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Immunization with the *Haemophilus ducreyi* Hemoglobin Receptor HgbA Protects against Infection in the Swine Model of Chancroid

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The etiologic agent of chancroid is *Haemophilus ducreyi*. To fulfill its obligate requirement for heme, *H. ducreyi* uses two TonB-dependent receptors: the hemoglobin receptor (HgbA) and a receptor for free heme (TdhA). Expression of HgbA is necessary for *H. ducreyi* to survive and initiate disease in a human model of chancroid. In this study, we used a swine model of *H. ducreyi* infection to demonstrate that an experimental HgbA vaccine efficiently prevents chancroid, as determined by several parameters. Histological sections of immunized animals lacked typical microscopic features of chancroid. All inoculated sites from mock-immunized pigs yielded viable *H. ducreyi* cells, whereas no viable *H. ducreyi* cells were recovered from inoculated sites of HgbA-immunized pigs. Antibodies from sera of HgbA-immunized animals bound to and initiated antibody-dependent bactericidal activity against homologous *H. ducreyi* strain 35000HP and heterologous strain CIP542 ATCC; however, an isogenic *hgbA* mutant of 35000HP was not killed, proving specificity. Anti-HgbA immunoglobulin G blocked hemoglobin binding to the HgbA receptor, suggesting a novel mechanism of protection through the limitation of heme/iron acquisition by *H. ducreyi*. Such a vaccine strategy might be applied to other bacterial pathogens with strict heme/iron requirements. Taken together, these data suggest continuing the development of an HgbA subunit vaccine to prevent chancroid.

Genital ulcer diseases (GUDs), including chancroid, are important risk factors for the heterosexual transmission of the human immunodeficiency virus (HIV) in certain African and Asian developing countries. This is especially true early in the course of HIV epidemics (10, 12, 41, 45, 60). Recent data show that GUDs can enhance HIV type 1 transmission by 3- to 10-fold (7, 17, 28, 30, 42, 48, 51). The World Health Organization acknowledges that the prevention of GUDs is an important strategy to control the spread of HIV infection (39, 40, 67).

Haemophilus ducreyi, a small fastidious gram-negative bacterium that infects only humans, is well known for its requirement for heme (23, 24, 52, 57, 61). *H. ducreyi* expresses two TonB-dependent receptors for heme compounds: the hemoglobin receptor (HgbA) and the heme receptor (TdhA) (19, 21, 23, 63). During in vitro growth in heme-limiting media, HgbA is induced to high levels of expression, but TdhA is induced only at modest levels. In vivo, both receptors are expressed to some extent, since antibodies are detected after natural (24) and experimental (14) infections. A role for HgbA in virulence was established by the inability of an *hgbA* mutant of *H. ducreyi* to initiate human experimental infection, even at a dose 10 times higher than the infective dose of the parent strain (3). Since the attenuated *hgbA* mutant expresses *tdhA*, it appears that the ability to acquire/transport heme from hemoglobin is more efficient than acquisition/transport of heme via TdhA. It can also be inferred that hemoglobin, as opposed to free heme, is the most important heme compound during experimental human infection. However, formal proof has not yet been obtained, since the *tdhA* mutant has not been tested for virulence.

The relative roles of the cellular and humoral arms of the immune system in resistance to chancroid infection are not well understood. Chancroid reinfection is common among patients, suggesting that a nonprotective immune response is generated as a result of natural infection (8, 25, 33–35, 37). It is well documented that early in the course of natural or experimental chancroid, an intense cellular infiltrate develops locally (27, 58, 59). This is characterized by an influx of mononuclear cells homing to affected tissues and perivascular cuffing (37). In untreated human chancroid, antibodies to *H. ducreyi* are detected after 3 weeks of natural infection, and the chancroid lesions resolve in about 6 weeks (11, 38, 50).

In vivo human studies confirm that *H. ducreyi* is primarily an extracellular pathogen (5, 6). The paradigm for immunity to extracellular pathogens is that an antibody response is critical (32, 47). Two animal models of chancroid infection, the rabbit model and the swine model, have been used for vaccine studies, and both are clearance models (14, 31, 49). In these two models, large infectious doses are required, limited bacterial multiplication occurs, and the rate of clearance is evaluated. A number of studies using the rabbit model of chancroid infection demonstrate that whole cells, crude outer membrane protein (OMP) preparations, or purified protein vaccines induce partial immunity to/protection against a challenge with *H. ducreyi* strain 35000 (15, 16, 18, 26, 29, 64, 65). None of the resulting antibodies generated in these studies were shown to be bactericidal or opsonophagocytic, two common features of effective vaccines. Recently, using a swine model of chancroid, Cole and colleagues demonstrated more rapid clearance

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after repeated infection with *H. ducreyi*. This resistance could be passively transferred with immune sera (14). These protective antibodies were modestly bactericidal and were directed against a number of OMPs of *H. ducreyi*.

Two classes of *H. ducreyi* strains, based on antigenic differences in a number of OMPs, have been described. In contrast to the dramatic differences seen between the DsrA and NcaA proteins from class I and class II strains, HgbA proteins from the two classes show greater than 95% identity (66). Moreover, HgbA is expressed on the surface of virulent *H. ducreyi* cells and is conserved functionally and structurally. These attributes suggest that HgbA might be an effective vaccine candidate. The objective of the present study was to determine the ability of native HgbA protein to elicit protective immunity in the swine model of *H. ducreyi* infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. ducreyi* type strain, 35000HP (class I strain, human-passaged variant), was obtained from Stanley Spinola, Indiana University, Indianapolis, IN. *H. ducreyi hgbA* mutant strain FX504 was previously described (19, 21), and *gmbH* mutant 35000.252 (a lipooligosaccharide [LOS] mutant of parent strain 35000HP whose LOS contains 2-keto-3-deoxy-octulosonic acid and lipid A only) (4) was obtained from Eric Hansen, University of Texas Health Science Center, Dallas, TX. CIP542 ATCC (class II strain) was obtained from the American Type Culture Collection, and C111 (class I strain) was obtained from William Albritton. DMC 111 (class II strain) was previously described (66). For routine growth, all strains were maintained at 34.5°C in 5% CO_2 on chocolate agar plates (CAP) containing gonococcal medium base (GCB; Difco, Detroit, MI), 1% bovine hemoglobin (Becton Dickinson, Sparks, MD), 1% IsoVitaleX (Becton Dickinson, Cockeysville, MD), and 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Except for FX504, all strains were grown under heme-limiting conditions (GCB with 1% IsoVitaleX and 5% FBS) as described previously (19). Since the FX504 mutant does not grow under the described heme-limiting conditions, 5 μg of hemin per ml was added to the above-described broth medium (44).

HgbA purification. HgbA protein was affinity purified as previously described (19) with the following modifications. Large-scale cultures of *H. ducreyi* strain 35000HP were grown under heme-limiting conditions in Fernbach flasks containing 1.5 liters of GCB broth with 5% FBS and 1% IsoVitaleX and incubated overnight at 35°C in the presence of 5% CO_2 with shaking. *H. ducreyi* growth and the lack of other bacterial or fungal contaminants were confirmed by plating aliquots of overnight cultures on CAP and GCB agar lacking heme, respectively. *H. ducreyi* cells from Fernbach flasks were harvested by centrifugation, and the pellet was suspended in 15 ml of 10 mM HEPES buffer, pH 8.0, containing 0.6 mg/ml lysozyme. Suspensions were subjected to two cycles of cell disruption in a French press and then centrifuged at 9,200 × *g* for 20 min. The pellet, containing broken cells and insoluble cell components, was incubated with 1% *N*-lauroyl-sarcosine (#L-5125; Sigma) for 30 min at 37°C with mixing. The sarcosyl-insoluble fraction, containing primarily outer membrane components, was recovered after centrifugation at 100,000 × *g* for 1 h and dissolved with 1% Zwittergent 3,14 (Zw) (Calbiochem) in TEN (20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.0). Zw-insoluble material was removed by centrifugation at 100,000 × *g* for 1 h. The Zw-soluble fraction was incubated overnight with hemoglobin-agarose (preparation described below) at 4°C with gentle mixing to allow binding of HgbA to the hemoglobin. The next day, the hemoglobin-agarose was poured into a column and washed with 10 volumes of 0.5% Zw in TEN. The detergent was exchanged by washing with 1% octylglucoside (OG) in TEN. HgbA was eluted in 1% OG in 200 mM glycine HCl, pH 2.3, and immediately neutralized with 2 M Tris, pH 8.0. HgbA was concentrated using a Centriprep YM-50 Amicon column (Millipore, Bedford, MA) and washed with 1% OG in phosphate-buffered saline (PBS). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (catalog [cat] #23225; Pierce, Rockford, IL). Aliquots of purified HgbA were stored frozen at −70°C until needed.

Previously, we used a commercial source of hemoglobin-agarose (cat #H8756, lot #072k 7082; Sigma) to purify native HgbA, but recent lots did not perform satisfactorily. Therefore, we prepared hemoglobin affinity supports by covalently attaching bovine hemoglobin (cat #H2500, lot #010K7618; Sigma) to Affigel 15 (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's directions. After preparation, we mock eluted the hemoglobin-agarose using OG-glycine HCl, pH 2.3, to remove any noncovalently bound hemoglobin that might subsequently elute and contaminate our HgbA preparation.

Animals. Yorkshire Cross pigs were obtained at 3 weeks of age and housed at ambient temperature (20 to 25°C) in AAALAC-accredited biosafety level 2 containment facilities at the North Carolina State University School of Veterinary Medicine. Each pig was kept in an individual enclosure. Pigs received water and an antibiotic-free high-protein growth ration ad libitum for 6 weeks prior to immunization and throughout the period of immunization and infection with *H. ducreyi* (31). The experimental procedures described herein were reviewed and approved by the Institutional Animal Care and Use Committees of UNC and NCSU. For inoculation and biopsy procedures, pigs were sedated with 2 mg of ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 2 mg xylazine (Miles Laboratories, Shawnee Mission, KS) per kg of body weight administered intramuscularly.

Immunization. Two independent experiments were performed. For each iteration, two pigs were immunized with HgbA and two were mock immunized. The animals were immunized on day 1 and subsequently received booster immunizations on day 21 and day 42. The immunogen was prepared by adding 500 μl HgbA (0.5 mg/ml) in 0.5% OG to 0.5 ml complete Freund's adjuvant (cat #F5881; Sigma) for the initial immunization. For the booster immunizations, 500 μl of HgbA (0.5 mg/ml) in 0.5% OG was emulsified in 0.5 ml incomplete Freund's adjuvant (cat #F5506; Sigma). The control group of mock-immunized pigs received injections of the adjuvant and the buffer on the same days. Each immunization was performed in two intramuscular injections on opposite sides of the nuchal region.

Sera. Serum samples were obtained immediately before each immunization, before challenge and one week after challenge. Hereafter, we will refer to sera obtained prior to the first immunization and those obtained after three immunizations as "preimmunized" and "postimmunized" sera, respectively. Individual or pooled sera from HgbA-immunized or mock-immunized animals were heat inactivated at 56°C for 45 min and filter sterilized for biological assays. Normal pig serum was obtained from unimmunized pigs. All samples were aliquoted and stored frozen at −80°C. Human serum was obtained by informed consent from four individuals without histories of chancroid. Sera were pooled, aliquoted, and stored at −80°C until use. Freezing and thawing of samples were kept to a minimum. Immunoglobulin G (IgG) was purified on protein G columns (Pierce, Rockford, IL) following the manufacturer's instructions.

Inoculum and infection. To challenge after immunization, we followed the published procedure for inoculation of swine (31), with the exception that the *H. ducreyi* cells were suspended in GCB instead of PBS. We used Multi-Test applicators (Lincoln Diagnostics, Decatur, IL), which deliver approximately 5×10^4 CFU of *H. ducreyi* (14, 54), to inoculate animals. In the first iteration of this experiment, swine were challenged on 16 sites on the dorsum of the right ear with a suspension containing 5×10^4 CFU of *H. ducreyi* and on 16 sites on the left ear with a suspension containing 10-fold fewer *H. ducreyi* cells (5×10^3 CFU). In the second iteration, only 5×10^4 CFU was used for inoculation at 16 sites on the right ear of each pig. Viability of the inocula was monitored prior to and after each experiment; there was no decrease in CFU detected. All animals were visually observed at 2, 5, and 7 days after inoculation.

Biopsy samples. One week after challenge with *H. ducreyi*, biopsy samples were taken from pig ears by using a 6-mm-diameter skin punch extractor (Acuderm, Lauderdale, FL). Each sample was bisected into 2 equal parts. One section was minced and cultured on CAP containing 3 μg/ml vancomycin. *H. ducreyi* colonies were identified by typical buff-yellow to gray colonial coloration and by their characteristic property of remaining intact when pushed across the agar surface. Gram stains confirmed bacteria as small pleomorphic gram-negative rods.

Statistical analysis was performed only with data from biopsy samples from animals for which the standard challenge dose of 5×10^4 CFU of *H. ducreyi* was used. To assess the effectiveness of the vaccine, we first determined the mean colony count from each pig. Because we anticipated that the mean colony counts would not be normally distributed, we used a Wilcoxon rank sum test to compare the two groups of pigs. With this approach, the multiple biopsy samples from each pig increase the precision of the estimates of the colony counts within each pig, but the comparison is based on the pig as the unit of analysis. Identical results were obtained using the median colony counts for each pig.

The second half of the biopsy sample was fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Twelve sections were made from each biopsy sample and stained with hematoxylin-eosin (H&E) for histologic analysis. Slides were coded and graded blindly on a scale from 1 to 5. This numerical scoring system was used to describe the stages of ulcer development as previously described (54). Briefly, skin of normal appearance was assigned a score of 1; the presence of perivascular and interstitial mononuclear cell infiltrate was assigned a score of 2; the presence of an intraepidermal pustule with neutrophils and

necrotic debris was assigned a score of 3; an epidermal pustule accompanied by keratinocyte cytopathology and diffuse mononuclear and polymorphonuclear dermal infiltrate was scored as 4; and ulceration or dermal erosion accompanied by confluence of immune cells was scored as 5 (54). An ECLIPSE E800 (Nikon Co., Tokyo, Japan) microscope and QCapture software 1394 (Quantitative Imaging Co., Burnaby, BC, Canada) were used to make high-performance digital images of H&E-stained sections.

Bactericidal assay. All strains used in this study are resistant to 50% normal human serum (NHS). The bactericidal activity of pig serum against the indicated *H. ducreyi* strains was determined by use of an immune bactericidal assay as previously described (1, 20, 22). Briefly, *H. ducreyi* cells were grown in GCB broth under heme-limiting conditions to induce expression of HgbA. The bacteria were harvested by centrifugation, and cell density was adjusted to an optical density at 600 nm (OD_{600}) of 0.2 in GCB without supplements. After a 10^4-to-10^5 dilution, 65 μl (approximately 100 to 300 CFU of viable *H. ducreyi* cells) was placed in the wells of a sterile 96-well plate (Falcon microtest tissue culture plate; Becton Dickinson, Franklin Lakes, NJ). Ten μl of preimmunized and postimmunized pig sera or GCB was separately added to the *H. ducreyi* cells and incubated for 30 min at 35°C to allow antibody binding. Twenty-five μl of NHS (the source of complement) was added to equal a total volume of 100 μl to allow complement activation, deposition, and bacterial killing. After 45 additional minutes, 80 μl was plated on CAP; colonies were counted after 48 h of incubation. The percent survival of bacteria was determined by dividing the number of colonies that survived in fresh complement by the number of colonies surviving in heat-inactivated NHS and multiplying by 100. Normal pig serum was also used as a source of complement in the bactericidal assay; since no significant difference in activity was noted, only the data obtained with human complement are presented. *Neisseria gonorrhoeae* strain F62 (55) or *H. ducreyi dsrA* mutant FX517 (22) was used as a positive control for NHS bactericidal activity. To ensure that killing activity was due to antibody and complement and that these antibodies were directed against HgbA, a number of controls were performed. First, *H. ducreyi* cells were incubated in fresh or heated complement source in the absence or presence of immune serum. Killing was observed only when both fresh complement and postimmunized pig serum or IgG were present. Second, the specificity of the bactericidal activity was assessed by use of the *H. ducreyi hgbA* mutant FX504 and *gmbH* 35000.252 (a LOS mutant) (4) as the target bacteria. Bactericidal data were compared with a paired *t* test, using Excel (Microsoft) software. A *P* value of <0.05 was accepted as the level of statistical significance.

ELISA. An enzyme-linked immunosorbent assay (ELISA) for detection of anti-HgbA antibodies from pig sera was performed in 96-well plates (Falcon Pro Bind, cat #3915; Becton Dickinson, Franklin Lakes, NJ). Plates were coated with HgbA (200 ng/well) in 0.1 M carbonate buffer, pH 9.4, overnight at 4°C. Residual binding sites were blocked with 2% bovine serum albumin (BSA) (Roche Diagnostics Co., Indianapolis, IN) in PBS with 0.01% azide (PBS/A) for 1 h at room temperature (RT). Serial fivefold dilutions of pig sera were prepared using 1% BSA in PBS. The final dilution of serum samples tested was $1:3 \times 10^6$. Each dilution was added (100 μl/well) to the coated/blocked ELISA plates and incubated at RT for 1 h. After washing three times with 0.05% (vol/vol) Tween 20 in PBS (PBS/T wash buffer), alkaline phosphatase-conjugated rabbit anti-pig IgG (cat #A1192; Sigma-Aldrich Inc., St. Louis, MO) diluted 1:10,000 (vol/vol) in PBS/T was added. Plates were incubated at RT for 1 h and washed five times with wash buffer. Color development was achieved by adding 50 μl/well of 1-Step *p*-nitrophenyl phosphate substrate (Pierce, Rockford, IL). The plates were incubated for 15 min at RT, and the reaction was stopped by the addition of 50 μl 2 M NaOH. Optical density was measured at 405 nm on a 1420 VICTOR^2 multilabel counter (Wallac Oy, Turku, Finland). The positive-control serum was rabbit anti-recombinant HgbA and the secondary antibody for this was goat anti-rabbit alkaline phosphatase (1:10,000). This serum was obtained by immunizing a New Zealand White rabbit three times with 200 μg of recombinant HgbA (rHgbA) (66). The first immunization used complete Freund's adjuvant and the second two immunizations used incomplete Freund's adjuvant. Specificity of the anti-rHgbA serum was shown in Western blots against 35000 and *hgbA* mutant FX504 (data not shown). The negative-control serum was rabbit anti-recombinant DsrA serum (13). The background was determined as the mean OD_{405} of wells not containing primary antibodies. The endpoint titer of antibodies was defined as the reciprocal of the highest serum dilution that gave a reading above an OD_{405} of 0.1 as previously described (9). Each sample was run in duplicate on at least three different days. The ability of anti-HgbA IgG to block hemoglobin binding to HgbA was evaluated with an ELISA format similar to that of the direct ELISA. After immobilization of HgbA, blocking, and washing, purified pig IgG (0.2 mg/ml in a volume of 100 μl) or sera (1:10) were added to each well, and incubation of the wells for 20 min at RT was followed by three washes with wash buffer. Human digoxigenin-labeled hemoglobin (1:100) was added to the wells, and the wells were incubated at RT for 1 h. After three washes with wash buffer, alkaline phosphatase-conjugated antidigoxigenin antibodies (cat #11327720; Roche Diagnostic, Indianapolis, IN) were added at a dilution of 1:5,000 (vol/vol) in PBS, and the wells were incubated for 1 h at RT. Color development was achieved as described above. Human hemoglobin was labeled according to the manufacturer's instructions (DIG protein labeling kit; Roche Diagnostic).

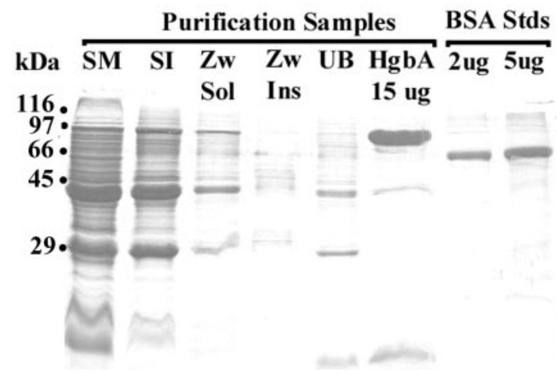

FIG. 1. Purification of HgbA. Various fractions from hemoglobin-agarose affinity purification of HgbA are shown in this Coomassie blue-stained 15% SDS-PAGE gel. Lanes: kDa, kilodalton (molecular marker lane); SM, French-pressed cell starting material; SI, sarcosyl-insoluble fraction; Zw Sol, Zwittergent 3,14-soluble fraction applied to a hemoglobin-agarose column; Zw Ins, Zwittergent 3,14-insoluble fraction; UB, unbound flowthrough fraction not bound to hemoglobin-agarose; HgbA (15 μg by BCA total protein determination); BSA Stds, purified bovine serum albumin used to semiquantitate intact HgbA protein. The purified HgbA shown here was the immunogen for the first vaccine experiment. Similar results were obtained in a second purification, which was used for the second vaccine iteration (not shown).

Western blots. Total cellular proteins of *H. ducreyi* strains (approximately 0.5×10^7 CFU per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred to nitrocellulose membranes and subjected to Western blotting as previously described (1). Blots were probed with individual or pooled pig sera diluted 1:10,000. The secondary rabbit anti-pig IgG alkaline phosphatase-conjugated antibodies (cat #A1192; Sigma) were diluted 1:15,000. Binding of secondary antibodies was detected using Lumi-Phos chemiluminescent substrate (Pierce, Rockford, IL). To test for the presence of contaminating LOS in purified HgbA immunogen preparations, the HgbA was digested with proteinase K and subjected to Western blotting using anti-LOS monoclonal antibodies (MAbs) MAHD6 (LOS epitope, DD-Hep*p*-1→6-β-D-Glc*p*) and MAHD7 (epitope is the common conserved inner core region of the LOS) (2) and wheat germ agglutinin-horseradish peroxidase (HRP). The secondary antibody was goat anti-mouse alkaline phosphatase (cat #A5153; Sigma). Known amounts of phenol-water purified LOS were used as standards for comparison (36).

Immunoglobulin G binding to whole *H. ducreyi* cells. Ten μl of diluted pig sera (1:50 in GCB, 1:500 final dilution) was incubated with a 90-μl suspension of *H. ducreyi* strains 35000HP, FX504, and CIP542 ATCC (OD_{600} = 0.4, approximately 5×10^7 CFU) or with 90 μl GCB in a MultiScreen filter plate (Millipore Corp., Bedford, MA) for 30 min. The plates were then suctioned and washed three times with GCB. Anti-pig IgG-HRP (cat #A7042; Sigma-Aldrich Inc., St. Louis, MO) was added and incubated for 30 min at RT; this was followed by suctioning and five washes. HRP substrate (ECL; Amersham Bioscience Corp., Piscataway, NJ) was added and chemiluminescence was detected by a 1420 VICTOR^2 multilabel counter (Wallac Oy, Turku, Finland). To ensure equal loading of class I *H. ducreyi* strains, we used an anti-rDsrA pig serum as an internal control. The antisera to DsrA were prepared by immunization of pigs using the same purified recombinant DsrA immunogen we previously described (13). Recombinant DsrA was mixed with Ribi adjuvant, and 500 μg was injected intramuscularly in the nuchal region. The two immunizations were separated by 3 weeks.

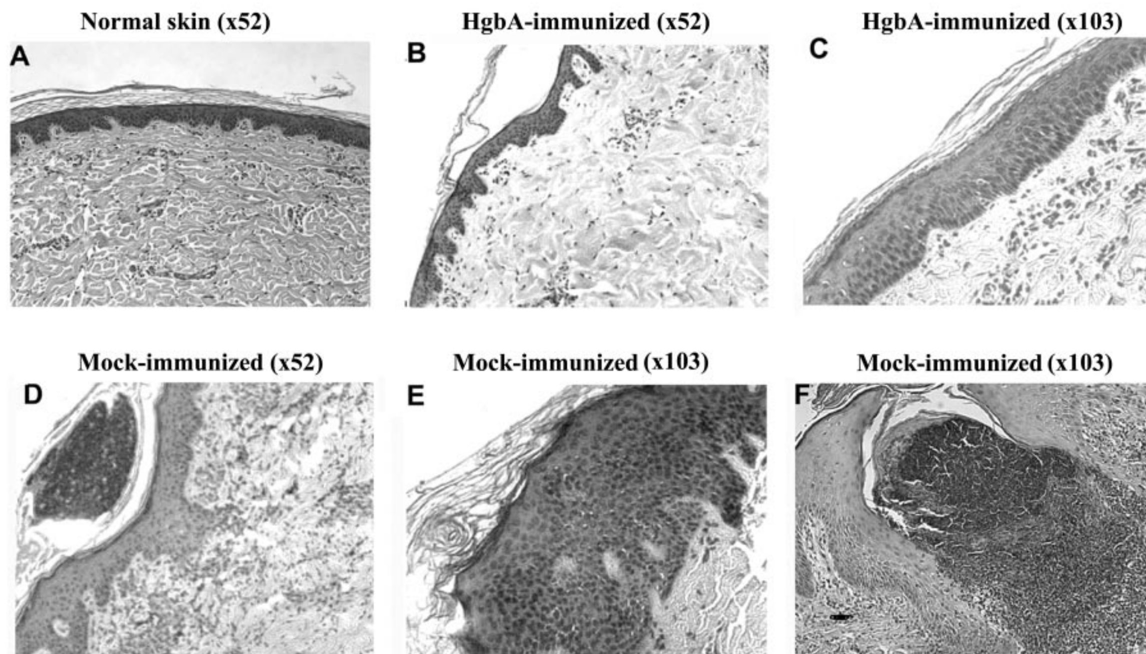

FIG. 2. Microscopic examination of lesions. Hematoxylin-eosin staining of skin biopsy sections of pigs 1 week after challenge with live *H. ducreyi* 35000HP cells. (A) Image of normal pig skin; (B and C) skin cross-sections from HgbA-immunized pigs; (D through F) skin sections of mock-immunized pigs. Magnification values are shown.

RESULTS

Purification of HgbA. We purified HgbA from type strain 35000HP using hemoglobin-agarose affinity chromatography as detailed in Materials and Methods. HgbA was >95% pure as judged by Coomassie blue staining of 15% SDS-PAGE gels (Fig. 1). Two minor additional bands of approximately 45 kDa and 20 kDa were also observed. The 45-kDa band reacted with independently derived anti-rHgbA rabbit sera. Moreover, an identically sized immunoreactive band is seen in whole-cell lysates of 35000 but not of FX504 (data not shown). Thus, this band may represent a breakdown product of HgbA. The 20-kDa protein band was not recognized by any HgbA antibodies, and the identity of this band is not presently known. We analyzed proteinase K-digested purified HgbA protein preparations in Western blots for the presence of contaminating LOS by use of anti-LOS MAbs and wheat germ agglutinin lectin. Comparison of the signal obtained with proteinase K-digested purified HgbA with the signals obtained with purified LOS standards indicated that less than 5 ng of LOS was present per μg of purified HgbA (data not shown). The concentration of total protein was measured by BCA, and the concentration of intact HgbA was estimated by comparing the staining intensity to BSA standards (Fig. 1).

Effect of HgbA immunization on the development of lesions in swine. In two separate experiments, four pigs were thrice immunized with the HgbA vaccine while four pigs were immunized with mock vaccine, each in Freund's adjuvant (see Materials and Methods for details). Three weeks after the third immunization, the dorsal skin of pig ears was inoculated with viable *H. ducreyi* strain 35000HP using an allergy-testing device. Each site was inoculated with approximately 5×10^4 CFU of *H. ducreyi* 35000HP. In the first experiment only, pigs from each group were also inoculated with 10-fold fewer CFU of *H. ducreyi* to examine the effect of a lowered dose on the development of lesions. The sites of infection were observed 2 and 7 days after inoculation. By day 2, papule-like lesions had developed on both HgbA-immunized and mock-immunized pig groups. On day 7, lesions from HgbA-immunized pigs appeared smaller on a macroscopic level. While macroscopic observations varied somewhat within each group, as a whole, lesions on immunized animals appeared smaller and less erythematous than those from mock-immunized animals.

Histopathology of skin lesions. The biopsy samples of lesions were sectioned, H&E stained, and analyzed microscopically by two independent, blinded observers. Representative sections are shown in Fig. 2. In general, sections from HgbA-immunized animals contained relatively mild inflammatory infiltrates and an intact basal lamina at the epidermal-dermal junction. Additionally, minor localized dermal perivascular and interstitial mononuclear cell infiltrate was observed. In contrast, sections from mock-immunized control pigs exhibited extensive thickening of the epidermis, intraepidermal pustule formation with diffuse hyperplasia, and acanthosis of epithelial cells. Sections from mock-immunized pigs showed a completely disorganized basal membrane layer. The relative severity of each lesion was graded and subjected to statistical analysis as previously described (54). The average histology score for lesions obtained from HgbA-immunized pigs was 2.0, while the score was 3.4 for mock-immunized pigs ($P = 0.035$, paired *t* test). Thus, immunization with HgbA reduced initial size and severity of lesion formation and/or increased the speed of healing.

Recovery of *H. ducreyi*. Seven days after challenge with live 35000HP cells, we recovered viable *H. ducreyi* cells from 17

TABLE 1. *H. ducreyi* recovery from pig ear biopsy samples

Experiment no.	Mock immunization data			HgbA immunization data		
	Pig no.	No. of biopsy samples	Bacterial recovery [median CFU[a] (range)]	Pig no.	No. of biopsy samples	Bacterial recovery [median CFU (range)]
1	1	4	5.0 (3, 10)	5	4	0 (0, 0)
	2	4	160.5 (138, 269)	6	4	0 (0, 0)
2	3	3	400.0 (41, 600)	7	6	0 (0, 0)
	4	6	11.5 (2, 37)	8	6	0 (0, 0)
Total		17	26.0 (2, 600)		20	0 (0, 0)[b]

[a] CFU per biopsy sample half.
[b] $P < 0.0139$ compared to mock immunization data by Wilcoxon rank sum test comparing bacterial recovery. $P = 0.029$ by Fisher's exact test comparing the number of infected pigs.

biopsy samples cultured from the four mock-immunized pigs. Strikingly, none of the biopsy samples ($n = 20$) from the four HgbA-immunized pigs yielded viable *H. ducreyi* cells (Table 1) ($P = 0.0139$, Wilcoxon rank sum). The numbers of *H. ducreyi* cells recovered from lesions of mock-immunized pigs varied considerably. In the mock-immunized group, an effect of challenge dose was observed; recovery was 19.1-fold higher from lesions inoculated with 5×10^4 CFU than from lesions inoculated with 5×10^3 CFU (data not shown). Therefore, HgbA immunization prevented the recovery of *H. ducreyi*.

Antibody response to HgbA. The antibody response to HgbA was monitored using ELISA, whole-cell binding, and Western blot assays. ELISA data (Fig. 3) indicated that all pigs were free of anti-HgbA antibodies before immunizations and that the levels of anti-HgbA IgG increased only in the sera of HgbA-immunized groups. After three immunizations with HgbA antigen, reciprocal endpoint titers of anti-HgbA antibodies in individual sera of HgbA-immunized animals varied from 5×10^5 to 3×10^7. All sera from mock-immunized animals had endpoint titers of less than 5×10^2. These data indicate that HgbA is a highly immunogenic protein.

In order to be protective, antibodies should bind to the surface of intact bacteria. To measure antibody binding to whole *H. ducreyi* cells, sera were mixed with *H. ducreyi* cells and antibody binding was assessed. Significant antibody reactivity with the surface of *H. ducreyi* 35000HP cells was observed only with postimmunized pig sera from HgbA-immunized swine (Fig. 4). To assess the cross-reactivity of antibodies elicited to the class I HgbA from *H. ducreyi* strain 35000HP, we measured binding to heterologous class II strain CIP542 ATCC. Anti-HgbA class I antibodies bound class II strain CIP542 ATCC almost as well as they bound strain 35000HP. As expected, *H. ducreyi hgbA* mutant FX504 was only weakly recognized with IgG from HgbA-immunized pig sera. This indicated that anti-

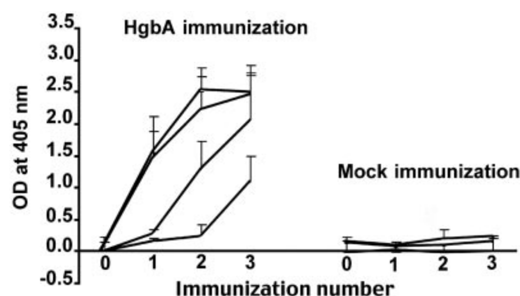

FIG. 3. ELISA. Profiles of specific IgG responses of individual pig sera diluted 1:5,000. The sera from four HgbA-immunized and four mock-immunized pigs were collected at immunization 0 (preimmunization) and 1, 2, and 3 (3 weeks after immunizations 1, 2, and 3, respectively). Sera were tested for IgG reactivity against immobilized HgbA. Bound IgG was detected with alkaline phosphatase-labeled anti-pig IgG. Each sample was run in duplicate on at least three different days. The results were expressed as units of optical density (OD) at 405 nm and represent the mean ± standard deviation (SD).

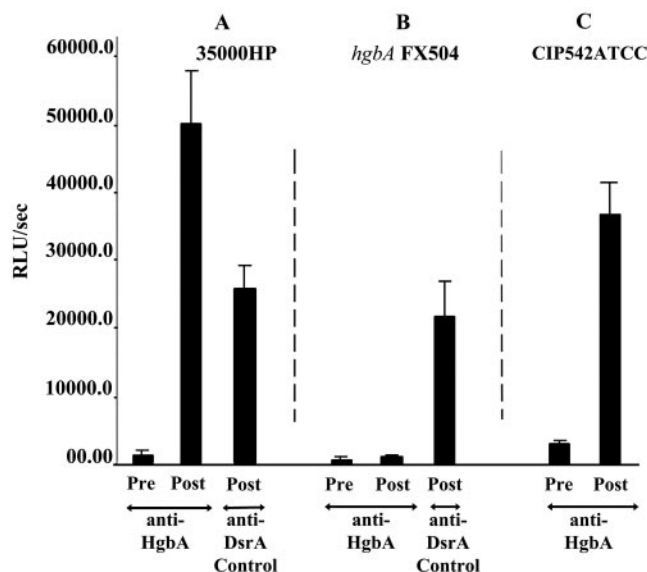

FIG. 4. Binding of anti-HgbA to whole *H. ducreyi* cells. A dilution containing 0.2 μl of each preimmunized (Pre) or postimmunized (Post) HgbA serum was mixed with 2×10^7 CFU of the indicated *H. ducreyi* strains in a total volume of 100 μl (final dilution of serum, 1:500). After binding for 30 min, bacteria were suctioned and washed and antibody binding was detected with a secondary HRP-conjugated anti-pig IgG antibody followed by chemiluminescence detection. The control anti-DsrA serum was made in a pig by using recombinant DsrA from strain 35000. The serum from each pig was individually tested for binding to whole cells in three separate experiments. The data were combined for each group (HgbA or mock immunized) and expressed as the mean ± SD. There was no significant binding of pre- or postimmunized sera from mock-immunized pigs to whole cells (data not shown). RLU/sec, relative light units/s.

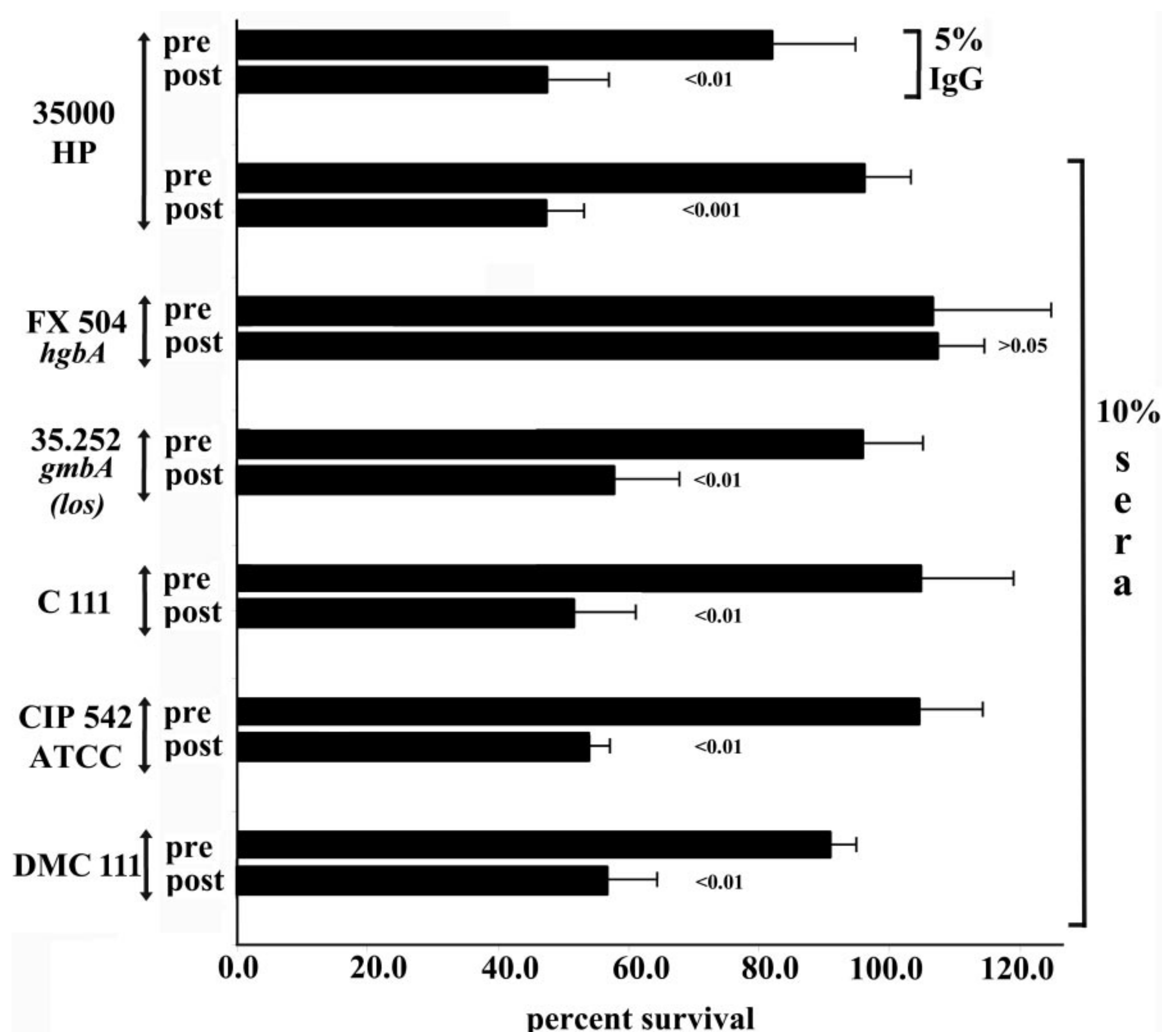

FIG. 5. Bactericidal killing of *H. ducreyi*. Survival of *H. ducreyi* in pooled pig serum in the presence of 25% NHS complement. Target strains are shown on the left. Purified pig IgG was used at 5% concentration relative to normal pig IgG serum levels (final concentration, 0.5 mg/ml). The data are compiled from separate experiments done on at least three different days and represented as the mean ± SD. Statistical difference (*P* value) was obtained by Student's paired *t* test.

bodies elicited to HgbA recognized primarily HgbA on the surface of 35000HP cells and not other surface-exposed antigens. Furthermore, IgG from sera of mock-immunized pigs did not show any significant binding with exposed bacterial antigens and resembled the preimmunized pig IgG (Fig. 4). Thus, antibodies elicited to HgbA were highly specific and bound to surface-exposed epitopes of HgbA.

To determine the specificity of the anti-HgbA IgG antibody, we subjected total cellular proteins to Western blotting under denaturing conditions. When probed with the anti-HgbA IgG, HgbA was recognized only weakly (data not shown). Binding to a molecule of approximately 20 kDa was also observed. This 20-kDa band was not LOS, since no signal was obtained when *H. ducreyi* cellular proteins were first treated with proteinase K prior to loading in Western blots (data not shown).

Bactericidal activity of sera and purified IgG from HgbA-immunized pigs. Bactericidal activity is commonly used as an in vitro correlate of protection in assessing the efficacy of gram-negative vaccines. Therefore, we tested the ability of HgbA-immunized pig serum to kill cells of virulent strain 35000HP. We observed more than 50% bacterial killing in 10% pooled sera from animals immunized with HgbA (Fig. 5). Little or no killing was observed in the preimmunized or mock-immunized pig sera. Killing required active complement, since heating the complement abrogated the killing effect. Moreover, purified IgG (final concentration, 0.5 mg/ml) from HgbA-immunized pigs killed as well as whole serum (Fig. 5). To examine whether the HgbA-immunized pig serum was bactericidal against other *H. ducreyi* strains, we tested heterologous strains in the bactericidal assay by use of pooled HgbA-immunized pig sera. Postimmunized pig sera killed heterologous strains C111 (class I), CIP542 ATCC (class II), and DMC 111 (class II) at modest levels (Fig. 5). Individual pig sera were also tested for bactericidal activity against strain 35000HP, and the survival varied between 30 and 70% (data not shown). Neither pooled pig sera (Fig. 5) nor individual sera killed the isogenic *hgbA* mutant FX504. Interestingly, killing of *H. ducreyi* 35000HP was similar to that of the deep rough LOS mutant 35000.252, consistent with anti-HgbA antibodies being

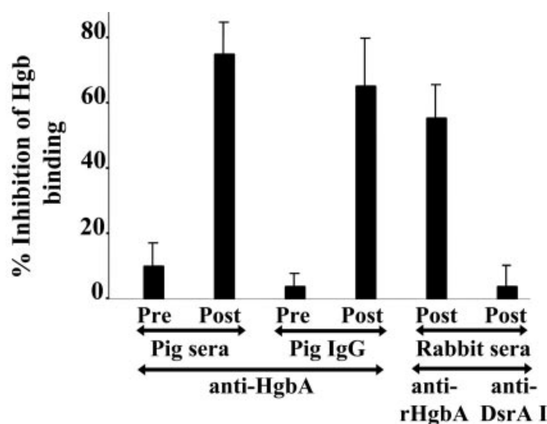

FIG. 6. Inhibition of hemoglobin (Hgb) binding by HgbA-immunized pig serum or pig IgG. Purified HgbA was coated onto ELISA plates, the plates were blocked, and the abilities of various antibodies to HgbA to block labeled hemoglobin binding to the receptor were tested. Pre, preimmune; Post, postimmune; Pig IgG, purified pig IgG; anti-rHgbA, rabbit anti-recombinant HgbA (positive control); anti-DsrA I, rabbit anti-recombinant DsrA (negative control). The data are compiled from separate experiments done on at least three different days.

responsible for killing as opposed to anti-LOS antibodies. Taken together, these data suggest that the modest bactericidal effect observed was due to anti-HgbA antibodies.

Effect of anti-HgbA IgG on hemoglobin binding. We previously demonstrated that the *hgbA* mutant FX504 is highly attenuated in the human experimental model of chancroid (3). This attenuation is thought to be attributed to its inability to bind hemoglobin and obtain heme for growth. This observation prompted us to examine the anti-HgbA pig antibodies to block hemoglobin binding to HgbA. In an ELISA format, immobilized HgbA was incubated with pre- or postimmunized pig serum; this was followed by binding of labeled human hemoglobin. Incubation of postimmunized anti-HgbA IgG with immobilized HgbA receptor inhibited subsequent hemoglobin binding, whereas little or no effect was seen with preimmune or irrelevant IgGs (Fig. 6).

DISCUSSION

A swine model of *H. ducreyi* infection was used in the present vaccine study because available evidence suggests that the histopathology of the lesions of the swine model closely resembles that of human chancroid (31) and that the swine immunology and skin structure closely resemble their human counterparts (53). Recently, it was shown that repeated infection with *H. ducreyi* in pigs elicited a form of immunity, since after the second and third challenges, lesions resolved more quickly and became sterile more rapidly than after a single challenge (14). Although the immune sera from thrice-infected pigs recognized a number of antigens, including HgbA, it was not clear which antigens might be responsible for the immunity or bactericidal activity of these sera. However, the immunity could be passively transferred, proving that the antibody-mediated protection in the swine model of infection is important (14).

In the present study, we demonstrate that HgbA may be an important vaccinogen. Immunization with native HgbA resulted in (i) reduced macroscopic and microscopic lesion development after *H. ducrey*i challenge, (ii) the absence of viable *H. ducreyi* cells in day 7 biopsies, (iii) the production of a vigorous antibody response, (iv) antibodies that bound intact bacteria, and (v) the development of bactericidal antibodies and antibodies that inhibited hemoglobin binding to HgbA.

Macroscopic and microscopic examination of lesions was consistent with a protective effect by HgbA. Microscopically, biopsy samples from lesions of HgbA-immunized animals resembled those of normal skin sections compared to biopsy samples from mock-immunized animals.

Perhaps the most important finding from this study is that HgbA completely prevented recovery of *H. ducreyi*. In contrast, mock immunization did not prevent recovery of *H. ducreyi*, and all cultured biopsy samples yielded organisms. In the first iteration, in addition to the standard inoculum, a 10-fold-lower inoculum level was also used. *H. ducreyi* cells were recovered from all lower-inoculum-level lesions of mock-immunized animals but not from HgbA-immunized animals. Thus, it appears that the HgbA vaccine protected against a challenge 10-fold higher than that required for initiating disease in the mock-immunized group.

Immunization with purified HgbA elicited antibodies that bound native HgbA in the context of the outer membrane of *H. ducreyi* cells. These antibodies bound poorly to the *hgbA* mutant, demonstrating specificity for HgbA. In Western blots, HgbA was weakly recognized with HgbA-immunized pig sera, probably due to its change in conformation after denaturation or poor transfer from the gel to nitrocellulose. Previously, we found that mouse monoclonal antibodies (44) and rabbit antisera (36) elicited to native HgbA also failed to bind in Western blots, suggesting that the conformation of HgbA is complex and critical to antibody binding. In Western blots, we also found that anti-HgbA sera recognized an unknown component of approximately 20 kDa. This reactivity is probably not to LOS, because this 20-kDa molecule was also present in the 35000.252 *los* mutant and reactivity was proteinase K sensitive. In studies not presented here, this molecule seemed to fractionate in the cytosolic fraction (data not shown). Furthermore, the 20-kDa antigen cannot account for the bactericidal activity we observed, since this molecule is present in *hgbA* strain FX504, which was not killed in bactericidal assays (data not shown). Moreover, LOS *gmbH* mutant 35000.252 shows a survival rate in 10% immune pig serum which is similar to that of *H. ducreyi* 35000HP, confirming the specificity of immunized pig antibodies to HgbA.

The mechanism of protection of the HgbA vaccine is presently not known. We found modest evidence of bactericidal killing of homologous strain 35000HP, initiated by anti-HgbA sera or IgG. Moderate bactericidal killing was also observed in heterologous *H. ducreyi* strains C111 (class I), DMC 111 (class II), and CIP542 ATCC (class II). Thus, since the HgbA deduced protein sequences are more than 95% identical (66) and if anti-HgbA antibodies are important, it is conceivable that a monovalent HgbA vaccine could protect against a wide variety of *H. ducreyi* strains.

An important part of mammalian innate immune systems is based on the concept of "nutritional immunity" (32, 43, 46, 56, 62). Mammals prevent growth of microbes by utilizing various proteins that bind and sequester heme and iron necessary for bacterial growth (23, 46). Obligate human bacterial pathogens

have evolved ingenious strategies to overcome these heme/iron-withholding systems. For example, gram-negative bacteria have evolved TonB-dependent receptors for heme, hemoglobin, transferrin, and lactoferrin that enable them to bind and utilize the heme/iron present in these proteins. We found that anti-HgbA IgG blocked hemoglobin binding to HgbA, suggesting that it is possible to induce antibodies that can block heme/iron acquisition. Thus, the protection observed in this study perhaps may be partly or wholly due to inhibition of bacterial growth by induced nutritional immunity.

The role of cellular immunity was not examined in this study. The possibility exists that the immunity we observed is due to a cellular response. Further studies are needed in this area.

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