            | --      |              | 10/10                  |

* The volume of antibody-containing ascitic fluid injected was 25 µl per mouse; the antibody concentration of four ascitic fluids was determined by quantitative precipitation with the appropriate antigen. ND, not determined.

** Dulbecco's balanced salt solution was substituted for diluted ascitic fluid for the control animals.

Antibodies against the complete type III antigen of the IgM, IgG2a, and IgA isotypes, however, were uniformly protective. All surviving mice were observed for at least 2 mo, and there were no additional deaths.

Discussion
The mouse hybridoma antibodies described in this study were specific for either the sialated or the nonsialated form of the GBS type III polysaccharide antigen. Antibodies of the two different specificities did not cross-react, either by ELISA or by double diffusion in agarose gels, and showed marked differences in functional properties in the mouse protection studies. Jennings et al. (12) reported previously that rabbits immunized with GBS type III produce two major populations of antibodies, one specific only for the sialated ("native") antigen and a second that precipitates both sialated and nonsialated ("core") antigens (12). None of our hybridoma antibodies showed this dual specificity.

Our mouse protection model was essentially that described by Fleming (9, 10), using mucin to enhance the virulence of GBS type III organisms by about four orders of magnitude. Although mucin has been used for over 50 years in
experimental infections in mice (13), the reason it increases virulence is not understood. Fleming showed that the ability of mucin to enhance the virulence of type III GBS varied with different strains of mice, suggesting that mucin affected host defense mechanisms rather than the bacteria. She found that the virulence of GBS type III (M732) was greatly enhanced by mucin in ICR Swiss outbred mice while C67BL/6 inbred mice were quite resistant to infection.

Fischer et al. (5) first noted the cross-reactivity between the nonsialated GBS type III antigen (made by hot acid extraction) and the pneumococcal type 14 polysaccharide and reported that antibody to the pneumococcal antigen was opsonic for type III GBS. In a passive protection model using infant rats, they further demonstrated that rabbit antisera to pneumococcus type 14 was also protective in vivo against GBS type III infection (5). Assuming that the nonsialated type III GBS antigen and the pneumococcal type 14 polysaccharides are structurally identical, as reported by Jennings et al. (14), it should follow that antibodies against the nonsialated GBS type III antigen should be similarly protective. Kasper et al. (4), however, reported that antibodies to the sialated native antigen correlated best with opsonophagocytosis of type III cells and with protection against disease, while those against the nonsialated antigen did not. In our studies, only those monoclonal antibodies directed against the sialated type III GBS antigen protected mice from an otherwise fatal challenge.

There are several possible explanations for these differences in functional activity reported for the antibodies of the various specificities. Though similar and cross-reactive, the pneumococcal and GBS antigens may not be precisely identical. The GBS type III organisms used in opsonophagocytic and protection tests may vary in the completeness of sialation of the exposed-type polysaccharides; the fine specificities of mouse monoclonal and rabbit polyclonal antibodies may differ; and the isotype of the antibody may also play different roles in protection for various animal species.

The ability of an IgA antibody to protect against infection in our mouse model was quite unexpected. Using a mouse model for pneumococcus type 3 infection, Briles et al. (15) found that IgA antibodies were not protective. Their model, however, was different in several respects, especially in the administration of a fairly large challenge (~10^5 organisms) by the intravenous route. Lowell et al. (16) have previously demonstrated IgA-mediated phagocytosis of *N. meningitidis* by peripheral blood monocytes in an in vitro system. It is possible that in our model the use of a small inoculum given intraperitoneally may have permitted the operation of a similar mechanism involving peritoneal macrophages. This is currently under investigation.

Summary

Mouse hybridoma antibodies of several major classes against group B streptococcus type III have been produced. Mice were immunized with either whole heat-killed or acid-treated organisms to obtain antibodies against both the complete (sialated) or incomplete (nonsialated) forms of the type III polysaccharide. Resulting monoclonal antibodies showed exclusive specificity for either the complete or incomplete antigen.

The ability of these antibodies to protect mice from a lethal challenge of live type III organisms was tested with a mucin model that permitted use of very
small inocula given intraperitoneally with antibody and mucin. Antibodies specific for the nonsialated antigen were not protective, whether of IgM, IgG2a, or IgG3 isotypes. Antibodies specific for the complete antigen were, however, highly protective, including monoclonals of IgM, IgG2a, and IgA isotypes. These mouse monoclonal antibodies against group B streptococci that are directed against either complete or incomplete antigenic determinants, and include isotypes other than IgM, should be particularly useful for studying the mechanism of protection against experimental infection.

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