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Murine Monoclonal Antibodies to Type Ib Polysaccharide of Group B Streptococci Bind to Human Milk Oligosaccharides

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The chemical structures of the repeating units of the type Ib polysaccharide of group B streptococci and of the desialylated form of this antigen are almost identical to those of some oligosaccharides in human milk and certain fetal antigens. The structural similarities suggested that the molecules may be immunologically cross-reactive. Mouse monoclonal antibodies to the sialylated and nonsialylated forms of the type Ib polysaccharide were produced and tested for their ability to bind to immobilized human milk oligosaccharides. One antibody, SMB19, reacted specifically with the sialylated form of the type Ib polysaccharide and was also bound by an affinity column containing immobilized sialyllacto-*N*-tetraose a. The antibody was eluted from the affinity column with EDTA, since its binding to the antigen was calcium dependent. A second monoclonal antibody, SIbD2, bound specifically to the nonsialylated form of the type Ib polysaccharide and also to immobilized lacto-*N*-tetraose. The antibody was eluted from the affinity column at an acidic pH and retained immunologic activity. These results further extend our previous observations that certain antibodies raised against group B streptococci can also react with normal human glycoconjugates.

Group B streptococci (GBS) elaborate type-specific capsular polysaccharides that share considerable structural similarity with known human glycoconjugates. The type Ib polysaccharide is of particular interest because, as illustrated in Fig. 1, its repeating unit (20) is virtually identical to those of certain oligosaccharides present in human milk (13, 21) and various fetal antigens (6, 19). Moreover, the immunologic response to the type Ib polysaccharide also involves the production of antibodies to the desialylated form of the antigen, which possesses a terminal galactose-containing determinant also present in various mammalian glycoconjugates. These observations must be considered as research is conducted towards developing vaccines for the prevention of GBS disease in neonates, since an essential requirement of any vaccine is that it does not induce antibodies that might be detrimental to mother, infant, or developing fetus. Cross-reactions of the type Ib polysaccharide and human glycoconjugates would be predicted on the basis of their chemical structures. The purpose of this study was to verify the existence of such cross-reactions by use of mouse monoclonal antibodies of defined specificities for the sialylated and desialylated determinants of the type Ib capsular antigen.

MATERIALS AND METHODS

Monoclonal antibodies. Monoclonal antibodies were produced by a modification of a procedure described previously (4). The vaccines were prepared from GBS grown in Todd-Hewitt broth to the log phase, washed in phosphate-buffered saline (PBS), heat killed at 80°C for 15 min, and diluted to an A_{590} of 0.6. A BALB/c female mouse was immunized intraperitoneally with 0.1 ml of a mixture composed of equal parts of the vaccine suspensions of strains H36B (type Ib), 18RS21 (type II), and 1073 (nontypeable). Another mouse was immunized with 0.1 ml of an H36B vaccine that had been treated with 2 N acetic acid at 80°C for 10 min to largely desialylate the type Ib polysaccharide. Mice were boosted intraperitoneally 24 days later and once again 22 days later, with half of the original vaccine being given intraperitoneally and half being administered intravenously. Four days later, the spleen cells were fused with Ag8.653 cells (12) as described previously (4). Hybridoma supernatants were screened for specific antibody production by an enzyme-linked immunosorbent assay (ELISA) (4, 7) and by an indirect immunofluorescence technique with freshly cultured GBS cells and GBS type Ib cells that had been desialylated as described above. In the latter technique, washed GBS cells were cytocentrifuged onto glass slides and heat fixed; 10 μl of hybridoma supernatant was placed on top of the fixed cells and incubated at 4°C for 30 min. After the slides were washed, 10 μl of 30-μg/ml fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was placed on top of the fixed cells and incubated at 4°C for 30 min. The slides were washed again, mounted in Gelvatol (Monsanto, Indian Orchard, Mass.), and examined under an epifluorescence microscope to identify positive hybridoma supernatants. Anti-GBS hybridomas were cloned by limiting dilution on peritoneal exudate feeder cells and grown in mice to produce ascites fluid containing monoclonal antibodies. The isotypes of the antibodies were determined by double diffusion of 20-fold-concentrated culture supernatants against isotype-specific anti-mouse immunoglobulin (Southern Biotechnology).

Chromatography of monoclonal antibodies on human milk oligosaccharide affinity columns. Immobilized sialyllacto-*N*-tetraose a (LST-a–Fractogel) and lacto-*N*-tetraose (LNT-Fractogel) were purchased from BioCarb Chemicals, Lund, Sweden. Ascites fluid containing monoclonal antibodies was diluted with an equal volume of running buffer (0.1 M Tris-HCl [pH 7.5], 1 mM $CaCl_2$), and 1 ml of the mixture was passed through a 1-ml column of immobilized oligosaccharide. The column was washed three times with 2 ml of running buffer and then eluted with 30 mM EDTA in Tris-HCl (pH 9), and 0.5-ml fractions were collected. The LNT-Fractogel column was also finally eluted with 0.1 M

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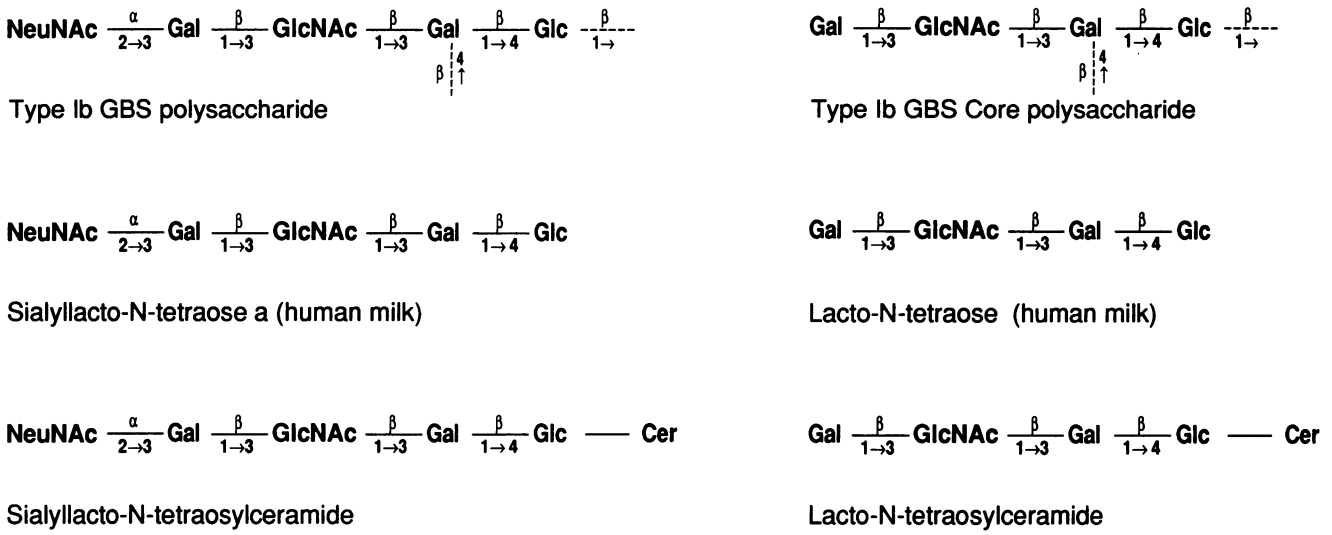

FIG. 1. Structures of the repeating units of the sialylated and nonsialylated forms of the GBS type Ib polysaccharide and the structurally similar human milk oligosaccharides and fetal antigens. NeuNAc, *N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Cer, ceramide.

sodium citrate buffer (pH 3.5), and the resulting fractions were immediately neutralized. The protein contents of the fractions were determined by use of the Bio-Rad protein assay with bovine gamma globulin (Sigma Chemical Co., St. Louis, Mo.) as a protein standard. Both the bound and unbound column fractions were subjected to double diffusion in a 1% agarose gel containing 0.1 M Tris-HCl (pH 7.5)–1 mM $CaCl_2$. Fractions from the LST-a–Fractogel column were also examined by electrophoresis on an 8% polyacrylamide gel run under reducing conditions for 55 min at 200 V and stained with 1% Coomassie blue (14).

RESULTS

Immunization of a mouse with a vaccine containing three different GBS strains made it possible for us to produce 31 different monoclonal antibodies of a variety of specificities. One of several antibodies with specificity for the type Ib polysaccharide was designated SMB19. It reacted specifically with GBS type Ib cells in the indirect immunofluorescence assay and only with the sialylated form of the GBS type Ib polysaccharide in the ELISA (data not shown). An additional 11 hybridomas were produced after immunization with desialylated GBS. One of them, designated SIbD2, which reacted only with the desialylated form of the GBS type Ib polysaccharide, was used in this study. Both SMB19 and SIbD2 monoclonal antibodies were immunoglobulin M (κ).

To determine whether monoclonal antibody SMB19 bound to the structurally similar human milk oligosaccharide, sialyllacto-*N*-tetraose a, we passed SMB19 ascites fluid through a column containing the immobilized milk oligosaccharide. Unbound material was washed through the column with buffer. Bound material was eluted with 30 mM EDTA, since a previous study demonstrated that the binding of SMB19 to the GBS type Ib polysaccharide was calcium dependent and could be inhibited by either EDTA or P_i (18). Figure 2 shows the results of affinity chromatography of SMB19. Unbound fractions 1 and 2 were pooled and, similarly, fractions 6, 7, and 8, containing bound material eluted by EDTA, were separately pooled. The two pools were analyzed by polyacrylamide gel electrophoresis (Fig. 3). Lanes 1 and 6 contained molecular weight standards, lane 5 contained mouse albumin, and lane 2 contained the original SMB19 ascites fluid. Lane 4 contained material that bound to the column and was subsequently eluted by EDTA. The two bands corresponded in molecular weight to the heavy and light chains of immunoglobulin M. Lane 3 contained unbound material that was washed through the column and was depleted of the two antibody bands shown in lane 4. Both unbound and EDTA-eluted fractions were studied by immunodiffusion against the GBS type Ib polysaccharide. Only the fraction eluted by EDTA produced a precipitin band (data not shown). To control for nonspecific binding of the antibody to the column, we also passed monoclonal antibody SMB19 through a similar column containing immobilized lacto-*N*-tetraose. None of the antibody bound to this col-

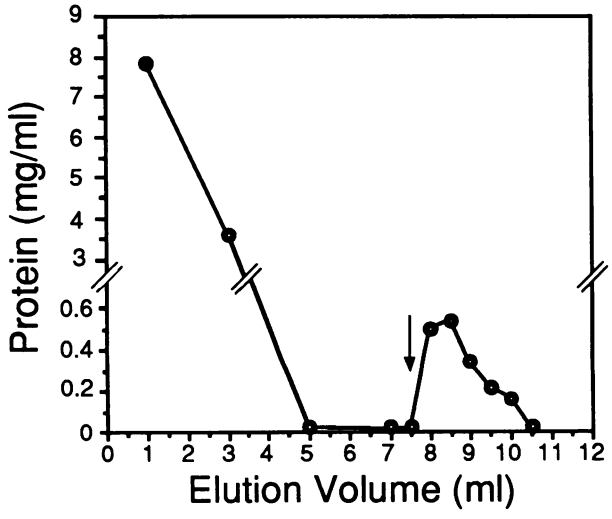

FIG. 2. Affinity purification of monoclonal antibody SMB19 on a column of immobilized sialyllacto-*N*-tetraose a. The arrow indicates the point at which elution was begun with 30 mM EDTA.

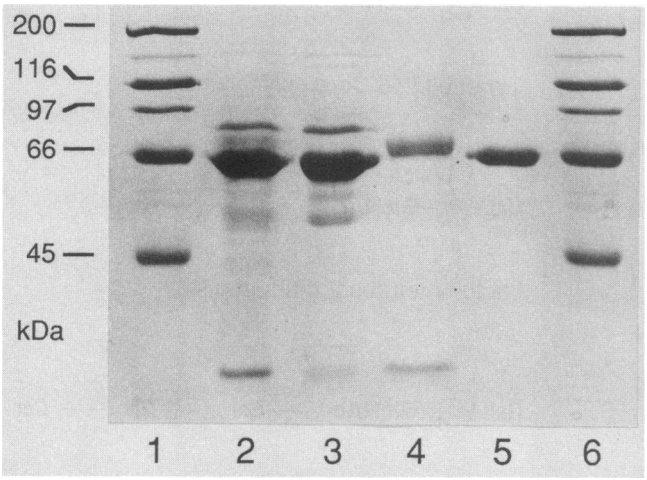

FIG. 3. SDS-PAGE protein profiles of fractions from the affinity purification of monoclonal antibody SMB19 on a column of immobilized sialyllacto-*N*-tetraose a. Lanes: 1 and 6, high-molecular-mass standards of the indicated sizes; 2, original SMB19 ascitic fluid; 3, ascitic fluid fraction not bound by the column (pooled fractions 1 and 2); 4, fraction bound to the column and eluted with EDTA (pooled fractions 6, 7, and 8); 5, mouse albumin.

umn, and no material could be eluted by EDTA (data not shown).

The second monoclonal antibody, SIbD2, which bound to acid-treated GBS type Ib cells in the indirect immunofluorescence assay, also bound specifically to the nonsialylated form of the GBS type Ib polysaccharide in the ELISA and produced a precipitin band against the nonsialylated antigen upon double diffusion in agarose (data not shown). Figure 4 shows the results of affinity chromatography of monoclonal antibody SIbD2. When SIbD2 ascites fluid was passed through the LNT-Fractogel column, the antibody was bound. Unlike monoclonal antibody SMB19, however,

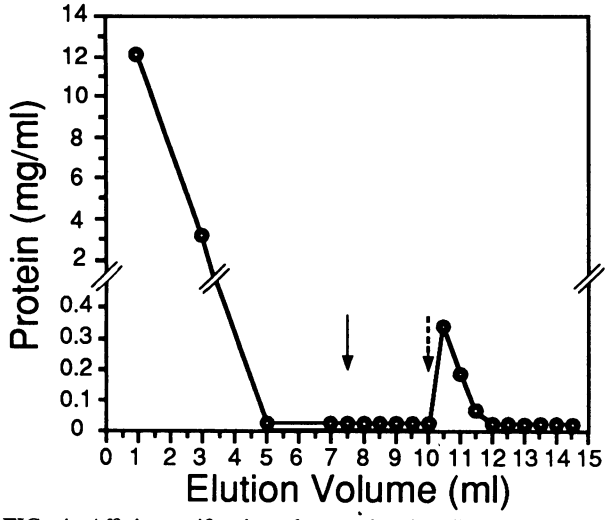

FIG. 4. Affinity purification of monoclonal antibody SIbD2 on a column of immobilized lacto-*N*-tetraose. The solid arrow indicates the point at which elution was begun with 30 mM EDTA, and the broken arrow indicates the point at which elution was begun with 0.1 M sodium citrate (pH 3.5).

monoclonal antibody SIbD2 could not be eluted by EDTA. This result was expected, since we had previously shown that the binding of this antibody was not calcium dependent (18). The antibody was eluted by 0.1 M sodium citrate buffer (pH 3.5) and retained immunologic activity, producing a precipitin band against the nonsialylated GBS type Ib polysaccharide upon double diffusion in agarose (data not shown).

DISCUSSION

The complex nature of antibody specificities for GBS polysaccharides have been recognized since Lancefield's original classification, which was based on rabbit antisera produced by immunization with whole killed organisms (15). Rabbits readily made antibodies to the acid-extracted, desialylated antigens used for serotyping, as well as to the labile, "complete" (sialylated) antigens and to a Iabc carbohydrate determinant common to both type Ia and type Ib (16, 22). In more recent immunochemical studies, Schifferle et al. found that their type Ib rabbit antiserum could not distinguish between the sialylated and desialylated forms of the type Ib polysaccharide (20). They postulated a conformational explanation for this finding, but it is also possible that it was simply a property of the particular hyperimmune rabbit antiserum used. The availability of murine monoclonal antibodies made it possible to demonstrate that antibodies can have an exclusive specificity for either the sialylated or the nonsialylated form of the Ib antigen (18). Monoclonal antibody SMB19 reacted only with GBS type Ib cells in an indirect immunofluorescence assay and only with the sialylated form of the GBS type Ib polysaccharide in the ELISA. SMB19 is highly protective in mice challenged with an otherwise lethal dose of GBS type Ib cells (18). In this study, we demonstrated that SMB19 binds to the human milk oligosaccharide sialyllacto-*N*-tetraose a bound to Fractogel. This same pentasaccharide has been reported to be linked to ceramide in human fetal tissues (6).

The calcium requirement for the binding of monoclonal antibody SMB19 was of particular interest. Negatively charged polysaccharide antigens are very likely to bind divalent metal ions, in turn possibly resulting in conformational changes critical for the binding of certain antibodies. We previously reported that some antibodies directed against GBS type antigens, the GBS group antigen, and teichoic acid appeared to require calcium for binding and were inhibited by EDTA and phosphate (18). We suspect that calcium-dependent antibody binding is a relatively common, although often unrecognized, property of many antigen-antibody systems involving acidic polysaccharides. In preliminary studies comparing various buffers in an ELISA, we found that most human sera with antibodies to GBS type Ib polysaccharide showed significantly more binding in Tris containing 1 mM calcium chloride than in Tris alone, phosphate, or borate (unpublished observations). The extent to which human antibodies to the various GBS polysaccharide antigens are calcium dependent is unknown but is currently under investigation.

The nonsialylated form of the GBS type Ib polysaccharide has a repeating unit corresponding to that of another human milk oligosaccharide, lacto-*N*-tetraose. Monoclonal antibody SIbD2, produced by immunizing a mouse with desialylated GBS type Ib cells, was found to bind to an affinity column of this milk oligosaccharide. The glycolipid form of this antigen, lacto-*N*-tetraosylceramide, has been reported to be restricted to a small number of cell types, including

human fetal intestinal and bronchial epithelial cells and embryonal carcinoma cells (6). It is not known whether humans make antibodies to these oligosaccharides, and no information has been published regarding human antibody responses to the desialylated antigen. We carried out a preliminary study in which antibody levels were assessed in an ELISA with type Ib polysaccharide desialylated with *Vibrio cholerae* neuraminidase (unpublished data). Significant antibody levels were detected in the sera from 28 adult women. This result was not surprising, in view of the observed mouse and rabbit responses. However, the fine specificities of the human antibodies have not been explored, and the extent to which they may cross-react with host glycoconjugates has yet to be determined.

The milk oligosaccharides used in this study are components of a mixture of neutral and sialylated human milk oligosaccharides whose biological functions are largely unknown. It seems likely that they do not serve a nutritional function, since they are largely excreted in infants' urine (3). One study found that nursing babies excreted 300 to 500 mg of milk oligosaccharides per day and that their mothers excreted 500 to 800 mg/day (3). The authors of this study also found that the neutral oligosaccharide fraction inhibited the adhesion of a pathogenic strain of *Escherichia coli* to uroepithelial cells, suggesting a possible role for the oligosaccharides in preventing urinary tract infections in mothers and infants. The sialylated milk oligosaccharides did not inhibit the adhesion of *E. coli* in their model. It is not known, however, whether sialylated milk oligosaccharides can inhibit the adhesion of GBS to uroepithelial cells.

This report has focused on the GBS type Ib polysaccharide, but the other three major type antigens of GBS are also very similar to many normal human glycoconjugates, and similar cross-reactions are likely to be found. The type Ia repeating unit is virtually identical to the oligosaccharide of the *i* blood group substance found on fetal erythrocytes and on pulmonary epithelia (10, 17). We previously reported that monoclonal antibodies to the GBS type II polysaccharide were inhibited by components in human milk and saliva (8) and showed that humans make antibodies to the two major determinants of the type II polysaccharide (8). We also noted that a monoclonal antibody to the desialylated form of GBS type III bound to a population of human lymphocytes (9).

It remains to be determined what precise antibodies are made in humans and whether immunization with GBS polysaccharides might give rise to antibodies that could be detrimental to mother or fetus. Häyrinen et al. studied this question by preparing glycopeptide fractions from various tissues, radiolabeling them, and using them in immunobinding assays with human antisera (11). These antisera were obtained from individuals who responded with significant antibody levels after vaccination with GBS type Ia, II, or III polysaccharides. No cross-reactions were found. However, antisera from these volunteers were collected 1 month to 6 years after immunization. Had autoantibodies been elicited by vaccination of these individuals, it seems unlikely that they would have persisted in the circulation for such a long time. Furthermore, not all humans immunized with polysaccharide vaccines mount a significant response. It has been reported, for example, that only about 60% of individuals respond to a GBS type III polysaccharide vaccine (1, 2, 5). It is possible that GBS vaccines are more likely to elicit autoimmunity in apparent nonresponders. Some antibodies may have been missed, since all the immunobinding assays of Häyrinen et al. were carried out in calcium- and magnesium-free PBS (11), conditions under which antibodies similar to SMB19 would not have bound. We remain hopeful that appropriate vaccines against GBS can be developed, but the possibility of inducing major cross-reactions with human glycoconjugates has not been excluded, and the complex immunochemistry of the GBS antigens merits further careful study.

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REFERENCES

1. **Baker, C. J., M. S. Edwards, and D. L. Kasper.** 1978. Immunogenicity of polysaccharides from type III, group B streptococcus. J. Clin. Invest. **61**:1107–1110.
2. **Baker, C. J., M. A. Rench, M. S. Edwards, R. J. Carpenter, B. M. Hays, and D. L. Kasper.** 1988. Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus. N. Engl. J. Med. **319**:1180–1185.
3. **Coppa, G. V., O. Gabrielli, P. Giorgi, C. Catassi, M. P. Montanari, P. E. Varaldo, and B. L. Nichols.** 1990. Preliminary study of breastfeeding and bacterial adhesion to uroepithelial cells. Lancet **335**:569–571.
4. **Egan, M. L., D. G. Pritchard, H. C. Dillon, Jr., and B. M. Gray.** 1983. Protection of mice from experimental infection with type III group B streptococcus using monoclonal antibodies. J. Exp. Med. **158**:1006–1011.
5. **Eisenstein, T. K., B. J. De Cueninck, D. Resavy, G. D. Shockman, R. B. Carey, and R. M. Swenson.** 1983. Quantitative determination in human sera of vaccine-induced antibody to type-specific polysaccharides of group B streptococci using an enzyme-linked immunosorbent assay. J. Infect. Dis. **147**:847–856.
6. **Fukuda, M. N., B. Bothner, K. O. Lloyd, W. J. Rettig, P. R. Tiller, and A. Dell.** 1986. Structures of glycosphingolipids isolated from human embryonal carcinoma cells. J. Biol. Chem. **261**:5145–5153.
7. **Gray, B. M.** 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. Methods **28**:187–192.
8. **Gray, B. M., M. L. Egan, and D. G. Pritchard.** 1988. Specificity of monoclonal antibodies against group B streptococcus type II and inhibition of their binding by human secretions. Pediatr. Res. **24**:68–72.
9. **Gray, B. M., M. L. Egan, and D. G. Pritchard.** 1990. The group B streptococci: from natural history to the specificity of antibodies. Semin. Perinatol. **14**:10–21.
10. **Hakomori, S.** 1984. Philip Levine Award Lecture: blood group glycolipid antigens and their modifications as human cancer antigens. Am. J. Clin. Pathol. **82**:635–648.
11. **Häyrinen, J., S. Pelkonen, and J. Finne.** 1989. Structural similarity of the type-specific group B streptococcal polysaccharides and the carbohydrate units of tissue glycoproteins: evaluation of possible cross-reactivity. Vaccine **7**:217–224.
12. **Kearney, J. F., A. Radbrush, B. Liesegang, and K. Rajewsky.** 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting cell lines. J. Immunol. **123**:1548–1550.
13. **Kobata, A., K. Yamashita, and Y. Tachibana.** 1978. Oligosaccharides from human milk. Methods Enzymol. **50**:216–220.

14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Lancefield, R. C., and E. H. Freimer.** 1966. Type-specific polysaccharide antigens of group B streptococci. J. Hyg. **64:** 191–203.
16. **Lancefield, R. C., M. McCarty, and W. N. Everly.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. J. Exp. Med. **142:**165–179.
17. **Loveless, R. W., and T. Feizi.** 1989. Sialo-oligosaccharide receptors for *Mycoplasma pneumoniae* and related oligosaccharides of poly-*N*-acetyllactosamine series are polarized at the cilia and apical-microvillar domains of the ciliated cells in human bronchial epithelium. Infect. Immun. **57:**1285–1289.
18. **Pritchard, D. G., M. L. Egan, B. M. Gray, and H. C. Dillon, Jr.** 1988. Immunochemical characterization of the polysaccharide antigens of group B streptococci. Rev. Infect. Dis. **10:**S367–S371.
19. **Rettig, W. J., C. Cordon-Cardo, J. S. C. Ng, H. F. Oettgen, L. J. Old, and K. O. Lloyd.** 1985. High molecular weight glycoproteins of human teratocarcinoma defined by monoclonal antibodies to carbohydrate determinants. Cancer Res. **45:**815–821.
20. **Schifferle, R. E., H. J. Jennings, M. R. Wessels, E. Katzenellenbogen, R. Roy, and D. L. Kasper.** 1985. Immunochemical analysis of the types Ia and Ib group B streptococcal polysaccharides. J. Immunol. **135:**4164–4170.
21. **Smith, D. F., D. A. Zopf, and V. Ginsburg.** 1978. Sialyl oligosaccharides from milk. Methods Enzymol. **50:**221–226.
22. **Wilkinson, H. W.** 1975. Immunochemistry of purified polysaccharide type antigens of group B streptococcal types Ia, Ib, and Ic. Infect. Immun. **11:**845–852.