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Swine Model of *Haemophilus ducreyi* Infection

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***Haemophilus ducreyi* is a strict human pathogen that causes sexually transmitted genital ulcer disease. We infected domestic swine with *H. ducreyi* 35000, resulting in the development of cutaneous ulcers histologically resembling human chancroid lesions. Intraepidermal lesions progressed from pustules to ulcers containing polymorphonuclear leukocytes and were accompanied by a dermal inflammatory infiltrate containing T cells and macrophages. *H. ducreyi* was recovered from lesions up to 17 days after inoculation, and pigs did not develop immunity to reinfection with the challenge strain. Features of the model include inoculation through abrasions in the epidermis, ambient housing temperatures for infected pigs, the ability to deliver multiple different inocula to a single host, and the availability of monoclonal antibodies against porcine immune cells permitting immunohistochemical characterization of the host immune response to *H. ducreyi* infection.**

Chancroid, an ulcerating cutaneous infection caused by *Haemophilus ducreyi*, is one of the most prevalent sexually transmitted diseases in developing countries (12, 34, 39) and is endemic in some areas of the United States (3, 5, 14, 31). *H. ducreyi* infection is a recognized cofactor for transmission of the human immunodeficiency virus (29, 37, 38, 40), making it the focus of considerable recent research aimed at understanding the molecular mechanisms of pathogenesis of this organism.

Potential *H. ducreyi* virulence factors include lipo-oligosaccharide (2, 10), pili (11, 50), and the production of extracellular cytotoxin(s) (27, 28, 42). The importance of these and other bacterial determinants in ulcer formation, and their roles in pathogenesis, may best be understood by using genetically defined mutant strains of *H. ducreyi* in an animal model of infection. Several models including the use of rabbits (41), humans (52) and primates (53) have been described, but each has limitations, ranging from artificial housing conditions and inoculation procedures for rabbits to the high cost of primates. In addition, ethical concerns preclude allowing the progression of lesions to the ulcerative stage in human volunteers, and the use of antibiotic-resistant bacteria in humans is prohibited under many circumstances.

H. ducreyi is primarily a cutaneous pathogen; while involvement of regional lymph nodes is not uncommon in chancroid, *H. ducreyi* is seldom recovered from bubo aspirates (25), and the bacteria do not invade deeper tissue or cause systemic disease (34). Because the skin of juvenile pigs closely resembles human skin both structurally and physiologically (6, 13, 23, 26, 32, 55, 56), we investigated the ability of *H. ducreyi* to cause dermal lesions in swine. Lesions histologically resembling natural human chancres developed in pig skin following inoculation through abrasions in the epithelium, providing a useful animal model for the study of *H. ducreyi* pathogenesis and host response to infection.

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MATERIALS AND METHODS

Animals. Crossbred (Yorkshire, Landrace, Hampshire, and Duroc Cross) or purebred (Landrace) conventionally reared swine were obtained at weaning (3 weeks old) and housed at ambient temperatures (20 to 25°C) in AAALAC-accredited P2 containment facilities at the North Carolina State University College of Veterinary Medicine. Pigs received water and an antibiotic-free, high-protein growth ration (77% corn meal, 21% soybean meal, 2% of a mineral and vitamin mixture) ad libitum for a minimum of 3 weeks prior to inoculation and throughout the period of infection with *H. ducreyi*. For inoculation and biopsy procedures, 6- to 10-week-old animals were sedated with approximately 2 mg each of ketamine HCl (Fort Dodge Laboratories, Fort Dodge, Iowa) and xylazine (Miles Laboratories, Shawnee Mission, Kans.) per kg of body weight administered intravenously. After inoculation, the pigs were kept in individual enclosures and observed daily for the first week and every 3 to 7 days thereafter.

Inoculum preparation. Stock cultures of *H. ducreyi* 35000 (ATCC 33922) were maintained at −80°C as described previously (10) and grown on chocolate agar plates (Difco GC agar base, 1% IsoVitaleX [Becton Dickinson, Cockeysville, Md.], 1% hemoglobin) with or without 3 μg of vancomycin per ml at 35°C in a humidified atmosphere with 5% CO_2. Cultures from frozen stocks were plated on chocolate agar, grown for 24 h, and passaged once onto fresh plates to obtain confluent 24-h growth for inoculation. *H. ducreyi* bacteria were scraped from two to three plates with wooden applicator sticks and suspended in 1 ml of sterile phosphate-buffered saline (PBS). Bacterial suspensions yielded between 5×10^8 and 3.2×10^9 [mean = $(1.4 \pm 1.2) \times 10^9$] CFU/ml on the basis of duplicate cultures of serial 10-fold dilutions. Solutions of *H. ducreyi*, particularly when prepared from plate-grown organisms, are difficult to quantitate accurately because the bacteria form clumps that cannot be dispersed uniformly. Therefore, the measurement of CFU per milliliter represents only an estimate of the actual concentration of viable *H. ducreyi*. Portions of the inoculum were killed by heating in a boiling water bath for 5 min. No *H. ducreyi* CFU were recovered from 100 μl of this suspension plated on chocolate agar.

Inoculation. For inoculation of pig ears, hair was shaved from the dorsal surface, when necessary, with a disposable razor. The skin was cleansed with 95% ethanol and allowed to dry. For inoculation on backs, hair was first removed with standard veterinary clippers, and then the back was shaved and cleansed as described above. We inoculated 10 μl of bacterial suspension containing approximately 10^7 CFU of live or heat-killed *H. ducreyi* onto prepared skin with a Multi-Test Applicator (Lincoln Diagnostics, Decatur, Ill.) as described by Spinola et al. (52). Each of the eight pads of the Multi-Test device contains an array (2 by 2 mm) of nine individual tines that penetrate approximately 2 mm when applied as described in the manufacturer's directions. Droplets were absorbed occasionally into the skin during the brief period of observation immediately after application, but frequently, beads of PBS remained on the surface of the skin at least 30 min after inoculation when the animals were beginning to regain consciousness. Visible clumps of *H. ducreyi* consistently remained lodged between the tines of the application device after inoculation of both live and heat-killed inocula. Undelivered material remained even after vigorous rinsing with PBS, precluding an accurate determination of the fraction of *H. ducreyi* loaded onto the device that was actually delivered to the pig skin.

Biopsy and sample preparation. Lesion biopsies were done with a 6-mm disposable skin punch (Acuderm, Ft. Lauderdale, Fla.), and the samples were cut in half. For recovery of *H. ducreyi*, sample halves were placed in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 10% fetal calf serum (HyClone Laboratories, Inc., Logan, Utah) 1% IsoVitaleX, and 50 μg of hemin per ml or 10 mg of hemoglobin per ml and transported to the laboratory. Specimens were minced with sterile scalpel blades and cultured on chocolate agar plates. *H. ducreyi* organisms recovered from lesions were identified by their distinctive colony morphology and resistance to 3 μg of vancomycin per ml.

Samples for routine histology were fixed in 10% formalin and embedded in paraffin. Thin sections were stained with hematoxylin-eosin (H&E), Giemsa, or Warthin-Starry (tissue silver stain) reagents by standard procedures. Specimens were evaluated independently by a dermatologist specializing in histopathology and a veterinary pathologist.

For immunohistochemistry, the tissue was frozen on dry ice immediately after biopsy and maintained at −80°C until 10-μm sections were cut with a cryostat and mounted on glass slides coated with poly-L-lysine (Polysciences, Warrington, Pa.). Frozen sections were fixed in cold acetone for 10 min, air dried, and probed with immunoglobulin G (IgG) monoclonal antibodies (MAbs) that bound to pig immune cells (VMRD, Pullman, Wash.) or *H. ducreyi*. Antibody binding was detected with the ABC peroxidase kit (Vector Labs, Burlingame, Calif.) and diaminobenzidine as a substrate after a blocking step with 5% normal horse serum plus 5% normal pig serum. Samples were counterstained with Giemsa. T cells were detected with MAb PG114A that recognizes porcine CD5. MAb DH59B binds to glycolipid GM1 on the surface of porcine and human macrophages and granulocytes. Swine polymorphonuclear leukocytes (PMNs) and macrophages bound DH59B and were distinguishable on the basis of nuclear morphology. MAb PIg45A was used to detect surface IgM on B cells; MAb MSA3 recognizes the porcine equivalent of the major histocompatibility complex (MHC) class II HLA-DR marker (36). MAbs 2C7 (51) and 5C9 (8) bind *H. ducreyi* outer membrane proteins (OMPs) and were kindly provided by Stanley Spinola. Omission of primary antibodies from tissue staining and antibody binding to freshly isolated pig peripheral blood lymphocytes served as negative and positive controls, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. *H. ducreyi* whole-cell sonicates were solubilized in Laemmli solubilizing buffer, and proteins were electrophoresed from a single preparative well in a 15% polyacrylamide gel. Prestained molecular weight markers were from Gibco-BRL (Gaithersburg, Md.). Separated proteins were transferred to BA-S nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.). Membrane strips were cut and probed with pig serum (1:250, 1:500, or 1:1,000), MAb 2C7 (hybridoma culture supernatant; 1:10), or MAb 5C9 (ascites, 1:20,000) after blocking with 2% skim milk powder in PBS containing 0.01% Tween 20. Primary antibody binding was visualized with horseradish peroxidase-conjugated rabbit anti-pig or goat anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.) and the enhanced chemiluminescent detection system (ECL; Amersham, Arlington Heights, Ill.).

RESULTS

Lesion development. We inoculated the dorsal surface of the ears of four Yorkshire Cross and two Landrace pigs with approximately 10^7 CFU of live or heat-killed *H. ducreyi* 35000 delivered with the Multi-Test allergy testing device as described in Materials and Methods. The imprint of the delivery device was evident at all sites immediately after application, and 24 h after inoculation, all sites were erythematous. We had conducted preliminary studies with the Multi-Test applicator loaded with PBS, into which wooden applicator sticks, scraped across sterile chocolate agar plates, had been swirled, to test for nonspecific inflammation produced by our inoculation procedure. Aside from the imprint of the delivery device immediately after application, there was no visible reaction to this procedure. We did not examine these sites histologically. Papules developed by 48 h at sites inoculated with live *H. ducreyi*. Occasionally, small papules were observed at 48 h at sites that received heat-killed inocula; these were visible only microscopically and were not accompanied by the histological changes described below for sites with live *H. ducreyi* organisms. Lesion development beyond 48 h occurred only at sites with live bacteria. Seven days after inoculation, individual pustules resulting from *H. ducreyi* delivered by the nine individual tines of the delivery device had coalesced into circular lesions approximately 5 mm in diameter. In contrast, sites inoculated with heat-killed bacteria were not measurable and were barely visible on day 7. By 14 days, the lesions were ulcerated, as defined by the complete erosion of the epidermis, with raised borders and a dry, crusty appearance. Figure 1 shows typical 18-day lesions on the ears of a Landrace pig. Lesions persisted for at least 21 days on the ears of Yorkshire pigs and 28 days on the Landrace animals but never developed into the exudative, open sores characteristic of human chancroidal ulcers. Resolution of ear lesions did not occur within the 4-week time frame of these experiments.

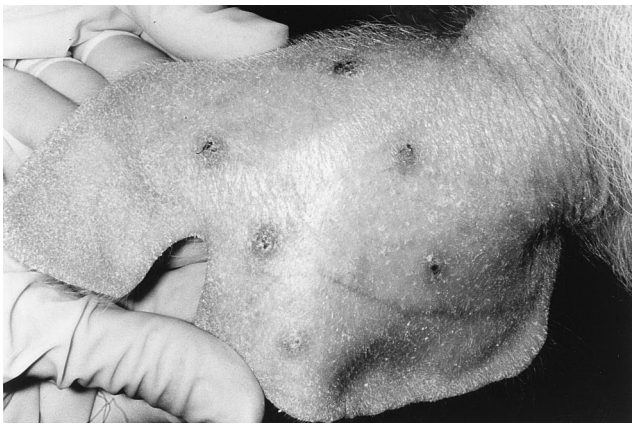

FIG. 1. Landrace pig ear inoculated with *H. ducreyi* 35000. Eighteen-day-old lesions resulting from inoculation of ~10^7 CFU of live *H. ducreyi* bacteria are visible. Six individual inocula were delivered through abrasions in the epidermis with an allergen-testing device.

We also inoculated the skin of the backs of 11 Yorkshire and 2 Landrace pigs exactly as described above. Lesion development at core body sites was more variable than that of ear lesions. The duration of back lesions ranged from 2 to 11 days (mean ± standard deviation, 5.5 ± 3.5 days). Of 10 back lesions examined histologically, only 2 had progressed beyond the pustule stage, and a single sample showed complete epidermal erosion. Thus, live *H. ducreyi* consistently caused ulcer development when inoculated onto the ears but not the backs of domestic swine.

Recovery of *H. ducreyi* from lesions. Four biopsy samples from ear sites inoculated with live *H. ducreyi* were cultured; three were positive (days 2, 14, and 17), and one was negative (day 7). *H. ducreyi* was isolated from four of five cultured biopsy samples from back sites inoculated with live organisms. Positive samples were obtained on days 1 (two independent samples), 4, and 11, and the negative culture was obtained on day 4. *H. ducreyi* was never recovered from biopsy sites that received heat-killed bacteria. Unidentified microbial skin contaminants were present consistently on recovery plates without vancomycin from both live and heat-killed samples; however, skin flora were not overgrown, and *H. ducreyi* was clearly not present in negative cultures. *H. ducreyi* isolated on nonselective media was confirmed by growth of subcultures on plates containing 3 μg of vancomycin per ml.

Histopathology of lesions. Figure 2A shows the histological presentation of normal porcine ear skin. The earliest biopsies of experimental infections on pig ears were done 2 or 4 days after inoculation, when lesions were clearly visible at sites that had received live *H. ducreyi* but not heat-killed bacteria. At these time points, papules containing PMNs were present consistently and confined to the upper layers of the epidermis in biopsy samples from pigs that had received live inocula and were sometimes present in samples from pigs that had received heat-killed inocula (Fig. 2B and C). The papule in Fig. 2C was sectioned near the center of the biopsy sample, making it

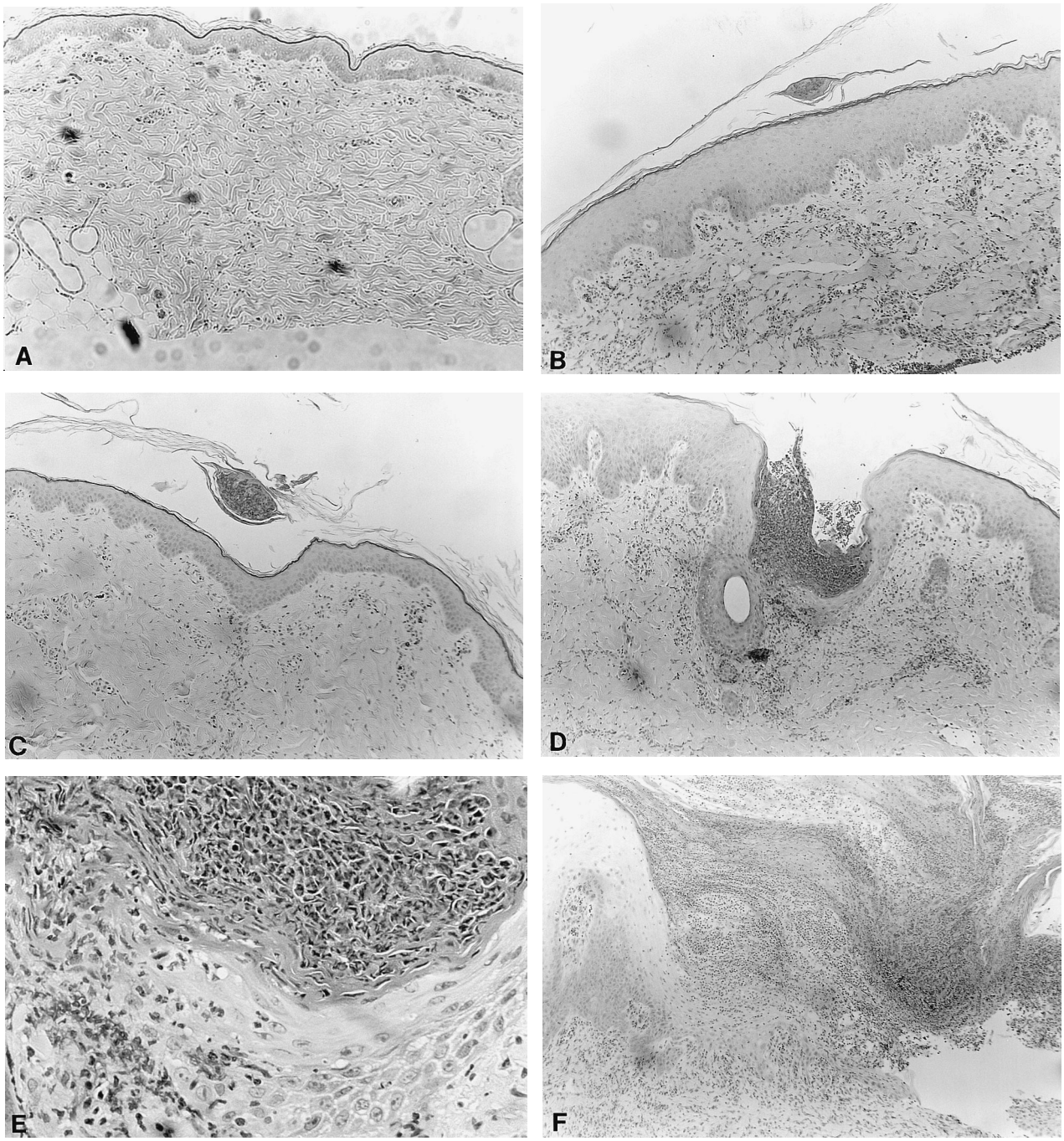

FIG. 2. Histologic appearance of normal and *H. ducreyi*-infected Yorkshire Cross pig ear skin. (A) Uninfected ear skin. (B and C) Lesions 4 days after inoculation with live (B) or heat-killed (C) *H. ducreyi* 35000. The pustule separation from the epidermis is a sectioning artifact. Epidermal thickening was noted only in biopsy samples from sites that received a live challenge (compare panels A, B, and C; H&E stain; magnification, ×60). (D and E) Ear lesions 1 week after inoculation with live *H. ducreyi* 35000 characterized by marked changes in the epidermis. Note the extensive thickening and plasticity of the epidermis in panel D. (H&E stain; magnification, ×60). The squamous epithelium beneath the intraepidermal lesion was disorganized and nearly eroded, and inflammatory cells had accumulated in the interstitial spaces below the lesion (E) (H&E stain; magnification, ×210). (F) Ear lesions 2 weeks after inoculation with live *H. ducreyi* 35000. Most of the squamous epithelium was replaced by PMNs in fibrin and cellular debris (H&E stain; magnification, ×210). The separation between the necrotic material and the dermis is an artifact of sectioning. Note the complete destruction of the epidermis beneath the lesion.

appear larger than the one in Fig. 2B, which was cut nearer the edge of the biopsy sample. Extensive thickening of the epidermis was noted only in biopsy samples taken from sites inoculated with live *H. ducreyi* organisms. While heat-killed bacteria produced a modest inflammatory response at these early times, the perivascular and interstitial infiltrate was more vigorous in response to live inocula.

Biopsy samples of ear lesions taken 1 week after inoculation with live *H. ducreyi* contained excessive keratotic layers interlaced with PMNs in the epithelium accompanied by marked hyperplasia and acanthosis of the adjacent epidermis (Fig. 2D). When present, the underlying basal keratinocytes were disorganized and interspersed with inflammatory cells beneath the lesion (Fig. 2E). Complete erosion of the squamous epithelium

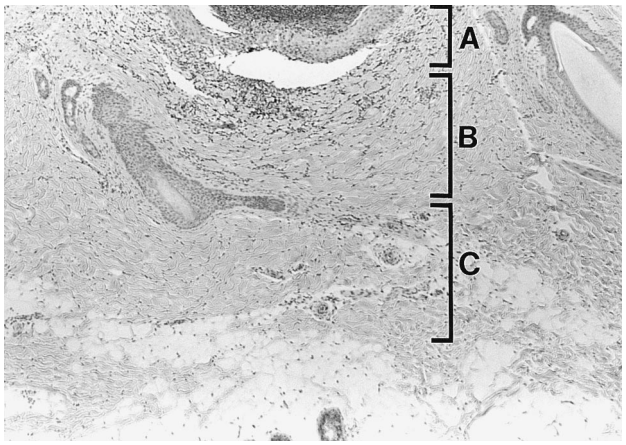

FIG. 3. Histologic appearance of a rare *H. ducreyi* lesion in Yorkshire Cross pig back skin 11 days after inoculation. This full-thickness biopsy sample shows three zones beneath the lesion: a narrow superficial zone containing necrotic tissue, PMNs, and fibrin (A); a broader middle zone characterized by inflamed tissue and a perivascular infiltrate (B); and a deep zone with a more diffuse infiltrate and thick-walled vessels (C). The separation between the superficial and middle zones is an artifact of sectioning. H&E stain; magnification, ×30.

was sometimes seen at this time point. We saw increased perivascular and interstitial accumulation of inflammatory cells in the dermis in comparison with that in younger lesions. Micropapules resulting from inoculation with heat-killed *H. ducreyi* were resolved by 1 week, and there was no further influx of inflammatory cells in the dermis (data not shown).

An ear biopsy sample taken 2 weeks after inoculation with live *H. ducreyi* (Fig. 2F) showed complete destruction of the epidermis beneath the lesion. The mononuclear infiltrate directly beneath the ulcer contained some histiocytes, but we were unable to locate intra- or extracellular bacteria in biopsy material with Giemsa or Warthin-Starry stain or immunoperoxidase staining with MAbs recognizing *H. ducreyi* surface proteins.

Figure 3 shows a rare but well-developed lesion 11 days after inoculation of back skin with *H. ducreyi*. In contrast to ear biopsy samples, from which we obtained only epidermis and the upper portion of the dermis to avoid completely piercing the pig ears, we obtained a full-thickness biopsy sample from this back lesion demonstrating the three classical histopathologic zones associated with human chancroid (15, 34, 48).

To determine the pig immune cell types involved in the host response to *H. ducreyi* infection, we used MAbs against swine lymphocyte markers for immunoperoxidase staining of samples taken 1 week after inoculation. GM1, a glycolipid expressed on swine and human granulocytes and macrophages, was present on cells in the inflammatory infiltrate (Fig. 4A) and on the PMNs within the intraepidermal lesion (data not shown). T cells were identified in the infiltrate by the CD5 marker (Fig. 4B). No immunoperoxidase staining was observed in the absence of primary antibody (data not shown). An MHC class II marker (MSA3) recognizing the porcine equivalent of the HLA-DR antigen stained epidermal dendritic cells but not the mononuclear inflammatory cells present in the dermal infiltrate (data not shown). We used an anti-IgM MAb in an effort to determine if B cells were present in biopsy samples; however, this reagent produced high background staining throughout the dermis, making identification of surface IgM-bearing cells impossible. Thus, although we cannot comment on the presence or absence of B cells, the cellular inflammatory response of pigs to *H. ducreyi* infection included T cells, macrophages, and granulocytes.

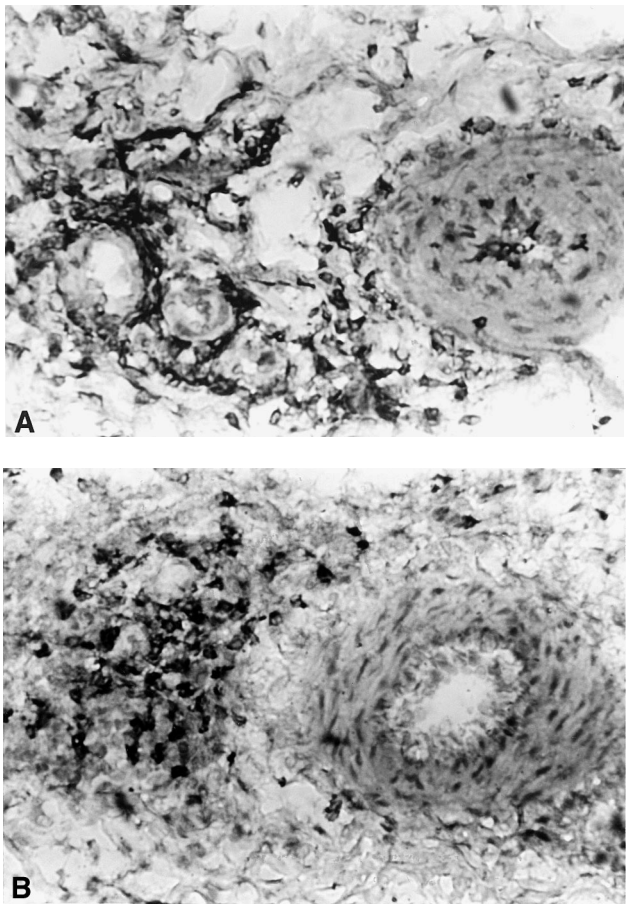

FIG. 4. Immunoperoxidase staining of the perivascular infiltrate beneath an ear lesion 1 week after inoculation with live *H. ducreyi* bacteria. (A) Glycolipid GM1 on macrophages and granulocytes was stained with MAb DH59B; (B) CD5 on T cells was identified with MAb PG114A (B). Giemsa counterstain; magnification, ×210.

Serologic response and lack of immunity to repeat challenge. Pooled normal pig serum (NPS) and serum collected from two infected animals 11 or 17 days after inoculation were used to probe Western blots of *H. ducreyi* 35000 proteins. All swine serum samples contained IgG antibodies that reacted with multiple bacterial proteins in whole-cell lysates, including a doublet that comigrated with two MAb 2C7-reactive bands (Fig. 5). MAb 2C7 binds the 39- to 42-kDa major OMP (MOMP) of *H. ducreyi* and other members of the family *Pasteurellaceae*; purified MOMP preparations contain multiple bands representing different conformations of the protein (51). Reactivities to the bands comigrating with the MOMP were similar in NPS and the serum from one infected pig (Fig. 5, lanes 3 and 4); however, reactivity for the doublet in 11-day serum from another infected pig was present at roughly a four-fold-higher titer than that of NPS (Fig. 5, lanes 2 and 5). Sera from both infected pigs, but not NPS, recognized a protein that comigrated with the *H. ducreyi*-specific 28-kDa OMP recognized by MAb 5C9 (8). The relative sizes of other bacterial proteins recognized by sera from infected pigs, but not NPS, are indicated in Fig. 5.

Two pigs, originally infected on the back, were reinoculated with live and heat-killed *H. ducreyi* 35000. One animal received the second infection at new sites on the back 27 days after the initial challenge, and the second pig was reinoculated on the ear 17 days after primary infection. Lesion development on the

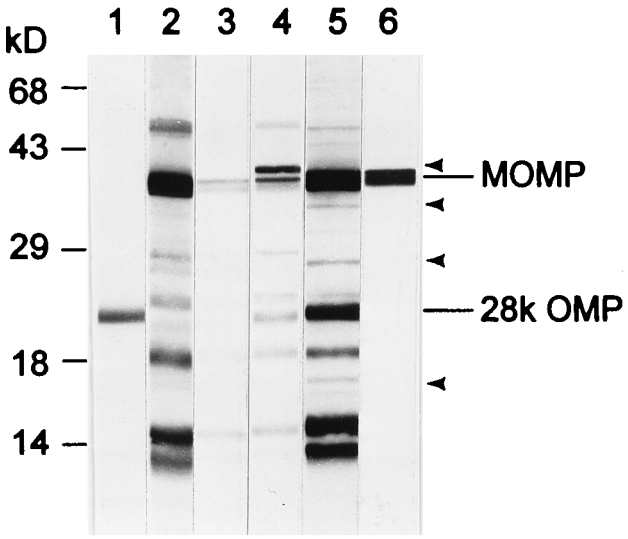

FIG. 5. Serologic response of Yorkshire Cross pigs to *H. ducreyi* infection. A Western blot of *H. ducreyi* 35000 whole-cell proteins probed with MAb 5C9 (lane 1), NPS diluted 1:250 (lane 2) or 1:1,000 (lane 3), serum collected from two pigs 17 (lane 4) or 11 (lane 5) days after infection, each diluted 1:1,000, and MAb 2C7 (lane 6) is shown. Size markers are indicated on the left, and the positions of two *H. ducreyi* OMPs are indicated on the right. The arrowheads mark unidentified *H. ducreyi* proteins recognized by IgG antibodies in serum from infected pigs but not in NPS.

back following repeat challenge with live *H. ducreyi* 35000 was indistinguishable from that from the original course of infection in that animal (data not shown). Ear lesions in the other pig were equivalent to those that developed on the ears of animals that received the same inoculum but had not been previously infected. While we did not examine biopsy material from lesions resulting from second infections, the ear sites were visually more dramatic than the original lesions on the back (data not shown). Thus, although infection with *H. ducreyi* resulted in increased serum IgG antibodies to bacterial antigens, pigs did not develop immunity to subsequent challenge with the same strain.

DISCUSSION

Experimental infection of domestic swine with *H. ducreyi* shares many clinical and histologic features with experimental human infection (52) and human chancroid disease. Following inoculation of live bacteria designed to simulate the natural route of infection through breaks in the epithelium, persistent lesions developed, from pustules to ulcers, containing a superficial zone of PMNs and extracellular debris accompanied by a perivascular and interstitial dermal infiltrate of T cells and macrophages. It is notable that while full lesion development did not occur following inoculation of heat-killed bacteria, a mild inflammatory response was observed at these sites. This attenuated host response may have been elicited by heat-stable bacterial components, such as lipo-oligosaccharide, present in the heat-killed inocula.

Experimental ulcers had raised, erythematous margins but never became soft or exudative, like chancroid lesions in humans. This result may reflect differences in host tissue responses or local parameters such as temperature, skin thickness, innervation, or vascularization, for which human genital skin and porcine ear skin likely differ. Indeed, inoculating pig backs, sites with thicker, less vascular, and warmer skin than the ears, resulted in less-consistent lesion development and generally reduced lesion duration. Furthermore, *H. ducreyi* delivered to the skin of the inner thigh of two pigs (a location approximating swine core body temperature of ~38°C) failed to produce any evidence of lesion development (data not shown), consistent with the sensitivity of this organism to temperatures exceeding 35°C. Whereas the temperature of pig ears may have been reduced sufficiently to allow ulcer development, temperature and other differences between human genital epithelia and pig ears may have contributed to the atypical outward appearance of experimental *H. ducreyi* lesions in swine.

The dose of *H. ducreyi* required to cause chancroid in natural sexually transmitted infection is not known. Because *H. ducreyi* bacteria adhere tightly to one another and to many surfaces (1, 15, 30), including the Multi-Test Applicator used in our inoculation procedure, it is difficult to determine the actual dose delivered to swine skin in our experiments. Although we loaded approximately 10^7 CFU of *H. ducreyi* onto the tines of the delivery device, undoubtedly only a portion of these bacteria penetrated the epidermis during inoculation. Studies using radiolabeled tuberculin suggest that the Multi-Test Applicator reproducibly delivers 0.2 to 0.5% of a solution loaded onto the tines (19a). Therefore, our inoculating dose may have been as low as 10^4 CFU. Lesions in the recently described chilled rabbit (41) and primate (53) models of *H. ducreyi* infection resulted from intradermal injection of ~10^5 CFU or between 10^7 and 10^8 CFU, respectively. Spinola et al. used the Multi-Test Applicator to inoculate the arms of the volunteers in recent human challenge studies (52) in which lesions developed from 10^4 to 10^6 CFU of *H. ducreyi* 35000 loaded onto the tines of the device. As few as 20 to 2,000 CFU of *H. ducreyi* may have initiated lesion development in that model. While we have not yet determined the minimum dose required to produce ulcers in the swine model, the inocula used in these pilot experiments were probably higher by an order of magnitude than those used in experimental human infection. Future experiments using lower challenge doses of *H. ducreyi* in pigs will provide a better basis for comparison of the numbers of *H. ducreyi* required for early lesion development and formation of persistent ulcers in the various available models.

We recovered *H. ducreyi* from pig lesions up to 17 days after inoculation of ears and 11 days after inoculation of back skin. We did not attempt to quantitate recovered bacteria, and given the difficulties in determining the numbers of *H. ducreyi* bacteria present in the skin immediately after inoculation, we cannot be certain that active bacterial replication occurred in the lesions. However, the presence of viable *H. ducreyi* in ulcers more than 2 weeks postinoculation, in the face of an inflammatory host response, suggests that the bacteria were capable of responding to environmental signals and expressing virulence determinants necessary for survival under those conditions. The fact that viable bacteria were required for lesion development is further illustrated by the failure of heat-killed *H. ducreyi* to produce ulcers in the swine model.

An advantage of the pig model and the use of the Multi-Test device for inoculation is the ability to simultaneously deliver multiple different inocula to the same animal host. The shaved backs of pigs easily accommodated up to 32 individual samples. Preliminary experiments were performed with two recent (1992 and 1993) clinical isolates from chancroid patients in Malawi and CIP542 (ATCC 33940), an isolate previously characterized as less virulent in an early rabbit model of *H. ducreyi* infection (18). The two independent clinical isolates produced lesions grossly equivalent to those formed by *H. ducreyi* 35000, whereas CIP542 produced lesions markedly less severe than those of *H. ducreyi* 35000, Thus, even though the lesions were less dramatic on the back than on the ear, lesion development

on the back was sufficient to distinguish between isolates differing in virulence properties. The extent of immune interactions between individual lesions has not been determined and is of some concern for comparisons of different inocula, particularly when isolates appear to elicit similar lesions. Nevertheless, reduced lesion development in this model, such as that which we observed with CIP542, is a potential indicator of reduced virulence. We hasten to point out that CIP542 is not completely avirulent, since it was originally a clinical isolate and has been shown recently to initiate ulcer formation upon accidental inoculation into human skin (54). However, others have demonstrated differences between CIP542 and more virulent strains, including *H. ducreyi* 35000, in an OMP profile (50) and in vitro cytotoxic activity against human fibroblasts (22), supporting its characterization as relatively less virulent than *H. ducreyi* 35000.

Even on pig ears, where lesions were more severe and persistent than on backs, we were able to deliver up to six individual inocula to each ear of the Yorkshire pigs and eight or more separate samples per ear of the Landrace pigs. In fact, the latter breed was chosen in part because they have larger ears than other pig breeds. In addition, the use of purebred pigs may reduce individual variation in the host immune response to *H. ducreyi*, providing a better standard for comparison between experiments using different inocula. Screening numerous *H. ducreyi* mutants or biological variants first on pig backs, followed by characterization of potentially interesting individual isolates on pig ears, may provide an efficient protocol for identification of bacterial virulence characteristics.

The histologic features of ulcers caused by *H. ducreyi* in swine were similar in many respects to those reported by Purcell et al. in rabbits housed at reduced temperatures (41), by Spinola et al. in volunteers (52), and, most recently, by Totten et al. in nonhuman primates (53). All of these models of infection result in intraepidermal lesions containing PMNs and a perivascular and interstitial accumulation of inflammatory cells, typical of human chancroid lesions. Using commercially available MAbs to pig immune cells, we were able to identify T cells, macrophages, and granulocytes among the infiltrating cells in the dermis beneath lesions in swine, consistent with observations from the human challenge study (52). Human inflammatory cells and keratinocytes examined in 3-day lesions also expressed the MHC class II HLA-DR antigen, whereas pig cells responding to *H. ducreyi* at day 7 did not. It is not clear whether this observation represents a fundamental difference between swine and human responses to *H. ducreyi* or is a consequence of the different stages of lesion development examined. Our initial antibody panel included the single MAb directed against the swine lymphocyte DR antigen; it is possible that other MHC class II antigens are expressed by pig cells in response to *H. ducreyi* infection. MAbs recognizing the porcine equivalents of HLA-DP and -DQ are available and will be used to better characterize the kinetics of the swine immune response to chancroid.

It is interesting that the swine T-cell population is unique regarding the expression of CD4 and CD8 antigens in circulating and tissue pools. In addition to the classical $CD4^+$ $CD8^-$ T-helper and $CD4^-$ $CD8^+$ cytolytic T cells, pigs have unusual extrathymic $CD4^-$ $CD8^-$ and $CD4^+$ $CD8^+$ subsets (43, 44, 47). Most of the so-called double-negative cells express the γδ TCR (20, 21, 45), and the double-positive cells constitutively express the MHC class II DR antigen (46). The specific functions of the $CD4^-$ $CD8^-$ and $CD4^+$ $CD8^+$ porcine T cells are unknown. The CD5 antigen is expressed on most swine T cells (4, 43), and immunoperoxidase staining with this marker identified many inflammatory cells in pig lesions caused by *H. ducreyi* (Fig. 3). Antibodies to porcine CD4, CD8, and γδ TCR markers are available, and we are currently using these reagents to further characterize the swine T-cell response to *H. ducreyi* infection. Preliminary observations suggest that both $CD4^+$ and $CD8^+$ pig cells are present in 7-day lesions, whereas γδ TCR^+ cells are rare. The experiments described in this report demonstrate the ability to characterize the porcine cellular immune response to *H. ducreyi* infection and ultimately to compare this response with that of humans.

NPS contained IgG antibodies that bound *H. ducreyi* whole-cell lysates; this may represent cross-reactivity of preexisting antibodies with antigens on other members of the *Pasteurellaceae* (9, 16, 51), several of which are common among swine herds (7, 17, 24, 33). Using Western blot analysis with sera from two *H. ducreyi*-infected pigs, we detected an increase in IgG antibodies to a protein comigrating with the *H. ducreyi*-specific 28-kDa OMP, and reactivity to proteins comigrating with the *H. ducreyi* MOMP was elevated in the serum from one infected animal. The role of humoral immune responses in chancroid is unclear. Several studies have shown that sera from chancroid patients have higher levels of antibody to *H. ducreyi* antigens than sera from controls without a history of chancroid (2, 35). It is difficult to compare the magnitude of the increased IgG reactivity we observed in Western blots with unadsorbed swine sera with published data from preadsorbed patient sera in solid-phase assays. However, the higher IgG titers in sera from infected swine against several *H. ducreyi* antigens are consistent with clinical data. Despite the increased antibody levels, we saw no evidence of protective immunity in pigs following repeated challenge with *H. ducreyi*. This is in contrast to the measure of protective immunity developed by rabbits after infection or immunization (19). The occurrence of repeated chancroid infections in human populations suggests that broadly protective immunity does not result from *H. ducreyi* infection. This lack of protective immunity was substantiated in rechallenge studies performed with human volunteers (52).

In conclusion, we have described a swine model of infection with *H. ducreyi* that resembles natural and experimental human infection. Advantages of this model include the use of ambient animal housing temperatures, a relevant route of inoculation, and lower purchase and housing costs for pigs than for primates. In addition, multiple bacterial strains, including antibiotic-resistant mutants, can be used to infect an individual animal, providing an effective means of comparing differences in virulence. We anticipate that this model will serve as a valuable tool in understanding *H. ducreyi* pathogenesis and host cellular immune responses involved in chancroid.

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