# HUMAN MONOCLONAL ANTIBODIES TO GROUP B STREPTOCOCCUS

## Reactivity and In Vivo Protection Against Multiple Serotypes

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Bacterial infections are frequently the direct or principal underlying cause of human neonatal deaths. The group B streptococci (GBS)[1] compose the predominant group of gram-positive bacteria responsible for severe or life-threatening infections. Infants born prematurely, and infants born more than 18 h after the amniotic membrane has ruptured, are at a higher risk for early-onset infection. Late-onset GBS infections occur in infected healthy newborns up to 2–3 mo of age. Regardless of the time of onset, a significant percentage of these infections result in death, or permanent disability (1).

GBS are distinguished from other streptococci by their conserved group-specific polysaccharide, and are further phenotyped based on the reactivity of their capsule polysaccharide with type-specific antisera (2). In humans, although any capsule type (Ia, Ib, II, and III) may cause early onset sepsis, type III GBS are associated with the majority of early onset meningitis, and late onset sepsis and meningitis (3). GBS capsule expression directly correlates with GBS virulence (4).

It is generally agreed that type-specific capsule, but not group polysaccharide-specific antibodies, provide GBS immunity (2, 5–8). Among healthy and infected newborns, the lowest infection rate correlates with elevated maternal anti-type-specific capsule titers (5, 9). Moreover, human maternal sera with the highest anticapsule activity passively protect GBS-infected rodents (9, 10). These data corroborate the protective activity of heterologous capsule antisera and murine anticapsule mAbs (2, 6, 9). Heterologous antisera and mouse mAbs reacting with the group B polysaccharide have consistently failed in animal protection studies (8, 10).

Human mAbs against specific pathogens may provide an effective and safe alternative, or adjunct treatment for neontal infections. Experiments using a protective human mAb against another common neonatal pathogen, *Escherichia coli* K1 (11), suggested mAbs against other prevalent bacteria might contribute towards reducing the mortality from neonatal infections. This report describes the development of human mAbs specific for the group B polysaccharide on GBS. The mAbs reacted with all GBS serotypes and provided therapeutic protection in neonatal rats infected with either type III or type Ia GBS clinical isolates.

[1] *Abbreviations used in this paper:* GBS, group B streptococci; XIEP, crossed immunoelectrophoresis.

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## Materials and Methods

*Bacterial Strains, Antigens, and Antisera.* The GBS reference strains and clinical isolates were obtained as follows: type Ia: SS-615, SS-800, SS-881; type Ib: SS-618; type Ic: SS-700; type II: SS-619; type III: SS-620 from Dr. R. Facklam (Centers for Disease Control (CDC), Atlanta, GA); 090R from American Type Culture Collection (ATCC, No. 12386, Rockville, MD). A type III GBS clinical isolate, COH 31r/s (rifampin and streptomycin resistant) and its isogenic, capsule-negative mutant COH 31-15 (3) used in crossed immunoelectrophoresis (XIEP) were provided by Dr. C. Rubens, Childrens Orthopedic Hospital, Seattle, WA. This insertion mutant was obtained using Tn*916* transposon mutagenesis of the COH 31r/s parent. The mutant was found to express the group B polysaccharide, but did not possess detectable capsule. The additional 132 isolates were obtained from Seattle area hospitals (Childrens Orthopedic Hospital, Harborview Medical Center, Group Health Hospital, and Veterans Administration Hospital), and from Dr. Joan Fung-Tomc (Bristol-Myers Company, Microbiology Culture Collection, Wallingford, CT). 29 of the clinical strains were isolated from blood or cerebrospinal fluid, primarily in neonates. All isolates were confirmed as GBS using a latex agglutination test kit (Streptex; Wellcome Diagnostics, Darford, England) and commercial (anti-group B; Difco Laboratories, Inc., Detroit, MI) or CDC reference antisera (generously supplied by Dr. R. Facklam, CDC).

Non-GBS reference strains were obtained from: *Pseudomonas aeruginosa* F2 (ATCC No. 27313); streptococcus group A (two isolates from Harboview Medical Center); streptococcus group C (vaccine strain SS-188 [CDC]); streptococcus group D (vaccine strain SS-499 [CDC] and a clinical isolate [Harboview Medical Center]); streptococcus group G (clinical isolate from Dr. F. Tenover [Veterans Administration Hospital]); streptococcus group G (vaccine strain SS-13 [CDC], ATCC No. 12394, six clinical isolates from Harborview Medical Center and five from Group Health Hospital); *Streptococcus mutans* (ATCC No. 27607); *Streptococcus sanguis* (ATCC No. 10557).

Serotype-specific antisera used in XIEP were raised in New Zealand white rabbits by Lancefield's procedure (6). Group-specific polysaccharide antigen was purchased (Difco Laboratories, Inc.). Bacteria were grown in Todd-Hewitt Broth modified for extra buffering capacity by increasing the disodium phosphate eightfold (12). Cell wall digests of logarithmic and stationary phase cultures were prepared by mutanolysin treatment (13).

*Chemical Reagents.* Unless otherwise noted, all chemical reagents were purchased from Sigma Chemical Co., St. Louis, MO.

*Lymphocyte Sources for Transformation.* B lymphocytes were obtained from the peripheral blood of normal humans, or cystic fibrosis patients hospitalized at Childrens Orthopedic Hospital, and from tonsil fragments obtained from routine tonsillectomies performed on otherwise normal patients at University Hospital, University of Washington, Seattle, WA.

*Viral Transformation for the Production of Human mAb.* Human mononuclear cells were separated from heparinized whole blood or tonsil cell suspensions by density gradient centrifugation through Lymphocyte Separation Media (Litton Bionetics, Charleston, SC) (14). The mononuclear cells were depleted of T lymphocytes using a modified E-rosetting technique (15). The E rosette-negative cells were washed once in Iscove's medium (Gibco Laboratories, Grand Island, NY) containing 15% (vol/vol) FCS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and resuspended in Iscove's-HAT (hypoxanthine [$10^{-4}$ M], aminopterin [$4 \times 10^{-7}$ M], and thymidine [$1.6 \times 10^{-7}$ M]).

The HAT-sensitive EBV-producing cell line, 1A2, was used for the transformations (16). 1A2 cells in logarithmic growth phase were combined with E rosette-negative mononuclear cells (30:1) in Iscove's-HAT medium. 200 μl of the cell mixture containing 1,000-2,000 E rosette-negative cells and 30,000-60,000 1A2 cells, were dispensed into each well of several 96-well round-bottomed microtiter plates. The cultures were incubated at 37°C in a humidified chamber containing 6% $CO_2$, and were fed every 3-4 d by replacing one-half the culture supernatant with fresh HAT medium. After 12-14 d, vigorous growth was generally apparent in 100% of the wells. After the culture supernatants were collected for assaying antibody activity, the cultures were fed with Iscove's medium without HAT.

*Antibody Screening Assay.* A standard ELISA protocol was used to screen culture supernatants for anti-GBS binding activity. This protocol has been previously described (11).

*Lymphoblastoid Cell Cloning.* Lymphoblastoid cells producing GBS antibodies were cloned by sequential limiting dilution platings. Cells, diluted in Iscove's medium containing 15% FCS, were seeded at densities between 20 and 2 cells/microtiter well in the absence of feeder cells. After one to two rounds of plating at gradually reduced cell input, cells showing good growth and antibody production were cloned by plating in 72-well Terasaki plates and visually identifying wells containing single cells (17).

*mAb Reactivity with Clinical Isolates.* The human GBS mAbs were assayed by "dot blot" analysis for reactivity with clinical isolates (11). A total of 132 GBS clinical isolates and five Lancefield reference strains were tested in this manner.

*Antibody Purification.* High cell density ($5 \times 10^5$ to $1 \times 10^6$ cells/ml), nutrient-exhausted culture supernatant was concentrated by Minitan tangential flow ultrafiltration (Millipore Corp., Bedford, MA) using PTHK 100,000 nominal molecular weight limit membranes. The mAbs were purified from concentrates by affinity chromatography on a murine anti-human IgM mAb column (11). Purity was examined by SDS-PAGE followed by silver nitrate staining (18), and antibody activity was assessed by ELISA as described above. Purified antibody preparations were assayed for pyrogen using the Limulus Amebocyte assay QCL-100 (M. A. Bioproducts, Walkersville, MD).

*Crossed Immunoelectrophoresis and Immunoblotting.* 12 ml of 1% agarose (SeaKem HGT; GMC Corp., Rockland, ME) in Monthony buffer (19) was poured onto an 84 × 94 mm glass plate. Wells punched in the solidified gel were filled flush with soluble antigen, and the first XIEP dimension was run on a Multiphore electrophoresis unit (LKB Instruments, Inc., Gaithersburg, MD) at 200 V, 10°C, for 1.5–2 h. The electrophoretically separated antigens were precipitated during electrophoresis into the second dimension antibody containing resolving gel (10–15 μl antiserum/cm$^2$) at 10°C, 2 V/cm for 18 h. After repeated washing in saline and press/blotting, gels were either dried onto Gelbond (GMC Corp.) and stained with Crowle's Double Stain (20) or used to prepare blots. XIEP gel protein precipitates were passively transferred to nitrocellulose. The pressed gels were reswelled in 0.1 M glycine-HCl, pH 2.5, for 15 min, removed from the glass plates, and sandwiched between nitrocellulose and blot paper. Sandwiches were prepared as follows: two sheets of Whatman 3MM blotting paper soaked in glycine-HCl were layered onto a glass plate, the reswollen XIEP gel was laid on top and was carefully overlaid with nitrocellulose sheets soaked in electrophoretic transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, with 20% methanol), and the nitrocellulose sheets were covered with four sheets of dry blotting paper and a glass plate. After 15 min, the nitrocellulose was blocked with PBS with Tween 20 (PBS-T) for at least 1 h. Gels blotted onto nitrocellulose paper were immersed in antibody containing culture supernatants for 1 h at room temperature. After washing, antibody binding was detected using the substrate system described for the dot blot analyses.

*Blocking Experiments with Monosaccharides.* Purified mAbs were mixed with individual monosaccharides (α-L-rhamose, D-glucitol, D-galactose, *N*-acetylglucosamine, or methyl-α-D-mannopyranoside at final concentrations of 0.1 μg/ml mAb and 20 mg/ml monosaccharide. Antibody and monosaccharide mixtures were incubated for 45 min at room temperature, and assayed in the standard ELISA (described above).

*Opsonophagocytic Studies.* The opsonic assays were performed essentially as described previously (11). Normal human serum adsorbed with GBS served as the complement source, and freshly isolated human neutrophils were used as the phagocytic cell source (21). To determine the percentage of bacterial survival, colony forming units (CFU) from experimental mixtures of bacteria, mAb-containing culture supernatant, complement, and neutrophils were compared with CFU from control mixtures lacking one or more of the components. For the control mixtures, a non-GBS-reactive mAb was used in place of the GBS mAb, heat-inactivated complement was used in place of active complement, and buffer was used in place of neutrophils. The data are reported as follows: 100 × 1− [(cfu remaining after incubation with PMN, complement, and test mAb)/(CFU remaining after incubation with PMN, complement, and negative mAb)].

*Protection Tests.* For all experiments, initial broth culture tubes were inoculated using overnight stationary phase cultures started from frozen maintained stock cultures. At logarithmic growth phase, the tubes were centrifuged at 22°C, 4,550 *g*, for 10 min, washed once with

25 ml broth, and resuspended in same to the appropriate bacterial density. For each experiment, dilutions of the bacterial source were plated on trypticase soy agar plates to quantitate the challenge dose, and on blood agar plates to confirm culture purity.

2–3-d-old outbred Sprague-Dawley (BK:SD) rat pups and their dams were purchased from Bantin and Kingman (Fremont, CA). Individual dams and their pups were housed in polycarbonate microisolator rat cages (Lab Products, Inc., Maywood, NJ), were given food and water ad libitum, and were exposed to a 12-h light/dark photoperiod.

For injections, a repeating Hamilton dispenser (10 μl/button depressor, ASP S9630-1) was loaded with a 0.5 ml Hamilton syringe fitted with a Leur tip (ASP S9660-55) attached to a Butterfly® pediatric infusion set (25 × 3/4-inch needle with 12-inch tubing, No. 4506; Pedline Surgicals, Seattle, WA). To visualize movement of the colorless reagent solutions in the tubing, an air gap followed by trypan blue dye progressed behind the bacteria or mAb.

A neonatal rat infection model was performed in the following ways: (*a*) To determine whether the mAbs were protective if administered before infection (prophylactic), neonatal rats received antibody 24 or 4 h before bacteria challenge. To avoid indirect mixing of antibody and bacteria, mAb was administered to one dorsal thigh (40 μl at 1 mg/ml), followed by intraperitoneal infection with 5 $LD_{50}$ (100–4,000 CFU) of bacteria (40 μl).

(*b*) The prophylactic efficacy of the mAbs was also tested against infections caused by in vivo passaged GBS. 18 h after intraperitoneal infection with GBS (1 $LD_{50}$), bacteria (passaged) were recovered from the cardiac blood of rat pups exhibiting lethargy and pallor. An aliquot of blood was mixed with an equal volume of 0.8% trypan blue, and the CFU/ml of blood were calculated after microscopic counting. The blood was diluted with Todd-Hewitt Broth and 40 μl containing 5 $LD_{50}$ (100–1,000 CFU) was injected intraperitoneally into pups who 24 h previously had received prophylactic mAb (see above).

(*c*) The therapeutic activity was assessed in pups receiving mAbs after GBS challenge. Antibodies were administered intraperitoneally 4 h after challenge with 5 $LD_{50}$ (80–500 CFU) of in vitro-grown GBS. At the time of mAb injection, a sampling of infected pups were septic (500–1,000 CFU/ml blood) with the infecting GBS strain.

In all experiments, the 40-μl dose of purified mAb contained less endotoxin (20 pg) than the sensitivity limit of the colorimetric assay (see Materials and Methods above). Because few negative mAb control rats survived, it is unlikely the observed protection was due to nonspecific macrophage activation, or other endotoxin mediated effects. Negative control mAbs were either *Pseudomonas aeruginosa*– (16) or *E. coli* K1– (11) specific human mAbs. Treated pups were examined twice daily for symptoms, and scored for survival.

*Statistical Analysis.* $LD_{50}$ values were calculated by the method of Reed and Muench (22), with 10 animals used for each bacterial concentration (data not shown). Significance of differences between mortality values in protection studies ($n$ = 10–12) was determined by Fisher's Exact Test of categorical data (23).

## Results

*Characterization of Group B Streptococcus Human mAbs.* Master well supernatants from 18 human B cell EBV transformations were screened by ELISA on microtiter wells coated with a pool of five GBS serotypes. Supernatants with binding activity were subsequently assayed on individual GBS serotypes to separate serotype-specific from cross-serotype reactions. From these transformations (>20,000 master wells), 104 master well supernatants reacted with all five GBS serotypes. In general, each supernatant reacted comparably on all serotypes (Table I).

mAb 4B9 (IgM) was derived from the peripheral blood B cells of a donor with cystic fibrosis, and antibody 3D2 (IgM) from tonsillar B cells. Neither donor had a known history of GBS infection. These mAbs were used in all experiments with virtually identical results. However, in some cases, only data with the most frequently used mAb are presented. The mAbs were further characterized by testing for cross-reactivity against other streptococcal groups. Both mAbs reacted with typable and

TABLE I

*ELISA-based Crossreactivity of Group B Streptococcus Human mAb*

| Strain | Serotypes | ELISA reactivity | |
|---|---|---|---|
| | | Positive* | Negative‡ |
| SS-615 | Ia/ – | 2.33§ | 0.14 |
| SS-618 | Ib/c | 2.29 | 0.14 |
| SS-700 | Ia/c | ≥3.0 | 0.15 |
| SS-619 | II/ – | 2.01 | 0.13 |
| SS-620 | III/c | 2.13 | 0.13 |
| | No bacteria | 0.35 | 0.12 |

* Anti-group B *streptococcal* human monoclonal antibody (IgM).
‡ Anti-*P aeruginosa* human monoclonal antibody (IgM).
§ ELISA value at $OD_{490}$.

nontypable GBS, and group G streptococci, but not other streptococcal groups. Cross-reactivity between the group B and group G streptococcal polysaccharides has been reported (24).

*Clinical Isolate Reactivity.* The potential for binding to a large number of clinical isolates was investigated using a collection of GBS clinical isolates representing all serotypes (Table II). The immunoblot nitrocellulose dot assay allowed the simultaneous testing of 132 clinical isolates using only 2 ml of mAb containing spent culture supernatant. 4B9 and 3D2 reacted with 132/132 of the clinical isolates. From these data, it would appear the mAbs recognize a GBS epitope conserved among clinical isolates obtained from different patient populations hospitalized in several U. S. cities.

*Biochemical Analysis Using XIEP Immunoblotting.* XIEP is useful for identifying individual interactions between heterogeneous crude antigen samples and polyvalent antisera. Complex cell wall digests, or purified antigen preparations, are first separated horizontally (first dimension), and then immunoprecipitated by the antisera in the second, vertically run agarose slab (second dimension). After Crowle staining,

TABLE II

*Group B Streptococcus Human mAb Reactivity with Human Clinical Isolates*

| Serotype | Total tested | | Seattle* | Other U.S.‡ |
|---|---|---|---|---|
| Ia | 0 | (0)§ | 0 | 0 |
| Ib | 26 | (20) | 24 | 2 |
| Ic | 25 | (19) | 21 | 4 |
| II | 25 | (19) | 16 | 9 |
| III | 51 | (39) | 41 | 10 |
| NT‖ | 3 | (2) | 3 | 0 |
| II/III¶ | 2 | (1.5) | 1 | 1 |
| Total | 132 | | 106 | 26 |

* Isolates obtained from Seattle area hospitals.
‡ Isolates obtained from all other United States Hospitals.
§ Value in parentheses are the percentage of isolates represented by each serotype.
‖ Nontypable for type-specific capsule.
¶ Reactive with both type II and type III capsule typing reagents.

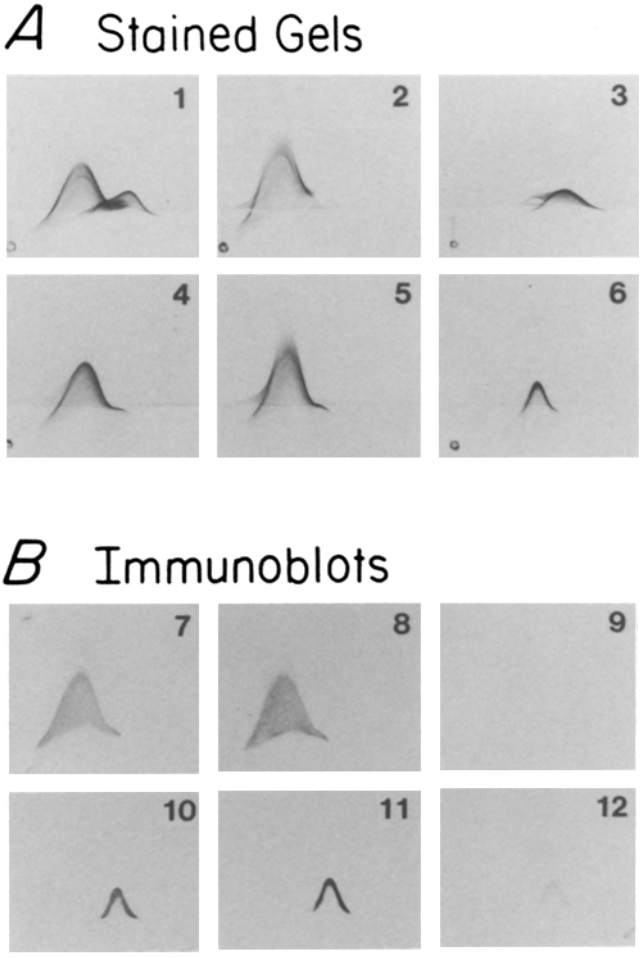


FIGURE 1. XIEP gels and immunoblots. (*A*) Stained XIEP gels of cell wall digests. Antigens run in the first dimension were cell wall digest of the type III clinical isolate COH 31r/s (panels *1, 2,* and *3*) or the capsule-deficient isogenic mutant COH 31-15 (*4* and *5*), or purified group B antigen (*6*). The second dimension gels contained the following antisera: anti-group B polysaccharide and anti-type specific (*1* and *4*), anti-group B polysaccharide only (*2, 5, 6*), or anti-type specific only (*3*). (*B*) Immunoblots of XIEP gels. Panels *7, 8,* and *9* contained digests of strain COH 31-15 and both monospecific sera. (*10, 11,* and *12*) Purified group B antigen and monospecific anti-group B sera. Vertical panel pairs (*7* and *10, 8* and *11*) were immunoblotted with the GBS mAbs 4B9 or 3D2, and (*9* and *12*) with negative control mAb (6F11).

the number and the localization of precipitant arcs help characterize antibodies and antigens. In addition, mAb-containing culture supernatants can be used to immunoblot antigens precipitated with antisera. If purified antigens are available as standards, a combination of methods can, at least indirectly, identify the antigen recognized by a mAb.

The relative locations of immunoprecipitated group B polysaccharide and type-specific capsule were determined by staining gels containing monospecific antisera. Purified group B polysaccharide, immunoprecipitated with monospecific group B antisera, was identified as a slower migrating molecule (Fig. 1 *A*, panel *6*). The clinical isolate COH 31r/s and its isogenic capsule-deficient mutant, COH 31-15 (3), provided GBS antigen sources differing only by their capsule expression. The purified group B carbohydrate arc corresponded to the reaction occurring when capsule-deficient COH 31-15 digests were precipitated in the same group B antisera (Fig. 1 *A*, panel *5*). Compared with the group B peak, a more anodal precipitate was observed between digests of the encapsulated COH 31r/s strain and monospecific type

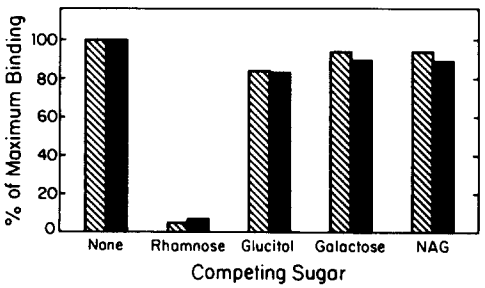


FIGURE 2. Monosaccharide competition assays to determine the ability of different monosaccharides to block GBS-mAb binding. For each bar, the percentage maximum binding (ordinate) was obtained using the formula: 100 × 1 − [(ELISA value in absence of competing monosaccharide)/(ELISA value in presence of test competing monosaccharide)]. Type Ia (strain H227, *hatched bar*) and type III (strain I334, *solid bar*) clinical isolates were used to assay binding activity after incubating the mAb with the test monosaccharides.

III capsule antisera (Fig. 1 *A*, panel *3*). Other reference mAb and antigen combinations ensured there was no interference when several reactions were possible (Fig. 1 *A*, panels *1* and *2*). The relative positions of the two principal GBS surface antigens served as references for the identification of antigens reacting with immunoblotted mAbs.

The 4B9 and 3D2 mAbs were immunoblotted against precipitated immune complexes passively transferred to nitrocellulose paper. Both mAbs reacted with an antigen in a similar location as antigen detected by the group B polysaccharide monospecific antisera and digests of strain COH 31-15 (compare Fig. 1 *A*, panel *5* with Fig. 1 *B*, panels *10* and *11*), or purified group B polysaccharide (compare Fig. 1 *A*, panel *6* with Fig. 1 *B*, panel *7* and *8*). The negative control mAb, 6F11 (anti-*P. aeruginosa*), did not react with the purified antigen, but did display a slight nonspecific reaction against the cell wall digest (Fig. 1 *B*, panels *9* and *12*, respectively). When blotted against cell wall digests from the other capsule serotypes (Ia, Ib, and II), the mAbs identified a similarly migrating molecule (not shown). These observations provide strong, albeit indirect, evidence that the GBS mAbs react with a highly conserved group B polysaccharide epitope.

*Monosaccharide Competition of Group B Streptococcus mAbs.* Monosaccharide competition assays with the mAbs further characterized their GBS target. On GBS, only the group B polysaccharide possesses α-L-rhamnose as a major structural component. Group B polysaccharide antisera, mixed with α-L-rhamnose, is effectively blocked from binding to bacteria-associated antigen (7). Purified mAbs were used at 0.1 μg/ml, an antibody concentration typically representing 50% saturation against intact bacterial ELISAs. After combining with 20 mg/ml of rhamnose, glucitol, galactose, or *N*-acetylglucosamine, the mAb and monosaccharide mixtures were tested by ELISA for reactivity against intact GBS bacteria (Fig. 2). Only rhamnose (95% inhibition) significantly interfered with mAb reactivity against type Ia or type III isolates. These data, and those obtained by XIEP, provide strong evidence that mAbs 4B9 and 3D2 recognize an epitope on the group B polysaccharide.

*Opsonization by the Group B Streptococcus mAbs.* The functional activity of the mAbs was tested against viable GBS in an opsonophagocytic assay. Combinations of GBS mAbs, a human serum complement source, and human neutrophils were used against clinical isolates and typing strains representing the five GBS serotypes. Both mAbs effectively (80–97% reduction in CFU) mediated the opsonization and destruction of bacterial strains from each serotype (Fig. 3, data shown only for mAb 4B9). In other experiments using additional type Ia, Ib, and III strains (data not shown) the

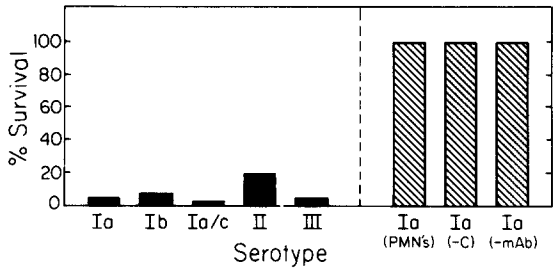


FIG. 3. Opsonophagocytosis assays using the GBS mAbs and five GBS serotypes. The strains used had the following designations: Ia (H227), Ib (SS-618), Ia/c (I546), II (F180), and III (I334). For each bar, the percentage survival (ordinate) was obtained using the formula: 100 × [(CFU remaining after incubation with PMN, complement, and test mAb)/(CFU remaining after incubation with PMN, complement, and negative mAb)].

Three control conditions for serotype Ia are represented by the hatched bars labeled Ia (*-PMN's*), Ia (*-C*), and Ia (*-mAb*). These control mixtures each lacked one active component: (*-PMN's*) buffer replacing neutrophils; (*-C*) heat-inactivated complement; and (*-mAb*) negative control human mAb replacing GBS mAb.

mAb consistently enhanced opsonization (80–95% reduction in CFU). In each control condition, a different active reagent was omitted. Substituting active components with a negative control human mAb, heat-inactivated human complement, or PBS for neutrophils, each resulted in complete removal of opsonic activity. Therefore, effective opsonic activity required both a GBS mAb and active complement. The IgM class mAbs were not anticipated to be opsonic alone. Further, mAbs and complement without neutrophils were ineffective in mediating direct bacteriolysis. These assays clearly show the group B polysaccharide mAbs facilitate complement-dependent opsonization of strains representing each GBS serotype.

*Neonatal Rat Protection Studies.* The ability of the mAbs to protect if given before (prophylactic) or after (therapeutic) infection was examined in a neonatal rat model. Attempts to avoid potential artefacts (e.g., reduced capsule production resulting from in vitro growth) necessitated using different variations of a standard rat model. mAb was administered either (*a*) before infection with in vitro grown bacteria, (*b*) before infection with in vivo passaged bacteria, or (*c*) after infection with in vitro grown bacteria. In these experiments, rat pups were infected intraperitoneally with 5 $LD_{50}$ of each GBS strain and received 40 μg of purified mAb, either subcutaneously or intraperitoneally. Litter-to-litter variation was minimized by dividing pups from individual litters (four to six litters/experiment) so all treatment groups were represented. This protocol provided internally controlled litters and larger experimental groups when the data from several litters were pooled. Each protection experiment (Fig. 4) represents the percentage survival from four to six identically treated litters.

The prophylactic activity of the mAbs was tested in pups receiving mAbs 24 h before infection with in vitro grown GBS. 40 μl of GBS or negative control mAb (see Materials and Methods) were injected subcutaneously 24 h before challenge with either a type III (1,000 CFU) or a type Ia (500 CFU) clinical isolate (Fig. 4, *A* and *B*). Against both isolates, only the GBS mAb protected 100% ($p < 0.001$) of the type Ia, and 90% ($p < 0.001$) of the type III-infected pups. In similar experiments using other type Ia and III clinical isolates, 90–100% protection was consistently observed (not shown). These data suggest that the group B polysaccharide

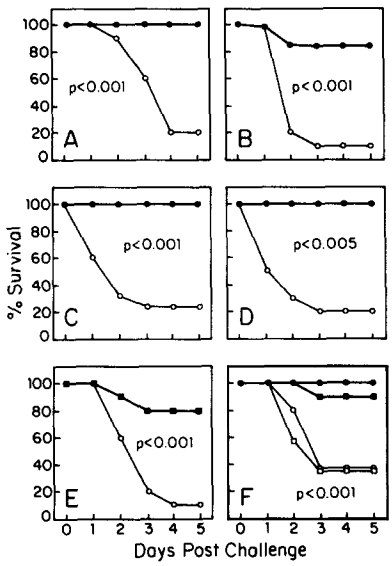


FIGURE 4. Neonatal rat protection trials using the GBS mAbs against type Ia (strain H227) and type III (strain I334) clinical isolates. The human mAbs 3D2 (*closed circle*) and 6F11 (*open circle*) were used in three protection models in which neonatal rats were infected with type Ia or type III GBS bacteria. Results from the three models are shown as follows: prophylactic (*A*) type Ia and (*B*) type III; in vivo-passed bacteria (*C*) type Ia and (*D*) type III; and therapeutic (*E*) type Ia and (*F*) type III. Also shown in *F* is a prophylactic experiment with mAb administered 4 h before infection, 3D2 (*closed square*) and 6F11 (*open square*).

mAbs provide prophylactic protection against infections caused by several in vitro grown GBS strains.

Because in vitro growth may affect their sensitivity to the GBS mAbs, the clinical isolates used above were first passaged through neonatal rats, and without subsequent in vitro growth, were used to infect rats. This approach was also intended to provide the opportunity for enhanced in vivo dependent capsule production that might decrease the accessibility of the group B polysaccharide to antibody. Therefore, to be protective, the GBS mAbs must opsonize bacteria demonstrating in vivo virulence. In fact, the $LD_{50}$ of in vivo-passaged bacteria were reduced by 50%, compared with the same strains grown in vitro. Blood from rats exhibiting lethargy and pallor 18 h after a 1 $LD_{50}$ infection, was used to infect pups that had previously received mAb (24 h before). After microscopically counting the GBS, blood samples were diluted to the appropriate bacteria concentration (CFU) and without additional processing, injected into several litters of rats pups. Comparable to the results using in vitro grown bacteria, the GBS mAbs (40 μg) were protective against infections caused by in vivo passed GBS (Fig. *4*, *C* and *D*). Complete protection was observed ($p < 0.001$) among rats that received the GBS mAbs, and were infected with either a type Ia (500 CFU) or a type III (300 CFU) strain. These data demonstrate that bacteria surviving short-term in vivo growth remain sensitive to the GBS mAbs.

The most stringent challenge for a protective mAb is the ability to protect or cure septic animals. In contrast to the prophylactic model, a therapeutic mAb should aid in clearing bacteria that have most likely disseminated into several organs. To test their therapeutic efficacy the mAbs were administered 4 h after infection with 100 or 200 CFU (5 $LD_{50}$). Cardiac blood samples drawn at the time of mAb injection (900 CFU/ml of blood for the type Ia and type III strains) confirmed the rats were bacteremic. In these experiments (Fig. 4, *E* and *F*), mAb administered 4 h after infection protected 80% ($p < 0.001$) of the type Ia and 100% ($p < 0.001$) of

the type III-infected rats. Negative control mAb-treated animals were not significantly protected (10% and 20%, respectively). Although 40 μg of each mAb were used in these experiments, mAb titration experiments usually showed 5 μg of mAb were able to protect 80% of the infected pups (not shown). These protection experiments provide convincing evidence that GBS anti-group B polysaccharide mAbs possess therapeutic protective activity against two GBS capsule types. In addition, these mAbs have provided protection against other GBS strains (Dr. Harry Hill, personal communication), strongly suggesting the mAbs have protective activity against a broad distribution of clinical strains.

## Discussion

This report describes the first example of a group B polysaccharide mAb protective against infections caused by multiple GBS serotypes. The antibody-producing cell lines were developed by EBV transformation of human B cells obtained from donors without histories of previous GBS infections. The mAbs recognized 132/132 of the clinical isolates screened, indicating the epitope is conserved on GBS infecting different patient types hospitalized in several geographical regions.

The antigen recognized by the cross-protective mAbs was shown to be the group B polysaccharide using XIEP with immunoblotting, and monosaccharide competition binding assays. Encapsulated (COH 31r/s) and unencapsulated (COH 31-15) isogenic GBS strains (3), and a purified group B polysaccharide preparation provided reference standards helpful in localizing capsule and group B polysaccharide XIEP patterns. Immunoblots against strain COH 31-15 cell wall digests revealed the GBS mAbs bound to molecules migrating to the same region as the group B polysaccharide. These immunoblot patterns also corresponded to patterns obtained using purified group B polysaccharide. These data strongly suggest the GBS mAbs recognize a conserved epitope on the group B polysaccharide, which appears to be expressed independently of capsule production (strain COH 31-15).

Although there have been other attempts (6, 8), this is the first report showing a GBS antibody is able to opsonize GBS strains with different capsule types. Using human mAbs with human serum (complement) and neutrophils, the GBS mAbs opsonized GBS strains representing the five serotypes. To mediate bacteriocidal or opsonic activity, antibody must interact with antigen (group B polysaccharide) in a fashion leading to complement binding and activation. Even though the GBS mAbs and complement were not bacteriocidal, the accessibility of the group B polysaccharide to antibody must still be critical if complement is to be activated leading to phagocytosis. Proving these assumptions will require additional studies exploring the physical relationship of the group B polysaccharide with capsule polysaccharide.

The bacterial strains used in all neonatal rat protection trials were isolated from the blood or cerebrospinal fluid of infected human neonates. Isolates of each capsule type were compared for their $LD_{50}$ in neonatal rats. Strains with the lowest $LD_{50}$, generally 10-100 CFU were specifically selected for in vivo trials. In total, prophylactically treated rats were challenged with two strains of each of the Ia, Ic, and III capsule types. Several type II isolates were screened; however, none had $LD_{50}$ below our 10,000 CFU exclusion limit. Type III encapsulated strains, the most frequent cause of GBS meningitis in the United States, were a primary target for therapeutic

trials. However, because a different serotype distribution occurs in other countries, animal studies included a type Ia strain (25).

The neonatal rat model was selected for in vivo studies because some aspects of the neonatal rat's early immunological development apparently resemble that of human neonates (26, 27). In this model, the GBS mAbs were clearly able to cross-protect against infections caused by clinical isolates expressing different capsule types. Protection against in vivo-passed bacteria suggests the mAbs are able to reach their target even if bacteria are grown under in vivo selective pressures. Moreover, the mAbs were therapeutically protective when administered 4 h after infection, at a time when GBS bacteria could be isolated from blood samples.

The antigen specificity and functional properties of mAbs typically reflect activities present in polyclonal antisera. However, immune human sera or heterologous antisera have not been described as possessing cross-protective activity against infections caused by GBS with different capsule types. Typically, GBS antisera raised by immunization with one serotype are protective only against infections caused by GBS with homologous capsule types (2, 5, 10, 28). Thus it is reasonable to assume that either cross-protective group B antibodies are not generated by active immunization, or if present, their activity is below detectable levels. In humans, because mAbs have been produced, it is more likely the relative concentration of functional cross-protective antibody is below the sensitivity limits of standard assays. Unfortunately, the majority of studies using human sera possessing anti-GBS binding activities did not specifically address the potential for protective group polysaccharide antibodies (6, 29, 30). Our laboratory also has not assayed human sera for functional anti-group B activity, particularly due to the difficulty preparing affinity matrices that selectively remove type-specific, but not group-specific antibodies. In the absence of studies using anti-capsule and anti-group B polysaccharide serum derived antibodies, it should not be concluded that sera is poorly representative of protective group B polysaccharide antibodies. However, if sera do not generally possess anti-group B polysaccharide protective activity, it is surprising that transformable anti-group B polysaccharide B cells are so prevalent. These data suggest human mAb technology may provide a means to identify nonimmunodominant epitopes eliciting therapeutic antibodies during exposure to bacterial pathogens.

EBV cell-driven transformation of human B cells was previously used in our laboratory to generate K1 capsule-specific *E. coli* mAbs (11). Identifying K1 mAbs was unexpected based on the very low *E. coli* K1 serum antibody levels typically found in adults (31). These observations suggest human mAb technology has the potential for generating mAbs with activities not apparent in sera. The GBS and *E. coli* K1 (*Neisseria meningitidis* group B, reference 11) human mAbs provide the working basis for a human mAb product formulation.

## Summary

Group B streptococcal (GBS) infections cause significant mortality and morbidity among infants. Passive antibody immunotherapy has been proposed as treatment for infected infants. To this end, two human mAb-secreting cell lines were produced by EBV immortalization of human B cells. The mAbs were specific for the group B polysaccharide and bound to strains of all five serotypes as demonstrated by ELISA

and crossed immunoelectrophoresis. The mAbs reacted and opsonized 100% (132/132) of the clinical isolates tested which represented all four capsule types. Both prophylactic and therapeutic protection with these mAbs were demonstrated in neonatal rats given lethal infections of types Ia and III human clinical isolates. These data indicate that a single human mAb directed against the group B carbohydrate can protect against GBS infections caused by the different serotypes. This antibody may be useful in the passive immunotherapy of infants infected with GBS.

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