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(57) Abrégé/Abstract:
This invention relates to antigenic conjugate molecules comprising the capsular polysaccharide of Group B streptococcus type II which are covalently linked to protein. This invention further relates to antigenic conjugate molecules comprising the capsular polysaccharide of Group B streptococcus type V which are covalently linked to protein. This invention also relates to vaccines and methods of immunizing mammals, including humans against infection by Group B streptococcus type II (GBS II) and/or Group B streptococcus type V (GBS V). Multivalent vaccines comprising the conjugate molecules of this invention and antigens to other pathogenic bacteria are also claimed.

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(54) Title: GROUP B STREPTOCOCCUS TYPE II AND TYPE V POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

(57) Abstract

This invention relates to antigenic conjugate molecules comprising the capsular polysaccharide of Group B streptococcus type II which are covalently linked to protein. This invention further relates to antigenic conjugate molecules comprising the capsular polysaccharide of Group B streptococcus type V which are covalently linked to protein. This invention also relates to vaccines and methods of immunizing mammals, including humans against infection by Group B streptococcus type II (GBS II) and/or Group B streptococcus type V (GBS V). Multivalent vaccines comprising the conjugate molecules of this invention and antigens to other pathogenic bacteria are also claimed.

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GROUP B STREPTOCOCCUS TYPE II AND TYPE V POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

FIELD OF THE INVENTION

This invention relates to antigenic conjugate molecules comprising the capsular polysaccharide of Group B streptococcus type II or V which are covalently linked to protein. This invention also relates to vaccines and methods of immunizing mammals, including humans against infection by Group B streptococcus type II (GBS II) and/or type V (GBS V). Multivalent vaccines comprising the conjugate molecules of this invention and antigens to other pathogenic bacterial are also claimed.

BACKGROUND OF THE INVENTION

Infections due to group B streptococci (GBS) are the most common single cause of sepsis and meningitis in newborns in developed countries (3, 31). Recent reports from some centers in the United States reflect a lower mortality (9 to 13%) than in series from the 1970s, perhaps as a result of earlier diagnosis and intensive care (1, 10). Nonetheless, fatal infections still occur, and equally important, up to 50% of survivors of GBS meningitis have chronic neurologic injury ranging from deafness and mild learning disabilities to profound motor, sensory, and cognitive impairment (3). Prevention rather

than improved diagnosis or therapy is likely to have the most significant impact in further reducing GBS-related morbidity and mortality.

Because GBS capsular polysaccharide-specific antibodies appear to protect both experimental animals (23, 24) and human infants (4, 5) from GBS infection, some of the polysaccharides have been purified and tested in healthy adults as experimental vaccines (6). Were a safe and efficacious GBS vaccine available, it could be administered to women before or during pregnancy to elicit antibodies that would transfer to the fetus in utero and provide protection against neonatal infection. Of the three GBS polysaccharides (types Ia, II, and III) tested in volunteers, type II had the highest rate of immunogenicity, eliciting a type II-specific antibody response in 88% of previously nonimmune recipients (6). Although a variety of GBS serotypes have previously been recognized as contributing significantly to the percent of patients with GBS infection, type V has not been considered to be responsible for a significant percent of GBS infection. In neonates, the level of specific antibody required for protection against type II GBS infection is not precisely defined but has been estimated at 2 or 3 μg/ml (6). In a vaccine recipient who achieves an antibody response only slightly above the minimum required for protection, the amount of maternal antibody transferred across the placenta may be inadequate to protect a premature infant, because of the incomplete transfer to the fetus of material immunoglobulin G (IgG), or an infant with late-onset infection, since the half-life of maternal IgG antibodies in the newborn is about 25 days (13). Many of the infants in these two groups of patients might be protected if the magnitude of the specific antibody response to vaccination were higher. The transfer of maternal IgG to the fetus increases

throughout the third trimester, so a higher maternal antibody level would provide protection earlier, i.e., to a more premature infant (16). Similarly, higher maternal levels would result in longer persistence of maternal antibodies in the infant, thereby providing protection against late-onset disease, as well.

The immunogenicity of several bacterial polysaccharide antigens has been increased by the attachment of suitable carrier proteins to polysaccharides or to derivative oligosaccharides (2, 14, 17-19, 22, 27, 29, 30, 34). Desirable properties of polysaccharide-protein conjugate vaccines include enhanced immunogenicity of the polysaccharide, augmented hapten-specific antibody response to booster doses, and a predominance of IgG class antibodies. Recently, we have been successful in developing a GBS III-Tetanus Toxoid (TT) vaccine by using the side chain sialic acid moieties as sites of directed protein coupling (33). The III-TT vaccine elicited GBS type III-specific, opsonically active antibodies, while the unconjugated type III polysaccharide was nonimmunogenic in rabbits (33).

SUMMARY OF THE INVENTION

This invention provides antigenic conjugate molecules comprising the capsular polysaccharide derived from Group B streptococcus type II and a protein component, or Group B streptococcus type V and a protein component, wherein two or more side chains terminating in sialic acid residues of the type II or type V polysaccharide component are each linked trough a secondary amine bond to the protein and the polysaccharide has a molecular weight of at least 5,000 daltons.

Also provided is a method of preparing the conjugate molecules of a capsular polysaccharide of Group B streptococcus type II or type V and a protein comprising:

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(a) subjecting the Group B streptococcus type II or type V capsular polysaccharide to periodate oxidation sufficient to introduce an aldehyde group into two or more terminal sialic acid residues linked to the backbone of the polysaccharide;

(b) coupling the oxidized Group B streptococcus type II or type V capsular polysaccharide to a protein through reductive amination to generate a secondary amine bond between the capsular polysaccharide and the protein.

Conjugate molecules prepared according to the method described above are also provided.

This invention further provides vaccines comprised of each of the conjugate molecules described above. In addition, this invention provides multivalent vaccines comprising the conjugate molecules of the invention and at least one other immunogenic molecule capable of eliciting the production of antibodies to a pathogenic substance other than Group B streptococcus type II or type V. In particular, in addition to comprising the GBS type II and/or GBS type V conjugate molecules,the multivalent vaccine, according to the invention, further comprises other immunogenic molecules capable of eliciting the production of antibodies to pathogens selected from the group consisting of Group B streptococcus types Ia, Ib, III, IV and Haemophilus influenzae type b and E. coli type K1.

In another embodiment of this invention, a method of immunizing neonates is provided wherein the vaccine comprising the conjugate molecules of the invention is administered in an immunogenic amount to a female human so as to produce antibodies capable of passing into a fetus conceived prior to or after administration of the vaccine in an amount sufficient to

produce protection against infection in the neonate at birth.

A method of immunizing adults wherein a vaccine comprising the conjugate molecules of the invention is administered in an immunogenic amount to a human adult is also provided. In addition, a method of immunizing adults wherein a vaccine comprising the conjugate molecules of the invention is administered in an immunogenic amount to a person at risk for being infected by Group B streptococcus type II or type V is provided.

In another embodiment of this invention, a vaccine comprising the conjugate molecules of the invention is administered in an immunogenic amount to volunteer vaccine recipients who could donate serum or plasma which could be passively administered to the above groups. This invention also provides a method of immunizing persons including neonates, children and adults at risk for being infected by Group B streptococcus type II or type V. Those at risk may include persons whose immune system is suppressed for a variety of reasons including but not limited to cancer or diabetes.

Another embodiment of this invention is a method of immunizing dairy herds against bovine mastitis consisting of administering a vaccine comprising the conjugate molecules of the invention in an immunogenic amount to dairy cows.

According to one aspect of the present invention, there is provided an antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type II bacteria or Group B streptococcus type V bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

According to another aspect of the present invention, there is provided an antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type II bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

According to still another aspect of the present invention, there is provided a method of preparing a conjugate molecule of a capsular polysaccharide of Group B streptococcus type II bacteria and a protein, which method comprises: (a) subjecting the capsular polysaccharide to periodate oxidation sufficient to introduce an aldehyde group into two or more terminal sialic acid residues linked to a backbone of the polysaccharide; and (b) coupling the oxidized capsular polysaccharide to the protein through reductive amination to generate a secondary amine bond between the capsular polysaccharide and the protein.

According to a further aspect of the present invention, there is provided a vaccine comprising the conjugate molecule as described above or as prepared by the

method as described above, and a pharmaceutically acceptable carrier.

According to yet a further aspect of the present invention, there is provided a multivalent vaccine comprising: the conjugate molecule as described above or as prepared by the method as described above, and at least one other immunogenic molecule capable of eliciting the production of antibodies to a pathogenic substance other than Group B streptococcus type II, wherein the other immunogenic molecule is capable of eliciting the production of antibodies to a pathogen selected from the group consisting of Group B streptococcus types Ia, Ib, III, IV and V; Haemophilus influenzae type b; and E. coli type K1.

According to still a further aspect of the present invention, there is provided an antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type V bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

According to another aspect of the present invention, there is provided a method of preparing a conjugate molecule of a capsular polysaccharide of Group B streptococcus type V bacteria and a protein, which method comprises: (a) subjecting the capsular polysaccharide to periodate oxidation sufficient to introduce an aldehyde group into two or more terminal sialic acid residues linked to a backbone of the polysaccharide; and (b) coupling the oxidized capsular polysaccharide to the protein through reductive amination to generate a secondary amine bond between the capsular polysaccharide and the protein.

According to yet another aspect of the present invention, there is provided a conjugate molecule prepared according to the method as described above.

According to yet another aspect of the present invention, there is provided a multivalent vaccine comprising: the conjugate molecule as described above or as prepared by the method as described above, and at least one other immunogenic molecule capable of eliciting the production of antibodies to a pathogenic substance other than Group B streptococcus type V, wherein the other immunogenic molecule is capable of eliciting the production of antibodies to a pathogen selected from the group consisting of Group B streptococcus types Ia, Ib, II, III and IV; Haemophilus influenzae type b; and E. coli type K1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Structure of the heptasaccharide repeating unit of type II GBS capsular polysaccharide (20).

FIGURE 2. GBS type II polysaccharide competitive ELISA. GBS type Ia (O), type II (●), and type III (▲) polysaccharides were used as inhibitors of II-TT vaccine antibody binding to plates coated with type II

polysaccharide. Results are expressed as percentages of inhibition relative to that of control wells that lacked inhibitor.

FIGURE 3. GBS type II polysaccharide competitive ELISA. Native (●) and desialylated or core (□) type II polysaccharides were used as inhibitors of antibodies elicited by II-TT vaccine. Datum points are means (with standard deviations) of triplicate determinations. Results are expressed as percentage of inhibition relative to that of control wells that lacked inhibitor.

FIGURE 4. Structure of the repeating unit of type V GBS capsular polysaccharide.

FIGURE 5. GBS type V polysaccharide competitive ELISA. GBS type Ia (-▲-), type Ib (-▲-), type II (- -), type III (-0-), type V (-■-), and type VI (-□-) polysaccharides were used as inhibitors of antibodies elicited by V-TT vaccine. Datum points are means of duplicate determinations. Results are expressed as percentage of inhibition relative to that of control wells that lacked inhibitor.

FIGURE 6. In vitro opsonophagocytic killing by human peripheral blood lymphocytes of GBS type V strain CJB 111 opsonized by rabbit antisera raised to GBS type V-TT conjugate vaccine. C′ is complement.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to antigenic conjugate molecules comprising a capsular polysaccharide component and a protein component.

Bacterial strains. GBS type II strain 18RS21 and type Ia strain 090 were originally obtained from the late Rebecca Lancefield of Rockefeller University and were maintained as frozen cultures at -80°C. Strain 18RS21 was used in in vitro and in vivo assays and was the source of

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type II capsular polysaccharide used in the conjugate vaccine. Two GBS type II clinical isolates (strains S16 and S20) and type III strain M781 were obtained from the Channing Laboratory's culture collection.

Although previously not recognized as significantly contributing to the number of GBS infections in humans, recent evidence now indicates that the type V serotype contributes to about 15% of GBS infections. To prepare a GBS-type V conjugate vaccine, GBS type V strain 1169-NT1 was obtained from Dr. J. Jelinkova of The Institute of Hygiene And Epidemology, Prague, Czech Republic and was maintained as frozen cultures at -80°C. Strain 1169-NT1 was used in in vitro and in vivo assays and was the source or type V capsular polysaccharide used in the conjugate vaccine. GBS type V strain CJB 111 was originally obtained from Dr. Carol Baker of Baylor University.

Conjugation of GBS type II polysaccharide or type V polysaccharide to TT. Type II capsular polysaccharide was purified from strain 18RS21 cells by methods described previously for purification of type III polysaccharide (33). The conjugation of type II polysaccharide to monomeric TT was performed by using techniques detailed previously for the conjugation of TT to GBS type III polysaccharide (33). In brief, native type II polysaccharide was size fractionated on a Sepharose* CL-6B column (1.6 by 85 cm; Pharmacia Fine Chemicals). The material eluting at the center of the major peak was pooled, dialyzed against water,and lyophilized to yield material with a relative molecular weight of 200,000. Analysis of this material by ^{1}H-nuclear magnetic resonance spectroscopy at 500 MHz confirmed the native type II polysaccharide structure (20) and the absence of group B antigen (26). Polysaccharides suitable for use in the conjugate molecules of this

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invention may vary in molecular weight over a broad range. A preferred molecular weight range for the polysaccharide component is between about 5,000 and 1,000,000 daltons. A more preferred range is between 50,000 and 500,000, and a still more preferred range is between 100,000 and 300,000. Within the range of 100,000 to 300,000 daltons, polysaccharides having molecular weight of about 200,000 daltons are preferred.

The size-fractionated type II polysaccharide was subjected to mild oxidation with sodium *meta*-periodate (18). This procedure resulted in the conversion of a portion of the sialic acid residues on the polysaccharide to the eight-carbon analog of sialic acid, 5-acetamido-3,5-dideoxy-D-galactosyloctulosonic acid (33). The percentage of sialic acid residues oxidized was estimated by gas chromatography-mass spectroscopy of the sialic acid residues and their oxidized derivatives as described previously (33). Preferably between about 5 and 50% of the sialic acid residues of each GBS type II polysaccharide are modified and available to be linked to protein. Most preferably, between about 5 and 25% of the sialic acid residues of the GBS type II polysaccharide are modified and available for linkage to protein. Modification of between about 5 and 10% of the GBS type II polysaccharides is most preferred.

Oxidation of the GBS type V polysaccharide to achieve between about 5 and 80% modification of the terminal sialic acid residues to form reactive aldehydes is preferred. Modification of between about 10 to 50% of the terminal sialic acid residues is more preferred.

The protein component of the conjugate molecules of the invention may be any physiologically tolerated protein. Among the preferred proteins are tetanus toxoid, diphtheria toxoid and cross reactive materials (CRA) such as CRM_{197}.

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The oxidized type II polysaccharide was linked to monomeric tetanus toxoid ("TT") (Institute Armand Frappier, Montreal, Canada) by reductive amination as described previously (33). The TT was purified to its monomer from by gel filtration chromatography also as described previously (33). In brief, 10 mg of the periodate-treated type II polysaccharide and 10 mg of purified TT were dissolved in 0.6 ml of 0.1 M sodium bicarbonate (pH 8.1). Sodium cyanoborohydride (20 mg) was added to the mixture and incubated at 37°C for 5 days. The progress of the conjugation was monitored by fast protein liquid chromatography (FPLC) of small aliquots of the reaction mixture analyzed on a Superose* 6 (Pharmacia) gel filtration column. The reaction was terminated when the height of the peak eluting at the void volume of the column (representing the high-molecular-weight conjugate) remained constant. The conjugate was purified by chromatography on a column of Biogel* A, 0.5 M (Bio-Rad Laboratories, Richmond, Calif.) as described previously (33). The protein content of the vaccine was estimated by the method of Lowry et al. (25), with bovine serum albumin as a standard. The carbohydrate content was assessed by the method of Dubois et al. (11), with purified type II polysaccharide as a standard.

Type V capsular polysaccharide was purified from GBS type V strain 1169-NT1 cells by the methods described previously for the purification of type III polysaccharide (33). The conjugation of type V polysaccharide to monomeric TT was performed using techniques detailed previously for the conjugation of TT to GBS type III polysaccharide (33).

Native type V polysaccharide had a relative molecular weight of 200,000 Daltons. Analysis of this material by H-nuclear magnetic resonance spectroscopy at

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500 MHZ confirmed the native type V structure (2) and the absence of group B antigen (26).

To introduce reactive aldehyde groups at the terminal sialic acid residues of the type V polysaccharide for linking to protein, type V polysaccharide was subjected to mild oxidation with sodium meta-periodate (18). This procedure resulted in the conversion of a portion of the sialic acid residues on the polysaccharide to the eight-carbon analog of sialic acid, 5-acetimido - 3,5-dideoxy-D-galactosyloctulosonic acid (33). The percentage of sialic acid residues oxidized was estimated by gas chromatography - mass spectroscopy of the sialic acid residues and their oxidized derivatives.

The oxidized type V polysaccharide was linked to monomeric TT (Institute Armand Frappier, Montreal, Canada) by reductive amination as described previously (33). The TT was purified to its monomer from by gel filtration chromatography also as described previously (33). In brief, 8.6 mg of the periodate-treated type V polysaccharide and 8.7 mg of purified TT were dissolved in 0.5 ml of 0.1 M sodium bicarbonate (pH 8.2). Sodium cyanoborohydride (31.5 mg) was added to the mixture and incubated at 37°C for 10 days. The progress of the conjugation was monitored by fast protein liquid chromatography (FPLC) of small aliquots of the reaction mixture analyzed on a Superose* 6 (Pharmacia) gel filtration column. The reaction was terminated when the height of the peak eluting at the void volume of the column (representing the high-molecular-weight conjugate) remained constant. The conjugate was purified by chromatography on a 2.6 x 91 cm column of Sephacryl* S-300 HR (Pharmacia) using 0.01M phosphate, 0.15M NaCl, pH7 plus 0.01% thimerosal as the running buffer. The protein content of the vaccine was estimated by the method of Lowry et al. (25), with bovine serum albumin as a

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standard. The carbohydrate content was assessed by the method of Dubois et al. (11), with galactose as a standard.

Vaccination of rabbits with II-TT vaccine and V-TT vaccine. Groups of three New Zealand White female rabbits (Millbrook Farms, Amherst, Mass.), each rabbit weighing approximately 3 kg, were vaccinated subcutaneously at four sites on the back with 50 μg of either uncoupled native type II polysaccharide or II-TT vaccine, each emulsified with complete Freund's adjuvant in a total volume of 2 ml. These animals received booster injections (50 μg) of vaccine prepared with incomplete Freund's adjuvant on days 20 and 41. Serum was obtained from each animal on days 0, 20, 34, 41, 55, and 70; filtered sterile; and stored at -80°C.

A New Zealand white female rabbit (Millbrook Farms, Amherst, Mass.) weighing approximately 3 kg was vaccinated subcutaneously at four sites on the back with 50 μg of GBS type V-TT conjugate vaccine mixed with alum in a total volume of 2 ml. This animal received booster injections (50 μg) of vaccine prepared with alum on days 22 and 42. Serum was obtained from each animal on days 0, 35, 56 and 91; filtered sterilized and stored at -80°C.

ELISAs. GBS type II-specific rabbit antibodies were quantified by enzyme-linked immunosorbent assay (ELISA) with goat anti-rabbit IgG conjugated to alkaline phosphatase (Tago Inc. Burlingham, Calif.) at 1/3,000 dilution. Microtiter plates (Immulon* 2; Dynatech Laboratories, Inc., Chantillty, Va.) were coated with 100 ng of purified type II polysaccharide linked to poly-L-lysine per well as described before (15, 33). Antibody titers were recorded as the reciprocal dilution that resulted in an A_{405} of ≥0.3 when wells containing the reference serum (rabbit antiserum raised to whole BGS 18RS21 cells) at a dilution of 1/800 reached an A_{405} of

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0.5. The amount of antibody specific for the protein portion of the conjugate vaccine was estimated by ELISA by using plates coated with monomeric TT (100 ng per well). TT-specific IgG titers were recorded as the reciprocal dilution that resulted in an A_{405} of ≥0.3 35 min after addition of the substrate, *p*-nitrophenyl phosphate (Sigma* 104 phosphatase substrate tablets; Sigma Chemical Co.).

GBS type V-specific rabbit antibodies were also quantified by ELISA with goat anti-rabbit IgG conjugated to alkaline phosphatase (Tago Inc. Burlingham, Calif.) at 1/3,000 dilution. Microtiter plates (Immulon* 2; Dynatech Laboratories, Inc., Chantillty, VA) were coated with 100 ng of purified type V polysaccharide linked to poly-L-lysine per well as previously described (15). Antibody titers were recorded as the reciprocal dilution that resulted in an A_{405} of ≥0.3 when wells containing the reference serum (rabbit antiserum raised to whole type v strain 1169-NT1) at a dilution of 1/3000 reached an A_{405} of 0.3.

Separation of IgG and IgM from Immune rabbit serum. Protein A-agarose affinity column chromatography (Pierce Chemical Co., Rockford, Ill.) was used to separate immunoglobulins (IgG and IgM) from 0.5 ml of pooled immune rabbit serum, obtained on day 70, raised to II-TT vaccine as described elsewhere (28). Separation of antibody classes was confirmed with type II polysaccharide-coated ELISA plates with goat anti-rabbit IgG (γ and light chain specific; Tago) used at 1/500 dilution and goat anti-rabbit IgM (μ chain specific; Sera-Lab, Westbury, N.Y.) used at a 1/200 dilution.

Competitive ELISA. The specificity of rabbit serum raised to the II-TT vaccine was determined by competitive ELISA with homologous (type II) and heterologous (types Ia and III) polysaccharides as inhibitors. Epitope specificity of vaccine-induced pooled

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rabbit serum (obtained on day 70) was examined with native and desialylated type II polysaccharide and β-*O*-methylgalactopyranose as inhibitors of antibody binding. Native type II polysaccharide was desialylated by treatment with 6% acetic acid at 80°C for 2 h. Polysaccharide inhibitors were serially diluted 4-fold and mixed with an equal volume (75 μl) of pooled rabbit serum (diluted 10,000-fold) obtained on day 70 after vaccination with II-TT vaccine. This mixture (100 μl) was then added to type II polysaccharide-coated ELISA wells. Alkaline phosphatase-conjugated anti-rabbit IgG was used as the secondary antibody at a dilution of 1/3,000. Results are expressed as follows: % inhibition = [A_{405} with no inhibitor - A_{405} with inhibitor)/A_{405} with no inhibitor] x 100.

The serotype specificity of GBS V-TT rabbit antiserum was evaluated using GBS type-specific polysaccharides Ia, Ib, II, III, V and VI. ELISA wells were coated with 100 ng/well with GBS type V polysaccharide linked to poly-L-lysine. Rabbit antisera to V-TT vaccine, diluted 1/6,000, was incubated with each of the polysaccharide inhibitors. Incubated antiserum was added to ELISA wells and the plate processed with goat anti-rabbit IgG alkaline phosphatase conjugate (diluted 1/3000), and substrate as detailed previously (28).

In vitro antibody-mediated killing of GBS. The ability of vaccine-induced rabbit antibodies to opsonize GBS cells for subsequent killing by human peripheral blood leukocytes was assessed by an in vitro opsonophagocytosis assay (7, 8).

The reaction assay for determining the opsonophagocytic activity of GBS type V reactive antibodies was conducted by incubating 1. 5 x 10^7 white blood cells (WBC) in 300 μl of buffer; 3 x 10^6 CFU of type V GBS stain CJB 111 in 50μl; human complement

(absorbed with GBS type V strain cells) (50μl); antibody - anti V-TT-heat inactivated (50μl); modified Eagle's Medium (50μl). The mixture was incubated with mixing for 60 min at 37°C. The difference in GBS Colony Forming Units (CFU) was determined following incubation at 37°C for 1 hour by the standard plate count method.

Passive protection of mice by vaccine-induced rabbit antibodies. Groups of 10 Swiss-Webster outbred female mice (Taconic Farms, Germantown, N.Y.), each mouse weighing 18 to 20 g, were injected intraperitoneally with 0.2 ml of pooled serum (day 70) from rabbits vaccinated with either type II polysaccharide or II-TT vaccine. The titer, as measured by ELISA, of the pooled serum obtained on day 70 from rabbits immunized with type II polysaccharide was 100, and that of II-TT vaccine was 12,800. Control groups of five mice received pooled preimmunization rabbit serum or pooled antiserum raised to uncoupled TT (27). Twenty-four hours later, mice were challenged with type II strain 18RS21 (1.5×10^5 CFU per mouse) in a total volume of 1.0 ml of Todd-Hewitt broth. The challenge dose for each strain was previously determined to be lethal for >90% of mice of similar weights and ages. Surviving mice were counted daily for three subsequent days.

Statistical Analysis. Fisher's exact test was used to compare the abilities of different rabbit sera to passively protect mice against lethal GBS infection.

RESULTS

Preparation and composition of II-TT vaccine and V-TT vaccine. GBS II-TT vaccine and GBS V-TT vaccine were prepared by methods detailed previously for the construction of the GBS type III conjugate vaccine (33). Controlled periodate oxidation of type II GBS polysaccharide resulted in the modification of 7% of the

polysaccharide's sialic acid residues as determined by gas chromatography-mass spectrometry analysis. Monomeric TT was covalently linked to modified sialic acid sites on the type II polysaccharide by reductive amination. The purified II-TT vaccine contained 32% (wt/wt) protein and 68% (wt/wt) carbohydrate. The final yield of II-TT vaccine was 7.8 mg, or 39%.

Controlled periodate oxidations of type V GBS polysaccharide resulted in modification of about 8%, 25% and 50% of the sialic acid residues in the polysaccharide. Monomeric TT was covalently linked to the modified sialic acid residues on the type V polysaccharide by reductive amination. Polysaccharides oxidized so that about 8% of their sialic acid residues were modified, resulted in conjugates having a low polysaccharide to protein molar ratio. Polysaccharides in which 25 or 50% of the terminal sialic acid residue were modified resulted in conjugate molecules having a more preferred polysaccharide to protein molar ratio. A GBS type V polysaccharide in which 25% of the sialic acid residues were modified, was used to produce conjugate vaccines having the biochemical characterization shown in Table I.

TABLE 1. Composition of the GBS type V-TT Vaccine

Polysaccharide size (M^r)	200,000
Sialic acid residues oxidized (%)	25
Amount of vaccine as tetanus toxoid (% dry wt.)	38
Amount of vaccine as carbohydrate (% dry wt.)	62
Mol carbohydrate/mol protein[a]	0.56

[a]Determined M^r of tetanus toxoid monomer was 150,000.

Immunogenicity of II-TT vaccine and V-TT vaccine in rabbits. The immunogenicities of the II-TT vaccine and native type II polysaccharide were compared in rabbits. An increase in type II-specific antibody was seen following the primary dose of II-TT vaccine (Table 2). A booster dose of vaccine further increased the antibody response. Antibody levels remained unchanged or rose slightly following a second booster, dose on day 41 and were sustained throughout the remainder of the study (Table 2). In contrast to the II-TT vaccine, uncoupled native GBS type II polysaccharide failed to elicit a specific antibody response (Table 2). Animals vaccinated with the II-TT vaccine also developed antibodies to TT, achieving approximately a 3-$\log_{10}$ increase over preimmunization levels.

TABLE 2. GBS type II polysaccharide-specific antibody titers of rabbits vaccinated with native type II polysaccharide or II-TT vaccine

Vaccine and rabbit	Antibody titer in ELISA as day:[a]					
	0	20[b]	34	41[b]	55	70
Native type II polysaccharide						
1	100	100	100	200	100	100
2	100	100	100	100	100	100
3	200	100	100	100	100	100
II-TT						
1	100	400	3,200	3,200	6,400	12,800
2	100	1,600	3,200	6,400	6,400	25,600
3	100	6,400	12,800	25,600	12,800	12,800

[a] A value of 100 indicates an antibody titer of ≤ 100. Values are the means of duplicate determinations. Rabbits were vaccinated subcutaneously with 50 μg of vaccine emulsified with complete Freund's adjuvant on day 0.

[b] Booster doses with incomplete Freund's adjuvant were administered.

The immunogenicity of the V-TT vaccine (polysaccharide oxidized to 25%) was also assessed in rabbits. Antibody (IgG) levels increased following the first and second dose of type V-II vaccine and were sustained for the course of the study (Table 3).

TABLE 3. GBS type V polysaccharide-specific IgG titer.

	IgG titer as determined by an ELISA at day[a]:			
Vaccine	0	35	56	91
$V_{25\%\alpha}$-TT				
Rabbit 1[a]	100	800	1,600	1,600

[a] This animal received three-50 μg doses of vaccine mixed with alum at 3 week intervals beginning at day 0

Antigenic properties of vaccine-induced rabbit sera. The conjugation of polysaccharide to a protein carrier should not alter important antigenic epitopes found on the polysaccharide in its native form. We tested the specificity of II-TT vaccine-induced antibodies by competitive ELISA using homologous and heterologous GBS polysaccharides as inhibitors. Native type II polysaccharide at a concentration of 450 ng/ml inhibited 50% of the binding of rabbit antibodies raised to II-TT vaccine (Fig. 2). GBS type Ia and type III polysaccharides did not inhibit binding of serum raised to II-TT vaccine even at concentrations as high as 500 μg/ml. (Fig. 2). These result verify the serotype specificity of II-TT vaccine-induced antibodies for the target antigen and indicate preservation of antigenic epitopes of the polysaccharide portion of the conjugate vaccine.

To determine whether the epitope influenced by sialic acid was maintained during the preparation of II-TT vaccine, native and desialylated type II polysaccharides were used in a competitive ELISA as inhibitors of binding of rabbit antibodies raised to II-TT vaccine. Desialylation of the polysaccharide was accomplished by treatment with 6% acetic acid at 80°C for 2h. Quantitative removal of sialic acid residues was confirmed by the thiobarbituric acid assay (32) with N-acetylneuraminic acid (Sigma) as the standard. The K_{av}

of native type II polysaccharide before acid treatment was 0.49, whereas the K_{av} of acid-treated polysaccharide was 0.52 on a Superose* 6 FPLC column (LKB-Pharmacia, Sweden), indicating a slight reduction in molecular size of the polysaccharide due to the loss of the side chain sialic acid residues that make up 20% of the native polysaccharide by weight. Even 200 μg of desialylated GBS type II polysaccharide per ml inhibited by 33% of the binding of II-TT vaccine antibodies to native type II polysaccharide (Fig. 3). The relatively poor recognition of the desialylated or core type II polysaccharide by II-TT vaccine antiserum was confirmed by immunoelectrophoresis gels, which showed a precipitin band formed with the native but not the core type II polysaccharide (not shown). Binding of II-TT vaccine antisera to native type II polysaccharide could not be inhibited with β-O-methylgalactopyranose used at a concentration range of 0.01 to 10 mg/ml (not shown).

The specificity of GBS type V-TT vaccine-induced antibodies was tested by competitive ELISA using homologous and heterologous GBS polysaccharides as inhibitors. Native type V polysaccharide effectively inhibited binding of rabbit antibodies raised to type V-TT vaccine (Figure 5). GBS type Ia, type Ib, type II, type III an type VI did not inhibit binding of serum raised to type V-TT vaccine (Figure 5). These results demonstrate the specificity of type V-TT vaccine induced antibodies for the target antigen.

In vitro activity of GBS vaccine-induced antibodies. The ability of immune serum to opsonize GBS for killing by human peripheral blood leukocytes in vitro has correlated with protective efficacy against GBS in animal protection experiments (27,33). Antibodies raised in the three rabbits vaccinated with II-TT vaccine enhanced the killing of GBS type II strain 18RS21 by ≥1.8

$\log_{10}$ (Table 4). Preimmunization rabbit serum or serum from rabbits vaccinated with native GBS type II polysaccharide or uncoupled TT failed to enhance the in vitro killing of GBS (Table 4). Vaccine-induced rabbit antibodies promoted the killing by human blood leukocytes of two GBS type II clinical isolates (strains S16 and S20) by ≥1.8 $\log_{10}$ compared with preimmunization rabbit serum (Table 5). Rabbit serum to type II-TT vaccine was determined to be serotype specific, as it failed to promote the in vitro killing of heterologous (types Ia and III) GBS strains (Table 5).

TABLE 4. In vitro opsonophagocytic killing of GBS type II strain 18RS21 by rabbit antiserum raised to native type II polysaccharide, II-TT vaccine, or TT

Serum source (day of collection)	CFU at[a]: 0 min	CFU at[a]: 60 min	GBS killed ($\log_{10}$)
Type II polysaccharide (70)[b]	4.3×10^6	6.6×10^6	-0.19
II-TT vaccine (0)	6.0×10^6	6.4×10^6	-0.03
II-TT vaccine (70)			
Rabbit 1	4.0×10^6	5.7×10^4	1.85
Rabbit 2	4.3×10^6	2.7×10^4	2.20
Rabbit 3	3.9×10^6	4.3×10^4	1.96
TT (70)	4.2×10^6	6.9×10^6	-0.21
None	3.9×10^6	7.1×10^6	-0.26

[a] Reaction mixture contained serum (at a final assay concentration of 1%) to be tested, type II GBS-absorbed human serum as a source of complement, human peripheral blood leukocytes, and type II GBS 18RS21. Values are means of duplicate determination.

[b] Rabbit serum collected following the primary dose of native type II polysaccharide in complete Freund's adjuvant.

TABLE 5. In vitro opsonophagocytic killing of GBS strains by preimmunization and immune rabbit serum raised to II-TT vaccine

GBS type and strain	GBS killed (log_{10})[a]	
	Preimmunization (day 0)	Immune II-TT vaccine (day 70)
II		
18RS21	-0.36	1.98
S16	0.96	2.78
S20	-0.01	1.84
Ia 090	ND	-0.51
III M781	ND	-0.54

[a]CFU (log_{10}) at 60 min - CFU (log_{10}) at 0 min. Reaction mixture contained serum to be tested, type II GBS-absorbed human serum as a source of complement, human peripheral blood leukocytes, and type II GBS I8RS21. Values are means of duplicate determinations. ND, not done.

Protein A-affinity-purified IgG and IgM were obtained from pooled serum raised to type II-TT vaccine (28). The specificity of each Ig fraction was confirmed by ELISA with class-specific secondary antibody. The A_{405}S of the IgM and IgG fractions (diluted 1/100) were 0.384 and 0.009, respectively, with μ-chain-specific conjugate and 0.086 and 2.367, respectively, with goat anti-rabbit IgG (γ and light chain specific). Isolated IgM and IgG were tested for their abilities to enhance opsonic killing of type II GBS by human blood leukocytes. Unfractionated sera raised to type II-TT vaccine diluted 1:100 and an equivalent 1:100 dilution of the IgG fraction from the same sera promoted killing of type II GBS by 1.65 ± 0.22 and 0.95 ± 0.09 log_{10}, respectively. In contrast, type II GBS were not killed but grew in the presence of preimmunization serum (-0.39 ± 0.13 log_{10}) and IgM-

enriched fraction from serum raised to type II-TT vaccine (-0.28 ± 0.09 $\log^{10}$) in the opsonophagocytic assay.

The ability of GBS type V-TT vaccine-induced rabbit antisera to promote the killing of a GBS-type V strain (CJB 111) by human blood leukocytes was also assessed by the in vitro opsonophagocytic assay (8). As shown in Figure 6, GBS type V-TT antisera at a concentration of 10%, effectively optimized GBS type V strain CJB 111 relative to controls. Control reactions conditions were: complement (10%) without antisera; antiserum (10%) without complement; antiserum (1%) and complement.

Mouse protection assay. To test the in vivo protective abilities of vaccine-induced antibodies, mice were passively immunized with pooled II-TT vaccine sera (day 70) 24 h prior to challenge with type II GBS 18RS21. Previously, the challenge does was determined to be lethal for 90 to 100% of mice tested. Complete (100%) protection was afforded to groups of mice that received serum raised to GBS II-TT vaccine, whereas only one of five mice receiving prevaccination serum survived (Table 6). There were no survivors among mice that received serum from rabbits vaccinated with either uncoupled type II polysaccharide or uncoupled TT (Table 6).

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TABLE 6. Passive protection of Swiss-Webster outbred mice against GBS type II strain 18RS21 with sera from rabbits vaccinated with native type II polysaccharide II-TT vaccine, or TT[a]

Rabbit serum[b] (day of collection)	No. of survivors/ total no. of mice[c]	% Survival
II-TT vaccine (70)	10/10	100[d]
II-TT vaccine (0)	1/5	20
Type II polysaccharide (70)	0/10	0
TT (70)	0/5	0

[a] Mice were given a 90% lethal dose (1.5×10^5 CFU per mouse) of GBS.
[b] Serum samples from three rabbits were pooled.
[c] Survival was determined 72 h after challenge.
[d] $P = 0.0037$ compared with preimmunization (day 0) values.

The coupling strategy used with GBS type III polysaccharide, first developed for meningococcal polysaccharide (18), may be applicable to all GBS capsular polysaccharide antigens, since they all contain sialic acid. However, unlike the other GBS polysaccharides that have sialic acid as the terminal saccharide of di- or trisaccharide side chains, the GBS type II polysaccharide has a repeating unit that bears sialic acid as the sole sugar on one of the two monosaccharide side chains (9, 20). In constructing the GBS type II conjugate vaccine, we oxidized 7% of sialic acid residues on the type II polysaccharide and used these as sites for coupling the polysaccharide to TT. Purified II-TT vaccine eluted in the void volume of a Superose* 6 column (compatible with a molecular weight of > 10^6) and was composed of 68% (wt/wt) carbohydrate and 32% (wt/wt) protein.

Type II-TT vaccine emulsified with adjuvant was immunogenic in rabbits in contrast to uncoupled native type II polysaccharide, which failed to elicit type II

polysaccharide-specific antibody. Two of three rabbits immunized responded strongly 3 weeks after a single dose of II-TT vaccine. Optimal type-specific antibody was achieved in all three rabbits 3 weeks after a booster dose of II-TT vaccine. Further increases in type II-specific antibody titer were not seen after the third dose of II-TT vaccine. Results from in vitro and in vivo experiments indicated that antibodies raised to II-TT vaccine were functionally active against type II GBS. Serum from each of three rabbits vaccinated with II-TT vaccine promoted the in vitro killing of type II GBS by human peripheral leukocytes and provided outbred mice with complete (100%) protection against a lethal dose of type II GBS. II-TT vaccine antiserum was opsonically active against homologous GBS strains (18RS21, S16, and S20) but not heterologous GBS serotypes (types 1a and III) tested.

Whereas native type II polysaccharide inhibited binding of II-TT vaccine antisera to type II polysaccharide-coated ELISA wells, <40% inhibition was obtained with desialylated type II polysaccharide, even when it was used at a concentration of 200 μg/ml. This result suggests that an important antigenic determinant of type II polysaccharide is dependent on the presence of sialic acid residues. This result corroborated those obtained with rabbit antisera raised to whole type II organisms (21). However, ELISA inhibition experiments using β-O-methylgalactopyranose as an inhibitor indicated that rabbit antisera raised to II-TT vaccine did not contain galactose-specific antibodies. No inhibition of binding of II-TT vaccine antiserum to type II native polysaccharide was obtained even with β-O-methylgalactopyranose at a concentration of 10 mg/ml. Therefore, the side chain galactose does not appear to be an immunodominant epitope of type II polysaccharide when the polysaccharide is coupled to TT. These results are in

contrast to immunochemical studies performed with rabbit sera raised to whole type II GBS organisms (12, 21) in which the galactose side chain appeared to be one of two immunodominant sites, along with a sialic acid-dependent epitope, of type II polysaccharide. Whole type II GBS cells used in previous studies (12, 21) and not cultured in pH-controlled conditions may possess polysaccharides that, to some degree, lack sialic acid. Under these circumstances, the side chain galactose might be major antigenic epitope. The source of type II polysaccharide used to synthesize the II-TT vaccine was a culture of type II GBS maintained at a pH of 7.0; a final analysis confirmed that sialic acid constituted ~20% (wt/wt) of the polysaccharide. We cannot exclude the possibility that coupling type II polysaccharide to TT altered the conformation of the polysaccharide, thereby rendering the galactose epitope unavailable for recognition by the host immune system. Although II-TT vaccine-induced rabbit antiserum lacked galactose-specific antibodies, it was fully functional in vitro and in vivo against type II GBS organisms. Neither chemical modification of some of the sialic acid residues nor the subsequent binding of TT to these sites altered critical antigenic epitopes necessary to elicit functional type II-specific antibody. That purified IgG from rabbit sera raised to II-TT vaccine promoted killing in vitro of type II GBS by human leukocytes suggests that not only was the immunogenicity of type II polysaccharide increased by conjugating it to TT but also that functional IgG antibodies were elicited.

The GBS type V capsular polysaccharide has a repeating unit structure consisting of a trisaccharide backbone containing two distinct side chains on adjacent sugar residues. (Reference 35; Figure 4). In one of the GBS type V-TT conjugate vaccine preparations, 25% of the sialic acid residues on the longer side chain (i.e. 3

sugar residues) in the type V polysaccharide were oxidized. The oxidized sialic acid residues were used as sites to couple the type V polysaccharide to TT. The purified type V-TT vaccine was composed of 38% (wt/wt) TT and 62% (wt/wt) carbohydrate. The V-TT vaccine mixed with alum was shown to be immunogenic in rabbits. A strong response was observed after the first dose and booster doses of type V-TT vaccine. The second booster dose also resulted in a further increase in the type V polysaccharide IgG titer. The serospecificity of the type V-TT induced assays was demonstrated by competitive ELISAs. Rabbit antiserum raised against the V-TT vaccine are functionally active as evidenced by the ability to kill type V GBS in vitro in the presence of human white blood cells and complement.

Like III-TT vaccine, II-TT vaccine demonstrated improved immunogenicity in rabbits compared with active polysaccharide and elicited opsonically active IgG in rabbits despite difference in polysaccharide structure, position of the sialic acid on the polysaccharide to which TT was linked, and vaccine composition. The type V-TT vaccine also proved immunogenic in rabbits and to be capable of inducing functionally antibodies in vivo. We anticipate that GBS polysaccharide-protein conjugates of this design will ultimately constitute components of a multivalent GBS vaccine capable of providing protection against the GBS serotypes most often associated with disease in humans.

In addition, we anticipate that vaccines can be produced from the conjugate molecules of this invention, by adding the conjugate molecules to a pharmaceutically acceptable carrier. Preferably, the degree of cross-linking in the antigenic molecules of this invention is controlled to an extent sufficient to provide vaccines which may be filter sterilized through a 0.2 micron

filter. Such vaccines preferably are soluble. Immunization may be effected by injecting the recipient mammal with the vaccine once followed by subsequent booster injections as necessary. A sufficient dose of the vaccine to be administered can be based on the amount of polysaccharide present: a range of 3-80 μg of the polysaccharide component of the conjugate vaccine may be administered.

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While we have hereinbefore described a number of embodiments of this invention, it is apparent that the basic constructions can be altered to provide other embodiments which utilize the methods of this invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

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CLAIMS:

1. An antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type II bacteria or Group B streptococcus type V bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

2. An antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type II bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

3. The conjugate molecule according to claim 2, wherein between 5 and 50% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

4. The conjugate molecule according to claim 3, wherein between 5 and 25% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

5. The conjugate molecule according to claim 4, wherein between 5 and 10% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

6. The conjugate molecule according to claim 5, wherein 7% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

7. The conjugate molecule according to any one of claims 2 to 6, which is capable of being filter sterilized through a 0.2 micron filter.

8. The conjugate molecule according to any one of claims 2 to 7, wherein the capsular polysaccharide has a molecular weight of between 5,000 and 1,000,000 daltons.

9. The conjugate molecule according to claim 8, wherein the capsular polysaccharide has a molecular weight of between 50,000 and 500,000 daltons.

10. The conjugate molecule according to claim 8, wherein the polysaccharide has a molecular weight of between 100,000 and 300,000 daltons.

11. The conjugate molecule according to claim 8, wherein the polysaccharide has a molecular weight of 200,000 daltons.

12. The conjugate molecule according to any one of claims 2 to 11, wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid and cross reactive material (CRM) 197.

13. The conjugate molecule according to any one of claims 2 to 6, wherein the polysaccharide is derived from Group B streptococcus (GBS) type II strain 18RS21, strain S16 or strain S20, has a molecular weight of between 100,000 and 300,000 daltons and lacks Group B antigen; and the protein is monomeric tetanus toxoid (TT).

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14. A method of preparing a conjugate molecule of a capsular polysaccharide of Group B streptococcus type II bacteria and a protein, which method comprises:

(a) subjecting the capsular polysaccharide to periodate oxidation sufficient to introduce an aldehyde group into two or more terminal sialic acid residues linked to a backbone of the polysaccharide; and

(b) coupling the oxidized capsular polysaccharide to the protein through reductive amination to generate a secondary amine bond between the capsular polysaccharide and the protein.

15. The method according to claim 14, wherein between 5 and 50% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

16. The method according to claim 14, wherein between 5 and 25% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

17. The method according to claim 14, wherein between 5 and 10% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

18. The method according to claim 14, wherein 7% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

19. The method according to claim 14, wherein between 5 and 50% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

20. The method according to claim 14, wherein between 5 and 25% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

21. The method according to claim 14, wherein between 5 and 10% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

22. The method according to claim 14, wherein 5% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

23. The method according to any one of claims 14 to 22, wherein the coupling of the oxidized polysaccharide to the protein is conducted by adding sodium cyanoborohydride to an aqueous solution containing the oxidized polysaccharide and the protein.

24. A conjugate molecule prepared according to the method of any one of claims 14 to 23.

25. A vaccine comprising the conjugate molecule as defined in any one of claims 1 to 13 or as prepared by the method defined in any one of claims 14 to 23, and a pharmaceutically acceptable carrier.

26. A vaccine comprising the conjugate molecule as defined in any one of claims 1 to 13 or as prepared by the method defined in any one of claims 14 to 23, and a vaccine adjuvant.

27. A multivalent vaccine comprising:

the conjugate molecule as defined in any one of claims 2 to 13 or as prepared by the method defined in any one of claims 14 to 23, and

at least one other immunogenic molecule capable of eliciting the production of antibodies to a pathogenic substance other than Group B streptococcus type II, wherein the other immunogenic molecule is capable of eliciting the production of antibodies to a pathogen selected from the group consisting of Group B streptococcus types Ia, Ib, III, IV and V; Haemophilus influenzae type b; and E. coli type K1.

28. The vaccine according to claim 26 or 27, which also comprises a pharmaceutically acceptable carrier.

29. The vaccine according to any one of claims 25, 26, 27 and 28, which is for a female human so as to produce antibodies capable of passing into a fetus conceived prior to or after immunization to produce protection against infection in a neonate at birth.

30. The vaccine according to claim 29, which is for immunization before pregnancy.

31. The vaccine according to claim 29 or 30, wherein the female human is 10 to 50 years old.

32. The vaccine according to claim 29, which is for immunization during pregnancy.

33. The vaccine according to any one of claims 25, 26, 27 and 28, which is for a human.

34. The vaccine according to any one of claims 25, 26, 27 and 28, which is for an adult person who is at risk for being infected by Group B streptococcus type II.

35. The vaccine according to claim 34, wherein the person has a suppressed immune system.

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36. The vaccine according to claim 35, wherein the suppressed immune system is due to a disease selected from the group consisting of cancer and diabetes.

37. The vaccine according to any one of claims 25, 26, 27 and 28, which is for immunizing dairy cows against bovine mastitis.

38. An antigenic conjugate molecule comprising a capsular polysaccharide derived from Group B streptococcus type V bacteria and a protein, wherein two or more side chains terminating in sialic acid residues of the polysaccharide are each linked through a secondary amine bond to the protein and wherein the polysaccharide has a molecular weight of at least 5,000 daltons.

39. The conjugate molecule according to claim 38, wherein between 5 and 50% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

40. The conjugate molecule according to claim 39, wherein between 5 and 25% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

41. The conjugate molecule according to claim 40, wherein between 5 and 10% of the terminal sialic acid residues of each polysaccharide are available to be linked to the protein.

42. The conjugate molecule according to any one of claims 38 to 41, which is capable of being filter sterilized through a 0.2 micron filter.

43. The conjugate molecule according to any one of claims 38 to 42, wherein the capsular polysaccharide has a molecular weight of between 5,000 and 1,000,000 daltons.

44. The conjugate molecule according to claim 43, wherein the capsular polysaccharide has a molecular weight of between 50,000 and 500,000 daltons.

45. The conjugate molecule according to claim 38, wherein 25% of the terminal sialic acid residues of each polysaccharide are linked to the protein.

46. The conjugate molecule according to claim 44, wherein the polysaccharide has a molecular weight of between 100,000 and 300,000 daltons.

47. The conjugate molecule according to claim 44, wherein the polysaccharide has a molecular weight of 200,000 daltons.

48. The conjugate molecule according to any one of claims 38 to 41 and 45, wherein the polysaccharide is derived from Group B streptococcus (GBS) type V strain 1169-NT1, has a molecular weight of between 100,000 and 300,000 daltons and lacks Group B antigen; and the protein is monomeric tetanus toxoid (TT).

49. A method of preparing a conjugate molecule of a capsular polysaccharide of Group B streptococcus type V bacteria and a protein, which method comprises:

(a) subjecting the capsular polysaccharide to periodate oxidation sufficient to introduce an aldehyde group into two or more terminal sialic acid residues linked to a backbone of the polysaccharide; and

(b) coupling the oxidized capsular polysaccharide to the protein through reductive amination to generate a secondary amine bond between the capsular polysaccharide and the protein.

50. The method according to claim 49, wherein between 5 and 50% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

51. The method according to claim 49, wherein between 5 and 25% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

52. The method according to claim 49, wherein between 5 and 10% of the sialic acid residues of each polysaccharide are oxidized to form aldehyde groups capable of coupling to the protein.

53. The method according to claim 49, wherein between 5 and 50% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

54. The method according to claim 49, wherein between 5 and 25% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

55. The method according to claim 49, wherein between 5 and 10% of the sialic acid residues of each polysaccharide are covalently linked to the protein.

56. The method according to claim 49, wherein 25% of the terminal sialic acid residues of each polysaccharide are linked to the protein.

57. The method according to any one of claims 49 to 56, wherein the coupling of the oxidized polysaccharide to the protein is conducted by adding sodium cyanoborohydride to an aqueous solution containing the oxidized polysaccharide and the protein.

58. A conjugate molecule prepared according to the method of any one of claims 49 to 57.

59. A vaccine comprising the conjugate molecule as defined in any one of claims 38 to 48 or as prepared by the method defined in any one of claims 49 to 57.

60. A vaccine comprising the conjugate molecule as defined in any one of claims 38 to 48 or as prepared by the method defined in any one of claims 49 to 57 and a vaccine adjuvant.

61. A multivalent vaccine comprising:

the conjugate molecule as defined in any one of claims 38 to 48 or as prepared by the method defined in any one of claims 49 to 57, and

at least one other immunogenic molecule capable of eliciting the production of antibodies to a pathogenic substance other than Group B streptococcus type V, wherein the other immunogenic molecule is capable of eliciting the production of antibodies to a pathogen selected from the group consisting of Group B streptococcus types Ia, Ib, II, III and IV; Haemophilus influenzae type b; and E. coli type K1.

62. The vaccine according to any one of claims 59, 60 and 61, which also comprises a pharmaceutically acceptable carrier.

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63. The vaccine according to any one of claims 59, 60, 61 and 62, which is for a female human so as to produce antibodies capable of passing into a fetus conceived prior to or after immunization to produce protection against infection in a neonate at birth.

64. The vaccine according to claim 63, which is for immunization before pregnancy.

65. The vaccine according to claim 63 or 64, wherein the female human is 10 to 50 years old.

66. The vaccine according to claim 63, which is for immunization during pregnancy.

67. The vaccine according to any one of claims 59, 60, 61 and 62, which is for a human.

68. The vaccine according to any one of claims 59, 60, 61 and 62, which is for an adult person who is at risk for being infected by Group B streptococcus type V.

69. The vaccine according to claim 68, wherein the person has a suppressed immune system.

70. The vaccine according to claim 69, wherein the suppressed immune system is due to a disease selected from the group consisting of cancer and diabetes.

71. The vaccine according to any one of claims 59, 60, 61 and 62, which is for immunizing dairy cows against bovine mastitis.

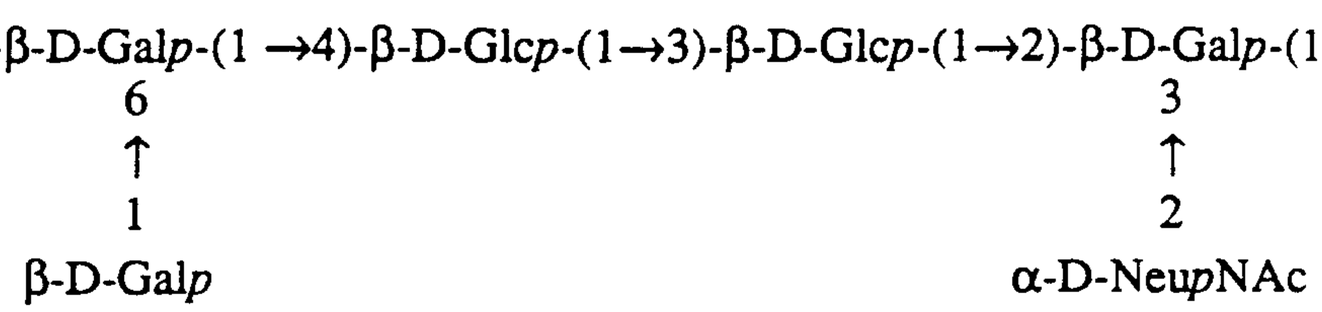
[→4)-β-D-Glcp NAc-(1→3)-β-D-Galp-(1 →4)-β-D-Glcp-(1→3)-β-D-Glcp-(1→2)-β-D-Galp-(1 →]n
6
↑
1
β-D-Galp
3
↑
2
α-D-Neup NAc

FIG. 1

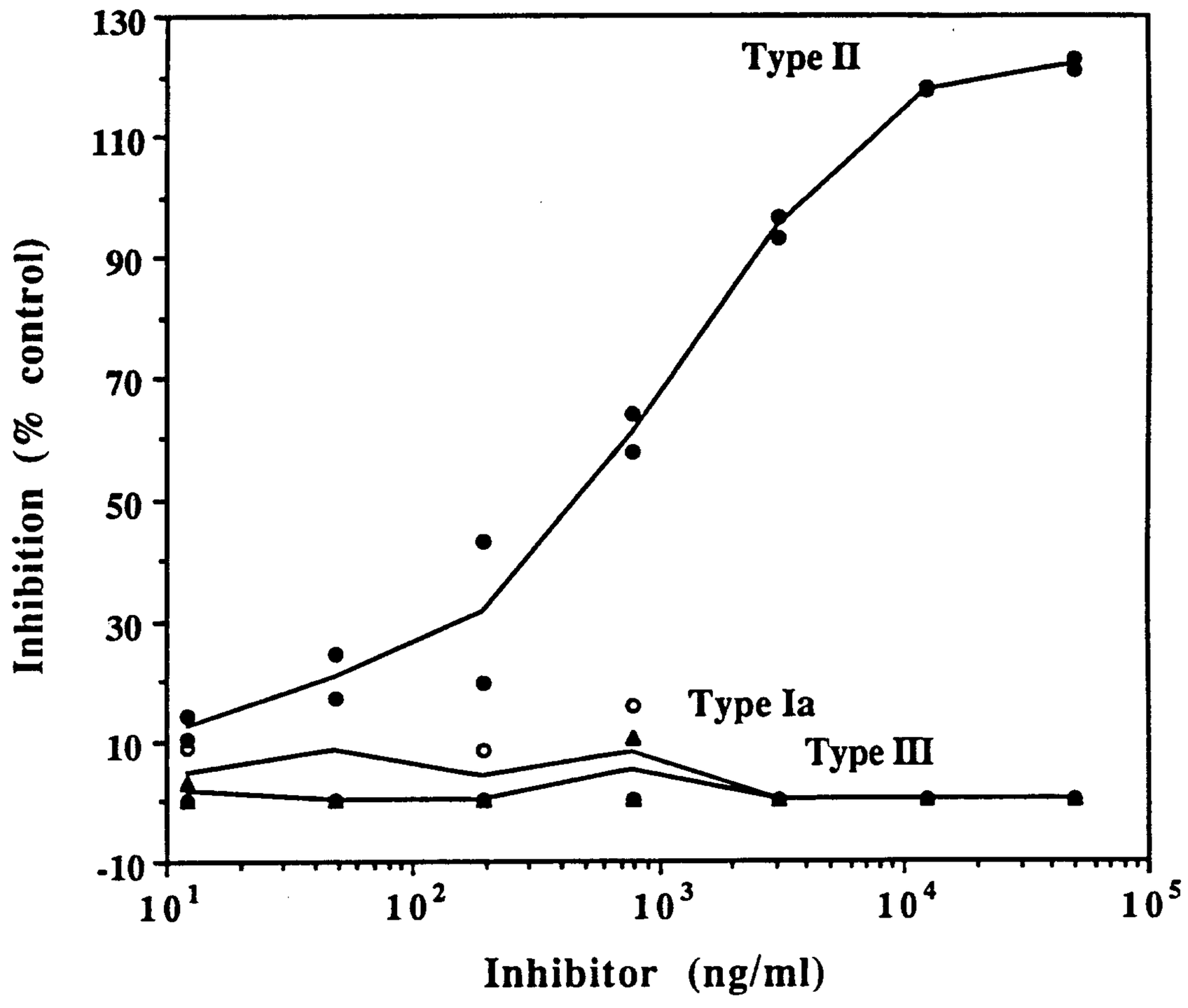
130
110
90
70
50
30
10
-10
Inhibition (% control)
Type II
Type Ia
Type III
10^1
10^2
10^3
10^4
10^5
Inhibitor (ng/ml)

FIG. 2

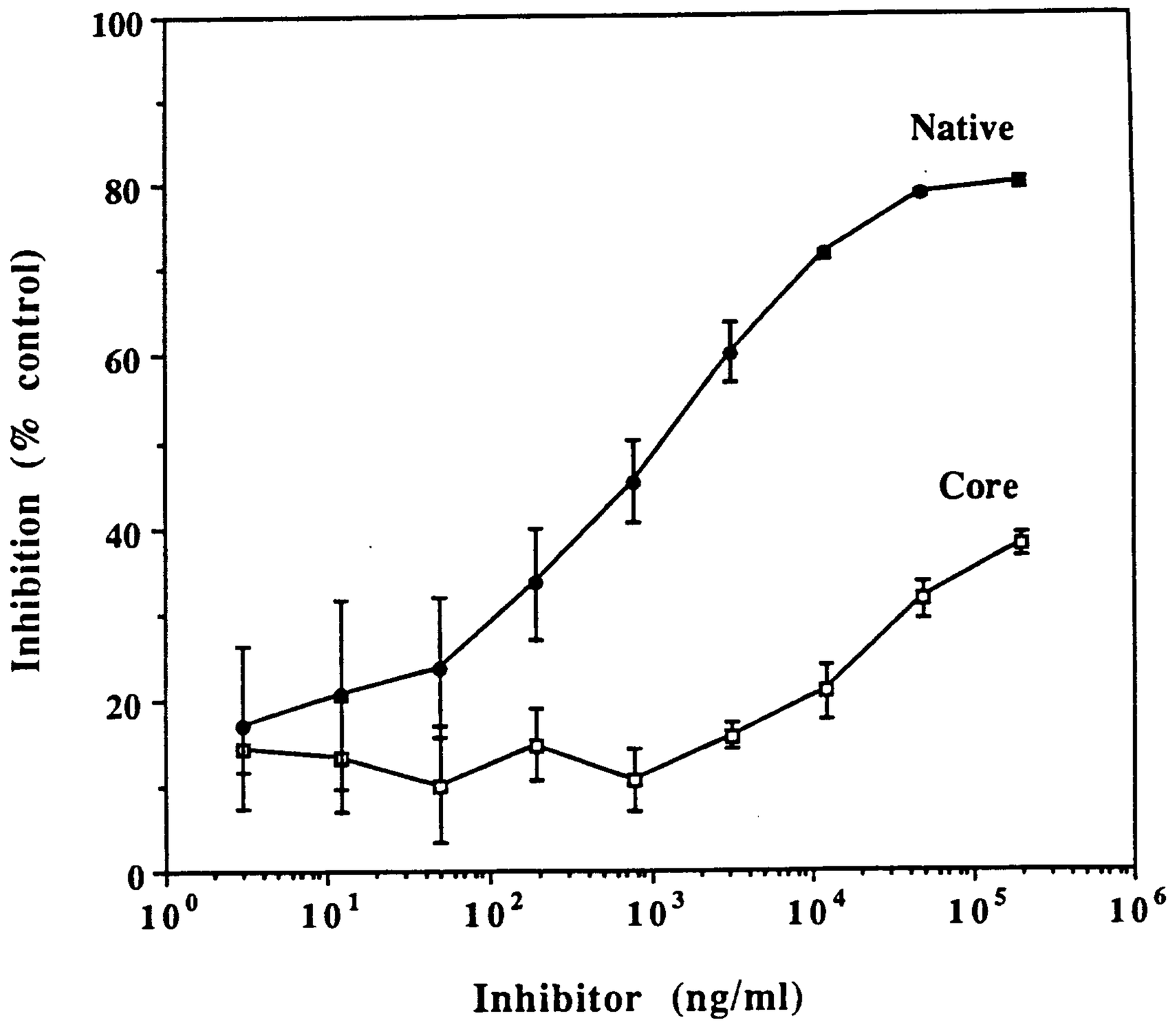
100
80
60
40
20
0
Inhibition (% control)
Native
Core
10^0
10^1
10^2
10^3
10^4
10^5
10^6
Inhibitor (ng/ml)

FIG. 3

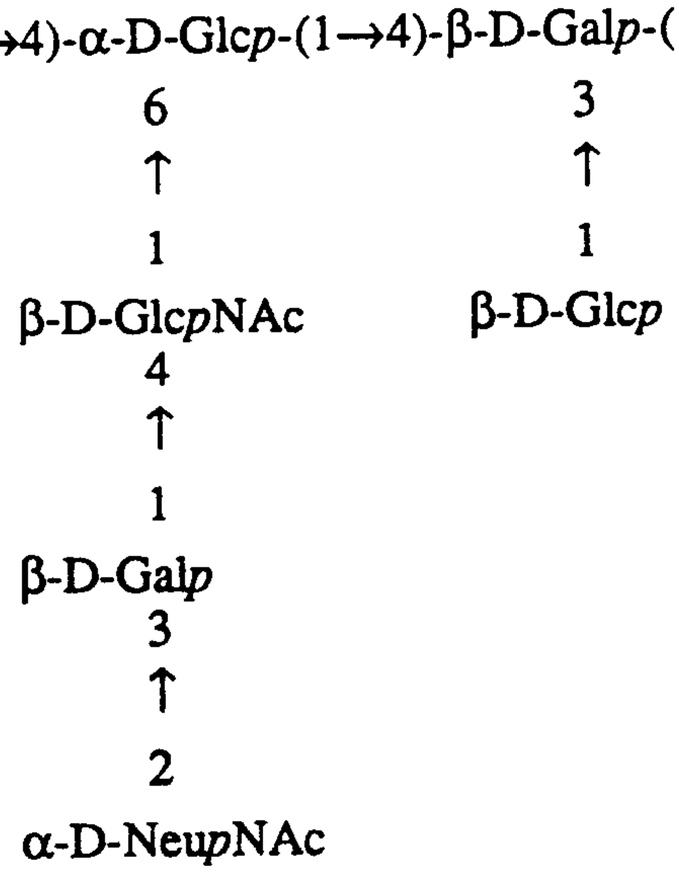
[→4)-α-D-Glcp-(1→4)-β-D-Galp-(1→4)-β-D-Glcp-(1→]n
6
↑
1
β-D-GlcpNAc
4
↑
1
β-D-Galp
3
↑
2
α-D-NeupNAc
3
↑
1
β-D-Glcp

FIG. 4

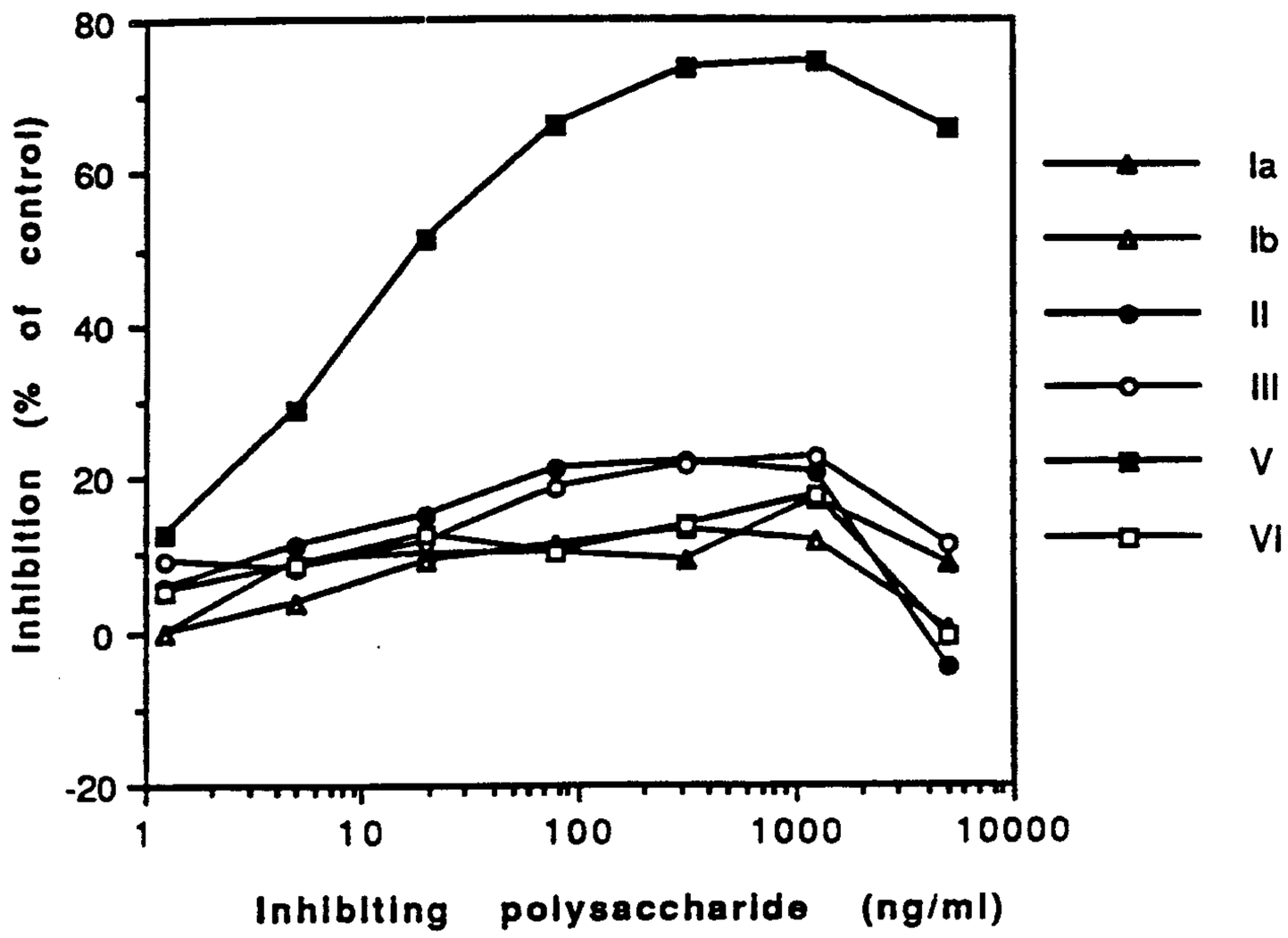
80
60
40
20
0
-20
Inhibition (% of control)
1
10
100
1000
10000
Inhibiting polysaccharide (ng/ml)
Ia
Ib
II
III
V
VI

FIG. 5

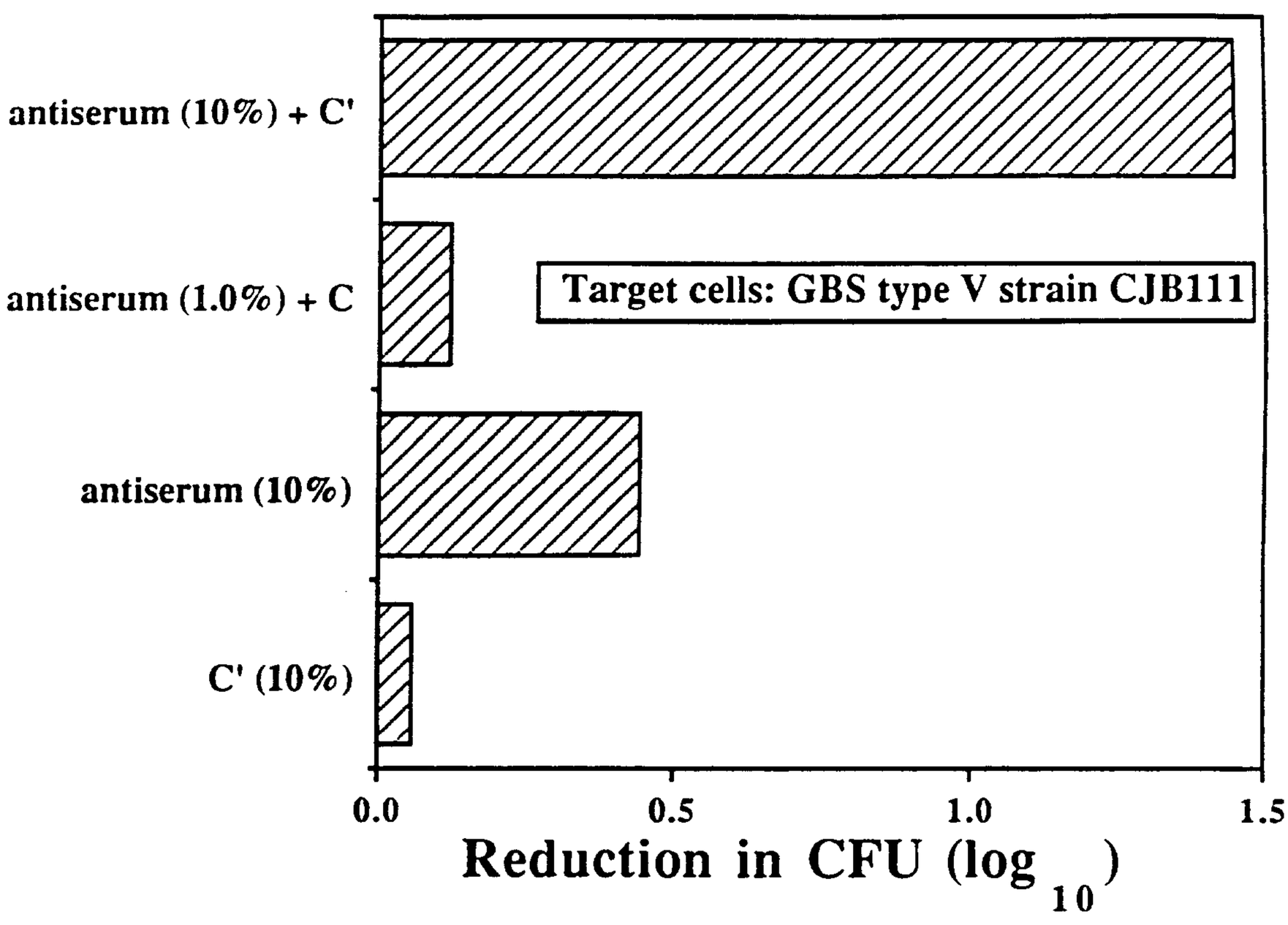
antiserum (10%) + C'
antiserum (1.0%) + C
antiserum (10%)
C' (10%)
Target cells: GBS type V strain CJB111
0.0
0.5
1.0
1.5
Reduction in CFU (log 10)

FIG. 6