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# Inducible Immunity with a Pilus Preparation Booster Vaccination in an Animal Model of *Haemophilus ducreyi* Infection and Disease

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**Using the temperature-dependent rabbit model of *Haemophilus ducreyi* infection as a quantitative virulence assay, we tested the abilities of two bacterial antigen preparations to induce protection against subsequent infection and disease. Lipooligosaccharide (LOS) and a pilus preparation were purified from *H. ducreyi* 35000 and were used in a booster immunization procedure. The serologic response to each immunogen was monitored by enzyme immunoassay. *H. ducreyi* virulence was assayed by intraepithelial inoculation and subsequent measurement of disease for homologous strain 35000 or clinical isolate RO-34. LOS and the pilus preparation induced humoral responses. The kinetics of the LOS antibody response suggest a type 1 T-independent response, whereas the pilus preparation induced an anamnestic response. An inoculum of $10^5$ CFU of *H. ducreyi* 35000 or RO-34 consistently produced ulcerative chancroidal lesions in naive rabbit controls. Immunization with LOS did not modify the virulence of *H. ducreyi* 35000. Immunization with the strain 35000 pilus preparation significantly reduced the severity of disease and the duration of infection and disease compared with controls, with either homologous or heterologous strain infection. The histology of lesions from pilus preparation-vaccinated rabbits compared with that of lesions from controls revealed accelerated lymphoid cell recruitment, more prominent plasma cell infiltrate, and reduction in subsequent histiocytic infiltration. We conclude that both LOS and the pilus preparation are immunogenic and that the latter induces homologous and heterologous strain protection in this animal model of infection and disease.**

*Haemophilus ducreyi* is the causative agent of chancroid, a sexually transmitted genital ulcer disease characterized by painful ulcerative lesions. Although relatively uncommon in most industrialized nations, it is endemic in many developing countries in association with prostitution (20). In sub-Saharan Africa chancroid accounts for up to 80% of all genital ulcers (23). Chancroid has become a major public health concern primarily because it is a biological cofactor or promoter of sexually transmitted human immunodeficiency virus (HIV) (4, 10, 25). Enhanced *H. ducreyi* virulence is also observed in the presence of HIV infection (2, 4).

Control of chancroid still relies on detection through clinical diagnosis and antibiotic therapy (14). Although control of outbreaks has been achieved in Winnipeg, Canada, and Orange County, California, condom promotion has had limited success in established reservoirs of disease in developing countries (23). To address compliance in treatment-based control programs, the use of single oral dose therapies, such as trimethoprim and sulfonamide combination and quinolone administration, has been successful (14). However, with increased incidences of microbial resistance (9, 14) and single-dose treatment failures associated with HIV seropositivity despite drug sensitivity (3, 27), alternative control strategies have become important.

If feasible, vaccination for *H. ducreyi* infection would benefit chancroid control and, it is hoped, indirectly augment HIV control efforts in areas of endemicity. Effective vaccination would offer an attractive control tactic through durable, practical, cost-effective chancroid prevention.

The lack of information concerning the nature and relevance of the immune response to *H. ducreyi* infection is an obstacle for vaccine development strategies. With the introduction of the temperature-dependent rabbit model of infection by Purcell and coworkers (24), some of these questions are now addressable. A method for use of this model of infection as a quantitative in vivo virulence assay, in which homologous strain-inducible immunity has been detected, has been developed (18). Recently, protective immunity to homologous and heterologous *H. ducreyi* strain infection in this model was demonstrated by vaccination of rabbits with cell surface extracts (12). By defining the specific antigens responsible for protective immunity, some problems associated with the use of a crude fraction for vaccine development can be averted. For example, purified extracts may minimize the presence of decoy antigens and suppressor epitopes (15). Here we demonstrate that protection from homologous and heterologous strain infection and disease was inducible in rabbits immunized with a pilus preparation purified through stepwise solubilization and crystallization of sheared pili, but not with lipooligosaccharide (LOS).

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## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *H. ducreyi* 35000 was initially reported as an isolate from a 1975 Winnipeg outbreak and was subsequently characterized (11). This strain has been shown to be virulent in the temperature-dependent rabbit model (18, 24). *H. ducreyi* RO-34 is a Kenyan clinical isolate.

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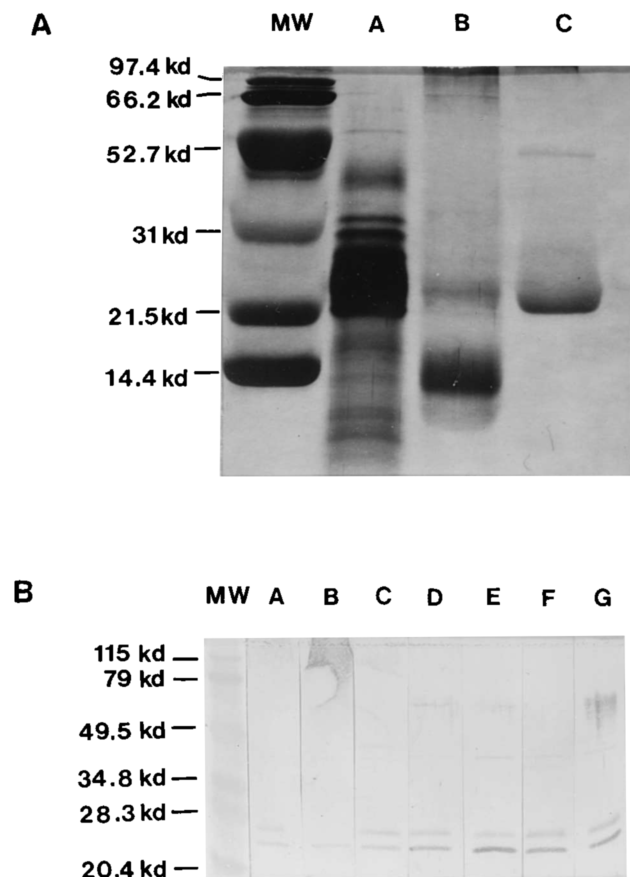


FIG. 1. (A) SDS-PAGE of the three antigen preparations used for immunization of rabbits. MW, molecular mass markers (in kilodaltons). Lane A, uncharacterized carbohydrate extract; lane B, LOS extract; lane C, pilus preparation. Extracts were run on a 12.5% acrylamide gel and stained with silver stain. (B) Western blot analysis of sera taken 21 days after booster immunization of seven rabbits with the pilus preparation (lanes A through G). MW, molecular mass standards.

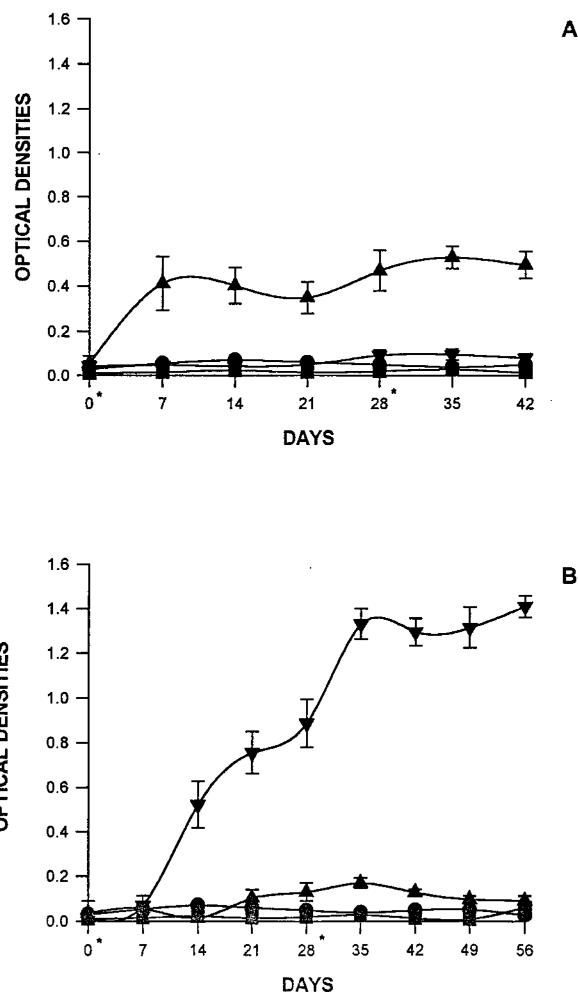


FIG. 2. IgG and IgM serologic responses in rabbits immunized with LOS (A) and the pilus preparation (B) from *H. ducreyi* 35000. Rabbits were immunized with 100 μg of each antigen and given boosters 4 weeks later as indicated by the asterisks. IgG (closed inverted triangles) and IgM (closed triangles) serologic responses to LOS (A) or the pilus preparation (B) and IgG (closed circles) and IgM (closed squares) of naive control rabbits were monitored by EIA.

Growth of *H. ducreyi* was carried out on chocolate agar supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and 5% fetal bovine serum or in broth (α minimal essential medium and Mueller-Hinton agar with 5% fetal bovine serum [1:1 ratio {vol/vol}]. Plates were incubated for 48 h at 33°C in 5% $CO_2$, and broth cultures were incubated for 16 h at 33°C in an environmental shaker. Inocula for subsequent infection were prepared by harvesting *H. ducreyi* from the late mid-log phase of growth in broth by centrifugation at 3,000 × *g* for 20 min. Pellets were washed once in phosphate-buffered saline (PBS; 0.01 M $NaHPO_4$, 0.1 M NaCl [pH 7.2]) and resuspended in Mueller-Hinton broth.

**Antigen preparation.** The pilus preparation from *H. ducreyi* 35000 was extracted as previously described by Spinola et al. (26). The protein concentration was determined by the Bio-Rad (Richmond, Calif.) protein determination assay. Aliquots were stored at −70°C. LOS from *H. ducreyi* 35000 48-h plate cultures was isolated by the hot phenol extraction method of Westphal and Jann (28). Final purification was done with three 3-h ultracentrifugation cycles at 105,000 × *g*. Pellets containing LOS were resuspended in double-distilled water, lyophilized, and stored at 4°C. LOS and the pilus preparation were evaluated for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% acrylamide and stained with Bio-Rad silver stain. Outer membrane proteins were isolated for comparison from *H. ducreyi* strains 35000 and RO-34 as previously described (22). Distinct protein profiles were compared by SDS-PAGE on a 12.5% acrylamide gel with Coomassie blue stain.

**Animals.** A total of 41 2.5-kg New Zealand White rabbits were purchased from Charles River Canada and housed in an 11.7-$m^2$ room. The ambient temperature was kept at 14 ± 1°C with a Thermo Air Plus air conditioning unit. Rabbits for all experiments were age matched from acquisition and housed under identical conditions for the duration of experiments.

**Immunization with LOS and the pilus preparation.** Six and 11 New Zealand White rabbits were immunized with 100 μg of LOS and 100 μg of the pilus preparation, respectively, in PBS (pH 7.2), suspended in Freund's complete adjuvant (FCA). One half of the 500-μl dose was administered intramuscularly, and the other half was administered subcutaneously in the back of the neck. At 4 weeks after primary immunization rabbits were given boosters of identical doses of each antigen suspended in Freund's incomplete adjuvant (FIA) by the same route of administration. Control rabbits included seven naive unimmunized rabbits and two rabbits similarly sham immunized with PBS in FCA and FIA. Seven pilus preparation-immunized rabbits and six LOS-immunized rabbits were ear bled weekly for measurement of immunoglobulin G (IgG) and IgM serological responses to each immunizing antigen by enzyme immunoassay (EIA) and detection of IgG antibody by Western blotting (immunoblotting) (see below).

**Virulence of *H. ducreyi*.** After the peak serologic response for each antigen (8 weeks for LOS and 9 weeks for the pilus preparation) (see Fig. 2), homologous strain virulence was assayed by challenge with five triplicate 100-μl doses of $10^7$ to $10^3$ CFU of late mid-log phase, broth-grown live *H. ducreyi* 35000. CFU counts for each inoculum in each rabbit were directly determined in duplicate by plating 100 μl of inoculum from the injecting syringe onto agar plates for colony counts. Inocula were delivered by intraepithelial injection in triplicate in a grid across the shaven backs of the animals. As $10^5$ CFU has been found to be the minimum inoculum consistently producing ulcers (18), comparisons of lesions produced at this inoculum were performed by measuring the transverse lesion diameter and scoring the lesion for severity on a scale of 0 to 4 (0, nil; 1, inflammation; 2, induration [>2 mm]; 3, suppuration; and 4, ulceration). The third lesion at each inoculum size was cultured to determine the presence or absence of *H. ducreyi* by lateral injection of 0.1 ml of sterile PBS and aspiration of lesion debris back into the syringe. Aspirates were plated onto chocolate agar and incubated at 33°C for

TABLE 1. Homologous strain protection at $10^5$ CFU in rabbits immunized with the pilus preparation or LOS

| Animal group | Inoculum size (CFU)[a] | No. of days culture positive[a] | Lesion size (mm)[a] | | Peak lesion score[a] | No. of ulcerous lesions/total | Ulcer duration (days)[a] |
|---|---|---|---|---|---|---|---|
| | | | Cumulative (21 days) | Average | | | |
| Control ($n = 9$) | 1.12 ± 0.424 | 16.9 ± 1.7 | 148.7 ± 25.7 | 7.1 ± 1.2 | 4 ± 0 | 7/7 | 11.4 ± 1.215 |
| LOS vaccinated ($n = 6$) | 2.45 ± 0.75 ($P = 0.26$)[b] | 16 ± 0.632 ($P = 0.72$)[b] | 135.83 ± 29.7 ($P = 0.52$)[b] | 6.46 ± 1.7 ($P = 0.057$)[b] | 4 ± 0 | 6/6 | 9.0 ± 1.4 ($P = 0.9$)[b] |
| Pilus preparation vaccinated ($n = 11$) | 2.5 ± 1.4 ($P = 0.37$)[c] | 7.4 ± 3.1 ($P < 0.001$)[c] | 56.4 ± 16.2 ($P < 0.001$)[c] | 2.69 ± 0.7 ($P < 0.001$)[c] | 3.2 ± 0.6 ($P = 0.009$)[c] | 3/10 | 1.4 ± 2.7 ($P < 0.001$)[c,d] |

[a] Data are means ± standard deviations.
[b] Comparative evaluation of LOS-vaccinated rabbits versus naive controls (one-way ANOVA).
[c] Comparative evaluation of rabbits vaccinated with the pilus preparation versus naive controls (one-way ANOVA).
[d] Data represent the durations of five ulcers in three rabbits.

48 h. Identification of *H. ducreyi* was based on characteristic colonial morphology, the push test, and microscopic examination of isolates for the characteristic appearance of *H. ducreyi* (6). Lesions were cultured until they were negative for 4 consecutive days. Lesions which were used for culture were not scored or measured for virulence, because of the potential influence of serial injection of 0.1 ml of PBS and manipulations in culturing. Naive and sham-immunized adjuvant control rabbits were challenged with grid inoculation in an identical fashion.

Control rabbits, either naive or sham immunized, were age matched from the same batch of 2.5-kg (6 months old) rabbits acquired for each experiment. Control animal infections were run concurrently with each experimental run.

Eight rabbits immunized with the pilus preparation from *H. ducreyi* 35000 were challenged with the heterologous strain RO-34. Virulence was assayed as described above and concurrently compared with that observed for five naive control rabbits challenged with strain RO-34 and evaluated in parallel.

**Histopathology.** On days 4, 10, 15, and 21 after inoculation, four rabbits were given 0.1 ml of Innovar (Fentanyl-droperidol; Janssen Pharmaceutical, Mississauga, Ontario, Canada) per kg of body weight with 0.05 mg of atropine sulfate (MTC Pharmaceutical, Cambridge, Ontario, Canada) per kg intramuscularly. Biopsies of lesions produced at $10^5$ CFU on two naive controls and two rabbits immunized with the pilus preparation were taken with 6-mm-diameter disposable biopsy punches. No suturing or antibiotic ointment was necessary. Biopsy specimens were fixed in 10% formalin embedded in paraffin, thin sectioned, and stained with hematoxylin and eosin.

**EIA.** Serologic responses to immunization with LOS or the pilus preparation were measured by an enzyme-linked immunosorbent assay as previously described (8, 17) with slight modifications as follows.

LOS was suspended in PBS with 1 mg of chenodeoxycholate per ml and heated for 15 min at 95°C. Alternatively, the pilus preparation was suspended in carbonate buffer (0.1 M; pH 9.6). Polysorb U96 (96 wells) round-bottom plates (Nunc) were coated with 10 μg of either extract per ml (100 μl per well), incubated for 2 h at 37°C, and washed three times with wash buffer (PBS, 0.1% Tween 80). For LOS-coated plates, wells were blocked with 2% bovine serum albumin (100 μl per well) for 1 h at 37°C and washed three times with wash buffer. All plates were stored at 4°C for no longer than 2 weeks. Test serum was applied at 100 μl per well in serial twofold dilutions from 1:200 to 1:25,600 in PBS with 1% newborn calf serum–0.1% Tween 80, and the plates were incubated for 2 h at 37°C and washed five times with the wash buffer. A 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Tago, Burlingham, Calif.) and a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit IgM (Southern Biotechnology, Birmingham, Ala.) diluted in PBS (with 0.1% Tween 80–5% newborn calf serum) were added to each well (100 μl per well). Plates were incubated at 37°C for 30 min and washed five times with wash buffer. Bound conjugated antibody was detected with 100 μl of a solution containing 0.36 mM 2,2′ azino bis-(3-ethyl-benthizoline-6-sulfonate) (Boehringer Mannheim) and 0.03% $H_2O_2$ dissolved in citrate buffer (0.1 M citrate, 0.02 M sodium phosphate [pH 4.25]). Plates were kept at 20°C for 20 min and read spectrophotometrically at 405 nm.

**Western blotting.** After separation of the pilus preparation by SDS-PAGE (10 μg of antigen preparation was loaded into each well), protein antigens were electroblotted to nylon membranes (Boehringer Mannheim) at 100 V for 1 h at 4°C. Transfer buffer consisted of 25 mM Tris (Bio-Rad Laboratories), 195 mM glycine (Bio-Rad Laboratories), and 20% (vol/vol) methanol. Nylon membranes were blocked with 2% skim milk for 1 h at 37°C and then washed for 5 min five times with PBS–0.1% Tween 20. Individual lanes were cut into separate strips. Sera from the seven phlebotomized rabbits taken 49 days after primary immunization and prior to infectious challenge were used at a 1:200 dilution in 2% skim milk in PBS for incubation of the nylon strips for 2 h at 37°C. After being washed, the strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000 dilution) (Tago) diluted in 2% skim milk in PBS. Strips were washed with PBS, and color was developed with 0.8 mM 3 amino-9 ethyl carbazole and 0.1% $H_2O_2$. The reaction was stopped with three washes of 5 min each in double-distilled water.

**Statistical analysis.** Lesions produced by inocula of $10^5$ CFU were compared by averaging the scores of the two nonmanipulated lesions (unused for culture) in each rabbit to generate a single lesion size measurement and score for each rabbit. Statistical analyses of mean lesion sizes and scores serially measured during 21 days of observations with inocula of $10^5$ CFU were performed by using the one-way repeated measure of analysis of variance (ANOVA), with Bonferroni *t* test for pairwise multiple comparisons and the Wilcoxon signed rank test, respectively. Comparative evaluation of inoculum sizes, durations of culture positivity, lesion sizes, and peak lesion scores was done by the ANOVA.

## RESULTS

**Antigen extracts.** The purity of the LOS and the pilus preparation was assessed by SDS-PAGE with silver staining, and both were found to be relatively pure. Similar to the results of Spinola et al. (26), several minor protein bands, in addition to the 24-kDa pilus antigen, were seen (Fig. 1A). Outer mem-

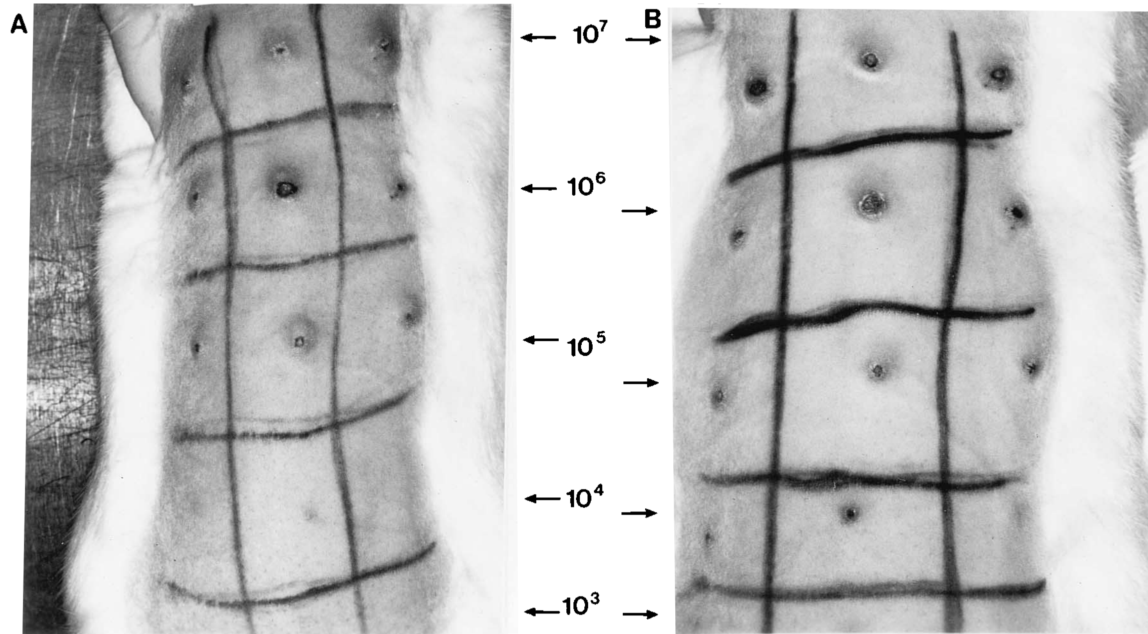


FIG. 3. Appearance of disease at day 7 on LOS-immunized (A) and control (B) rabbits challenged with the homologous strain 35000, showing no difference in disease severity.

brane protein profiles of both *H. ducreyi* 35000 and the clinical isolate RO-34 were found to be distinct (data not shown).

**Serologic response to immunization with LOS and the pilus preparation.** Immunization with purified LOS produced a moderate IgM response peaking at 7 days following primary immunization, and at 7 days after booster administration, with no detectable response or booster effect for IgG (Fig. 2A). No LOS-specific IgG or IgM was detectable by EIA in naive (Fig. 2A) or adjuvant control (data not shown) rabbits.

Immunization with the pilus preparation induced a strong IgG response detectable within 7 days and peaking at 35 days after primary challenge and 7 days after booster administration (Fig. 2B). Only a weak IgM response was observed for immunization with the pilus preparation (Fig. 2B). Again naive and adjuvant control rabbits were negative. The presence of IgG serologic reactions in all seven rabbits phlebotomized for serology is illustrated by the Western blot (Fig. 1B). Dominant and consistent reaction to the 24-kDa protein was present in all

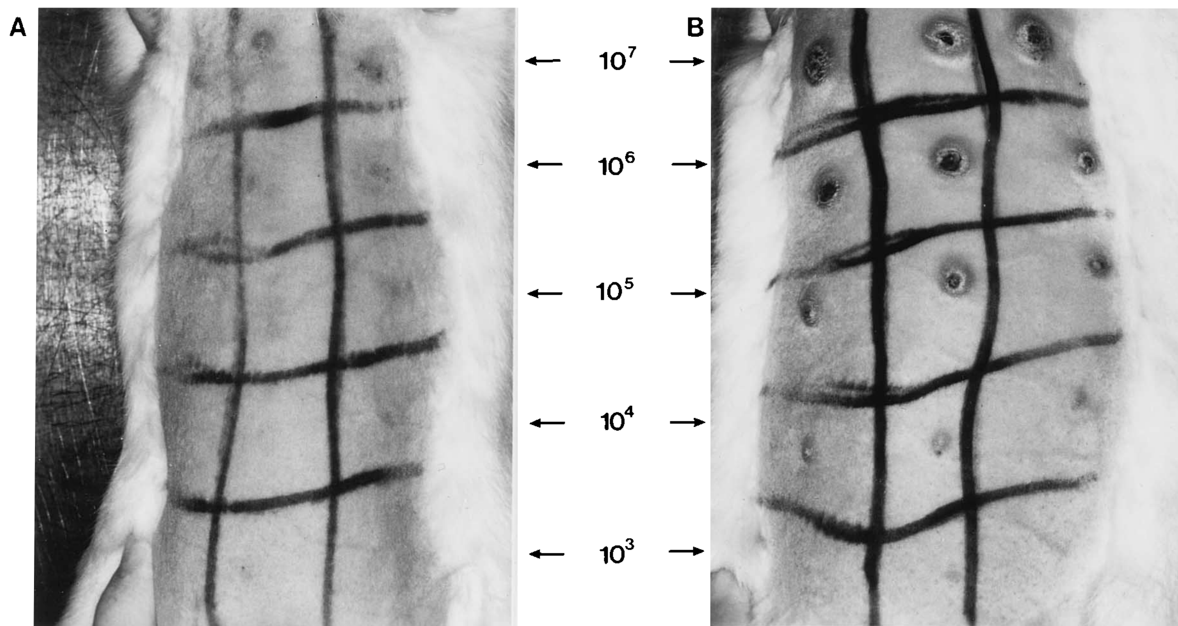


FIG. 4. Homologous strain disease protection of rabbits vaccinated with the pilus preparation. The appearance of disease at day 7 on immunized (A) and control (B) rabbits challenged with the homologous strain is shown. There was a significant reduction in the sizes and severity of lesions.

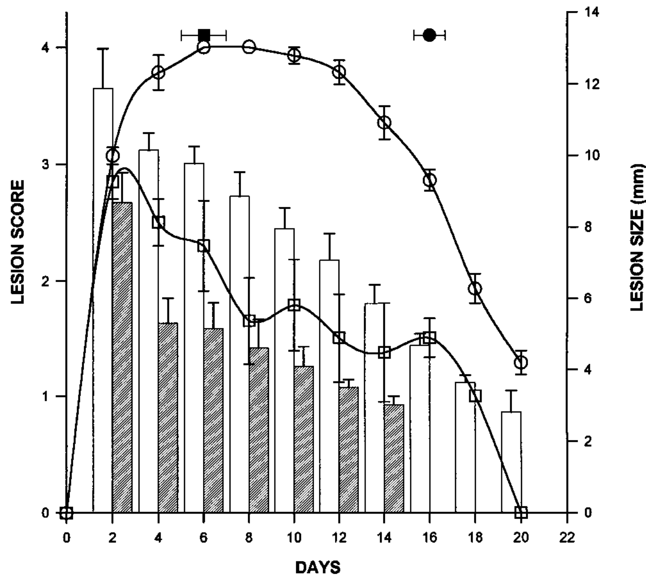


FIG. 5. Homologous strain (35000) protection at $10^5$ CFU produced by immunization with the pilus preparation. Lesion sizes (bar graph) and lesion scores (plot) for 11 rabbits vaccinated with the pilus preparation (shaded bars and open squares) and 7 control rabbits (open bars and open circles), with the last culture-positive day indicated for vaccinees (closed square) and controls (closed circle) in reference to numbers of days on the *x* axis, are shown. A significant reduction in lesion sizes and scores was observed over 21 days of observation ($P < 0.001$).

seven, with weaker inconsistent reaction to bands at 25 to 26 kDa and higher molecular masses.

**Infection with *H. ducreyi* 35000 in naive controls.** The onset of disease following challenge with *H. ducreyi* 35000 was similar to that described by Purcell et al. (24). Rabbits developed erythematous nodular indurated inflammatory lesions within 24 h of challenge with $10^5$ CFU of *H. ducreyi* 35000, and these became suppurative (score of 3) within 2 days. Lesions became ulcerative 3.8 ± 0.9 days after challenge, with a mean duration until reepithelialization of 11.4 ± 1.2 days (Table 1). All naive control rabbits developed ulcerative lesions with a scabbing surface and a thick, purulent, friable base (Fig. 3B and 4B). Lesions produced at $10^7$ and $10^6$ CFU were also ulcerative and were generally larger and more severe. Inocula at or below $10^4$ CFU failed to induce ulcerative lesions consistently. *H. ducreyi* was recovered from lesions from 24 h to 16.9 ± 1.7 days after challenge at $10^5$ CFU (Table 1). There was no significant difference in disease between naive control rabbits and adjuvant control rabbits after challenge with *H. ducreyi* 35000 (data not shown).

**Virulence of *H. ducreyi* in LOS-immunized rabbits.** Although rabbits did respond serologically to LOS, no significant protection against disease was observed. In six LOS-vaccinated rabbits challenged with the homologous strain, disease progressed and resolved in a manner similar to that in seven naive control rabbits. No significant difference in peak lesion size ($P = 0.097$) or score ($P = 0.244$) was observed over 21 days, or at any point of observation (Fig. 3; Table 1).

**Virulence of *H. ducreyi* in rabbits immunized with the pilus preparation.** Within 24 h of challenge with *H. ducreyi* 35000, rabbits vaccinated with the pilus preparation developed indurated lesions similar in size and severity to those in controls. However, only 3 of 10 vaccinated rabbits developed ulcerative lesions, lasting an average of 1.4 ± 2.7 days ($P < 0.001$; Table 1), at $10^5$ CFU. The duration and severity of disease were significantly reduced in all 10 rabbits (Table 1; Fig. 4 and 5). Lesion sizes and scores throughout the period of observation

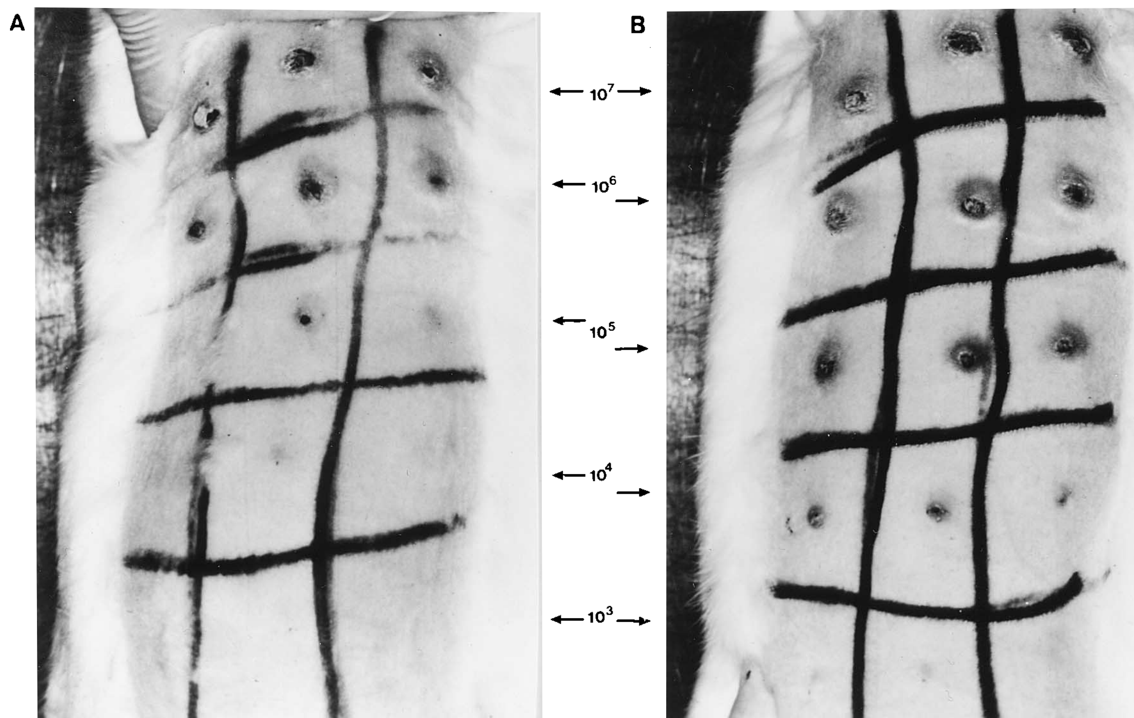


FIG. 6. Heterologous strain disease protection in rabbits immunized with the pilus preparation. The appearance of disease on rabbits immunized with the pilus preparation from *H. ducreyi* 35000 (A) and a naive control rabbit (B), all of which were challenged with the heterologous clinical isolate RO-34, shows a reduction in disease severity in vaccinees.

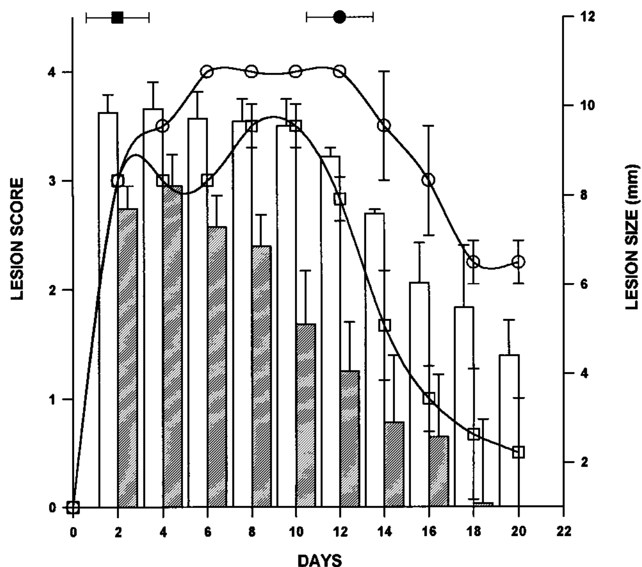


FIG. 7. Heterologous strain protection at $10^5$ CFU in rabbits immunized with the pilus preparation from strain 35000 and challenged with the clinical isolate RO-34. Lesion sizes (bar graph) and lesion scores (plot) for eight rabbits vaccinated with the pilus preparation (shaded bars and open squares) and five control rabbits (open bars and open circles) are shown. The last culture-positive day is indicated for controls (closed circles) and vaccinees (closed squares) in reference to numbers of days on the *x* axis. In a manner similar to that for homologous strain protection, there was a significant reduction in lesion sizes and scores over 21 days of observation ($P < 0.001$).

were both significantly reduced in pilus preparation-vaccinated rabbits compared with naive controls ($P < 0.001$) (Fig. 5).

**Heterologous strain protection.** At $10^5$ CFU, five naive control rabbits infected with the RO-34 clinical isolate developed suppurative lesions within 24 h, and these became ulcerative within 3.8 ± 0.4 days with a mean duration of 10.2 ± 1.9 days (Fig. 6; Table 2). At $10^5$ CFU all eight rabbits immunized with the pilus preparation from strain 35000 developed nodular indurated lesions within 24 h of challenge with the heterologous strain RO-34. Five rabbits developed ulcerative lesions within 7.0 ± 0.7 days, with an average duration of 3.0 ± 2.7 days (Table 2). Lesions produced at $10^5$ CFU were culture positive for 1.9 ± 2.6 days compared with 12.6 ± 1.5 days for naive controls ($P < 0.005$) (Table 2). Disease severity and duration were significantly reduced by immunization (Fig. 7). Lesion sizes and scores were significantly reduced for rabbits immunized with the strain 35000 pilus preparation and challenged with the heterologous strain RO-34 ($P < 0.001$).

**Histopathology of chancroidal lesions.** In both pilus preparation-vaccinated and naive rabbits, lesions observed at 4 days after infection with strain 35000 were similar and typical of chancroidal lesions, which are characterized by three architectural zones (13). The first zone consisted of an area of necrosis with cellular debris, degenerative polymorphonuclear leukocytes (PMNs), and fibrin corresponding to the ulcer surface. The second zone consisted of PMNs and histiocytic infiltrate characteristic of an acute inflammatory response. The third, a regenerative or edematous zone with PMNs, reactive fibroblasts, edema, and neovascular endothelial cells (Fig. 8A-1 and B-1), underlies zones 1 and 2. At 10 days after challenge, the lesions of naive rabbits were still characterized by an acute inflammatory infiltrate predominantly consisting of PMNs (Fig. 8A-2). Acute inflammatory cells were still present in lesions of rabbits vaccinated with the pilus preparation, but

TABLE 2. Heterologous strain protection at $10^5$ CFU in rabbits immunized with the pilus preparation from strain 35000 and challenged with the RO-34 isolate

| Animal group | Inoculum size (CFU)[a] | No. of days culture positive[a] | Lesion size (mm)[a] | | Peak lesion score[a] | No. of ulcerous lesions/total[b] | Ulcer duration (days)[a] |
|---|---|---|---|---|---|---|---|
| | | | Cumulative (21 days) | Average | | | |
| Control ($n = 5$) | 1.78 ± 0.56 | 12.6 ± 1.5 | 144 ± 41 | 6.85 ± 2 | 4 ± 0 | 5/5 | 10.2 ± 1.9 |
| Pilus preparation vaccinated ($n = 8$) | 1.62 ± 0.47 ($P = 0.35$)[c] | 1.88 ± 2.6 ($P < 0.005$)[c] | 83.5 ± 32 ($P < 0.01$) | 3.98 ± 1.5 ($P < 0.001$)[c] | 3.38 ± 0.35 ($P < 0.001$)[c] | 5/8 | 3 ± 2.7 ($P < 0.001$)[c,d] |

[a] Data are means ± standard deviations.
[b] An ulcerous lesion had a lesion score of 4.
[c] Comparison of mean values by the one-way ANOVA.
[d] Data represent the durations of six ulcers in five rabbits.

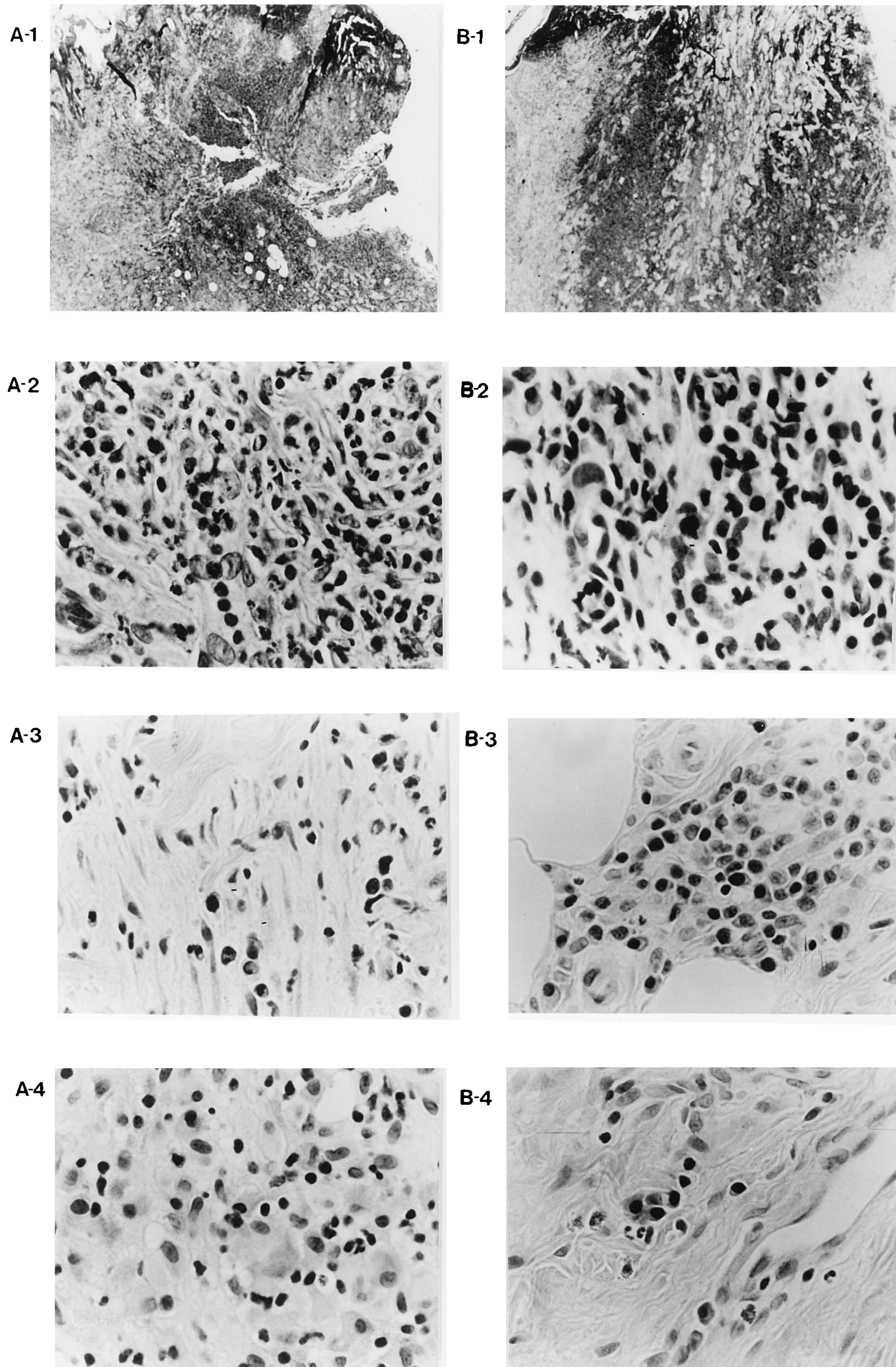


FIG. 8. Hematoxylin and eosin stain of biopsy samples of chancroidal lesions of naive control rabbits (A) and rabbits immunized with the pilus preparation (B). Biopsies were performed on days 4 (A-1 and B-1), 10 (A-2 and B-2), 15 (A-3 and B-3), and 21 (A-4 and B-4) following infection with the homologous strain 35000. The day 4 biopsy samples show typical organization of chancroidal lesions into three well-defined zones, whereas samples from the day 10, day 15, and day 21 biopsies are high-power views of the infiltrative zones. Accelerated recruitment of lymphoid and plasmacytoid infiltrate is illustrated, corresponding to more rapid healing of lesions in vaccinees.

there was a marked infiltration of lymphoid cells with scattered plasma cells corresponding to accelerated clinical healing of the lesions (Fig. 8B-2). By day 15, lesions of naive rabbits showed a gradual progression towards a chronic inflammatory response, with a prominent lymphoid cell infiltrate (Fig. 8A-3). Day 15 lesions of rabbits vaccinated with the pilus preparation had a prominent plasma cell infiltrate with scattered lymphoid cells and histiocytes (Fig. 8B-3). On day 21 after infectious challenge, lesions of naive rabbits were characterized by a heavy histiocyte infiltrate with very few plasma cells (Fig. 8A-4). Lesions of rabbits vaccinated with the pilus preparation had scattered plasma cells and neovascular endothelial cells at this time point with no clear differentiation of lesions into three zones (Fig. 8B-4).

## DISCUSSION

Although mechanisms involved in immune clearance of *H. ducreyi* infection in humans have not been identified, the establishment of the temperature-dependent rabbit model of infection may permit the identification of antigens as potential vaccine candidates by demonstration of the feasibility of vaccination and of inducible immunity to infection and disease. Using this model, we assessed the abilities of two purified bacterial fractions to induce measurable serologic immune responses and protective immunity in rabbits. Some characterization of the protective immune response may be made by definition of the class and kinetics of a serologic antibody response to immunization shown to be protective against infection and disease and by histopathology.

Immune responses to type 1, thymus-independent antigens such as LOS are dominated by IgM antibodies with little or no class switching, minimal affinity maturation, the lack of a booster effect, and no memory (19, 21). It is not surprising therefore that the kinetics of the serologic response to purified LOS immunization in rabbits resembles that of thymus-independent type 1 antigens (Fig. 2A). However, detection of LOS-specific IgG in sera of patients with chancroid is possible (1, 7). Immune response to LOS presented with bacterial protein antigens in the course of natural infection could lead to antibody class switching and affinity maturation. The role of IgG anti-LOS antibodies in the clearance of infection is unknown, and since vaccination did not provide protection against homologous strain infection, the suitability of purified LOS by itself as a candidate vaccinogen is likely limited. In addition, serologic recognition of LOS may not be consistent in natural infection, since only 40% of the chancroid sera reacted in an EIA to pooled LOS (16), perhaps because of antigenic heterogeneity of *H. ducreyi* LOS in nature.

In rabbits vaccinated with the pilus preparation, early-sterilizing protective immunity was apparent for both homologous strain infection and one heterologous unrelated clinical isolate. Immunization led to partial protection, with a significant reduction in lesion size, severity, and number of culture-positive days and accelerated healing in this animal model. At the ulcer-producing inoculum, the duration of infection was reduced, as measured by the number of days lesions remained culture positive. Comparative histology of these chancroidal lesions corroborated the differences in clinical outcome. The earlier resolution and reepithelialization of lesions in vaccinated rabbits corresponds to a switch from acute inflammatory response and neutrophilic infiltrate to a chronic lymphocytic infiltrate, which also preceded later convalescence for disease produced in naive control rabbits. Thus, vaccination with the pilus preparation accelerated the recruitment of lymphoid cells to the site of infection, with a more pronounced infiltration of plasma cells. Although lesions of naive rabbits were reepithelialized at day 21, a pronounced histiocyte infiltrate was still present, in contrast to vaccinated rabbits at the same time after infection. The histology during healing of chancroidal lesions in naive rabbits and vaccinees appeared to be consistent with cell-mediated immune responses.

The specific role of humoral mechanisms in vaccination with the pilus preparation cannot be defined here despite the more prominent plasma cell infiltrate in lesions of vaccinees and the presence of IgG antibody to the 24-kDa protein in sera of vaccinees before infection. The pilus preparation would exist in distinct forms, as monomers under denaturing conditions in immunoassays and as partially reassembled pili in solution at physiologic pH in immunization. The convalescent histology suggests a cellular immune response to infection, which may be the protective mechanism. Although antibody in serum and increased plasma cell infiltration were seen in vaccinees, humoral immunity may or may not be important in natural convalescence or disease protection.

A third uncharacterized carbohydrate extract obtained during LOS purification (Fig. 1) was also tested for antigenicity and protection against disease in this model. In all animals immunized with either 1, 10, or 100 μg of the extract in FCA and FIA, we failed to detect any serologic response to the antigen by immunoblotting or EIA (data not shown). Furthermore, none of these immunized rabbits showed any protection. Since *H. ducreyi* virulence was not altered in rabbits immunized with either PBS, carbohydrate, or LOS in the presence of FCA or FIA, it is unlikely that adjuvant-mediated immune activation (5) is responsible for the observed protection in rabbits immunized with the pilus preparation.

Because of the well-established link between HIV transmission and genital ulcer disease, measures to control chancroid could have a significant impact on the epidemiology of HIV. Previous work with the chancroid temperature-dependent rabbit model has demonstrated homologous and heterologous strain protective immunity by vaccination with cell envelope extracts. We have extended this work by examining two purified antigens, LOS and a pilus preparation. Of the two, the latter succeeded in producing partial homologous and heterologous strain protection from infection and disease in this model. The fact that protective immunity can be induced in this rabbit model of infection is a necessary first step in developing effective vaccination for human chancroid.

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