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Immunization with the *Haemophilus ducreyi* Hemoglobin Receptor HgbA with Adjuvant Monophosphoryl Lipid A Protects Swine from a Homologous but Not a Heterologous Challenge[▽]

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Haemophilus ducreyi*, the etiological agent of chancroid, has a strict requirement for heme, which it acquires from its only natural host, humans. Previously, we showed that a vaccine preparation containing the native hemoglobin receptor HgbA purified from *H. ducreyi* class I strain 35000HP (nHgbA$_I$) and administered with Freund's adjuvant provided complete protection against a homologous challenge. In the current study, we investigated whether nHgbA$_I$ dispensed with monophosphoryl lipid A (MPL), an adjuvant approved for use in humans, offered protection against a challenge with *H. ducreyi* strain 35000HP expressing either class I or class II HgbA (35000HP*hgbA$_I$* and 35000HP*hgbA$_{II}$*, respectively). Pigs immunized with the nHgbA$_I$/MPL vaccine were protected against a challenge from homologous *H. ducreyi* strain 35000HP*hgbA$_I$* but not heterologous strain 35000HP*hgbA$_{II}$*, as evidenced by the isolation of only strain 35000HP*hgbA$_{II}$* from nHgbA$_I$-immunized pigs. Furthermore, histological analysis of the lesions showed striking differences between mock-immunized and nHgbA$_I$-immunized animals challenged with strains 35000HP*hgbA$_I$* but not those challenged with strain 35000HP*hgbA$_{II}$*. Mock-immunized pigs were not protected from a challenge by either strain. The enzyme-linked immunosorbent assay (ELISA) activity of the nHgbA$_I$/MPL antiserum was lower than the activity of antiserum from animals immunized with the nHgbA$_I$/Freund's vaccine; however, anti-nHgbA$_I$ from both studies bound whole cells of 35000HP*hgbA$_I$* better than 35000HP*hgbA$_{II}$* and partially blocked hemoglobin binding to nHgbA$_I$. In conclusion, despite eliciting lower antibody ELISA activity than the nHgbA$_I$/Freund's, the nHgbA$_I$/MPL vaccine provided protection against a challenge with homologous but not heterologous *H. ducreyi*, suggesting that a bivalent HgbA vaccine may be needed.

Chancroid is one of the genital ulcer diseases and is transmitted through sexual contact. Lesions caused by chancroid initially appear as papules, which evolve within several days into pustules. If left untreated, chancroid pustules develop into painful, bleeding ulcers with soft, irregular borders. Chancroid is prevalent in certain developing countries but is rarely found in the United States (47, 62). Several studies have shown that chancroid serves as an important independent cofactor in the heterosexual transmission of HIV where both diseases are endemic (29, 35, 48, 51, 66). Commercial sex workers serve as the reservoir of chancroid, and control of disease in this population strikingly reduces the number of cases of chancroid in their male clients (58). Thus, one possible approach to control chancroid is to implement a limited vaccination program to control infection in this reservoir; however, no vaccine for chancroid currently exists.

Haemophilus ducreyi, the etiologic agent of chancroid, is a fastidious Gram-negative bacterium and a strict human pathogen. An interesting biologic feature of *H. ducreyi* is its obligate requirement for heme. Heme (for *H. ducreyi*) and iron are critical nutrients required for most pathogenic bacteria. Many Gram-negative bacteria obtain heme/Fe through systems that include TonB-dependent outer membrane receptors specific for heme/Fe compounds. The relatively small genome of prototypical *H. ducreyi* strain 35000HP encodes only three TonB-dependent receptors; in comparison, other bacterial genomes can encode more than 30 (14). Using isogenic mutants, the Spinola and Elkins laboratories surveyed the ability of *H. ducreyi* TonB-dependent receptor mutants to initiate infection in the human experimental model of chancroid. An *H. ducreyi* mutant that does not express the gene encoding the hemoglobin (Hb) receptor, *hgbA*, did not establish human infection (3). In contrast, an isogenic double mutant lacking the genes encoding the two other TonB-dependent receptors of *H. ducreyi*, *tdhA* and *tdX*, was fully virulent, indicating that HgbA is the only TonB-dependent receptor of *H. ducreyi* to be a virulence factor in the human experimental model of chancroid (38). Since HgbA is required for the utilization of heme from Hb by *H. ducreyi* (17), these data suggest that Hb is the most important source of heme in the early stages of the human experimental model of chancroid and that HgbA is a potential vaccine candidate.

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HgbA is a large, 100-kDa outer membrane protein that has a complex structure similar to that of other TonB-dependent receptors whose structure has been solved (10, 12, 20, 21, 40). HgbA is believed to contain 22 transmembrane beta sheets and 11 putatively surface-exposed loops. A recent study in our laboratory using *H. ducreyi* mutants expressing single loop deletions in HgbA provided evidence for surface exposure of loops 4, 5, 6, and 7 (44). Moreover, deletions of loops 5 and 7 but not of the other 9 loops of HgbA abrogated the binding of human Hb to HgbA. We also found that IgG from pigs immunized with native HgbA (nHgbA) bound loops 4, 5, and 7 and that antibodies directed at loops 4 and 5 partially blocked Hb binding to HgbA *in vitro*. Thus, a central domain of the primary sequence of HgbA is immunogenic, required for binding Hb, and surface exposed.

H. ducreyi strains exist in two groups, designated class I and class II, based on striking primary sequence differences in certain outer membrane proteins, such as DsrA (*ducreyi* serum resistance A) and NcaA (necessary for collagen adhesion A), and on their lipooligosaccharide (LOS) structures (49, 50, 53, 67). In contrast, the HgbA proteins of different classes of *H. ducreyi* strains are more than 95% identical. Prototypical *H. ducreyi* strain 35000HP, a class I isolate, is the strain used for most studies, including isogenic mutant construction and the experimental human model of chancroid. 35000HP is the only *H. ducreyi* strain whose genome has been sequenced.

Previously, we showed that immunization of swine with native HgbA from class I strain 35000HP ($nHgbA_I$) in Freund's adjuvant provided complete protection from a homologous challenge infection with *H. ducreyi* strain 35000HP (1). The antibodies elicited by $nHgbA_I$/Freund's showed modest bactericidal activity, bound to the cell surface of both class I and class II *H. ducreyi* strains, and partially blocked Hb binding to nHgbA (1). $nHgbA_I$ antisera did not recognize the surface of, nor did they show bactericidal activity against the isogenic *hgbA* mutant, demonstrating specificity of the humoral response to HgbA.

In the current study, we pursued two objectives. First, we investigated the effectiveness of monophosphoryl lipid A (MPL), an adjuvant approved for use in humans, to elicit an immune response to the $nHgbA_I$ vaccine that is protective against an *H. ducreyi* challenge in the experimental swine model of chancroid. Second, we examined the ability of this vaccine to protect swine from a challenge infection with *H. ducreyi* strain 35000HP expressing either class I *hgbA* (*hgbA*$_I$) or class II *hgbA* (*hgbA*$_{II}$) from *H. ducreyi* strain DMC111 (homologous versus heterologous challenge, respectively).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. ducreyi* strain 35000HP (human-passaged variant, class I strain) (4, 25) used in this study was obtained from Stanley Spinola, Indiana University, Indianapolis, IN. *H. ducreyi* class II strain DMC111 is an isolate from Bangladesh (67). The construction of isogenic *hgbA*$_I$ mutant *H. ducreyi* strain FX547 was previously described (44). FX547 contains a complete deletion of the *hgbA*$_I$ open reading frame, replaced with a chloramphenicol resistance cassette insert, and cannot grow on Hb plates (100 μg/ml) as a sole source of heme (44). For routine growth, *H. ducreyi* strains were maintained on chocolate agar plates containing gonococcal medium base (GCB; Difco, Detroit, MI) and 1% bovine Hb (Becton Dickinson, Sparks, MD) and supplemented with 1% GGC (0.1% glucose, 0.001% glutamine, 0.026% cysteine) and 5% Fetalplex (Gemini Bio-Products, West Sacramento, CA). Cultures were incubated at 34.5°C with 5% CO_2.

Construction of *H. ducreyi* strain 35000HP expressing the *hgbA*$_{II}$ gene from strain DMC111. A class I *H. ducreyi* strain expressing the class II *hgbA* gene (*hgbA*$_{II}$), 35000HP*hgbA*$_{II}$, was constructed in the following fashion: PCR was used to amplify *hgbA*$_{II}$ from *H. ducreyi* strain DMC111 using class II-specific *hgbA* primers that contain restriction sites (underlined) (5′-TAACACTTAAGGAATACGTAATGAAAACGAATAAACTC-3′ and 5′-GAGAAATCATATGACAAAAAAGGGCACCGAAGT-3′) and cloned into the AflII and NdeI sites of pUNCH1411 (44) to create pUNCH1413. pUNCH1413 contains the open reading frame of class II *hgbA* with class I *hgbA* flanking sequences. pUNCH1413 DNA was digested with EcoRI and XhoI, and the *hgbA* fragment isolated. This DNA fragment was electroporated into *H. ducreyi* FX547, and cells plated on Hb agar plates. Transformants that grew on Hb as a sole source of heme were screened for the expression of HgbA by Western blotting. A single colony was chosen, isolated, and sequenced. Two mutations were detected during sequencing. The first mutation was silent and the second mutation changed amino acid 21 of the mature protein from valine to glutamic acid. This change is in the "plug" region of mature HgbA and is thought not to be surface exposed. The $HgbA_{II}$ protein functions normally for growth on medium with Hb as the sole heme source in 35000HP, and *H. ducreyi* strain 35000HP*hgbA*$_{II}$ is fully virulent in the swine model. For the purpose of this report, wild-type *H. ducreyi* strain 35000HP will be referred to as 35000HP*hgbA*$_I$. To ensure that $HgbA_{II}$ was expressed at the same level as $HgbA_I$ in the 35000HP background, outer membrane proteins were prepared from low-heme liquid cultures of strains 35000*hgbA*$_I$, 35000HP*hgbA*$_{II}$, and the *hgbA* isogenic null mutant 35000HPΔ*hgbA* and subjected to SDS-PAGE followed by Coomassie staining. $HgbA_{II}$ is expressed at a level similar to that of $HgbA_I$ in the 35000HP background in both whole cells and outer membrane protein preparations (data not shown).

HgbA purification. Purification of $nHgbA_I$ and $nHgbA_{II}$ from *H. ducreyi* strains 35000HP*hgbA*$_I$ and 35000HP*hgbA*$_{II}$ was performed as previously described (1). The purity of both $nHgbA_I$ and $nHgbA_{II}$ was greater than 95% (data not shown).

Animals. Eight Yorkshire Cross pigs were used in two separate immunization experiments (four pigs per experiment). Pigs were obtained at 3 weeks of age and housed at ambient temperature (20° to 25°C) in individual pens at the North Carolina State University (NCSU) School of Veterinary Medicine. Pigs were given water and antibiotic-free high-protein feed *ad libitum* starting 6 weeks prior to the start of and continuing throughout the study. During inoculation and biopsy procedures, pigs were sedated with 2 mg of ketamine HCl (Fort Dodge Laboratories, Fort Dodge, IA) and 2 mg of xylazine (Miles Laboratories, Shawnee Mission, KS) per kg of body weight, injected intramuscularly. Pigs at challenge generally weighed between 70 and 100 lbs. The Institutional Animal Care and Use Committee (IACUC) at NCSU approved the use of animals for these experiments.

Immunization protocol. The immunization protocol used in this study was exactly replicated from the original $nHgbA_I$ vaccine trial so that the results from the current study would be directly comparable to the one from the previous study (1). In each of 2 experiments, 2 pigs were mock immunized with buffer (1% octylglucoside [Calbiochem, La Jolla, CA] in phosphate-buffered saline [PBS]) and adjuvant alone and 2 pigs received $nHgbA_I$ in buffer and adjuvant. The MPL adjuvant (Sigma, St. Louis, MO) was prepared according to the manufacturer's instructions. The immunogen was prepared by mixing 250 μg of $nHgbA_I$ in 500 μl PBS containing 1% octylglucoside with 500 μl of MPL adjuvant. Each pig received three intramuscular injections in the nuchal region in two sites 21 days apart. Pigs were bled prior to each of the three immunizations, as well as before challenge and biopsies. Pigs were euthanized immediately following biopsies in accordance with IACUC protocols.

Challenge infection. Challenge infection was performed 21 days after the third immunization. *H. ducreyi* strains 35000HP*hgbA*$_I$ and 35000HP*hgbA*$_{II}$ were grown overnight at 34.5°C and 5% CO_2 on chocolate agar plates supplemented with 1% GGC and 5% FetalPlex. For each strain, a cell suspension at an optical density at 600 nm (OD_{600}) of 1.5 (approximately 1×10^9 CFU/ml) was prepared in GC broth, along with a 1:10 dilution (approximately 1×10^8 CFU/ml). Multi-Test skin test applicators (Lincoln Diagnostics, Decatur, IL) were used to inoculate 10 μl of each bacterial suspension at multiple sites on the pig ears. Since the efficiency of dose delivery by the Multi-Test skin test applicator has been estimated at 1:1,000, 10 μl of a 10^9 CFU/ml bacterial suspension delivers approximately 10^4 CFU, and 10 μl of a 10^8 CFU/ml bacterial suspension delivers an estimated 10^3 CFU dose (55). Prior to inoculation, the ears of the pigs were cleaned thoroughly with alcohol wipes and lesion sites were marked with an ethanol-resistant pen. Each pig was inoculated with *H. ducreyi* strain 35000HP*hgbA*$_I$ on the left ear and strain 35000HP*hgbA*$_{II}$ on the right ear. The viability of bacterial suspensions was determined prior to and after each experiment, and no decrease in CFU was observed.

Biopsy processing. Pig ear lesions were biopsied 1 week after infection using disposable 6-mm skin biopsy punches (Acuderm, Ft. Lauderdale, FL). Biopsied tissues were either placed in 0.5 ml of GC broth for bacterial recovery or in 0.5 ml of 4% paraformaldehyde for histological studies. In order to recover *H. ducreyi* from biopsied lesions, tissues were thoroughly minced in GC broth using a sterile scalpel and spread on chocolate agar plates supplemented with 1% GGC, 5% FetalPlex, and 3 μg/ml vancomycin to reduce contaminants (1). Plates were incubated at 34.5°C with 5% CO_2 for up to 72 h, the presence/absence of *H. ducreyi* colonies was noted, and representative colonies were subcultured for confirmatory PCR.

Sections of biopsy specimens preserved in 4% paraformaldehyde were stained with hematoxylin and eosin (H&E) (Histology Laboratory, College of Veterinary Medicine, North Carolina State University). Each slide was coded and graded blindly by 2 authors according to a previously described protocol (1, 52). The appearance of normal skin received a score of 1; the presence of perivascular and interstitial mononuclear cell infiltrate received a score of 2; the presence of an intraepidermal pustule with neutrophils, fibrin, and necrotic debris received a score of 3; the presence of an epidermal pustule with keratinocyte cytopathology and mononuclear and polymorphonuclear infiltrate received a score of 4; and ulceration or epidermal necrosis and dermal erosion with confluence of immune cells was scored a 5. All slides were viewed using a Leica DM IRB inverted microscope (Leica Microsystems, Bannockburn, IL), and images were saved using QCapture software (QImaging, Surrey, BC, Canada). The scores were compared using Cohen's kappa, which indicated that the similarity between the evaluators ($\kappa = 0.541$) was in moderate agreement.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (1), except that plates were coated with 100 ng/well of $nHgbA_I$ instead of 200 ng/well. Each sample was run in duplicate, and the assay was performed on at least 3 different days.

Hb-blocking ELISA. An ELISA was used to determine the ability of anti-$HgbA_I$ to block Hb binding to HgbA (1). One hundred nanograms of $nHgbA_I$ or $nHgbA_{II}$ in 0.1 M carbonate buffer (pH 9.5) was added to wells and incubated overnight at 4°C. The next morning, wells were blocked for 1 h with 2% bovine serum albumin (BSA)-PBS. Twenty micrograms of anti-$nHgbA_I$ IgG was added to wells before a 30-min incubation at room temperature. Digoxigenin (DIG)-labeled human Hb (400 ng in 1% BSA-PBS) was added to wells, and plates were incubated at room temperature for 1 h. Three washes with PBS–0.05% Tween 20 (PBS-T) were followed by the addition of alkaline phosphatase-conjugated anti-DIG antibodies (100 μl, 1:5,000 in PBS) (Roche Diagnostics, Indianapolis, IN). The plates were incubated for 1 h at room temperature and washed thrice with PBS-T. One hundred microliters of alkaline phosphatase substrate (1-Step PNPP [*p*-nitrophenyl phosphate]) (Pierce, Rockford, IL) was added to wells, and the optical density at 405 nm was measured after 45 min using a 1420 VICTOR^2 multilabel counter (Wallac Oy). The Hb was labeled with DIG according to the manufacturer's instructions (DIG protein labeling kit; Roche Diagnostics, Indianapolis, IN). IgG was purified from pig antisera using protein A/G resin according to the manufacturer's instructions (ExAlpha Biologicals, Watertown, MA).

Whole-cell binding ELISA. *H. ducreyi* strains 35000HP*hgbA*$_I$ and 35000HP*hgbA*$_{II}$, isogenic *hgbA*$_I$ mutant FX547, and *H. ducreyi* class II strain DMC111 were grown overnight at 34.5°C with 5% CO_2 and shaking for 15 h in GC broth supplemented with 1% GGC and 5% Fetalplex. The whole-cell binding ELISA was performed as previously described (1) with the following changes: two hundred microliters of each strain of *H. ducreyi* ($OD_{600} = 0.2$) was added in triplicate to a Multiscreen HTS 96-well plate (Millipore, Billerica, MA). Fifty microliters of anti-nHgbA antisera was added to wells (final dilution 1:2,000 in 0.25% Tween 20-GC broth) and incubated for 90 min at room temperature with gentle rocking. Wells were washed 4 times with 0.1% Tween 20-GC broth using a vacuum manifold. One hundred microliters of rabbit anti-pig IgG-horseradish peroxidase (HRP) (product no. A5670; Sigma, St. Louis, MO), diluted 1:20,000 in 0.25% Tween 20-GC broth, was added to wells, and the plate incubated at room temperature for 60 min with gentle rocking. Wells were washed 4 times with 0.1% Tween 20-GC broth, and 100 μl of HRP substrate (Amersham ECL; GE Healthcare, Buckinghamshire, United Kingdom) was added to wells. Chemiluminescence was detected using the Wallac 1420 VICTOR^2 plate reader.

Bactericidal assay. In order to determine the bactericidal activity of the $nHgbA_I$ antisera against *H. ducreyi* strains, an immune bactericidal assay was performed as previously described (1, 18). Percent killing was determined by dividing the number of colonies that survived in fresh normal human serum (NHS) by the number of colonies that survived in heat-treated NHS and multiplying by 100. There was no decrease in CFU for the heated NHS control compared to the count in the GC broth control.

PCR amplification of loop 4 of the *hgbA* gene. To confirm strain identity, *H. ducreyi* colonies obtained from biopsy specimens were subjected to PCR using primers specific for the conserved flanking regions of loop 4 *hgbA*$_I$ and *hgbA*$_{II}$. The upstream and downstream *hgbA* primers (5′-CTAACCCTTCTGGGCTATAC-3′ and 5′-GCTAGGTAAATACACACGGC-3′, respectively) generated a PCR product of approximately 350 bp from either strain. The PCR steps used for amplification are as follows: an initial 5-min 94°C denaturing step was followed by 30 cycles of 1 min of denaturation at 94°C, annealing for 30 s at 45°C, and extension for 30 s at 72°C. A final polishing step for 5 min at 72°C was used prior to completion. PureTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, United Kingdom) were used according to the manufacturer's instructions. Ten picomoles of PCR primers were used, along with 1 μl of cells (OD_{600} of 0.2 in 50 μl of water) as template. PCR products were purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA) and sequenced using the upstream PCR primer at the UNC Genome Analysis Facility.

Whole-cell immunoprecipitation. *H. ducreyi* strains were grown overnight in heme-limiting conditions to induce the expression of HgbA at 34.5°C in 50-ml broth cultures of GC broth, 5% Fetalplex, 1% GGC in the presence of 5% CO_2 (17). Whole-cell immunoprecipitation was performed as previously described with the following modifications (26, 44, 60): *H. ducreyi* cultures were centrifuged and the pellets suspended to an OD_{600} of 1.0 (approximately 5×10^8 CFU/ml) in GC broth. Ten microliters of serum was added to 1 ml of the bacterial suspension and rocked at room temperature for 20 min. Bacterial cells were centrifuged, and the supernatant discarded to remove unbound antibody and serum components. The washed cell pellet was resuspended in 100 μl PBS, and 1 ml of 2% Zwittergent 3-14 (ZW 3-14) (Calbiochem) in TEN buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, pH 8.0) was added to solubilize proteins. After incubation at 37°C with agitation for 1 h, the tube was centrifuged for 10 min at 14,000 rpm to remove insoluble debris. The supernatant containing ZW 3-14-soluble total cellular proteins and antigen/antibody complexes was moved to a new tube containing 25 μl of a 50% slurry of protein A/G agarose beads (ExAlpha Biologicals, Shirley, MA). The tubes were incubated for 2 h or overnight to allow binding of antigen/antibody complexes to the protein A/G beads. The tubes were centrifuged and washed thrice using 0.5% ZW 3-14 in TEN. The agarose pellet was then resuspended in 1 ml TEN, moved to a fresh tube, and centrifuged, and the supernatant discarded. Forty microliters of Laemmli sample buffer lacking any reducing agents was added to the washed agarose, the tubes boiled for 5 min at 95°C, and 20 μl of the mixture subjected to SDS-PAGE and Coomassie staining.

Western blotting. Samples were subjected to SDS-PAGE and transferred onto nitrocellulose, which was blocked in 0.5% Tween 20–PBS for 1 h. Rabbit anti-N-terminal HgbA peptide antiserum diluted at 1:500 was used as the primary antibody (17). The sequence of the peptide used to generate the anti-N-terminal antiserum is identical in both classes of HgbA proteins and should therefore bind $HgbA_I$ and $HgbA_{II}$ equally. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) diluted at 1:20,000 was used as the secondary antibody. Protein bands were visualized using Lumi-Phos (Thermo Scientific, Rockford, IL).

Statistics. Statistical analyses were performed using the SigmaStat program (version 3.5; Systat Software, CA). A Mann-Whitney rank sum test was performed for the nonparametric data obtained in the ELISAs and for histology scoring. Fisher's exact test was used to compare bacterial recovery at the animal and lesion levels (Table 1).

RESULTS

Construction of *H. ducreyi* strain 35000HP expressing the *hgbA* gene from class II strain DMC111. In preliminary swine challenge experiments using *H. ducreyi* strains other than 35000HP, all tested strains from either class were less infectious than strain 35000HP (data not shown). Furthermore, *H. ducreyi* wild-type strain 35000HP grows faster than the class II strains. Therefore, because of these and other phenotypic differences between classes of *H. ducreyi* strains, we engineered an isogenic 35000HP strain expressing the class II *hgbA* gene from strain DMC111 (35000HP*hgbA*$_{II}$) to determine if the $nHgbA_I$ vaccine would be protective against a class II HgbA-expressing strain. In preliminary pig virulence studies, lesions produced by strain 35000HP*hgbA*$_{II}$ were indistinguishable from lesions caused by the 35000HP parent strain and the

TABLE 1. Recovery of *H. ducreyi* 35000HP*hgbA*$_I$ and 35000HP*hgbA*$_{II}$ from biopsy specimens

HgbA class	Expt	Mock immunization			HgbA immunization		
		Pig	No. of *H. ducreyi*-positive specimens/total no. of specimens		Pig	No. of *H. ducreyi*-positive specimens/total no. of specimens	
			10^4 CFU inoculum	10^3 CFU inoculum		10^4 CFU inoculum	10^3 CFU inoculum
I	1	1	2/3	3/3	3	0/4	ND[a]
		2	3/3	2/3	4	0/4	ND
	2	5	4/4	2/4	7	0/4	0/4
		6	4/4	2/4	8	0/4	0/4
		Total	13/14[b]	9/14[d]		0/16[b]	0/8[d]
II	1	1	2/3	3/3	3	3/4	ND
		2	3/3	2/3	4	1/4	ND
	2	5	4/4	4/4	7	3/4	4/4
		6	1/4	1/4	8	3/4	1/4
		Total	10/14[c]	10/14[e]		10/16[c]	5/8[e]

[a] ND, not determined.
[b] $P < 0.001$, Fisher's exact test.
[c] $P = 0.709$, Fisher's exact test.
[d] $P = 0.006$, Fisher's exact test.
[e] $P = 1.000$, Fisher's exact test.

$HgbA_{II}$ protein was expressed at the same level as $HgbA_I$ in the 35000HP background (data not shown).

The $nHgbA_I$/MPL vaccine reduced lesion severity after a homologous challenge. Pigs were immunized and challenged as described in Materials and Methods using a protocol identical to that of the previous HgbA vaccine study (1). The following parameters were analyzed: pig ear lesions were examined at both the macroscopic and microscopic levels; recovery of *H. ducreyi* was determined; and antisera from $nHgbA_I$-immunized animals were subjected to several *in vitro* immunological assays, including different types of ELISAs, to measure antibody activity to $nHgbA_I$, binding to whole cells of *H. ducreyi*, and the ability of IgGs from immunized animals to block Hb binding to both $nHgbA_I$ and $nHgbA_{II}$. The bactericidal activity of anti-$nHgbA_I$ antisera was measured using a classic bactericidal assay.

Figure 1A shows representative photographs of lesions from pigs challenged with both strains of *H. ducreyi* taken immediately prior to biopsy. In animals immunized with $nHgbA_I$, inoculation with homologous strain 35000HP*hgbA*$_I$ produced small lesions (Fig. 1A1), and in some cases, lesions were undetectable, save for puncture wounds from the applicator device. In contrast, infection of mock-immunized animals with both strains of *H. ducreyi* (Fig. 1A2 and 4) produced raised, visible lesions, much larger than those found in $nHgbA_I$-immunized animals challenged with strain 35000HP*hgbA*$_I$, indicating that both challenge strains are virulent in the experimental swine model of chancroid. The lesions of $nHgbA_I$-immunized animals challenged

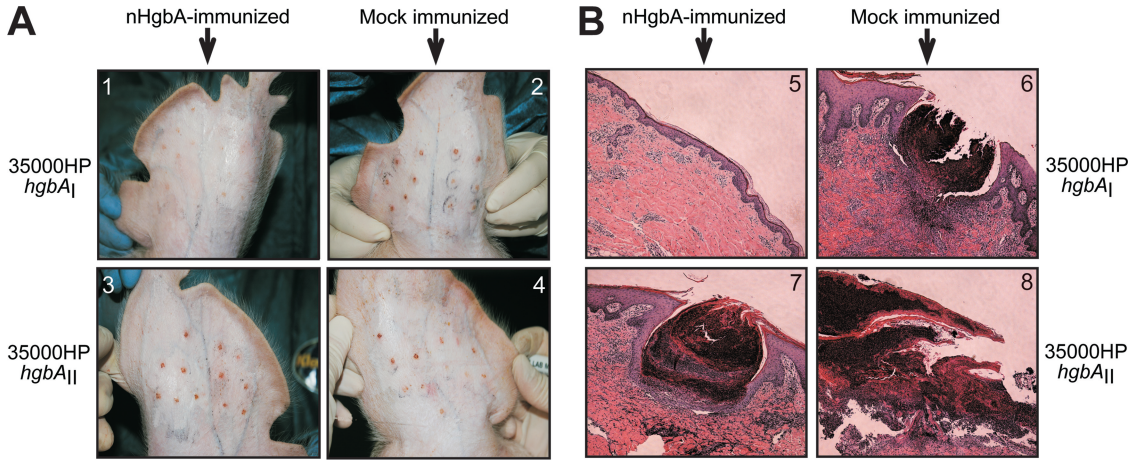

FIG. 1. $nHgbA_I$/MPL vaccine reduces lesion severity of experimental chancroid at the macroscopic and microscopic levels. (A) Photographs of pig ear lesions. Pigs were either mock immunized with MPL adjuvant only (2 and 4) or immunized with $nHgbA_I$/MPL (1 and 3) and challenged with either *H. ducreyi* strain 35000HP*hgbA*$_I$ (1 and 2) or 35000HP*hgbA*$_{II}$ (3 and 4). Photos were taken 1 week after challenge and immediately prior to biopsy. (B) H&E-stained biopsy sections from pig ears 1 week after infection. Pigs were either mock immunized with MPL adjuvant only (6 and 8) or immunized with $nHgbA_I$/MPL (5 and 7) and challenged with either *H. ducreyi* strain 35000HP*hgbA*$_I$ (5 and 6) or 35000HP*hgbA*$_{II}$ (7 and 8) (magnification, ×50).

with heterologous strain 35000HP*hgbA*$_{II}$ (Fig. 1A3) appeared similar to those from mock-immunized pigs.

Figure 1B shows representative histological sections of lesion biopsy specimens stained with H&E. In nHgbA$_{I}$-immunized animals challenged with homologous strain 35000HP*hgbA*$_{I}$ (Fig. 1B5), a low level of inflammatory cell infiltrate was present in the lesions, and the basement membrane was intact, resembling healthy sections (52). In mock-immunized animals challenged with either strain (Fig. 1B6 and 8), there was a dense infiltrate of neutrophils and the epidermal-dermal border was completely destroyed, which left the dermis exposed, similar to previous results (1) and comparable to natural and experimental infection in humans (34, 56). In the nHgbA$_{I}$-immunized heterologous challenge group (Fig. 1B7), the lesions appeared to be similar to those of mock-immunized animals.

We used a previously described grading system based on a 1-to-5 range (for lesion scores, see Materials and Methods) to judge the severity of lesions in each group of animals (52). The mean lesion grade of mock-immunized animals challenged with the homologous strain was 4.0 ± 0.76, whereas the nHgbA$_{I}$-immunized group challenged with the same strain had a score of 2.6 ± 0.78 ($P = 0.002$). The mean lesion grade for the control group challenged with the heterologous strain was 4.1 ± 1.3, compared to a score of 3.7 ± 1.3 for nHgbA$_{I}$-immunized animals ($P = 0.442$).

Viable *H. ducreyi* cells were not recovered from lesions of nHgbA$_{I}$-immunized pigs challenged with a homologous *H. ducreyi* strain. The recovery of viable *H. ducreyi* cells from biopsy specimens is shown in Table 1. At both challenge inocula (10^4 and 10^3 CFU), no *H. ducreyi* cells were isolated from nHgbA$_{I}$-immunized animals challenged with homologous strain 35000HP*hgbA*$_{I}$, whereas all mock-immunized animals yielded viable *H. ducreyi* ($P < 0.001$). In heterologous challenge experiments, all pigs, whether nHgbA$_{I}$ or mock immunized, yielded strain 35000HP*hgbA*$_{II}$ ($P = 1.000$).

Analysis of bacterial recovery at the lesion level revealed striking differences between nHgbA$_{I}$- and mock-immunized animals infected with the homologous *H. ducreyi* strain. In the control group, 13 of 14 lesions yielded *H. ducreyi* strain 35000HP*hgbA*$_{I}$ at an inoculum of 10^4 CFU. In the nHgbA$_{I}$-immunized group, 0 of 16 lesions yielded *H. ducreyi* ($P < 0.001$). At the lower dilution (10^3 CFU), 9 of 14 lesions in mock-immunized animals yielded *H. ducreyi* strain 35000HP*hgbA*$_{I}$, whereas 0 of 8 lesions yielded *H. ducreyi* strain 35000HP*hgbA*$_{I}$ in nHgbA$_{I}$-immunized animals ($P = 0.006$). Thus, the nHgbA$_{I}$ vaccine provided protection against a bacterial challenge of at least 10 times the minimum infectious dose. In heterologous challenge experiments, 10 of 14 lesions in the control group yielded *H. ducreyi* strain 35000HP*hgbA*$_{II}$, compared to 10 of 16 lesions from the nHgbA$_{I}$-immunized group at a 10^4 CFU inoculum ($P = 0.709$). Therefore, based on macroscopic examination, histological sections, and recovery of *H. ducreyi* cells, there was homologous protection against strain 35000HP*hgbA*$_{I}$ but no protection against the genetically engineered heterologous strain 35000HP*hgbA*$_{II}$ by the nHgbA$_{I}$ vaccine.

The activity of the antiserum from nHgbA$_{I}$/MPL-immunized animals was lower than the activity of antiserum of nHgbA$_{I}$/Freund's-immunized pigs. We analyzed the humoral immune response of nHgbA$_{I}$-immunized animals in a number of *in vitro* assays. An ELISA was performed to compare the antibody activity developed in response to our current MPL adjuvant HgbA vaccine to that of antiserum generated from the previous study using Freund's adjuvant (Fig. 2). The Freund's antiserum activity was approximately 7-fold higher than the MPL antiserum activity prior to the second immunization ($P = 0.029$) and approximately 2 to 3 times higher than the MPL activity after the third immunization (prior to infection) ($P = 0.057$). Of note, one of the four pigs immunized with nHgbA$_{I}$/MPL (pig 4M) showed ELISA activity twice as high as that found for the other three MPL-immunized pigs after the third immunization, indicating that different animals can respond differently to the vaccine preparation. Preimmune and mock-immune pig sera were free of anti-nHgbA$_{I}$ antibodies (data not shown).

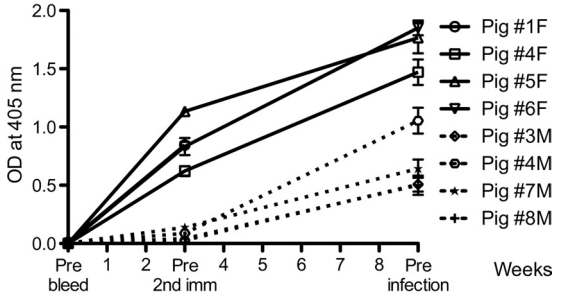

FIG. 2. The activity of the nHgbA$_{I}$/MPL antisera is lower than that of the antisera from nHgbA$_{I}$/Freund's-immunized animals. Sera were collected preimmunization (prebleed), before the second immunization, and prior to challenge infection (three weeks after the third immunization). Data are expressed as OD_{405} readings and given as median ± variance. Solid lines represent data from each pig immunized with nHgbA$_{I}$/Freund's adjuvant (1F, 4F, 5F, and 6F) (1), whereas dotted lines represent data from pigs immunized with nHgbA$_{I}$/MPL adjuvant (3M, 4M, 7M, and 8M). ELISA data for week 6 (prior to 3rd immunization) are not shown due to an incomplete data set for nHgbA$_{I}$/Freund's antiserum samples. *P* values comparing anti-nHgbA$_{I}$ antibodies using Freund's and MPL adjuvants after one and after three immunizations (just prior to challenge) were 0.029 and 0.057, respectively, and were determined using the Mann-Whitney rank sum test ($n = 3$).

Two different assays were employed to determine whether antisera from nHgbA$_{I}$/MPL-immunized pigs recognized surface-exposed epitopes of HgbA in the context of whole cells of *H. ducreyi*. The first technique, a whole-cell binding ELISA, measured the relative amounts of antibodies bound to the surface of different *H. ducreyi* strains expressing either class I (wild-type strain 35000HP*hgbA*$_{I}$) or class II (strain 35000HP*hgbA*$_{II}$) HgbA or expressing class II HgbA in the native class II *H. ducreyi* background (wild-type strain DMC111), as detected by a secondary antibody conjugate (Fig. 3) and compared to an *H. ducreyi* strain that does not express HgbA (strain 35000HPΔ*hgbA*). Antisera from all four nHgbA$_{I}$/MPL-immunized pigs and pooled anti-nHgbA$_{I}$/Freund's antisera showed greater reactivity with strain 35000HP*hgbA*$_{I}$ than with strains 35000HP*hgbA*$_{II}$, 35000HPΔ*hgbA*, or DMC111 (Fig. 3). As was observed in the solid-phase ELISA, there was also a statistically significant difference between the reactivity of pooled nHgbA$_{I}$/Freund's antiserum and all four nHgbA$_{I}$/MPL antisera with strain 35000HP*hgbA*$_{I}$ (*P* values range from < 0.001 to 0.005) (Fig. 3). Antisera taken from mock-immunized pigs and prebleeds from nHgbA$_{I}$/MPL-immunized pigs reacted poorly with all strains of *H. ducreyi* tested (data not shown).

The second method used to examine the interaction between

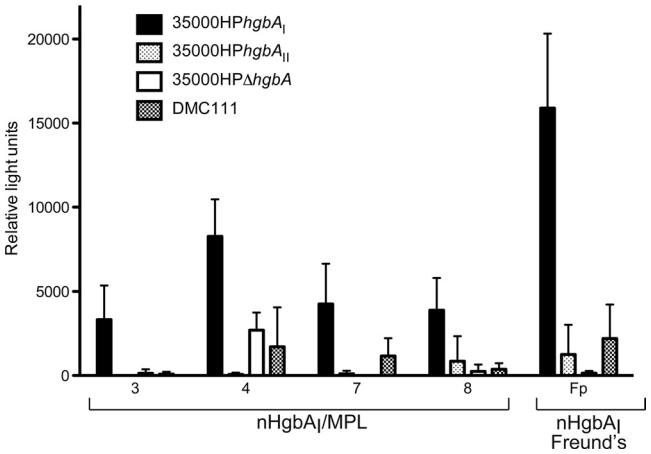

FIG. 3. nHgbA$_I$/MPL antisera bind HgbA$_I$ but not HgbA$_{II}$ in the context of whole *H. ducreyi* cells. Antisera from individual pigs (pigs 3, 4, 7, and 8), obtained after three immunizations with nHgbA$_I$/MPL, and pooled antiserum from pigs immunized with nHgbA$_I$/Freund's (Fp; obtained from a previous study) (1) were tested for binding to whole cells of *H. ducreyi* strains 35000HP*hgbA$_I$*, 35000HP*hgbA$_{II}$*, 35000HPΔ*hgbA*, and DMC111. The antisera used in these assays were from preinfection bleeds. Experiments were performed in triplicate on at least three different days. Data are expressed as relative light units and given as median ± variance. A *P* value of 0.005 was found for the difference between the binding of nHgbA$_I$/MPL and nHgbA$_I$/Freund's antisera to whole cells of *H. ducreyi* strain 35000HP*hgbA$_I$* (Mann-Whitney rank sum test).

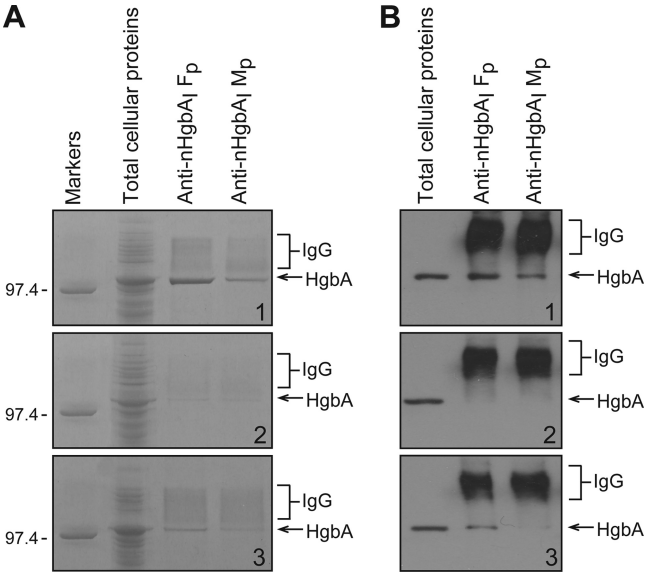

FIG. 4. nHgbA$_I$/MPL antiserum immunoprecipitates only HgbA$_I$ from whole cells of *H. ducreyi*. The ability of anti-nHgbA$_I$ antisera to immunoprecipitate HgbA from different *H. ducreyi* strains is shown in a Coomassie blue-stained 10% SDS–PAGE gel (A) and a Western blot probed with an antiserum to full-length rHgbA$_I$ (19) (B). The *H. ducreyi* strains used in these immunoprecipitation assays were 35000HP*hgbA$_I$* (1), 35000HP*hgbA$_{II}$* (2), and DMC111 (3). Antisera used in these experiments were either pooled anti-nHgbA$_I$/Freund's (Fp) purified from antisera obtained in a previous study (1) or pooled anti-nHgbA$_I$/MPL (Mp). Molecular size markers are shown in kilodaltons.

anti-nHgbA$_I$ and surface-exposed epitopes on *H. ducreyi* whole cells was an immunoprecipitation assay. Immunoprecipitates were analyzed by Coomassie blue staining (Fig. 4A) and Western blotting (Fig. 4B). Pooled anti-nHgbA$_I$/MPL antisera precipitated nHgbA$_I$ from *H. ducreyi* strain 35000HP*hgbA$_I$* but with less reactivity than with pooled antisera from pigs immunized with nHgbA$_I$/Freund's (Fig. 4A1 and B1). Neither antibody preparation precipitated nHgbA$_{II}$ in a 35000HP background (Fig. 4A2 and B2). Only pooled anti-nHgbA$_I$/Freund's was able to immunoprecipitate nHgbA$_{II}$ from the wild-type strain DMC111 (Fig. 4A3 and B3).

Antiserum elicited by the nHgbA$_I$/MPL vaccine partially inhibited the binding of DIG-Hb to nHgbA. We examined the ability of purified anti-nHgbA$_I$ IgG to block the binding of Hb to immobilized nHgbA$_I$ or nHgbA$_{II}$ in an ELISA format (1). Pooled IgG purified from serum of nHgbA$_I$/Freund's-immunized pigs (1) blocked 59% of DIG-Hb/nHgbA$_I$ binding, while pooled IgG purified from nHgbA$_I$/MPL-immunized pigs blocked 48% of binding ($P = 0.180$) (Fig. 5). There was a significant difference between IgGs purified from nHgbA$_I$/Freund's-immunized (38.1% inhibition) and nHgbA$_I$/MPL-immunized pigs (20.6% inhibition) ($P = 0.002$) (Fig. 5) in blocking Hb/nHgbA$_{II}$ interactions. IgG purified from pooled sera of mock-immunized pigs failed to interfere with the binding of Hb to either nHgbA$_I$ or nHgbA$_{II}$ (data not shown).

Antisera from nHgbA$_I$/MPL pigs did not exhibit bactericidal activity. We performed immune bactericidal assays using purified IgG from nHgbA$_I$/MPL-immunized pigs as previously described (1, 18). We were unable to demonstrate bactericidal killing in the presence of human complement using either *H. ducreyi* strain as a target (data not shown).

DISCUSSION

Administering the nHgbA$_I$ vaccine with an adjuvant approved for use in humans was as effective as using Freund's adjuvant. This study is similar to a previous vaccine study conducted in our laboratory, which demonstrated complete protection using purified nHgbA$_I$ as immunogen (1). In the previous study, we used Freund's adjuvant, while MPL was used as adjuvant in the present study. Monophosphoryl lipid A

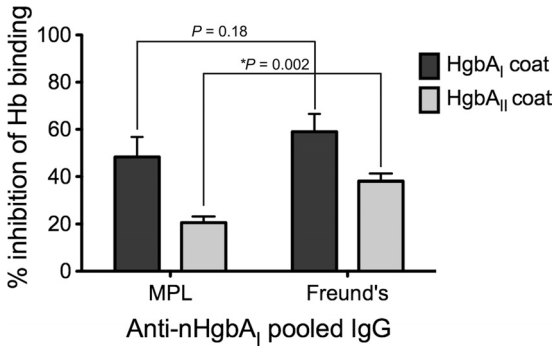

FIG. 5. IgGs from nHgbA$_I$/MPL-immunized animals partially inhibit Hb binding to nHgbA. The ability of pooled IgGs from pigs immunized with either nHgbA$_I$/MPL or nHgbA$_I$/Freund's to inhibit the binding of Hb to immobilized nHgbA$_I$ or nHgbA$_{II}$ was measured. *P* values comparing the ability of anti-nHgbA$_I$ IgG to block Hb binding to nHgbA$_I$ and nHgbA$_{II}$ were determined using the Mann-Whitney rank sum test. Pooled nHgbA$_I$/Freund's IgG was purified from antisera obtained in a previous study (1) ($n = 3$).

(MPL) is a detoxified lipopolysaccharide analog isolated from *Salmonella enterica* serovar Minnesota R595. The structure, mechanism of action, and immunologic responses to MPL have been the subjects of intense investigation for many decades (11), and the safety of MPL has been extensively documented (5, 65). MPL has been successfully utilized as an adjuvant in numerous human vaccines, including vaccines for human papillomavirus 16/18 (HPV) (27, 28, 46), hepatitis B virus (HBV) (36, 61), and herpes simplex virus (HSV) (57). In these trials, MPL was used in combination with alum in the GSK proprietary AS04 adjuvant system (23). MPL has also been examined as an adjuvant in vaccines against malaria (24, 30, 59), leishmania (9), anthrax (32), Epstein-Barr virus (EBV) (54), and HIV (39, 43) and against allergies to house dust mites (6), ragweed pollen (7), and grass pollen (16). All of these studies showed that MPL is a successful and safe adjuvant.

The nHgbA$_I$ vaccine administered with MPL protected only against a homologous *H. ducreyi* strain. The current study advances the findings from the original nHgbA$_I$ vaccine study in that we examined the ability of this vaccine to protect against strain 35000HP expressing a (heterologous) class II HgbA protein (HgbA$_{II}$). In alignments of HgbA from 10 strains of *H. ducreyi*, HgbA was highly conserved, showing ≥95% identity between strain classes (67). Based on this high degree of identity, we anticipated that nHgbA$_I$ would provide cross-protection against strains expressing HgbA from either class of *H. ducreyi* strains. Surprisingly, this is not what we observed. Immunization with the nHgbA$_I$/MPL vaccine offered full protection to pigs when challenged with *H. ducreyi* strain 35000HP*hgbA$_I$* (homologous), but not from a challenge with *H. ducreyi* strain 35000HP*hgbA$_{II}$* (heterologous). Since these are isogenic strains, except for the HgbA protein (HgbA$_I$ versus HgbA$_{II}$), two conclusions can be drawn from the results: (i) slight dissimilarities in the amino acid sequence between these 2 proteins are responsible for the difference in protection, and (ii) the observed protection cannot be the result of a protective response elicited by minor antigens contaminating the vaccine preparation.

In another of our previous studies, we found that loop 4 of HgbA is surface exposed and the largest, most immunodominant and variable loop among the 11 putatively exposed loops of HgbA (44). Including the highly conserved membrane-spanning segments, loops 4 of HgbA proteins are 91% identical between strains (16 of 186 amino acids are different). These results suggest that at least part of the protective response against HgbA$_I$ lies in exposed epitopes from loop 4. Such divergence between strains in protective epitopes of surface-exposed outer membrane proteins has been shown in the development of gonococcal and meningococcal protein vaccines (15, 42, 64) and resulted in the requirement for multivalent vaccines in the latter.

Because preliminary experiments showed that wild-type class II strains were either weakly or noninfectious in the swine model (data not shown), an *H. ducreyi* class I strain expressing a class II HgbA protein was constructed in the current study in order to determine the possibility of heterologous protection. However, the use of an isogenic class II construct has certain limitations. Class II strains typically grow more slowly than class I strains, which may explain why they were less infectious in the swine model. Moreover, class II strains have a truncated LOS compared to that of class I strains (50, 53, 67), and it is possible that HgbA$_{II}$ may be more exposed in a truncated LOS background. In data not presented here, anti-HgbA monoclonal antibody 1.51 binds poorly to whole cells of wild-type 35000HP*hgbA$_I$* but binds a 35000HP *gmhA* mutant (an isogenic mutant with a short LOS) (8) and two of four class II strains containing truncated LOS, including strain DMC111 (data not shown). These data suggest that the presence of a longer LOS molecule is responsible for the lack of monoclonal antibody 1.51 binding to HgbA on the surface of *H. ducreyi*. As shown by the results in Fig. 4, polyclonal anti-nHgbA$_I$ antibodies bound better to strain DMC111 than to strain 35000HP*hgbA$_{II}$*, consistent with this notion. Thus, if additional HgbA epitopes are exposed in the context of a class II LOS, then it is possible that the nHgbA$_I$ vaccine might offer some cross-protection for wild-type class II *H. ducreyi* strains.

Despite activity lower than that of antiserum from anti-nHgbA$_I$/Freund's immunization, antiserum from animals immunized with the nHgbA$_I$/MPL vaccine partially blocked DIG-Hb binding to HgbA. The ELISA activity of antiserum from HgbA$_I$/MPL-immunized pigs to nHgbA$_I$ was lower than the activity of antiserum from our earlier study using Freund's adjuvant after the first and third immunizations. These results are consistent with the results of two other immunization studies using other protein antigens in rabbits, which showed higher ELISA activity obtained using Freund's adjuvant (33, 41). We attempted to compare the antibody response of nHgbA$_I$/MPL antiserum to immobilized nHgbA$_{II}$ in an ELISA format to determine if this might correlate with protection. However, the two HgbA proteins did not appear to bind the ELISA plates equally or may not have been equally in native conformation, either of which may have skewed the results. Variability in binding and folding of the different classes of HgbA proteins also prevented us from making valid conclusions about the results obtained in the ELISA measuring blocking of Hb binding (Fig. 5), even though some comparisons were statistically significant. Further work is necessary to elucidate the causes of the different binding capabilities of classes of HgbA proteins.

Instead of making comparisons of antiserum binding to purified protein, we compared the binding of nHgbA$_I$/MPL antisera to whole cells of the isogenic pair of 35000HP strains. As predicted, the homologous strain bound more antibodies than the heterologous strain in both whole-cell ELISA and the immunoprecipitation assay (Fig. 3 and 4). Similar to the activity measured by ELISA, the whole-cell antibody binding activity from the previous nHgbA$_I$/Freund's immunization study was higher than the activity from the present nHgbA$_I$/MPL study.

The ability of antibodies to block Hb binding to immobilized nHgbA$_I$ and nHgbA$_{II}$ was measured using an ELISA-based assay. Antibodies from both MPL and Freund's adjuvant groups modestly blocked Hb binding to either class of HgbA protein (Fig. 5). Despite significantly higher ELISA activity with Freund's antiserum, there was no significant difference in the ability of either IgG pool to inhibit Hb binding to homologous HgbA$_I$. However, Freund's pooled IgG significantly inhibited the binding of Hb to heterologous HgbA$_{II}$ compared to the inhibition by MPL pooled IgG.

The lack of bactericidal and opsonophagocytic killing of *H.*

ducreyi is well documented in the literature (2, 22, 31, 37, 45, 63, 68). In this study, bactericidal activity was not observed using antisera from nHgbA$_I$/MPL-immunized pigs. This differed from the results of a previous study that showed modest bactericidal activity using antiserum from nHgbA$_I$/Freund's-immunized pigs (1). The lack of bactericidal activity using nHgbA$_I$/MPL antisera may be due to lower antibody titers, lower antibody affinity, or inability to fix complement.

We previously studied opsonophagocytosis by nHgbA$_I$/Freund's pooled antisera. In a luminol-enhanced chemiluminescence assay, human neutrophils were not significantly stimulated in the presence of nHgbA$_I$/Freund's antiserum and complement, whereas controls stimulated neutrophils (data not shown). Furthermore, human neutrophils failed to kill *H. ducreyi* cells opsonized with nHgbA$_I$/Freund's antiserum and complement, while control bacteria were killed (data not shown). Since nHgbA$_I$/Freund's antiserum, which has a higher activity than nHgbA$_I$/MPL antiserum, did not exhibit any bactericidal activity, we did not test the opsonic activity of nHgbA$_I$/MPL antiserum obtained in the current study.

Future studies. Anti-nHgbA$_I$ antibodies bound better to class I-specific epitopes and failed to protect against a heterologous challenge, which suggests that the protective epitopes lie in the divergent central domain of HgbA. Studies are under way to determine whether a vaccine containing the central domain of HgbA (loops 4 through 7) is protective for class I strains.

The mechanism of protection of the HgbA vaccine (cellular versus humoral) is not known. However, the results of a previous study using passively transferred serum from thrice-infected pigs suggested that the humoral response was sufficient to protect from an *H. ducreyi* challenge (13). Studies are under way in our laboratory to determine whether protection using passively transferred serum from nHgbA$_I$-immunized pigs is sufficient to provide protection.

Based on our results, it remains unclear whether a single HgbA protein will protect against both classes of *H. ducreyi*, and it is possible that a bivalent vaccine might be needed. While anti-nHgbA$_I$ antibodies did not bind well to class II HgbA in the genetically engineered 35000HP background, they bound moderately well to class II HgbA in the wild-type class II (DMC111) background, possibly due to differences in LOS or other antigens. Thus, based on our previous (1), present, and a third study under way (unpublished data), binding to whole cells and blocking Hb binding to HgbA are the two *in vitro* assays that correlate with the protection of the nHgbA$_I$ vaccine.

The immune response to nHgbA$_I$/MPL was relatively modest, similar to the results of other studies using different antigens. Newer vaccines using MPL have overcome this problem by including alum in the vaccine mixtures with MPL (termed AS04), and we will examine this in our system. Despite these limitations, we have proof of concept that nHgbA$_I$ in conjunction with an adjuvant approved for use in humans can provide complete homologous protection in a highly relevant animal model, paving the way toward phase I clinical trials.

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