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

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OBJECTIVE: 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 streptococcus (GBS) infection.

STUDY DESIGN: C5a peptidase was encapsulated in semipermeable microspheres of polylactide-coglycolide (PLGA). Female ICR mice were immunized with 0, 10, or 30 μg 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 enzyme-linked immunosorbent assay at weeks 4, 8, 11, and 40. Vaginal challenges with GBS types Ia, III, and V were performed at week 12.

RESULTS: Thirty microgram 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 with mice that received empty microspheres.

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

Key words: antigen encapsulation, C5a peptidase, group B streptococcus, microparticle, microsphere, mouse, poly(lactide-coglycolide), vaccine

nal 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. A recent study found 91% of strains isolated were resistant to erythromycin. 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 PPROM or protect against late-onset disease caused by GBS infection.

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 target only the serotype from which it was derived. Furthermore, polysaccharide-based vaccines were unable to elicit significant mucosal immune responses. 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 further evaluated 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. C5a peptidase (ScpB) expressed by GBS is 98% identical in sequence to that expressed by GAS. Structurally, C5a peptidase contains 5 domains including a subtilisin-like protease domain, a protease-associated domain, and 3 fibronectin type III domains. The enzymatic activity of the peptidase is highly specific for C5a, cleaving the chemotaxin at its polymorphonuclear leukocyte binding site.

Recent evidence also suggests that C5a peptidase may bind fibronectin to promote cellular invasion. We have previously shown that encapsulating C5a peptidase within microspheres composed of a copolymer of lactic and glycolic acids, poly(lactide-co-glycolide) (PLGA), was able to induce systemic and mucosal immune response in mice. Furthermore, this vaccine provided protection in mice against GBS serotype III in vaginal and pup challenge studies. 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. The antigen release profile by PLGA microsphere–based vaccines is largely dependent on the lactide/glycolide ratio. Copolymers with a higher lactide/glycolide ratio have a longer degradation profile because lactic acid is hydrophobic.

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 30 μg) 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, guanosine monophosphate prepared and greater than 98-99% pure, was generously provided by Pfizer (Groton, CT). The C5a peptidase was microencapsulated in 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). Polyvinyl alcohol (PVA; 87-89% hydrolyzed, molecular weight 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. 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% (weight/volume) 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 microtip 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 5000 × g for 10 minutes, washed 3 times with deionized water, and lyophilized overnight.

To quantitate encapsulation efficiency of protein for dosing purposes, 30 mg of lyophilized PLGA microspheres containing C5a peptidase were dissolved in 3.0 mL of 1 M NaOH containing 5.0% (weight/volume) sodium dodecyl sulfate and incubated for 24 hours at room temperature. After centrifugation (4000 × g for 10 minutes at room temperature), the supernatant was assayed for protein...
concentration using the bicinchonic acid assay (Thermo Scientific, Swedesboro, NJ) following the manufacturer’s protocol. All the measurements were done in triplicate.

**In vitro release profile**

Thirty to 40 mg of C5a-peptidase–loaded PLGA microspheres were incubated in 2-3 mL phosphate buffered saline (PBS; pH 7.4). Two hundred microliters of samples were withdrawn at predetermined time intervals. The sample was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was analyzed using a bicinchoninic assay to determine C5a content. The sedimented microspheres were dispersed in 200 µL 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 Breeding Laboratories, Portage, MI) 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 100 µL into the right upper leg. For all doses of the intranasal administration, 50 µL of vaccine was administered into each nostril (100 µL 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, Lincoln Park, NJ) per the manufacturer’s recommendations, frozen, and stored at –80°C. Concurrently, vaginal washes were obtained by pipetting 100 µL of PBS 40-50 times. Washes were frozen and stored at –80°C. Colorimetric enzyme linked immunosorbant assay (ELISA) was used to measure the C5a peptidase–specific immunoglobulin (Ig) G 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 optical density (OD)405 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 Institutional Animal Care and Use Committee–approved protocols.

**Vaginal colonization studies**

At 12 weeks, 1 × 10⁶ colony-forming units of GBS serotypes Ia, III, and V (ATCC 12400, 12403, and 700046, respectively, American Type Culture Collection, Manassas, VA) were pipetted into the vagina of 5 mice of each vaccination group. After 48 hours, vaginal washings were obtained and 2 dilutions were plated on blood agar plates. Plates were incubated for 24 hours at 37°C with 5% CO₂. After 24 hours, 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. Cyclic adenosine monophosphate 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, 0 µg).

**Pup protection studies**

At 48 hours of age, pups were injected with a 70% lethal dose of GBS serotype V (1.8 × 10⁷ colony-forming units) intraperitoneally. These pups were born to dams who received the 50:50 30 µg for-
mulation of the vaccine. Pup survival was assessed at 48 hours after injection.

Statistical analysis
Descriptive statistics described and compared the characteristics of our study groups. For continuous variables, a Student t test or Mann Whitney U test was utilized for comparisons. For differences in proportions of dichotomous variables, a \( \chi^2 \) 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, Point Richmond, CA).

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 \( \mu \)m (Figure 1, A and B). 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 milligram of PLGA (50:50, 0.41 dL/g) was 12 \( \mu \)g, whereas that of PLGA (75:25, 0.51 dL/g) was 16 \( \mu \)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, whereas the 75:25 formulation is approximately 60% at that point (Figure 2). By day 30, the microspheres were completely degraded (Figure 1, C and D).

Immune response
To compare the strength and duration of C5a peptidase–specific IgG and IgA immune responses of mice vaccinated with various microsphere 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 30 \( \mu \)g 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 vaginal washes (Figure 3). The 75:25 30 \( \mu \)g dose led 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 30 µg and 50:50 30 µg PLGA microspheres by 4 weeks and were sustained through 40 weeks for serum C5 peptidase–specific IgG, whereas the 75:25 10 µg titer dropped to 1000 at 40 weeks.

In serum, the C5a-IgA response was not detectable for the 75:25 30 µg dose until 8 weeks; by 11 weeks both the 75:25 30 µg and 50:50 30 µg doses were 1:100,000. By 40 weeks these serum titers were reduced to 1:10,000 for the 30 µg doses and were not detectable for the 10 µg dose. The vaginal C5a-specific titers were inconsistent.

The vaginal washes of mice inoculated with the 50:50 30 µg and 75:25 30 µg 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 10 µg. However, at 40 weeks, the vaginal washes had titers of 1:100,000 and 1:10,000 with 75:25 30 µg and 50:50 30 µg, 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 found only at week 8 in the vaginal wash samples.

**Protection by immunization**

In previous work, we demonstrated that the 50:50 30 µg dose administered intranasally was able to prevent GBS colonization of the vaginal vault by serotype III.28 We hypothesized that the 30 µg doses of the 75:25 and 50:50 PLGA microsphere formulations would be able to protect against multiple serotypes of GBS. We used 15 mice from each group and inserted $1 \times 10^6$ colony-forming units of serotypes Ia, II, and V ($n = 5$ per serotype per vaccine group).

The 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 with mice that received the empty microspheres (18 of 30 positive) ($P = .005$). The intramuscular 30 µg doses of the 75:25 and 50:50 formulations were able to prevent colonization by serotype Ia and III (0 of 5 positive) (Figure 4). When all serotypes were considered together, the intranasal 30 µg doses of the 75:25 and 50:50 PLGA microsphere formulations trended toward significantly inhibited vaginal colonization in mice ($P = .06$).

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 intraperitoneally with a 70% lethal dose of serotype V.

In comparing survival of pups between nonvaccinated (3 of 10) and vaccinated mice (4 of 5), we observed a notable higher

### Table 1

<table>
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<tr>
<th>Variable</th>
<th>4 weeks, n</th>
<th>8 weeks, n</th>
<th>11 weeks, n</th>
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<td>50:50 30 µg</td>
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<td>Vaginal C5-IgG</td>
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<tr>
<td>75:25 10 µg</td>
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<td>1000 (11)</td>
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</table>

Titers are shown as 1:Y (Y represents the dilution). A Student t test was used to determine the significant titer. The largest dilution tested that was significantly different from mice receiving 75:25 0 µg is the reported titer.

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survival for pups born to vaccinated dams (30% vs 80%). Although this was not a statistically significant difference \((P = .11)\) 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.\(^{28}\)

**TABLE 2**

<table>
<thead>
<tr>
<th>Variable</th>
<th>4 weeks, n</th>
<th>8 weeks, n</th>
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<td>75:25 30 μg</td>
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</table>

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 0 μg is the reported titer.

**TABLE 3**

<table>
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<th>Vaccine formulation</th>
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<td>0/5 (n/a)</td>
<td>0/5 (n/a)</td>
<td>0/15 (n/a)</td>
</tr>
</tbody>
</table>

Values represent 2-tailed Fisher exact comparing the 75:25 0 μg dose of each route of administration against each of the other vaccination groups. GBS: group B streptococcus; IM, intramuscular; IN, intranasal; n/a, not available.

**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 whether 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 30 μg) 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 30 μg 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 30 μg 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 30 μg vaccine compared with the 50:50 30 μg 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 with 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 10 μg, 75:25 30 μg, and 50:50 30 μg) were significantly protected
from vaginal colonization compared with mice that received empty microspheres (75:25 0 μg). 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 because the number of colonies was innumerable on many of the plates from mice receiving the empty microspheres. Although 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 likely are 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.

We may have experienced less variable results if we had isolated the vagina and extracted the antibodies from the tissue. 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 kill 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 titers at 4, 8, and 11 weeks. 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 up 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 to 14 days after the last booster dose. In contrast, we administered our last booster dose at week 8, and our last titer was measured at week 40, more than 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. Sero
type V has also been shown to induce low levels of antibodies in older human patients. Our future studies will work to determine the mechanisms of serotype V to evade an immune response. Although 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 with 30%) from vaccinated dams.

Now that we have identified the most optimal formulation for a PLGA 50:50 peptidase vaccine against GBS, we can work to compare this vaccine with other potential GBS vaccines. In addition, our future work will also investigate combining 2 or more antigens within the vaccination to determine whether 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.

The results presented here contribute greatly to the field of GBS vaccine development as well as 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 in addition to methods of evasion by different GBS serotypes.

### REFERENCES


![FIGURE 4
Vaginal GBS serotype Ia challenge](image-url)
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