Published in final edited form as:

Am J Obstet Gynecol. 2011 September ; 205(3): 249.e1–249.e8. doi:10.1016/j.ajog.2011.06.024.

Efficacy of polymeric encapsulated C5a peptidase–based group B streptococcus vaccines in a murine model

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Abstract

Objectives—The purpose was to examine in mice the efficacy of various polymeric encapsulated C5a peptidase vaccine formulations in eliciting a long-term immune response and preventing Group B Streptococci infection.

Study Design—C5a peptidase was encapsulated in semi-permeable microspheres of poly(lactide-co-glycolide) (PLGA). Female ICR mice were immunized with 0, 10, or 30ug of encapsulated C5a peptidase within 2 different formulations of PLGA polymers. Booster doses were given at weeks 4 and 8. Antibody responses were measured by ELISA at weeks 4, 8, 11, and 40. Vaginal challenge with GBS types 1a, III, and V were performed at week 12.

Results—30ug doses of the 75:25 and 50:50 PLGA formulations generate the highest and most sustained C5a peptidase specific immune responses. Mice that received encapsulated C5a peptidase were significantly protected from vaginal colonization compared to mice that receive empty microspheres.

Conclusion—Encapsulated C5a peptidase elicited significant immune responses and protection against GBS challenge. C5a peptidase microsphere encapsulation has potential as a GBS vaccine.

Keywords

antigen encapsulation; C5a peptidase; Group B *Streptococcus*; microparticle; microsphere; mouse; PLGA; vaccine

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A portion of the results reported here were presented at the Society for Maternal-Fetal Medicine Annual Meeting in 2011

DISCLOSURE: The authors report no conflict of interest.

Introduction

Group B streptococci are gram positive diplococci that produce polysaccharide capsules.¹ There are 10 different GBS serotypes, based on the antigenic variation of the capsular polysaccharides. These serotypes are Ia, Ib, and II through IX. A recent international study most commonly isolated serotypes III and V from patients.² GBS can colonize the intestinal and urogenital tract of healthy adults without causing any complications. However, GBS infections can cause serious problems in pregnancy.

In 1985, the Institute of Medicine from the National Academies of Sciences indicated that a vaccine for Group B Streptococcus (GBS) should be a high priority. In a 1999 report, they again listed a GBS vaccine for high risk adults and pregnant women in the top tier for vaccine development.³ They suggest the vaccine would have the greatest effect if given as part of routine prenatal care for all women in their first pregnancy. Maternal complications of GBS infections in pregnancy include urinary tract infections, chorioamnionitis, postpartum wound infection, pyelonephritis, postpartum endometritis and sepsis. For the neonate, preterm premature rupture of membranes (PPROM), neonatal pneumonia, sepsis, meningitis or death can occur as a consequence of GBS.⁴⁻⁸

Because GBS infection continues to be a serious concern during pregnancy, the updated guidelines from the Centers for Disease Control continue to recommend universal culture-based screening of all pregnant women at 35–37 weeks gestation.^{9, 10} Currently if a pregnant woman screens positive for GBS, she will receive intravenous antibiotics during labor to prevent early-onset GBS disease in the child which can include sepsis and death.⁹ Recent studies demonstrate that up to 24% of all pregnant women receive antibiotic prophylaxis for GBS. Between 50–75% of neonates exposed through the birth canal of GBS-infected mothers will be colonized. Many shortcomings exist in the current therapy of antibiotic prophylaxis. These shortcomings are especially evident in cases in which a woman has a lack of prenatal care, delivers before being screened, delivers before the culture results return, has a rapid labor and does not finish receiving all of the antibiotic dose(s), or is allergic to antibiotics. In addition, the development of antibiotic resistance is an increasing problem. Penicillin-tolerant strains of GBS have been identified and resistance to other antibiotics has been documented.^{8, 11} A recent study found 91% of strains isolated were resistant to erythromycin.¹² The consequences of a GBS infection in pregnancy require that treatment be given. However, this large scale use of antibiotics is contributing to the development of antibiotic resistance. Without prevention strategies, such as vaccination, our first line antibiotic therapies are going to become useless against GBS. Most importantly, this traditional approach does not prevent preterm delivery or preterm premature rupture of membranes (PPROM) or protect against late-onset disease caused by GBS infection.¹³ A GBS vaccine could overcome these pitfalls.

Development of a vaccine for GBS has been hindered by several factors. First, there are 10 serotypes of GBS that are based on antigenic variation of the capsular polysaccharides. Purified capsular polysaccharides, without adjuvants, have elicited weak immune responses in vaccines and a multivalent vaccine would be necessary to provide protection against the multiple GBS serotypes because each polysaccharide can only target the serotype from which it was derived. Furthermore, polysaccharide-based vaccines were unable to elicit significant mucosal immune responses.¹⁴⁻¹⁶ A mucosal immune response would be critical in completely eliminating maternal GBS colonization. Eliminating colonization by all GBS serotypes would give the best chance of preventing the infection from being vertically passed to the child during the birthing process.

In the current study, we are further evaluating the use of streptococcal C5a peptidase as the vaccine antigen. C5a peptidase is a highly conserved multifunctional surface protein that is expressed on the surface of all serotypes of both group A streptococcus (GAS) and group B streptococcus tested.^{17–20} C5a peptidase (ScpB) expressed by GBS is 98% identical in sequence to that expressed by GAS.^{21, 22} Structurally, C5a peptidase contains 5 domains including a subtilisin-like protease domain, a protease-associated domain, and 3 fibronectin type III domains.²³ The enzymatic activity of the peptidase is highly specific for C5a, cleaving the chemotaxin at its polymorphonuclear leukocyte binding site.²⁴ Recent evidence also suggests that C5a peptidase may bind fibronectin to promote cellular invasion.^{25, 26} Cheng *et al.* demonstrated that ScpB increased the immunogenicity of a GBS type III polysaccharide vaccine in mice when the two were coupled.²⁷ In addition they found that ScpB was also found to induce the production of GBS serotype-independent antibodies.²⁷ We have previously shown that encapsulating C5a peptidase within microspheres composed of a co-polymer of lactic and glycolic acids, poly(lactide-co-glycolide (PLGA) was able to induce systemic and mucosal immune response in mice. Further, this vaccine provided protection in mice against GBS serotype III in vaginal and pup challenge studies.²⁸ The PLGA polymer based microspheres are able to act as an adjuvant to the vaccine and are safe for use in humans and has been used for many years in resorbable sutures, bone plates, and commercial depot drug delivery formulations.²⁹ The antigen release profile by PLGA microsphere based vaccines is largely dependent on the lactide:glycolide ratio. Co-polymers with a higher lactide:glycolide ratio have a longer degradation profile because lactic acid is hydrophobic.^{30, 31} PLGA microspheres have been studied for use in numerous vaccines.

We hypothesized that encapsulation of C5a peptidase within PLGA microspheres would induce specific systemic and mucosal immune responses that would afford protection against multiple serotypes of GBS. We further hypothesized that differences in antigen doses (0, 10, and 30ug) and PLGA microsphere lactide:glycolide formulations (75:25 and 50:50) would affect these immune responses and the ability of vaccinated mice to prevent GBS colonization and to pass GBS protection to pups of vaccinated dams.

Materials and Methods

C5a peptidase encapsulation

C5a peptidase, GMP prepared and greater than 98–99% pure was generously provided by Pfizer. The C5a peptidase was microencapsulated in poly (D,L-lactide-co-glycolide) (PLGA) microspheres. PLGA (50:50, Inherent Viscosity, 0.4 dL/g) and PLGA (75:25, Inherent Viscosity, 0.51 dL/g) were purchased from Lactel Absorbable Polymers (Cupertino, Ca). Poly vinyl alcohol (PVA, 87 – 89 % hydrolyzed, MW 30– 67,000 Da) was purchased from Sigma-Aldrich (St. Louis, MO). Encapsulation was done using a water-in-oil-in-water (w/o/w) double emulsion technique as described previously.^{16, 32, 33} Briefly, the internal aqueous phase consisted of 3.6 mg peptidase equivalent to 6.6 mg lyophilized powder C5a peptidase solubilized in 500 µL of 1% w/v aqueous solution of PVA as a surfactant. This was emulsified into an oil phase containing 200 mg of PLGA 50:50 or PLGA 75:25 dissolved in 5 mL dichloromethane (DCM) using a micro tip probe sonicator. This primary water/oil emulsion was then poured into 50 mL of external aqueous phase containing 1 % weight/volume PVA as a surfactant and rapidly homogenized using a high speed homogenizer at 9500 rpm for 30 seconds to form the secondary w/o/w emulsion. Stirring was then continued using a magnetic stirrer until complete evaporation of DCM. The microspheres were collected by centrifugation at 5000g for 10 minutes, washed three times with deionized water and lyophilized overnight.

To quantitate encapsulation efficiency of protein for dosing purposes, 30mg of lyophilized PLGA microspheres containing C5a peptidase were dissolved in 3.0 ml of 1M NaOH

containing 5.0% (w/v) sodium dodecyl sulfate and incubated for 24h at room temperature. After centrifugation (4000g for 10 minutes at room temperature), the supernatant was assayed for protein concentration using the bicinchonic acid assay (Thermo Scientific) following the manufacturer's protocol. All the measurements were done in triplicate.

***In Vitro* Release Profile**

30–40 mg of C5a-peptidase loaded PLGA microspheres were incubated in 2–3 mL PBS, pH 7.4. 200 μ L of samples were withdrawn at pre-determined time intervals. The sample was centrifuged at 10,000 rpm for 5 minutes and supernatant was analyzed using BCA assay to determine C5a content. The sedimented microspheres were dispersed in 200 μ L of PBS and replaced back instantly into the release samples. Scanning electron microscopy was performed as described previously at days 0 and 30 of the release profile assay.^{28,32, 33}

Administration of vaccine

Female ICR mice (Charles River) 5–7 weeks old were vaccinated either through an intramuscular or intranasal route. For all doses of the intramuscular vaccine, the vaccine was administered in 100ul into the right upper leg. For all doses of the intranasal administration, 50ul of vaccine was administered into each nostril (100ul total volume). Booster doses were administered in the same manner as the initial vaccination and were given at weeks 4 and 8. For the pup challenge experiment, the vaccine was administered intranasally and boosters were given at weeks 2 and 4.

Determination of Immune Response

Mice were bled via the submandibular route at weeks 4, 8, 11, and 40. Serum was isolated using serum separator tubes (Becton Dickinson) per the manufacturer's recommendations, frozen and stored at -80°C . Concurrently, vaginal washes were obtained by pipetting 100ul of Phosphate Buffered Saline (PBS) 40–50 times. Washes were frozen and stored at -80°C . Colormetric enzyme linked immunosorbant assay (ELISA) was used to measure the C5a peptidase-specific IgG and IgA antibody responses in serum and vaginal washes as described previously.²⁸ Samples producing a significant difference when further diluted are considered to have a larger immune response than samples in which a significant difference was observed in only smaller dilutions. The OD₄₀₅ reading for each dilution was compared between each vaccine formulation group and the empty microsphere control. The largest dilution that remained statistically significant in the OD₄₀₅ comparisons was considered the titer. The largest dilution tested was 1:100,000. Animals were housed at the University of Iowa and all experiments were performed according to IACUC-approved protocols.

Vaginal Colonization Studies

At 12 weeks, 1×10^6 colony forming units of GBS Serotypes Ia, III, and V (ATCC 12400, 12403, and 700046 respectively) were pipetted into the vagina of 5 mice of each vaccination group. After 48h, vaginal washings were obtained and 2 dilutions were plated on blood agar plates. Plates were incubated for 24 at 37°C with 5% CO_2 . After 24h, plates were assessed for growth of GBS. If no colonies were evident, plates were incubated for another 24 hours and growth of GBS colonies was again assessed. CAMP tests and gram staining were used to determine whether questionable beta-hemolytic colonies were GBS.³⁴ The presence of at least 1 GBS colony on a plate was counted as a positive plate. Results of each vaccine group were compared against the group of mice receiving empty microspheres (75:25 0ug).

Pup Protection Studies

At 48h of age, pups were injected with an LD₇₀ dose of GBS serotype V (1.8×10^7 CFU) intraperitoneally. These pups were born to dams who received the 50:50 30ug formulation of the vaccine. Pup survival was assessed at 48h post-injection.

Statistical Analysis

Descriptive statistics described and compared the characteristics of our study groups. For continuous variables, a Student's t test or Mann Whitney U was utilized for comparisons. For differences in proportions of dichotomous variables, Chi square or a Fisher's Exact Test was used. Statistical significance was designated at $\alpha=0.05$. All statistical analyses were performed with SigmaStat 11 software (Systat Software, Inc, California).

Results

Microsphere Formulation

Both the C5a-loaded PLGA (50:50, 0.41 dL/g) microspheres and the C5a-loaded PLGA (75:25, 0.51 dL/g) microspheres exhibited a mean particle size of 3–4 μ m (Figures 1a and 1b). There was no significant difference in the particle size of both the formulations.

In terms of entrapment efficiency, the amount of C5a peptidase loaded per mg of PLGA (50:50, 0.41 dL/g) was 12 μ g while that of PLGA (75:25, 0.51 dL/g) was 16 μ g. The overall entrapment efficiency was about 65%.

C5a peptidase release profile from PLGA microspheres

Both the 50:50 and 75:25 PLGA formulations demonstrated a similar biphasic burst rate *in vitro*. There was an initial burst in the first 2 days that resulted in the release of approximately 35–40% of the C5a peptidase. The 50:50 formulation is 95% degraded by day 25 while the 75:25 formulation is approximately 60% at that point (Figure 2). By day 30, the microspheres were completely degraded (Figure 1C and 1D).

Immune response

To compare the strength and duration of C5a peptidase specific IgG and IgA immune responses of mice vaccinated with various microspheres formulations and doses of encapsulated C5a peptidase, an ELISA was performed on serum and vaginal mucosal samples. When average titers were calculated regardless of route of administration, the 30ug doses of the 75:25 and 50:50 formulations elicited the highest titers at 40 weeks for C5a peptidase specific IgG responses in serum and in vaginal washes (Figure 3). The 75:25 30ug dose lead to the highest C5a peptidase specific IgA titer at 40 weeks in serum and vaginal washes.

When we analyzed the C5a peptidase specific antibody titers with respect to route of vaccine administration, we found that same results were achieved for mice vaccinated via the intramuscular route (Table 1). Titers of 1:100,000 were achieved by both 75:25 30ug and 50:50 30ug PLGA microspheres by 4 weeks and were sustained through 40 weeks for serum C5 peptidase specific IgG whereas 75:25 10ug titer dropped to 1000 at 40 weeks. In serum, C5a-IgA response was not detectable for the 75:25 30ug dose until 8 weeks; by 11 weeks both the 75:25 30ug and 50:50 30ug doses were 1:100,000. By 40 weeks these serum titers were reduced to 1:10,000 for the 30ug doses and were not detectable for the 10ug dose. The vaginal C5a specific titers were inconsistent. The vaginal washes of mice inoculated with the 50:50 30ug and 75:25 30ug vaccines had C5a-IgG titers of 1:100,000 at 40 weeks despite titers of 1:10,000 and 1:100 at 11 weeks, respectively. C5a peptidase specific IgA

antibodies were not detectable after 8 weeks in mice vaccinated with 75:25 10ug. However at 40 weeks, the vaginal washes had titers of 1:100,000 and 1:10,000 with 75:25 30ug and 50:50 30ug, respectively.

Intranasal administration resulted in more variable titers (Table 2). Each vaccine was able to generate a 1:100,000 C5a-IgG titer in serum by 4 weeks and sustain this titer through 11 weeks. However, all of the titers dropped by 40 weeks. Also in serum, the C5a-IgA titer reached the maximum dilution tested at 1:100,000 for all vaccines at week 8 and then dropped to 1:10,000 by week 11. In the vaginal washes, C5a-IgG titers of 1:100,000 were measured for each of the vaccine formulations at weeks 8 and 11; whereas the maximal 1:100,000 C5a-IgA titer was only found at week 8 in the vaginal wash samples.

Protection by Immunization

In previous work, we demonstrated that the 50:50 30ug dose administered intranasally was able to prevent GBS colonization of the vaginal vault by serotype III.²⁸ We hypothesized that the 30ug doses of the 75:25 and 50:50 PLGA microspheres formulations would be able to protect against multiple serotypes of GBS. We used 15 mice from each group and inserted 1×10^6 CFU of serotypes Ia, III, and V (n=5 per serotype per vaccine group). Results were compared against those from mice vaccinated with empty microspheres. Not all mice receiving empty microspheres were colonized (Table 3). Without regard to which encapsulated vaccine the mice received, the mice that received a vaccine were significantly protected against colonization (27 of 90 positive) in comparison to mice that received the empty microspheres (18 of 30 positive) ($p=0.005$). The intramuscular 30ug doses of the 75:25 and 50:50 formulations were able to prevent colonization by serotypes Ia and III (0 of 5 positive) (Figure 4). When all serotypes were considered together, the intranasal 30ug doses of the 75:25 and 50:50 PLGA microspheres formulations trended toward significantly inhibited vaginal colonization in mice ($p=0.06$). We also tested mice that were vaccinated with unencapsulated antigen and this vaccine did not significantly impede vaginal colonization (Table 3). Of note, most blood agar plates from mice that were colonized demonstrated colonies that were too numerous to count, often even in the smallest dilution plated; whereas in mice that were protected, the plates showed no evidence of GBS growth. Without being able to count colonies, it is impossible to perform statistical analysis based on the number of GBS colonies.

Because we did not observe any significant protection against serotype V in the vaginal colonization studies, we were interested in whether the IgG antibodies that cross the placenta in combination with any IgA antibodies in the dam's milk were able to afford protection to pups. Because we achieved high C5a-IgG titers in serum, we hypothesized that the C5a peptidase IgG antibodies that cross the placenta would be able to better protect pups than the weaker mucosal immune response that would be necessary to prevent vaginal colonization. In pup protection studies, pups were injected IP with an LD₇₀ dose of serotype V. In comparing survival of pups between non-vaccinated (3 of 10) and vaccinated mice (4 of 5), we observed a notable higher survival for pups born to vaccinated dams (30% vs. 80%). While this was not statistically significant difference ($p=0.11$) in a 2-tailed Fisher's exact test, we were limited by the number of female mice that bred within a similar time frame and by small litter sizes. However, these results were similar to the significant improvement in survival that we previously reported with serotype III.²⁸

Comment

In our previous work, we demonstrated that by encapsulating C5a peptidase within microspheres composed of PLGA, we were able to elicit antibody responses in serum and in the vagina of mice against GBS and that these responses were sufficient to protect against

vaginal colonization by serotype III.²⁸ This protection was also conferred to pups of vaccinated dams. In this study, our primary objective was to determine the duration of the immune response to various vaccine formulations and doses. In addition, our secondary goal was to determine if this univalent vaccine was able to protect against vaginal colonization by multiple serotypes of GBS. Furthermore, we wanted to compare whether different formulations of the PLGA microspheres vaccine (75:25 and 50:50) and different doses (0, 10, and 30ug) were more effective in protecting from vaginal colonization by GBS.

We achieved our main objective by determining whether there are differences in titers between vaccine formulations and doses. We have demonstrated that, in general, the 30ug doses of the 75:25 and 50:50 PLGA microsphere formulations generate the highest and most sustained C5a peptidase specific IgG and IgA antibody responses. At weeks 4, 8, and 11, we did not detect any significant differences in the IgG or IgA titers between the PLGA 75:25 and 50:50 microsphere formulations at the 30ug dose. At week 40, there were also no differences in the C5a peptidase specific IgG responses. The titers were higher at week 40 for the 75:25 30ug vaccine compared to the 50:50 30ug vaccine. Because we measured similar particle sizes and we adjusted our dosing based on the actual amount of protein encapsulated, these factors cannot account for this difference. For additional consistency, the same volume was used to administer each vaccine. The slower degradation rate of PLGA 75:25 in comparison to PLGA 50:50 may affect the IgA titers over a longer time period.

Furthermore, we found that mice receiving the encapsulated C5a peptidase (including 75:25 10ug, 75:25 30ug, and 50:50 30ug) were significantly protected from vaginal colonization compared to mice that receive empty microspheres (75:25 0ug). It is a strength of this study that we used very stringent guidelines for this experiment by identifying any number of GBS colonies as a positive plate. We could not statistically compare the number of colonies per plate as the number of colonies was innumerable on many of the plates from mice receiving the empty microspheres. While the vaginal challenge studies were able to demonstrate trends toward significance and promise for this vaccine to protect against serotypes 1a and III, the number of mice used per group was too small to detect any significant differences between vaccination subgroups. These results did indicate that this encapsulation approach and antigen have merit for use as a GBS vaccine and warrant future studies to further expand on their ability to protect against the 10 GBS serotypes. Because we have now identified the optimal vaccine formulation and dose, we will be able to utilize larger groups of mice for subgroup analysis in future studies.

We found more fluctuations in the antibody responses in the intranasal group than in the intramuscular group. These observations in the titers of vaginal washes are likely due to difficulties in equally distributing the mucus between dilutions in the ELISA as well to variations in mucosal IgG and IgA production with the mouse estrous cycle.³⁵ We may have experienced less variable results if we had isolated the vagina and extracted the antibodies from the tissue.³⁶ Because we used the mice to measure titers at several time points, it was not feasible for us to have a large enough sample group to sacrifice mice at each time point for vaginal tissue. These variations may have been minimized by one of the strengths of this study in that we used a large number of mice for the 4, 8, and 11 week titers. Our sample size was greatly reduced by week 40 because we used 15 mice from each of these groups for the vaginal challenge studies.

However, our study was one of the longest to follow the duration of the immune response and to use multiple serotypes as challenges to the vaccine. In recent studies, groups have examined the titers at dates ranging from 7–14 days after the last booster dose.^{36–39} In contrast, we administered our last booster dose at week 8 and our last titer was measured at week 40, over 220 days later. Knowledge of the duration of the immune response will be

especially important in knowing how often women will need to be vaccinated to at least be protected throughout pregnancy.

We and others have had difficulty protecting mice from serotype V.^{39–41} Serotype V has also been shown to induce low levels of antibodies in older human patients.⁴² Our future studies will work to determine the mechanisms of serotype V to evade an immune response. While we did not observe significant protection from vaginal colonization, the systemic IgG response passed from the dam to the pups appears to have led to the improved survival of challenged pups (80% compared to 30%) from vaccinated dams.

Now that we have identified the most optimal formulation for a PLGA C5a peptidase vaccine against GBS, we can work to compare this vaccine to other potential GBS vaccines. In addition, our future work will also investigate combining 2 or more antigens within the vaccination to determine if that provides better protection against GBS, particularly against serotype V. We may also try alternate routes of administration such as vaginal vaccination. Intravaginal immunization has recently been shown to elicit higher IgG antibody responses than other vaccination strategies.⁴³

The results presented here contribute greatly to the field of GBS vaccine development as well the use of PLGA microsphere based vaccines for mucosal immune responses. We are continuing to pursue the development of a GBS vaccine and to better understand the mucosal immune response to our vaccine as well as methods of evasion by different GBS serotypes.

Acknowledgments

This work was supported by the Eunice Shriver Kennedy National Institute of Child Health & Human Development at the National Institutes of Health 1R03HD056006 (to SKH), Children's Miracle Network and the University of Iowa Children's Hospital (to DAS), NIH T32 AI 007260 Interdisciplinary Immunology Postdoctoral Training Program (to DAS), NIH Interdisciplinary Training Program in Pain Research T32 NS045549 (to KKR), National Institutes of Health University of Iowa Institute for Clinical and Translational Science KL2 RR024980-2 (to MKS), Department of Obstetrics & Gynecology, the American Cancer Society RSG-09-015-01-CDD (to AKS), the National Cancer Institute at the National Institutes of Health 1R21CA13345-01/1R21CA128414-01A2/UI Mayo Clinic Lymphoma SPORE (to AKS), the Pharmaceutical Research and Manufacturers of America (PhRMA) Foundation (to AKS), and support from a Guillory Fellowship (to YK).

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Figure 1. Vaccine Microspheres. Scanning electron microscopy of vaccines at days 0 (A and B) and 30 (C and D) during the *in vitro* release profile. Microspheres from both formulations were round and of uniform size. The average size sphere at Day 0 was 3–4 μm. Microspheres were completely degraded by day 30 with debris of the low molecular weight fractions of lactic acid and glycolic acid left behind.

Figure 2.
Release Profile of Antigen. The release profiles demonstrate an initial burst phase followed by a longer release of antigen.

Figure 3.

Average C5a Peptidase Specific Titers. Serum and vaginal wash samples were obtained at weeks 4, 8, 11, and 40. The blue bars represent titers from all mice immunized with 75:25 10ug. Purple bars represent all mice immunized with 75:25 30ug doses. Green bars represent the titers of mice receiving the 50:50 30ug vaccine. These results are the average titers for the vaccine doses and are irrespective of route of administration of the vaccine.

Pre challenge swab
IM Vaccine	PLGA 75:25 0 ug	PLGA 75:25 10 ug	PLGA 75:25 30 ug	PLGA 50:50 30 ug
Post Challenge Swab
	GBS +	GBS +	GBS -	GBS -

Figure 4.

Vaginal GBS Serotype Ia Challenge. Prior to challenge at 12 weeks, vaginas of mice were washed and samples were assessed for GBS growth (not detected). 48h post-infection with serotype Ia, the vagina of each mouse was washed and samples were grown to assess growth of beta-hemolytic GBS colonies.

Table 1

Intramuscular Vaccine Administration Titers

Serum C5a-IgG	4 weeks (n)	8 weeks (n)	11 weeks (n)	40 weeks (n)
75:25 10ug	1000 (27)	100000 (27)	100000 (27)	1000 (11)
75:25 30ug	100000 (28)	100000 (26)	100000 (25)	100000 (4)
50:50 30ug	100000 (28)	100000 (27)	100000 (27)	100000 (6)
Serum C5a-IgA				
75:25 10ug	100000 (27)	100000 (27)	100000 (27)	0 (11)
75:25 30ug	0 (28)	10000 (26)	100000 (25)	10000 (4)
50:50 30ug	0 (28)	0 (27)	100000 (27)	10000 (6)
Vaginal C5-IgG				
75:25 10ug	100000 (27)	100000 (27)	100000 (27)	1000 (11)
75:25 30ug	10000 (28)	10000 (26)	100 (25)	100000 (4)
50:50 30ug	100000 (28)	10000 (27)	10000 (27)	100000 (6)
Vaginal C5a-IgA				
75:25 10ug	100000 (27)	100000 (27)	0 (27)	0 (11)
75:25 30ug	10000 (28)	100000 (26)	100 (25)	100000 (4)
50:50 30ug	1000 (28)	0 (27)	10000 (27)	10000 (6)

Titers are shown as 1:Y (Y represents the dilution). A Student's t-test was used to determine the significant titer. The largest dilution tested that was significantly different from mice receiving 75:25 0ug is the reported titer.

Table 2

Intranasal Vaccine Administration Titers

Serum C5a-IgG	4 weeks (n)	8 weeks (n)	11 weeks (n)	40 weeks (n)
75:25 10ug	100000 (29)	100000 (29)	100000 (26)	100 (10)
75:25 30ug	100000 (28)	100000 (27)	100000 (25)	100 (4)
50:50 30ug	100000 (29)	100000 (28)	100000 (27)	1000 (4)
Serum C5a-IgA				
75:25 10ug	10000 (29)	100000 (29)	10000 (26)	100000 (10)
75:25 30ug	100000 (28)	100000 (27)	10000 (25)	100000 (4)
50:50 30ug	0 (29)	100000 (28)	10000 (27)	0 (4)
Vaginal C5a-IgG				
75:25 10ug	10000 (29)	100000 (29)	100000 (26)	10000 (10)
75:25 30ug	100000 (28)	100000 (27)	100000 (25)	0 (4)
50:50 30ug	10000 (29)	100000 (28)	100000 (27)	10000 (4)
Vaginal C5a-IgA				
75:25 10ug	0 (29)	100000 (29)	10000 (26)	10000 (10)
75:25 30ug	100000 (28)	100000 (27)	10000 (25)	0 (4)
50:50 30ug	0 (29)	100000 (28)	10000 (27)	100 (4)

Titers are shown as 1:Y (Y represents the dilution). A Student's t-test was used to determine the significant titer. The largest dilution tested that was significantly different from mice receiving 75:25 0ug is the reported titer.

Table 3

Vaginal Colonization

			# mice colonized with GBS			
Vaccine Formulation	Dose	Route	Ia (p-value)	III (p-value)	V (p-value)	Total (p-value)
75:25	0ug	IM	3/5 (n/a)	2/5 (n/a)	3/5 (n/a)	8/15 (n/a)
75:25	10ug	IM	3/5 (1)	2/5 (1)	2/5 (0.58)	7/15 (1)
75:25	30ug	IM	0/5 (0.16)	0/5 (0.16)	3/5 (1)	3/15 (0.07)
50:50	30ug	IM	0/5 (0.16)	0/5 (0.16)	4/5 (0.54)	4/15 (0.27)
free C5a peptidase	30ug	IM	3/5 (1)	5/5 (0.08)	3/5 (1)	11/15 (0.45)
75:25	0ug	IN	4/5 (n/a)	2/5 (n/a)	4/5 (n/a)	10/15 (n/a)
75:25	10ug	IN	1/5 (0.21)	2/5 (1)	2/5 (0.29)	5/15 (0.08)
75:25	30ug	IN	1/5 (0.21)	1/5 (1)	2/5 (0.29)	4/15 (0.06)
50:50	30ug	IN	2/5 (0.29)	1/5 (1)	1/5 (0.21)	4/15 (0.06)
free C5a peptidase	30ug	IN	3/5 (0.58)	3/5 (1)	4/5 (1)	10/15 (1)

p-values represent 2-tailed Fisher exact comparing the 75:25 0ug dose of each route of administration against each of the other vaccination groups.