

DEMONSTRATION OF OPSONIC ACTIVITY
AND IN VIVO PROTECTION AGAINST GROUP B
STREPTOCOCCI TYPE III BY
STREPTOCOCCUS PNEUMONIAE TYPE 14 ANTISERA

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Life-threatening group B streptococcal infections in neonates have become a major problem throughout the United States and many other parts of the world (1). It has been estimated that within the United States, $\approx 15,000$ babies each year develop sepsis or meningitis due to this organism, with a high rate of morbidity and mortality (1). Why this disease has emerged as an increasing problem over the last 10 yr is not known. Recent studies have suggested that a deficiency of type-specific antibody in newborn infants may be an important factor in predisposing them to developing group B streptococcal disease (2).

Immunity to *Streptococcus pneumoniae* and *Streptococcus agalactiae* (group B streptococcus) is primarily related to their capsular polysaccharide antigens (3, 4). Opsonophagocytic studies have demonstrated type specificity of opsonic antibody directed against each of the five types of group B streptococci, although occasional cross-reactions have been detected (3).

Using immunoelectrophoresis, we recently demonstrated that antisera directed against *S. pneumoniae* type 14 reacts with the hot HCl-extracted polysaccharide antigen of group B streptococci type III (5). The present studies were designed to determine if antisera directed against *S. pneumoniae* type 14 are opsonic for group B streptococci type III in a neutrophile bactericidal assay and afford protection in a suckling rat model of neonatal group B streptococcal type III sepsis.

Materials and Methods

Streptococcal Strains. Group B streptococcal strain, IIINor, isolated from the cerebrospinal fluid of an infant with meningitis was used in most of these studies. Precipitin and opsonic analysis confirmed IIINor to be a type III strain. For neutrophiles to demonstrate bactericidal activity against IIINor, both type-specific antibody and complement were required. Group B streptococci type III, strain SS620, and group B streptococci 090R, a strain devoid of type-specific antigen (6), were kindly supplied by Dr. Hazel Wilkinson, Center for Disease Control, Atlanta, Ga. *S. pneumoniae* type 14 strain SP14 and type 3 strain SP42 were kindly supplied by Dr. Byrd Smith, also of the Center for Disease Control. Organisms were grown to mid log phase in Todd-Hewitt broth, and stored at -70°C until used.

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Preparation of Antisera. Adult New Zealand white rabbits weighing ≈ 3 kg were injected intravenously with formalin-killed group B streptococci type III, *S. pneumoniae* type 14, or *S. pneumoniae* type 3, according to the general protocol of Wilkinson and Moody (7). Sera from all animals were evaluated before immunization to insure the absence of opsonic antibody to the study organisms. The organisms were cultured overnight at 37°C in 200 ml of Todd-Hewitt broth. The cells were sedimented by centrifugation, washed, and resuspended in 0.2% formalin in phosphate-buffered saline for 5–7 days at 4°C. Cultures were performed daily to assess sterility. After dilution to restore the original concentration, 1.0 ml of the nonviable bacterial suspension was given daily for 5 days followed by a 1-wk rest. This series of injections were repeated three times. After a 1-mo rest, the animals were given two additional series of injections.

The animals were bled via cardiac puncture 4–5 days after the third and fifth series of injections. Serum was removed from the clotted blood, heat-inactivated at 56°C for 30 min, and stored in 0.5-ml aliquots at -70°C .

Neutrophile Opsonophagocytic Bactericidal Assay. Neutrophils were isolated from normal adult volunteers by dextran sedimentation followed by Ficoll-Hypaque density centrifugation (8). The cell population used consisted of $>98\%$ viable neutrophils. These cells were washed once in normal saline and then resuspended at a concentration of 25×10^6 cells/ml. The bactericidal capability of neutrophils was tested in a modified form of the assay of Hirsch and Strauss (9). Use of a microtiter plate technique to measure neutrophil-mediated bactericidal activity was developed by Dr. J. C. Sadoff.¹ The microtiter plate technique (modified so that bacteria could be adequately dispersed by vigorous vortex and/or sonication) described by Cross and Lowell² was used in this study with only minor modification. The technique requires a total volume of 0.1 cc/well and allows efficient testing of numerous variables using small amounts of sera and neutrophils. For the assay, bacteria were taken during mid log phase growth in Todd-Hewitt broth, washed once, and then appropriately diluted in Eagle's medium. 10 μl (containing $0.4\text{--}4.0 \times 10^6$ bacteria) were added to round-bottom microtiter wells along with 40 μl of neutrophils (containing $0.5\text{--}2.0 \times 10^6$ neutrophils), 10 μl of newborn rabbit serum (screened for absence of antibody activity against test organisms) as a source of active complement, and 40 μl of various dilutions of heat-inactivated pre- or post-immunization rabbit serum. Control wells in every experiment consisted of (a) neutrophils alone, (b) complement alone, and (c) neutrophils plus complement. In addition, serum with known opsonic activity and serum known to lack opsonic activity were included as positive and negative controls in each experiment. The microtiter plates were then sealed with pre-cut acetate sealing tape (Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Va.) and incubated at 37°C with constant vigorous shaking.

To determine neutrophil bactericidal activity, 10- μl samples were taken from each well at zero time and also after 1 and 2 h of incubation. Before plating the bacteria onto blood agar, the samples were appropriately diluted in test tubes containing 0.1% bovine serum albumin in distilled water; the samples were then vigorously vortexed. This ensured that neutrophils were selectively lysed and that bacteria were adequately dispersed.

Neutrophil bactericidal activity was calculated by the formula $100 - (100 \times (\text{number of bacteria at 60 or 120 min}) / (\text{number of bacteria in the initial inoculum}))$; 0% represents bacterial growth.

Absorption of Antisera. Sera collected after five series of injections were absorbed. Adsorbing bacteria were grown overnight on blood agar, scraped from the plate, suspended in normal saline, and then pelleted in 0.5-ml microfuge tubes to one-fifth the volume of the tube. After adding 0.4 ml of serum, the tubes were vortexed and then rotated at slow speed on an end-over-end tumbler (Fisher Rotorack model 343, Fisher Scientific Co. Pittsburgh, Pa.) at 4°C overnight. The following day the bacteria were sedimented in a microfuge tube and the supernatant sera were removed and filtered through a 0.2- μm membrane filter. The sterile sera were then used either directly or after storage at -70°C .

Neonatal Sepsis Model. Pregnant Wistar rats were obtained from a commercial breeder

¹ J. C. Sadoff. Manuscript in preparation.

² A. S. Cross and G. H. Lowell. Stimulation of polymorphonuclear leukocyte bactericidal activity by supernatants of activated human mononuclear cells. Manuscript submitted for publication.

(Charles River Breeding Laboratories, Wilmington, Mass.), housed on polycarbonate cages with hardwood litter, and given antibiotic free food and water ad libitum.

Bacteria inocula frozen in Todd-Hewitt broth were thawed and subcultured on blood agar plates the day before animal injection. Bacteria scraped from the blood agar plate were suspended in Todd-Hewitt broth to the desired optical density (0.03 at 650 nm using a Coleman spectrophotometer from Coleman Systems, Irvine, Calif.) and placed in a shaker bath at 37°C for $\approx 2\frac{1}{2}$ h. When the desired optical density (0.6) was achieved, the organisms were sedimented by centrifugation, washed once, and then resuspended in Todd-Hewitt broth to a concentration of $\approx 10^7$ colony-forming units/ml.

Suckling rats (2–3 days old) were injected subcutaneously with 0.02 ml ($\approx 10^5$ viable bacteria) of the group B streptococcal suspension administered just cephalad to the tail. Immediately after inoculation, the study animals were given an intraperitoneal injection (0.1 or 0.2 ml of antisera) directed against group B streptococci type III or *S. pneumoniae* types 3 or 14. Controls consisted of untreated littermates. All animals were observed daily for the study period of 5 days to determine survival. In this model, deaths are rarely encountered later than 72 h after infection.

Antisera used in these studies were of relatively low titer (obtained after three series of injections) except in one litter (no. 53) in which high titer antiserum (obtained after five series of injections) was utilized.

Results

Opsonophagocytic Bactericidal Studies

HOMOLOGOUS ANTISERA. The ability of neutrophiles to phagocytize and kill group B streptococci type III (IIINor) and *S. pneumoniae* type 14 was studied in the presence of antisera directed against these organisms. After 1 and 2 h of incubation, >90% of the initial inoculum were killed (Figs. 1 and 2). In the absence of serum, or in the presence of sera obtained before immunization, neutrophiles did not impede bacterial growth (Figs. 1 and 2).

HETEROLOGOUS ANTISERA. Antisera were analyzed for activity against heterologous organisms. Antisera directed against *S. pneumoniae* type 14 enabled neutrophiles to kill >90% of an inoculum of group B streptococci type III (Fig. 1). Significant killing by neutrophiles was also accomplished when *S. pneumoniae* type 14 were opsonized with type III group B streptococcal antisera (Fig. 2).

EFFECT OF DILUTION OF SERUM ON OPSONOPHAGOCYtic BACTERICIDAL ACTIVITY. The data in Table I are based on sera obtained after three series of injections and describe their activity against group B streptococci type III. These sera are of relatively low titer. At a dilution of 1:10, anti-group B streptococcal type III serum enabled neutrophiles to kill >85% of the initial inoculum after both 1 and 2 h of incubation.

When sera directed against *S. pneumoniae* type 14 were used, comparable neutrophile-mediated bactericidal activity was observed at a serum dilution of 1:5. In contrast, antisera directed against *S. pneumoniae* type 3 did not opsonize group B streptococci type III to allow significant neutrophile killing.

Since three series of injections resulted in relatively low titers of opsonophagocytic bactericidal activity, rabbits were immunized with two further series of injections. Sera obtained from these animals demonstrated significantly higher titers (Fig. 3). These data represent the averaged results of six separate experiments. Antisera induced by *S. pneumoniae* type 14 were virtually as effective as the homologous antisera in facilitating the killing of group B streptococci type III, e.g. >90% kill after 2 h incubation at 1:80 dilution.

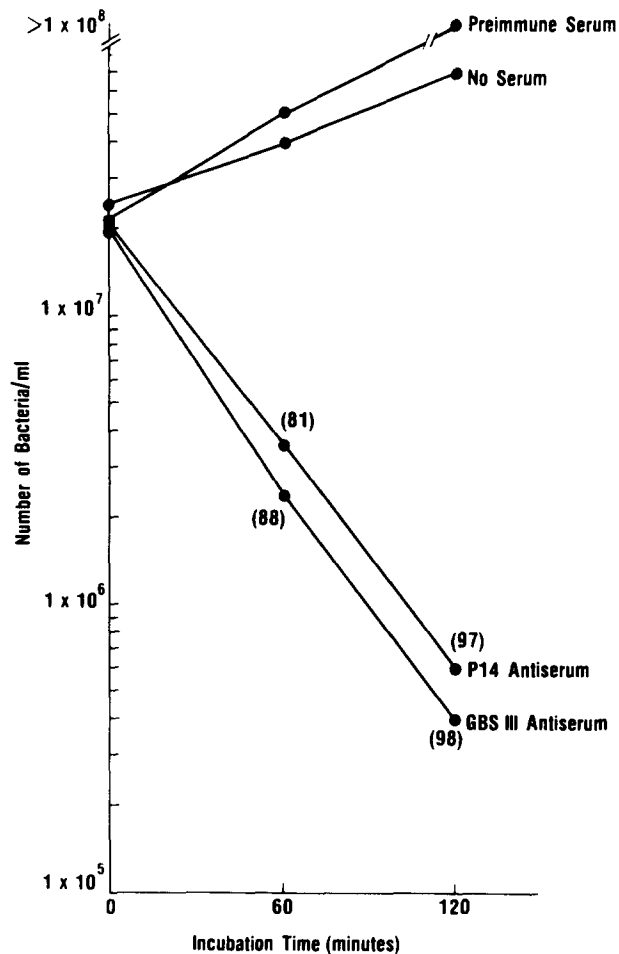


FIG. 1. Effect of rabbit antisera on group B streptococci type III in a neutrophilic bactericidal assay. Conditions for the assay were as follows: no serum given; serum obtained before immunization (preimmune); antisera directed against group B streptococci type III (GBS III); antisera directed against *S. pneumoniae* type 14 (P14).

Studies similar to those using group B streptococci type III were performed using *S. pneumoniae* type 14 as the test organism (Fig. 4). Review of the data presented in Figs. 3 and 4 shows that the heterologous activity of anti-*S. pneumoniae* type 14 serum against group B streptococci type III is comparable to the activity against *S. pneumoniae* type 14 itself. Interestingly, antisera directed against group B streptococci type III did not show the same degree of cross-reactivity.

ANTIGENIC SPECIFICITY OF RABBIT ANTISERA. Absorption studies were performed to determine the specificity of the opsonic reaction to group B streptococci type III (Table II). When antisera directed against *S. pneumoniae* type 14 was adsorbed with *S. pneumoniae* type 14 or group B streptococci type III organisms (IIINor or SS620), opsonophagocytic bactericidal activity against group B streptococci type III was abolished. In contrast, after absorption with group B streptococci 090R (a strain devoid of type-specific antigen), or with *S. pneumoniae* type 3, bactericidal activity

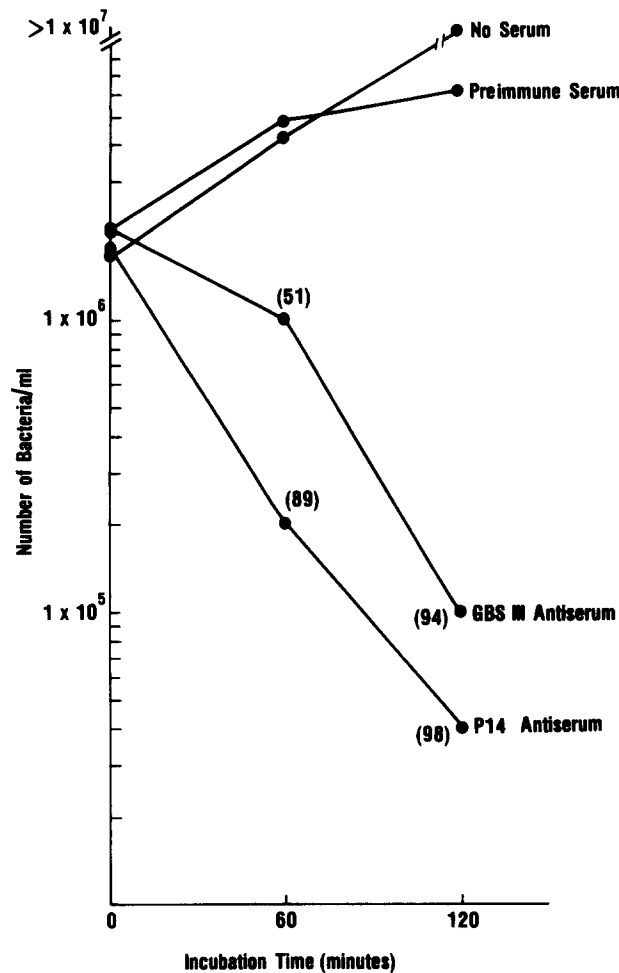


FIG. 2. Effect of rabbit antisera on *S. pneumoniae* type 14 in a neutrophilic bactericidal assay. Conditions for the assay were: no serum given; serum obtained before immunization (preimmune); antisera directed against group B streptococci type III (GBS III); antisera directed against *S. pneumoniae* type 14 (P14).

persisted. *S. pneumoniae* type 14 was as effective as group B streptococci type III in absorbing opsonic activity from serum directed against group B streptococci type III (Table II).

Protection Studies. Administration of rabbit antisera directed against the homologous group B streptococci type III (IIIINor) and administration of heterologous antisera against *S. pneumoniae* type 14 both resulted in significant survival in this model of group B streptococcal sepsis (Fig. 5). Neonatal rats given rabbit *S. pneumoniae* type 3 antisera and those animals not given antisera did not survive. 55 of 59 control animals (this includes animals given *S. pneumoniae* type 3 antisera as a serum control and those animals not treated with antisera) were dead within 48 h of infection and all control animals had died by 72 h. In contrast, 20 of 22 animals given antisera directed against group B streptococci type III and 17 of 18 animals given anti-pneumococcal type 14 antisera were still alive at the end of the 5-day study period.

TABLE I
The Effect of Low-Titer Rabbit Antisera on PMN Bactericidal Activity Against GBS III

Serum	Serum dilution	No. of viable bacteria/ml $\times 10^{-6}$			PMN bactericidal activity*	
		0 min	60 min	120 min	60 min	120 min
					%	
None	0	8.6	17.2	30.4	0	0
Preimmune	1/2.5	6.2	17.2	27.6	0	0
	1/5	7.0	14.0	22.0	0	0
	1/10	6.4	16.8	28.0	0	0
	1/20	6.0	16.4	22.8	0	0
	1/40	6.5	13.2	24.4	0	0
	Anti-GBS III	1/2.5	7.3	0.3	0.1	96
1/5		9.6	1.7	0.2	82	98
1/10‡		19.2	1.2	0.8	94	96
1/20§		14.0	3.7	6.0	74	57
1/40		24.0	16.4	9.2	32	61
1/80		11.2	6.5	30.8	0	0
Anti-P14	1/2.5	9.8	1.2	1.5	99	85
	1/5‡	14.8	1.9	0.8	87	95
	1/10	11.7	6.2	6.9	47	41
	1/20§	15.6	7.1	4.9	54	69
	1/40	8.0	6.7	8.6	16	0
	1/80	6.8	8.9	10.4	0	0
Anti-P3	1/2.5	7.0	3.2	64.0	55	0
	1/5	18.8	16.0	77.2	11	0
	1/10	14.4	9.2	85.6	36	0
	1/20	13.2	78.8	80.0	0	0
	1/40	11.6	30.8	80.0	0	0

GBS III, group B streptococci type III; P14, *S. pneumoniae* type 14; P3, *S. pneumoniae* type 3; PMN, polymorphonuclear neutrophils.

* Percent bacteria killed compared to number of viable bacteria present in the initial inoculum (0 min) according to the formula shown in Materials and Methods.

‡ Highest titer at which >80% of bacteria killed after both 60 and 120 min of test incubation.

§ Highest titer at which >50% of bacteria killed after both 60 and 120 min of test incubation.

Discussion

Over the last several years, group B streptococcal infections have become increasingly recognized as a major cause of neonatal infection (8-18).² In some areas, group B streptococci comprise the single most common cause of bacterial sepsis and meningitis in newborns (19-21). Recently, the absence of type-specific antibody in maternal and cord sera has been shown to be a major determinant for the development of invasive neonatal group B streptococcal disease (2). The observation with immunoelectrophoresis that the hot HCl-extracted polysaccharide antigen of group B streptococci type III reacted with antisera directed against *S. pneumoniae* type 14 (5) suggested that a common antigen was present in both organisms. The present studies demonstrate that antisera directed against *S. pneumoniae* type 14 not only facilitates opsonophagocytic bactericidal activity but also protects suckling rats against death due to type III group B streptococcal sepsis.

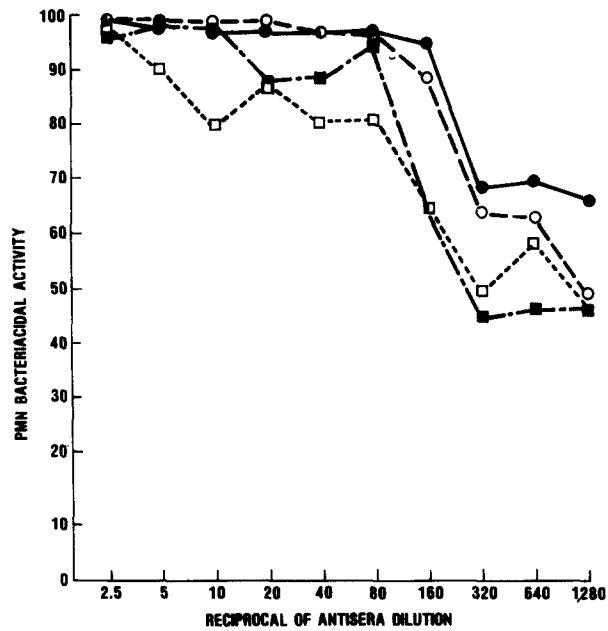


FIG. 3. The effect of high-titer rabbit antisera on neutrophil bactericidal activity against group B streptococci type III. Antisera directed against group B streptococci type III (GBS III) were incubated for 120 min (●) and 60 min (○). Antisera directed against *S. pneumoniae* type 14 (P14) were incubated for 120 min (■) and 60 min (□).

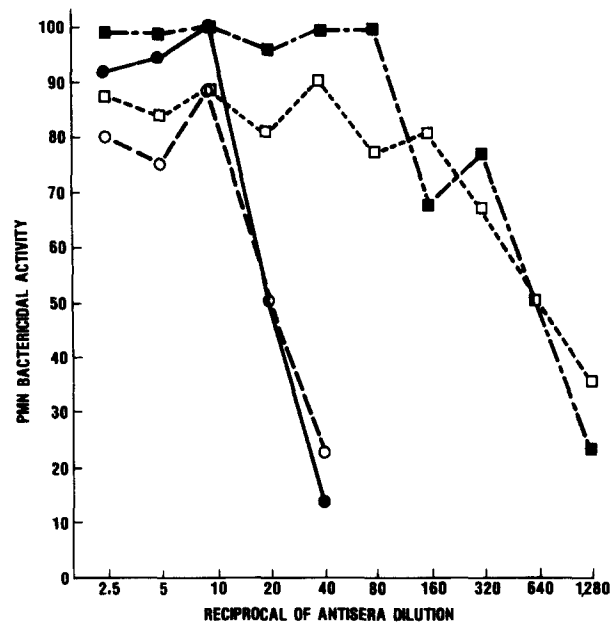


FIG. 4. The effect of high-titer rabbit antisera on neutrophil bactericidal activity against *S. pneumoniae* type 14. Antisera directed against group B streptococci type III (GBS III) were incubated for 120 min (●) and 60 min (○). Antisera directed against *S. pneumoniae* type 14 (P14) were incubated for 120 min (■) and 60 min (□).

TABLE II
The Effects of Adsorption with Homologous or Heterologous Bacteria on the Ability of Rabbit Antisera to Opsonize GBS III

Antiserum	Adsorbing bacteria	No. of viable bacteria/ml $\times 10^{-6}$		PMN bactericidal activity against GBS III at 120 min*
		0 min	120 min	
				%
P14‡	None	4.3	0.2	95
	P14	6.6	>8.0	0
	GBS III (Nor)	10.4	28.0	0
	GBS III (620)	5.0	>8.0	0
	GBS 090R	5.6	0.7	87
	P3	18.0	0.4	98
GBS III§ (Nor)	None	7.5	0.04	99
	GBS III (Nor)	5.8	>8.0	0
	P14	31.2	54.8	0

GBS III, group B streptococci type III; GBS 090R, group B streptococci strain 090R; P14, *S. pneumoniae* type 14; P3, *S. pneumoniae* type 3; PMN, polymorphonuclear neutrophils.

* Percent bacteria killed compared to number of viable bacteria in the initial inoculum (0 min) according to the formula shown in Materials and Methods.

‡ Serum titers used = 1/20.

§ Serum titers used = 1/10.

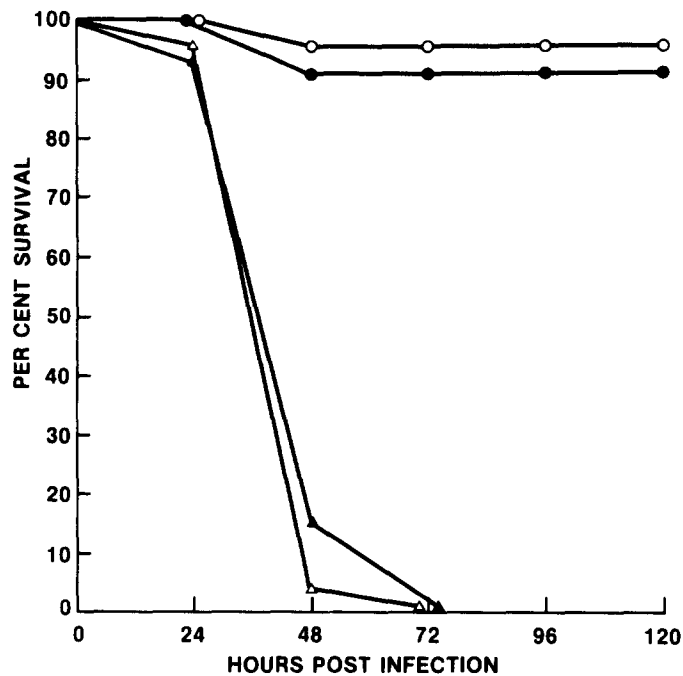


FIG. 5. Effect of type-specific antisera on the survival of suckling rats with experimental type III group B streptococcal sepsis. Antisera were directed against *S. pneumoniae* type 14 (P14) (○); group B streptococci type III (GBS III) (●); *S. pneumoniae* type 3 (P3) (▲); and no serum given (None) (△).

Absorption studies with both *S. pneumoniae* type 14 and group B streptococci type III demonstrated that the opsonic antibody in both antisera is directed against common antigenic determinants. The fact that group B streptococci type III, strain SS620 also absorbs out the bactericidal activity of antisera directed against *S. pneumoniae* type 14 further suggests that the common antigenic determinant is not strain-specific for the group B streptococci type III, III^{Nor}, clinical isolate. The 090R group B streptococci (devoid of type-specific antigen) and the *S. pneumoniae* type 3 did not remove the opsonic activity. This indicates that the cross-reacting antigen is type-specific. These data are consistent with the earlier findings of cross-reactivity between the antibody directed against *S. pneumoniae* type 14 and the hot HCl-extracted polysaccharide capsular antigen of group B streptococci type III (5).

The chemical composition of the capsular polysaccharide antigens of many types of pneumococci have been studied (4). The *S. pneumoniae* type 14 capsule has been shown to contain the amino sugar glucosamine and the neutral sugars galactose and glucose, and its structure has been elucidated (4, 22). Russell and Norcross (23) and Baker et al. (24) have reported that the type-specific polysaccharide antigen of group B streptococci type III also contains glucosamine, galactose, and glucose. The immunochemical basis for the cross-reaction we have described may be related to this similarity in capsular composition. Strains of other bacterial species cross-reacting with *S. pneumoniae* type 14 have also been reported (25). Numerous cross-reactions between pneumococci (non-type 14) and various streptococci (non-group B) have also been shown (26). The biochemistry of these cross-reactions, however, is not clear.

Protection studies using a suckling rat model of neonatal type III group B streptococcal sepsis demonstrated that antisera directed against *S. pneumoniae* type 14 is highly protective. Group B streptococci type III antisera provided similar protection in this model. Since antisera directed against *S. pneumoniae* type 3 were not effective, it seems unlikely that protection is related to a nonspecific serum factor. This inability of antisera directed against *S. pneumoniae* type 3 to afford in vivo protection is further evidence that the antigen against which protective antibodies are directed is closely associated with the capsular polysaccharide of type 14 pneumococci.

The protection studies reported in this paper were performed with rabbit antisera of relatively low titer collected after three series of injections. Preliminary data from our laboratories suggest that the anti-group B type III streptococcal opsonophagocytic bactericidal activity of the sera of most adults is of similarly low titer. Therefore, antibody in maternal serum directed against *S. pneumoniae* type 14 may be able to provide protection against group B streptococci type III in the newborn child.

With this information, one might speculate that naturally acquired immunity to group B streptococci type III may be related to the development of antibodies secondary to childhood pneumococcal infections. Interestingly, *S. pneumoniae* type 14 is one of the most common causes of pneumococcal infections in childhood (27). Since currently available pneumococcal vaccines have been shown to induce antibodies directed against *S. pneumoniae* type 14 (28), protection against neonatal group B streptococci type III disease may be possible by maternal immunization using these vaccines.

Summary

The present studies demonstrate that antisera directed against *Streptococcus pneumoniae* type 14 is opsonic for group B streptococci type III in a neutrophil-mediated

bactericidal assay. Specificity was demonstrated by the observations that group B streptococci type III and *S. pneumoniae* type 14 adsorbed the opsonic activity of anti-*S. pneumoniae* type 14 antisera. Group B streptococci strain 090R (devoid of type antigens) and *S. pneumoniae* type 3, did not remove the opsonic activity of anti-*S. pneumoniae* type 14 serum. In vivo studies using a suckling rat model of neonatal group B streptococcal type III sepsis demonstrated that antisera directed against *S. pneumoniae* type 14 was highly protective.

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