A randomized trial of conjugated group B streptococcal type Ia vaccine in a rabbit model of ascending infection

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OBJECTIVE: Maternal vaccination may become a central strategy in the prevention of early-onset group B Streptococcal sepsis. Unlike earlier group B streptococcal polysaccharide vaccines that were poorly immunogenic, newer vaccines conjugated to tetanus toxoid have been developed and have improved immunogenicity. We sought to evaluate a conjugated vaccine using our rabbit model of ascending infection.
STUDY DESIGN: Rabbit does were randomized to receive either conjugated group B streptococcal type Ia (Ia–tetanus toxoid) or conjugated group B streptococcal type III (III–tetanus toxoid) vaccine. Does were vaccinated 7 days before conception and 7 and 21 days after conception. On days 28 to 30 of a 30-day gestation, does were inoculated intracervically with 10^6 colony-forming units of type Ia group B *Streptococcus*. Labor was induced if does were undelivered after 72 hours. Does were observed up to 7 days after inoculation. Offspring were observed up to 4 days. We obtained maternal cultures from the uterus, peritoneum, and blood and offspring cultures from the mouth, anus, and blood. Antibody levels were also determined.
RESULTS: Offspring survival was significantly improved in the group receiving Ia–tetanus toxoid ($P = .047$). Outcomes such as maternal sepsis and severe illness, although not reaching statistical significance, showed a trend toward improved outcomes in the Ia–tetanus toxoid group.
CONCLUSIONS: This is the first study to evaluate the conjugated group B streptococcal vaccine by using any model of ascending infection. The Ia–tetanus toxoid vaccine led to improved survival and was immunogenic but fell short of its expected efficacy in preventing ascending group B streptococcal disease under these experimental conditions. (Am J Obstet Gynecol 1999;181:803-8.)

Key words: Group B *Streptococcus*, vaccination, intra-amniotic infection, rabbit, neonatal sepsis

Early-onset neonatal sepsis caused by group B *Streptococcus*, which is defined as onset within the first 7 days of life, accounts for approximately 80% of neonatal group B streptococcal disease and is caused by vertical transmission from colonized mothers. Several maternal risk factors for early-onset neonatal disease have been identified, including low levels of maternal antibody.[1] Recently, the Centers for Disease Control and Prevention have issued guidelines for screening for vaginal colonization with group B streptococci and intrapartum treatment with antibiotics during labor to prevent perinatal disease.[2]

Intrapartum chemoprophylaxis as a sole strategy for eradication of neonatal group B streptococcal disease has several shortcomings. It is invasive and may rarely result in serious allergic reactions. In addition, chemoprophylaxis is temporary, does not protect against late-onset group B streptococcal neonatal sepsis, and may select for development of resistant strains. In fact, there are recent reports of significant resistance of group B streptococci to antibiotics, such as clindamycin and erythromycin.[3] A successful vaccine strategy for eradication of group B streptococcal disease would overcome many of these problems. Although previous group B streptococcal polysaccharide vaccines were poorly immunogenic, newer vaccines with polysaccharide conjugated to tetanus toxoid have been developed. These newer conjugated vaccines have demonstrated improved immunogenicity in both laboratory animals and human subjects.[4]

Protective efficacy has been demonstrated in neonatal mice inoculated intraperitoneally after maternal immunization with group B streptococcal vaccine,[5] but there has been no assessment of vaccine efficacy in any model of ascending infection. We conducted a controlled trial of vaccination of pregnant rabbits to prevent neonatal group B streptococcal disease. Using our well-described model of ascending infection, we hypothesized that active type-specific vaccination of pregnant rabbits would lead to a significantly lower incidence of disease in their

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Table I. Group B *Streptococcus* type Ia capsular polysaccharide-specific IgG levels in does and their litters

	Does							
	*Prevaccination**				*Preinoculation†*			
Vaccine	*No.*	*GMC (μg/mL)*	*Range (μg/mL)*	*95% Confidence interval (μg/mL)*	*No.*	*GMC (μg/mL)*	*Range (μg/mL)*	*95% Confidence interval (μg/mL)*
Ia–tetanus toxoid	17	0.0	0.0-0.0	0.0-0.1	15	64.9	22.7-171.5	51.6-114.4
III–tetanus toxoid	17	0.0	0.0-0.3	0.0-0.1	16	9.2	0.22-50.0	7.9-19.9

GMC, Geometric mean concentration.
*Seven days before conception.
†95% gestation or 35 days after initial dose.

offspring after challenge with homologous serotype group B *Streptococcus*.

Our specific objectives were to determine (1) the effects of active maternal vaccination on neonatal survival after bacterial challenge, (2) the rates of group B streptococcal colonization in offspring as measured by oral-anal culture, (3) the rates of bacteremia, and (4) the maternal morbidities, including fever, positive uterine culture, and bacteremia. Finally, we sought to determine type-specific immunoglobulin (Ig) G levels in the does and the transplacentally acquired levels in the rabbit offspring.

Material and methods

The group B streptococcal type Ia polysaccharide vaccine conjugated to tetanus toxoid (Ia–tetanus toxoid) was chosen as the study vaccine, and the group B streptococcal type III vaccine conjugated to tetanus toxoid (III–tetanus toxoid) was chosen as the control vaccine. There was no apparent cross-reactivity of the type III polysaccharide with rabbit antisera to Ia–tetanus toxoid vaccine.[6]

Both the Ia–tetanus toxoid and the III–tetanus toxoid vaccines were prepared at the Channing Laboratory (Boston, Mass). Isolation and purification of the polysaccharide components, as well as purification of and coupling of the vaccine to tetanus toxoid, have been described.[6, 7]

The protocol was approved by the Animal Use and Care Committees of the University of Colorado Health Sciences Center and of Harvard Medical School. Breeding age nonpregnant female New Zealand White rabbits were obtained. Four proven breeder male New Zealand White rabbits were obtained for mating purposes (Myrtle's Rabbitry, Thompson Station, Tenn). The rabbit does, each weighing approximately 4 kg, were randomized to receive Ia–tetanus toxoid (the study vaccine) or III–tetanus toxoid (the control vaccine) by means of a computer-generated random numbers table. All investigators except one (S.L.) were blinded to the randomization.

Seven days before conception (day –7), all animals were sedated with ketamine (25 mg/kg) and xylazine (5.5 mg/kg). Blood was collected, and serum was frozen at –80°C. The animals were subsequently vaccinated with 50 μg of coupled capsular polysaccharide in complete Freund's adjuvant. The lyophilized vaccine was mixed with phosphate-buffered sodium chloride solution to make a 50-μg/mL solution and then emulsified with an equivalent volume of complete Freund's adjuvant (Gibco BRL, Grand Island, NY). Two milliliters of vaccine was given subcutaneously in 4 separate locations along each rabbit's back.

On days 0 to 2, female rabbits were mated by means of accepted breeding techniques. The does were vaccinated again on day 7, approximately 1 week after conception, and on day 21, approximately 3 weeks after conception. For the second and third immunizations, vaccines were emulsified with incomplete Freund's adjuvant (Gibco BRL, Grand Island, NY) and administered in a similar fashion to the first immunization.

At 95% gestation (days 28-30 of a 30-day gestation in the rabbit), the rabbits were again anesthetized with ketamine and xylazine. Blood was again collected, and serum was frozen at –80°C. After vaginal and perineal preparation with Betadine, the does were endoscopically inoculated (Karl Storz Endoscopy-America, Culver City, Calif) intracervically with 10^6 colony-forming units of type Ia group B *Streptococcus* (strain Eskens).[8] Rectal temperatures and animal weights were monitored twice daily.

Does were observed for spontaneous labor. If the animal remained undelivered 3 days after inoculation, labor was induced with 20 U of oxytocin administered by intramuscular injection. After delivery, oral-anal cultures were collected by using a sterile, sodium chloride solution–moistened, cotton-tipped applicator to swab the oral cavity and then the perianal area to determine group B streptococcal colonization status on all formed offspring.

Offspring were killed if ill or on the fourth day. They were killed if their color was pale, if they were in obvious respiratory distress, or if they did not move well. Offspring were killed with an intraperitoneal injection of pentobarbital 0.1 mL (260 mg/mL). Percutaneous intracardiac puncture was used to collect blood for antibody level and blood culture. If percutaneous collection was unsuccessful, the chest was opened by using a sterile technique, the heart was opened, and the myocardium was swabbed with a cotton-tipped applicator for culture only.

Does were killed 7 days after inoculation or sooner if they became ill. Maternal illness was defined as one or

Does							
Necropsy				Offspring			
No.	*GMC (μg/mL)*	*Range (μg/mL)*	*95% Confidence interval (μg/mL)*	*No.*	*GMC (μg/mL)*	*Range (μg/mL)*	*95% Confidence interval (μg/mL)*
17	84.9	6.3-313.5	75.3-164.6	33	51.9	18.4-104.8	49.3-67.2
15	13.0	0.8-64.3	11.0-28.2	32	6.7	6.4-8.7	0.9-15.6

more febrile episodes (rectal temperature ≥104.0°F), loss of >15% of body weight (food was supplemented if there was ≥10% weight loss), inability to eat or drink properly, or other signs of sepsis. Does were killed with 2.5 mL (260 mg/mL) pentobarbital given intravenously after anesthesia was obtained with intramuscular ketamine and xylazine. Before the animals were killed, blood was collected for blood culture and for measurement of antibody levels. After they were killed, the abdomen was opened by using a sterile technique, and peritoneal and intrauterine cultures were collected by using a sterile cotton-tipped applicator. Any fetuses remaining within the uterus at the time of necropsy were so counted and cultured.

Oral-anal cultures from the offspring were plated on blood and Columbia CNA (colistin and nalidixic acid) agars and inoculated into selective Todd-Hewitt broth (gentamicin, nalidixic acid, and blood). All maternal cultures, as well as offspring blood cultures, were plated on blood and Columbia CNA agars and inoculated into nonselective Todd-Hewitt broth. Plates and broth were incubated at 35°C in a 5% carbon dioxide atmosphere and checked daily. Plates were held up to 48 hours. Broth was held for 72 hours and subcultured on blood and Columbia CNA agars if the original plates had no growth. Colony counts were semiquantitative: No growth was classified as 0, <10 colonies as sparse (1+), 10 to 50 colonies as few (2+), >50 colonies but not confluent as moderate (3+), and confluence as many (4+). If growth was in broth only and original plates were negative, these were classified as sparse (1+). β-Hemolytic colonies were identified by Gram stain, colony morphologic features, and catalase and CAMP (Christie, Atkins, and Munch-Petersen) testing with known group A and group B streptococcal controls. Other species were identified by using standard microbiologic techniques.[9]

Levels of group B streptococcal type Ia–specific antibody were estimated with enzyme-linked immunosorbent assay on 96-well microtiter plates coated with type Ia polysaccharide–human serum albumin conjugate, as has been described elsewhere.[4] In addition, an in vitro opsonophagocytosis assay was performed on selected animals to evaluate functional activity.[6]

Our primary outcome was neonatal bacteremia. Other outcomes measured in offspring were neonatal survival (number of offspring born alive), colonization status (positive vs negative oral-anal culture), and health (poor if stillborn or found dead or ill in the first 2 days of life; good if survived ≥3 days). Maternal outcomes included fever (rectal temperature >104.0°F [>2 SD above mean for rabbits]), bacteremia, and severity of illness, with severe illness defined as bacteremia or need for early death because of illness.

For the power analysis, it was assumed that Ia–tetanus toxoid vaccine would reduce neonatal sepsis by 90% compared with the control vaccine, III–tetanus toxoid. By using 80% power, $P = .05$, and a 1-tailed t test, a sample size of 14 rabbits per group was needed. Assuming that not all animals would become pregnant, we chose to vaccinate a total of 34 animals because the first vaccination would occur before breeding. Data were analyzed with SAS (SAS Institute, Cary, NC) or Sigmastat (SPSS Inc, Chicago, Ill) statistical software. Categoric data were analyzed by means of the Fisher exact test or χ^2 test, and continuous data were analyzed by t test, with $P \leq .05$ considered significant. Spearman rank correlation was used to determine the association between maternal and neonatal antibody levels.

Results

The 34 does were randomized to receive the study vaccine, Ia–tetanus toxoid, or the control vaccine, III–tetanus toxoid. All 34 does were vaccinated. Twenty-five of the 34 does became pregnant. Serum was collected from nonpregnant does for measurement of antibody levels. One doe was discovered dead because of a presumed anesthesia overdose. Another doe was killed after a progressive bilateral hind-leg cellulitis thought to be unrelated to vaccination. A third doe delivered spontaneously on the morning before inoculation. Thus 22 animals were available for inoculation, 10 of which had previously been randomized to Ia–tetanus toxoid and 12 to III–tetanus toxoid. There were 83 offspring born to the 10 does in the Ia–tetanus toxoid group and 97 offspring born to the 12 III–tetanus toxoid does. There were 34 stillborn offspring in the Ia–tetanus toxoid group and 55 stillborn in the III–tetanus toxoid group. At the time of doe necropsy, there were 2 offspring in situ in the Ia–tetanus toxoid group and 8 in situ in the III–tetanus toxoid group.

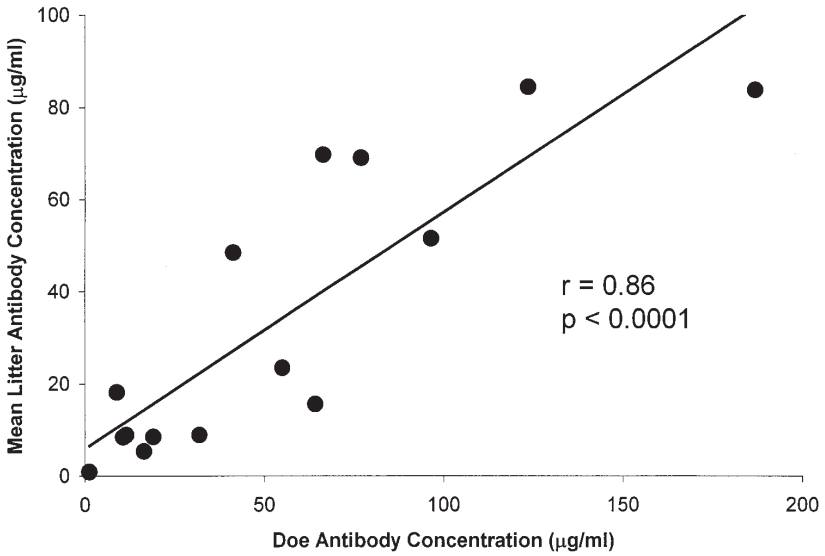

Fig 1. Correlation between doe antibody and mean offspring litter group B streptococcal type Ia IgG antibody levels (in micrograms per milliliter).

The immune response of vaccinated rabbit does is shown in Table I at the 3 predetermined time points—prevaccination, preinoculation, and necropsy. Serum samples were not available for 3 does at the preinoculation time point because one died (presumed anesthesia overdose), one was put to death (because of a progressive bilateral hind-leg cellulitis), and one delivered spontaneously before inoculation. Similarly, 2 animals in the III–tetanus toxoid group did not have serum available at the time of necropsy—1 was found dead and no serum was obtained from the second animal.

In those animals with serum samples available, mean group B streptococcal type Ia IgG antibody levels conferred by the Ia–tetanus toxoid vaccine were significantly higher than those levels conferred by the III–tetanus toxoid vaccine at both the preinoculation time point and necropsy ($P < .001$). In the 15 paired samples there was a high degree of correlation between doe and mean litter antibody levels (Fig 1). Offspring antibody levels were approximately half of doe levels.

In Table II we show various neonatal outcomes of interest. There were significantly more offspring born alive in the Ia–tetanus toxoid group ($P = .047$). There were no significant differences between the Ia–tetanus toxoid and the III–tetanus toxoid groups in the other offspring outcomes, specifically, bacteremia, oral-anal colonization, and timing of neonatal death.

With respect to maternal outcomes, which are summarized in Table III, maternal fever was an almost universal finding, with 88.8% in the Ia–tetanus toxoid group and 83.3% in the III–tetanus toxoid group having at least one episode of fever. There were no significant differences between the groups regarding positive uterine cultures or bacteremia. However, bacteremia and severe illness were only seen in the does receiving III–tetanus toxoid, although the numbers did not reach significance.

An in vitro opsonophagocytosis assay was performed to evaluate the functional capacity of the antibody in a rabbit vaccinated with III–tetanus toxoid compared with a rabbit vaccinated with Ia–tetanus toxoid. Sera were selected from 2 animals with similar Ia polysaccharide IgG antibody levels, as measured by enzyme-linked immunosorbent assay. This opsonophagocytosis assay measures the ability of antibody to opsonize group B *Streptococcus* type Ia for killing by human peripheral blood leukocytes in the presence of exogenously supplied complement. The serum from the rabbit vaccinated with Ia–tetanus toxoid resulted in a 1.8 $\log_{10}$ killing of group B streptococcal colony-forming units over 1 hour of incubation at 37°C. The animal vaccinated with III–tetanus toxoid resulted in a 0.71 $\log_{10}$ reduction compared with 0.41 $\log_{10}$ growth in the prevaccinated serum. These results demonstrate a high level of opsonic activity toward group B *Streptococcus* type Ia by serum raised by the animal vaccinated with III–tetanus toxoid, albeit less than half of the killing by the animal vaccinated with Ia–tetanus toxoid.

Because there were significant Ia antibody levels raised by the III–tetanus toxoid vaccine, we also examined Ia antibody levels in both the Ia–tetanus toxoid and III–tetanus toxoid groups versus neonatal outcome. Offspring colonized with group B *Streptococcus* had significantly higher antibody levels (44.13 ± 6.66 μg/mL) than did the noncolonized offspring (21.19 ± 3.63 μg/mL; $P = .004$). At offspring necropsy, there was no association between mean antibody levels and offspring health (25.75 ± 4.67 μg/mL in those with good health compared with 36.58 ± 5.93 mg/mL in those with poor health). In addi-

Table II. Selected neonatal outcomes

Outcome	Ia–tetanus toxoid		III–tetanus toxoid		Statistical significance*
	No.	%	No.	%	
Pups born alive	49/83	59.0	38/97	39.2	$P = .047$
Pups with positive oral-rectal culture	59/77	76.6	77/91	84.6	NS
Pups with positive blood culture	34/76	44.7	43/89	48.3	NS

NS, Not significant.
*χ^2 and Fisher exact test.

Table III. Selected maternal outcomes

Outcome	Ia–tetanus toxoid		III–tetanus toxoid		Statistical significance*
	No.	%	No.	%	
Does with fever	8/9	88.8	10/12	83.3	NS
Does with positive uterine culture	6/9	66.7	9/12	75.0	NS
Does with positive blood culture	0/9	0	2/12	16.8	NS
Does with severe illness	0/9	0	4/12	33.3	NS

NS, Not significant.
*χ^2 and Fisher exact test.

tion, antibody levels of bacteremic offspring were not significantly different from nonbacteremic offspring (27.11 ± 13.53 μg/mL vs 31.13 ± 3.95 μg/mL).

Comment

This is the first study to evaluate the conjugated group B *Streptococcus* vaccine in any model of ascending infection. This is a unique animal model in which to test the group B *Streptococcus* vaccine because early-onset neonatal sepsis in the human is thought to arise from acquisition during labor in colonized women. In the maternal immunization model in the mouse, female mice are vaccinated before becoming pregnant. After delivery, offspring are challenged with an intraperitoneal injection of homologous serotype group B *Streptococcus* to which they were vaccinated. Superior survival rates have been seen in those receiving the conjugated compared with unconjugated polysaccharide vaccine and sodium chloride solution–vaccinated controls.[5, 10]

The Ia–tetanus toxoid vaccine was highly immunogenic in our rabbit model, as has been demonstrated previously in mice and nonpregnant rabbits. There was no evidence of native immunity to group B *Streptococcus* in the rabbit in this experiment. The vaccine schedule of 3 doses given once every other week beginning just before conception was modeled to maximize antibody levels in preparation for the bacterial challenge in late gestation. In addition, previous vaccine pharmacokinetic studies had shown optimal antibody levels after the third vaccination.[6] Our study demonstrated excellent correlation between maternal and neonatal antibody levels.

Statistical significance was reached in our study only with respect to neonatal survival as measured by offspring born alive. However, our primary outcome, neonatal sepsis, was not different between the Ia–tetanus toxoid and III–tetanus toxoid groups. Some of the maternal parameters showed a trend toward improved outcomes in the Ia–tetanus toxoid group. A larger sample size might allow these outcomes to achieve statistical significance.

We recognize several limitations of our experiment. First, the control-vaccinated rabbits, the III–tetanus toxoid group, had a significant rise in their measurable type Ia–specific IgG, which was in contrast to previous findings.[6] We speculate that our 39% survival rate in the offspring receiving III–tetanus toxoid, a much higher survival than we anticipated, resulted from the cross-protective antibodies. In addition, a generalized enhancement of immune function may also have resulted from the carrier protein tetanus toxoid.[10]

Second, survival in the Ia–tetanus toxoid group was much lower than anticipated, perhaps because of the overwhelming intrauterine bacterial challenge of 10^6 colony-forming units. In a previous study, survival was only 50% after challenge with homologous strain group B *Streptococcus* in passively immunized rhesus monkeys.[11]

Third, conception rates in our study were significantly lower than expected (unpublished observations from Myrtle's Rabbitry). There is anecdotal information about a foul odor associated with use of complete Freund's adjuvant, which results in lower spontaneous conception rates in laboratory animals.

Although the exact concentration remains to be determined, protective levels of antibody in human neonates may be as low as 1 to 2 μg/mL.[12] This experiment did not have sufficient power to do breakpoint analysis to determine a protective level of antibody in the rabbit. Nonetheless, 95% of does achieved this level of antibody

because of the cross-reactivity of the III–tetanus toxoid vaccine.

Several modifications of the experimental design may achieve statistical significance of secondary outcomes. First, a different control vaccine should be chosen. Unconjugated group B streptococcal polysaccharide vaccine or a non-group B streptococcal vaccine conjugated to tetanus toxoid, such as *Haemophilus influenzae*, could be chosen. Similarly, a sodium chloride solution placebo should be used concurrently to illustrate any protection conferred by the group B streptococcal polysaccharide protein or the tetanus toxoid carrier protein. Second, a smaller inoculum size may more closely mimic early ascending infection. We could inoculate the does vaginally rather than intracervically, but this approach leads to a lower rate of intrauterine infection[8] and would require a larger sample size. Third, a different adjuvant, such as alum, might be chosen to perhaps improve conception rates, as well as to better simulate experimental conditions in human subjects. Fourth, in future work we plan to examine mucosal immunity as measured by vaginal secretory IgA levels.

This rabbit model of ascending infection is useful for assisting in the development of group B streptococcal vaccines for use in human subjects because experimental conditions allow for controlled assessments of inoculum size, timing of infection, and intrauterine quantitative cultures. Results with an animal model are available in a relatively short period of time. In comparison, the low prevalence of disease in human subjects demands a much larger sample size. Additionally, animal experimentation avoids some of the ethical problems of the natural history of disease in human research.

In summary, definitive conclusions regarding the efficacy of group B streptococcal conjugate vaccines in preventing maternal morbidity and improving neonatal survival in this rabbit model of ascending group B *Streptococcus* infection await further experimentation.

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