Protective Levels of Human Immunoglobulin G Antibody to Group B Streptococcus Type Ib

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We studied the concentration of circulating human immunoglobulin G (IgG) antibody to the native capsular polysaccharide of group B streptococcus (GBS) type Ib necessary to protect mice against lethal challenge by laboratory and clinical GBS Ib strains. Antibody was measured by an enzyme-linked immunosorbent assay in which native polysaccharide antigen coupled to human serum albumin was used. The assay was standardized by a quantitative precipitation test, using native antigen and specific human IgG antibody purified by affinity chromatography. IgG anti-GBS Ib antibody level-protection curves for 90% lethal dose challenge of mice were sigmoidal. The curves of whole serum and affinity-chromatographed IgG anti-GBS Ib were superimposable. The serum concentrations of human antibody required for complete protection of mice varied with the infecting strain and ranged from 0.038 to 0.175 $\mu\text{g/ml}$. Protective levels of human IgG anti-GBS Ib were lower than those we found previously for homologous protection against GBS Ia challenge (range, 0.25 to 1.0 $\mu\text{g/ml}$).

Type I strains of group B streptococci (GBS) are subdivided into two serotypes, types Ia and Ib, based on related but antigenically and biochemically distinct capsular polysaccharides (9, 13). A third serotype, designated type Ic, contains the GBS Ia polysaccharide antigen and a protein, Ibc, which is also found in GBS Ib strains (20, 22). GBS Ib strains account for 5 to 8% of neonatal GBS infections (3, 21, 23).

Low or undetectable levels of antibody to the type-specific native polysaccharide antigens have been identified as a risk factor in neonatal GBS Ia (12) and GBS III (4, 5) infections. In a mouse protection test, we have shown that serum concentrations of $\geq 1.0 \mu\text{g}$ of human immunoglobulin G (IgG) antibody per ml to the type-specific native polysaccharide antigen of GBS Ia protect against 90% lethal dose (LD_{90}) challenge by stock and clinical strains of GBS Ia (12). Concentrations of type-specific antibody in sera from 11 infants infected with GBS Ia were all $< 1.0 \mu\text{g/ml}$. In the present study, we have determined the concentration of human IgG antibody to the native polysaccharide of GBS Ib necessary to protect mice against lethal challenge by stock and clinical strains of GBS Ib.

MATERIALS AND METHODS

Bacteria. GBS Ib stock strain SS618 (identical to Lancefield strain H36B/60/2 [13]) was obtained from Hazel Wilkinson at the Centers for Disease Control, Atlanta, Ga., and virulized by 17 additional mouse passages. In subsequent text, strain SS618 designates the original stock strain, and strain SS618/17 designates the passaged organism. GBS Ia stock strain SS615 (identical to Lancefield strain 090/14 [13]) also was obtained from Hazel Wilkinson and virulized by 28 additional mouse passages. In challenge experiments, this strain is designated SS615/28. Clinical GBS Ib strains No. Gr, Co, and Pry were blood isolates from infants with neonatal sepsis. All bacteria were grown to late-logarithmic phase in Todd-Hewitt broth and stored in portions at -70°C .

Groups of 8 to 12 21-day-old Cox/Swiss mice (Laboratory Supply Co., Indianapolis, Ind.) were challenged with various inocula to determine approximate LD_{90} s (Table 1).

Sera. Sera were obtained from healthy adult volunteer blood donors and screened for IgG antibody to GBS Ib by a semiquantitative immunofluorescent (IF) assay (19). Four positive sera, BB7, BB26, BB58, and TB, with titers between 1:160 and 1:640, were selected for use in these studies. Two of these sera, BB58 and TB, had low levels of IgG antibody to GBS Ia by an enzyme-linked immunosorbent assay (ELISA), < 0.03 and $0.20 \mu\text{g/ml}$, respectively (16). Sera BB7 and BB26 had high levels of IgG antibody against GBS Ia, 26.8 and $35.2 \mu\text{g/ml}$, respectively.

ELISA. Development of the ELISA was based on techniques previously described for GBS Ia (16). Using the method of Kane and Karakawa (10), we purified native GBS Ib polysaccharide from strain SS618. Monosaccharides were analyzed by gas-liquid chromatography after methanolysis, re-N-acetylation, and conversion to trimethylsilyl ethers (8). Desialylated type Ib core antigen was prepared by hot HCl extraction of bacterial cell pellets. The native antigen was coupled to human serum albumin with cyanogen bromide before adsorption to polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). The integrity of the coupled antigen relative to the native and core antigens was ascertained by Ouchterlony immunodiffusion analysis. The optimal amount of antigen added to ELISA wells was determined by checkerboard titration to be $0.625 \mu\text{g}$ of anthrone-reactive carbohydrate (15). For antibody assay, antigen-coated plates were rinsed with 0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline; test sera were added in $50\text{-}\mu\text{l}$ volumes and incubated at 37°C in a humidified chamber for 3 h. After washing three times with phosphate-buffered saline-Tween, $50 \mu\text{l}$ of appropriately diluted goat anti-human IgG (Atlantic Antibodies, Scarborough, Maine) conjugated with alkaline phosphatase was added as the secondary antibody. After rinsing, paranitrophenyl phosphate (Sigma Chemical Co.) was added as the substrate, and optical density was read at 405 nm (OD_{405}) in a Dynatech Microelisa reader. Wells

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TABLE 1. Stock and clinical strains of group B streptococcus serotype Ib used in passive protection experiments

Strain	Source	Manifestations	LD ₅₀ (CFU)
SS 618/17 ^a	Stock	— ^b	3 × 10 ³
No	Clinical	Late-onset sepsis, nonfatal	3 × 10 ¹
Gr	Clinical	Late-onset sepsis, nonfatal	6 × 10 ⁴
Pry	Clinical	Late-onset sepsis, nonfatal	4 × 10 ⁶
Co	Clinical	Early-onset sepsis, fatal	1 × 10 ⁸

^a Lancefield stock strain H36B/60/2, virulized by 17 additional mouse passages.

^b Original isolate obtained from the blood of an infected newborn infant (13).

primed with human serum albumin alone were used as a background control, which was subtracted from the optical density with coupled antigen.

The ELISA was standardized by quantitative precipitation of GBS Ib native antigen with affinity-purified IgG antibody to GBS Ib from two donors (TB and BB26) as previously described for IgG antibody to GBS Ia (16). The IgG fractions were obtained by 18% Na₂SO₄ precipitation and DEAE-cellulose chromatography and applied to a column containing GBS Ib-human serum albumin coupled to cyanogen bromide-Sepharose 4B. The column was washed with phosphate-buffered saline (pH 7.2) to an optical density at 280 nm of <0.01 (~10 column volumes), and purified IgG to GBS Ib was then eluted with 3 M NaSCN. Antigen-antibody reaction mixtures were incubated at 4°C for 3 days before centrifugation at 3,000 × g for 20 min. The precipitates were washed twice with cold normal saline, dissolved in 0.1 N NaOH, and assayed for protein relative to a human gamma globulin standard. After the supernatant fluids were precipitated with antigen, they were tested by ELISA to determine the proportion of nonprecipitating antibody. The proportion of nonprecipitating antibody at equivalence was used to correct the total antibody measurable by ELISA according to the following formula: protein in precipitate at equivalence/1 - (IgG anti-GBS Ib in supernatant fluid at equivalence/IgG anti-GBS Ib in supernatant fluid without antigen). A standard for ELISA was prepared by diluting IgG anti-GBS Ib from serum TB to a concentration of 10.0 µg/ml with human agammaglobulinemic serum. To compare the ELISA system with IF, we used ELISA to examine 49 sera from selected pregnant women previously tested by IF (19), with reciprocal antibody titers ranging from <1 (undetectable) to 320.

The specificity of the antibody reaction in ELISA was assessed by inhibition of assayable IgG anti-GBS Ib in serum BB26 with purified GBS Ib and Ia native polysaccharides at 37°C for 30 min over a carbohydrate concentration range from 0.5 to 125 µg/ml. Specificity of the assay was assessed by comparison of levels of IgG antibodies against GBS Ia and Ib in 50 random human sera measured by ELISA.

Mouse protection assay. Mice were given various amounts of human IgG antibody to GBS Ib intramuscularly in 0.1-ml injection volumes either as heat-inactivated whole serum (BB58 or TB) or as affinity-purified IgG anti-GBS Ib derived from serum BB7. Twenty-four hours after passive immunization, groups of four mice at selected dosages were bled by carotid transection, and serum from the pooled blood specimens was assayed for human IgG antibody to GBS Ib by ELISA. Serum pools were tested at four dosage levels in mice passively immunized with serum BB58, at five dosage levels in mice passively immunized with serum TB, and at

one dosage level in mice passively immunized with affinity-purified serum BB7. From the concentrations of human IgG anti-GBS Ib antibody in the pooled mouse sera, a log-log regression line of antibody concentration versus amount of antibody administered was developed by the least-squares method. Levels of human IgG antibody to GBS Ib in mice passively immunized with various doses were then calculated from the equation for the best linear fit of the data (2).

Groups of 6 to 10 experimental and control mice were challenged 24 h after passive immunization with LD₅₀ doses of stock and clinical strains of GBS administered intraperitoneally. Mice were observed for 5 days after bacterial challenge, and deaths were recorded. Antibody level-mortality curves were developed according to the percentage of deaths by day 5. Protective levels were estimated based on the mouse serum concentration of human IgG anti-GBS Ib antibody necessary for 100% protection against experimental challenge. We also compared the protection of mice from an LD₅₀ challenge by stock strain SS618/17, using comparable dosages of IgG anti-GBS Ib in whole and affinity-chromatographed sera.

RESULTS

GBS Ib antigens. Native GBS Ib polysaccharide antigen derived from strain SS618 contained galactose, glucose, *N*-acetylglucosamine, and sialic acid in a molar ratio of 2.00:1.06:1.02:0.99, respectively, as determined by gas-liquid chromatography. These results are close to the theoretical molar ratio of 2:1:1:1 for the GBS Ib antigen (9). The preparation contained 2.1% protein, 0.7% nucleic acids, and 0.5% group B antigen. Immunodiffusion with hyperimmune rabbit antisera to GBS Ia, Ib, II, and III showed complete identity with antisera to GBS types Ia and Ib and no reactions with antisera to GBS types II and III. The monosaccharide content of the antigen by gas-liquid chromatography after coupling to human serum albumin agreed with precoupling values within 10%. On Ouchterlony immunodiffusion with rabbit antiserum to GBS Ib, coupled antigen showed a single line of identity with native and core GBS Ib polysaccharides.

ELISA standardization. In quantitative precipitation of IgG anti-GBS Ib affinity purified from TB serum (Fig. 1), equivalence was attained when 1.06 µg of GBS Ib carbohydrate was precipitated by 11.4 µg of IgG. Since 46% of ELISA-assayable antibody was present in this reaction supernatant fluid, the preparation was calculated to contain 21.1 µg of IgG anti-GBS Ib per ml. A similarly high proportion of nonprecipitating antibody was found in assays with affinity-purified serum BB26 (30%) and whole serum TB (34%). A standard curve was developed relating the ELISA optical density at 405 nm to IgG anti-GBS Ib antibody content over a range of serial dilutions of affinity-purified TB serum in human agammaglobulinemic serum (Fig. 2). Serial dilution of whole TB serum in phosphate-buffered saline yielded a comparable curve.

ELISA sensitivity and specificity. IgG anti-GBS Ib antibody was detectable by ELISA to a sensitivity limit of ca. 0.015 µg/ml. Antibody content in selected sera as measured by ELISA and IF correlated well ($r = 0.85$; $P < 0.001$), but we noted a small number of sera with detectable antibody by ELISA that were negative by IF, and vice versa (Fig. 3).

In inhibition experiments, a starting IgG anti-GBS Ib concentration of 4.8 µg/ml was reduced to 1.2 µg/ml (24%) with 5 µg of native Ib polysaccharide per ml and to 0.26 µg/ml (5.5%) with 50 µg of native Ib polysaccharide per ml. With 50 µg of Ia native polysaccharide per ml, the antibody

FIG. 1. Specific precipitation of anti-GBS Ib antibodies from affinity-chromatographed serum TB. Precipitated antibodies (●) and the proportion of ELISA-assayable antibodies in supernatant fluids (○) are designated.

concentration was reduced to 1.8 µg/ml (38%). There was no correlation between levels of IgG antibodies against GBS Ia and Ib measured by ELISA in random human sera (Fig. 4; $r = 0.20$; $P > 0.10$).

Quantitation of antibody after passive immunization. Human IgG antibody to GBS Ib was detectable at concentrations of 0.014 and 0.017 µg/ml in the sera of mice 24 h after intramuscular administration of as little as 0.037 µg of antibody. Concentrations of antibody measured in mice passively immunized with serially diluted human sera were linear (Fig. 5; $r = 0.996$; $P < 0.001$).

Passive protection from challenge with GBS Ib strains. The amount of antibody required for protection of mice against an LD₉₀ challenge varied with the strain (Fig. 6A). LD₉₀ inocula varied over a wide range from 3×10^1 to 1×10^8

CFU. A lower level of antibody, 0.038 µg/ml, was necessary for protection against strains SS618/17 and No, which had LD₉₀s of $<10^4$ CFU. Greater concentrations of antibody, 0.082 and 0.175 µg/ml, were needed for protection against the three clinical strains requiring higher inocula for an LD₉₀. Animals were completely protected against LD₉₀ doses of all strains with an antibody concentration of ≥ 0.175 µg/ml.

Passive protection with type-specific IgG antibody. Whole serum and affinity-chromatographed IgG antibody to GBS Ib yielded superimposable antibody level-mortality curves when animals were challenged with strain SS618/17 (Fig. 6B). Purified antibody gave 100% protection at a concentration of 0.030 µg/ml.

Passive protection against heterologous challenge. Passively

FIG. 2. ELISA standard curve. Symbols: ○, serially diluted IgG anti-GBS Ib from affinity-chromatographed serum TB, mean of 4 to 10 determinations $\pm$ standard deviation; ●, IgG anti-GBS Ib in serially diluted whole serum TB, mean of 4 to 10 determinations $\pm$ standard deviation.

FIG. 3. Comparison of IgG antibody to GBS Ib, measured by ELISA and IF assay in 49 selected human sera ($r = 0.85$; $P < 0.001$).

immunized mice challenged with LD₅₀ doses of GBS Ia strain SS615/28 were not protected by up to 0.82 µg of IgG anti-GBS Ib per ml (Table 2). Conversely, IgG anti-GBS Ia in concentrations of up to 2.0 µg/ml did not protect against GBS Ib challenge.

Comparison with type-specific passive protection against GBS Ia. Protection curves of homologous challenge by GBS Ia and GBS Ib stock and clinical strains are compared in Fig. 6C. Lower levels of homologous antibody were required for protection against GBS Ib challenge than were necessary for homologous protection against GBS Ia challenge in comparable experiments (12).

DISCUSSION

Human immunity to GBS Ib has previously been assayed by indirect, semiquantitative techniques in which whole bacteria, with either fluoresceinated anti-human IgG (18, 19) or radiolabeled staphylococcal protein A (7), were used as secondary reagents. In this study, we have extended the use of a quantitative ELISA system to the determination of human IgG antibody to GBS Ib and documented the striking relationship between levels of circulating antibody and protection against lethal challenge in the mouse model.

The importance of antibody in protection against experimental GBS infection was clearly demonstrated in the classic passive protection studies of Lancefield in the 1930s, in which hyperimmune rabbit sera were used (13). Although other host immune factors, such as complement activation and phagocytic capacity, and microbial factors, such as inoculum size, organism virulence, and route of infection, are confounding variables (6), the concept of a critical protective level of antibody is implicit in the work of

FIG. 4. Comparison of IgG antibodies to GBS Ia and Ib, measured by ELISA in 50 random human sera ($r = 0.20$; $P > 0.1$).

FIG. 5. Levels of IgG anti-GBS Ib in mice passively immunized with serially diluted serum BB58 (●), serum TB (◆), and affinity-chromatographed serum BB7 (△). Least-squares best fit for mouse serum levels: $\log_{10}y = 1.107 \log_{10}x - 0.460$; $r = 0.996$; $P < 0.001$.

Lancefield and in several recent studies of infection by other encapsulated organisms (11, 14). The definition used for protection in the present studies is somewhat arbitrary; however, we have found that it applies to human immunity to GBS Ia (12). In view of the paucity of neonatal invasive infections by GBS Ib, the present study in a model system provides a useful adjunct to studies of antibody levels in human populations (Gotoff et al., in press).

The ELISA used in the present study, although identical in concept, differed somewhat in its standardization and specificity from our previous ELISA experience with GBS Ia. We were struck by the large proportion (30 to 46%) of affinity-purified antibody against GBS Ib polysaccharide which was nonprecipitating. It is conceivable that insufficient incubation time of quantitative precipitation could account for this finding. However, in standardizing our assay for antibody against GBS Ia, we incubated our quantitative precipitation reaction mixtures for 24 h less than that in the present work and found no more than 5% of nonprecipitating antibody at equivalence. Regardless of mechanism, ELISA of antibody in supernatant fluid after precipitation permitted accurate quantitation of total antibody activity.

Although the ELISA system may be more sensitive than the IF assay, the finding that some sera determined to be negative by ELISA were positive by IF emphasizes the greater diversity of surface antigens of GBS Ib, including Ibc protein, than of GBS Ia. Although these observations suggest that antibodies against Ibc may occur naturally, the low IF titers in the sera and the superimposability of protection curves for affinity-purified and whole serum rule against a major contribution of these antibodies to natural immunity against GBS Ib.

The observation that unabsorbed rabbit antisera against GBS Ia and Ib yielded precipitation lines of identity with

purified GBS Ib antigen is similar to the findings of Tai et al. (17) and is consistent with the presence of the Iabc determinant in the purified polysaccharide molecule (1, 9). Cross-reacting antibodies against this determinant do not appear to

FIG. 6. Passive protection of mice against LD₉₀ challenge. (A) Protection against GBS Ib strain SS618 (●) and four clinical GBS Ib strains (○) provided by whole serum, according to mouse serum levels of human IgG anti-GBS Ib. (B) Protection against GBS Ib strain SS618/17 provided by whole (●) and affinity-chromatographed (○) sera, according to mouse serum levels of human IgG anti-GBS Ib. (C) Protection of mice against LD₉₀ challenge by stock and clinical strains of GBS Ia (■, □) and GBS Ib (●, ○), respectively, provided by whole serum, according to mouse serum levels of homologous antibody.

TABLE 2. Homologous and heterologous challenge experiments with stock strains of group B streptococcus serotype Ia and Ib^a

Mouse serum concn of IgG-anti-GBS (μg/ml)		Mouse mortality with LD ₉₀ challenge by GBS ^b			
		Ia (SS 615/28)		Ib (SS618/17)	
Ia	Ib	D/I	%	D/I	%
		31/32 ^c	97	32/35	91
0.03		15/16	94	ND	
0.06		13/16	81	5/6	83
0.12		9/16	56	3/6	50
0.25		1/16	6	5/6	83
0.50		0/15	0	4/8	50
1.0		0/8	0	7/8	87
2.0		0/8	0	4/6	67
	0.002	ND		15/16	94
	0.004	ND		12/16	75
	0.008	6/6	100	16/16	100
	0.018	6/6	100	6/14	43
	0.038	6/6	100	0/24	0
	0.08	4/6	67	0/6	0
	0.18	6/6	100	0/8	0
	0.38	5/6	83	0/8	0
	0.82	3/4	75	0/12	0

^a Experiments were done with stock strains of group B streptococcus serotype Ia (strain SS615/28) and Ib (strain SS618/17) in 21-day-old mice protected with whole sera JM (IgG anti-GBS Ia level, 40.0 μg/ml; anti-GBS Ib level, <0.03 μg/ml) and BB58 (IgG anti-GBS Ib level, 13.9 μg/ml; anti-GBS Ia level, <0.03 μg/ml).

^b D/I, Number dead/number injected on day 5 of observation. ND, Not done.

^c Controls received no passive protection.

have an important effect on the specificity of the GBS Ib ELISA in human sera, however, since inhibition experiments demonstrated a marked discrepancy between inhibition of IgG anti-GBS Ib antibody by native Ia and Ib polysaccharides. Assay of random sera yielded no correlation between Ia and Ib antibody content. Moreover, moderate concentrations of human antibody to GBS Ib failed to provide cross protection in mice lethally challenged with GBS Ia, and vice versa.

Because of the specificity of the ELISA system for human IgG antibody to GBS Ib, we were able to document levels of antibody in mouse serum at the time of lethal challenge, as we have previously done with GBS Ia. In contrast to our previous work with GBS Ia clinical strains, we found a wide range of dosages of GBS Ib clinical strains necessary for an LD₉₀. Despite the varied virulence of challenge strains, critical protective levels of IgG anti-GBS Ib were remarkably similar. In general, highly virulent strains that required a low infectious challenge for an LD₉₀ required somewhat less antibody for protection than did strains of low virulence that required larger inocula for comparable lethality.

Differences in organism virulence, however, did not appear to account for the clear differences in critical protective levels of homologous antibody required for protection against strains of GBS Ia and GBS Ib. We considered the possibility that these results were caused by an error in ELISA standardization, but restandardization of the GBS Ib assay yielded results comparable with our original standard. Thus, these differences in critical protective levels suggest that the antibody requirements for each GBS serotype may vary and should be determined experimentally. Based on the present studies, IgG anti-GBS Ib antibody concentrations of ≥0.2 μg/ml appear to be a conservative estimate of critical protective levels.

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