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Strain typing and vaccine development for chancroid

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STRAIN TYPING AND VACCINE DEVELOPMENT FOR CHANCROID

AUTHOR: Isabelle Leduc

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease shown to be involved in the heterosexual transmission of the human immunodeficiency virus. Heterogeneity of *H. ducreyi* lipooligosaccharide (LOS), complex sugars present in the bacterial membrane, was investigated to explore a potential basis for a classification system. A panel of clinical *H. ducreyi* isolates could be classified into 7 groups (1 to 7) according to the number and intensity of the LOS bands after electrophoresis, and grouped into 5 categories (A through E) according to the specific reaction and cross-reaction of antisera raised to outer membrane proteins (OMP). Carbohydrate and mass spectrometry analyses confirmed that all strains studied expressed many different LOS glycoforms at the cell surface, and that strains belonging to serologic categories B and D expressed truncated LOS molecules and lacked the characteristic DD-heptose found only in *H. ducreyi* strains.

The vaccine potential of the native (nHgbA) and recombinant forms (rHgbA) of the hemoglobin receptor of *H. ducreyi* as well as the recombinant form of the outer membrane protein D15 (rD15) were evaluated in the temperature-dependent rabbit model of chancroid. Rabbits were immunized twice, 4 weeks apart, with 100 µg of the immunogens (nHgbA, rHgbA, rD15, PBS and rFetA) in Freund's adjuvant, and challenged 4 weeks after the booster immunization with strains 35000 (homologous) and V1157 (heterologous) in doses ranging from 10^3 to 10^5 CFU. nHgbA vaccination modified the course of a homologous challenge at an inoculation of 10^3 CFU: fewer rabbits developed any ulcers, which were culture positive for a shorter period of time than in controls. After heterologous infection with 10^4 CFU of broth-grown strain V1157, none of the lesions of nHgbA-vaccinated rabbits developed into ulcers and lesion aspirates were sterile, while 2/3 of controls had ulcers. The rHgbA vaccine also modified the course of an experimental homologous infection, although results were very inconsistent from experiment to experiment. Indeed, across all experiments only the duration of ulcers was significantly reduced in rHgbA-vaccinated animals compared to controls at a 10^4 CFU inoculum. After a heterologous challenge with 10^4 CFU of *H. ducreyi* strain V1157, no lesions ulcerated in rHgbA-vaccinated animals. Modest protection similar to the rHgbA vaccine was obtained when animals were immunized with rD15. In terms of protection, there appeared to be different performance in different experiments: although rD15 provided partial protection against a homologous challenge infection in some experiments, it failed to protect animals against disease in others. After a homologous challenge with 10^4 CFU of *H. ducreyi* strain 35000, only the duration of ulcers were reduced in rD15-vaccinated animals compared to controls. However, across all experiments, better protection in rD15-vaccinated animals was associated with a greater humoral response to vaccine. In conclusion, nHgbA seems to be the best of these vaccine candidates against chancroid, while more experiments are needed to determine the potential of rHgbA and rD15 vaccines.

ABSTRACT

Haemophilus ducreyi is a fastidious Gram-negative bacillus that causes chancroid, a painful sexually transmitted genital ulcer disease (GUD), which has been shown to be a promoter of heterosexual transmission of the human immunodeficiency virus (HIV). Progress has been made in the past ten years in new experimental diagnostics for chancroid, selective culture media for *H. ducreyi*, and study of the pathogenesis has identified novel virulence factors of *H. ducreyi*. However, a simple discriminate typing system has yet to be established. Moreover, as treatment-based chancroid control programs have worked in outbreaks, emergence of antibiotic resistance in this pathogen has made the development of a vaccine against chancroid more important for control strategies, both for HIV control and for chancroid itself.

It has been reported that *H. ducreyi* expresses diverse lipooligosaccharides (LOS) at its surface. This diversity has never been systematically studied. I investigated electrophoretic, serologic and structural heterogeneity of *H. ducreyi* LOS, to explore a potential basis of a typing system in a panel of clinical *H. ducreyi* isolates. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), we grouped 91 *H. ducreyi* isolates into 7 groups (1 to 7) according to the number and intensity of the LOS bands on a silver-stained SDS-PAGE gel. Rabbit antisera were raised against outer membrane protein (OMP) from each SDS-PAGE group and were assayed against purified LOS in Enzyme-linked Immunosorbent Assay (EIA) and Western blots with adsorption by cross-reactive strains. *H. ducreyi* strains from each of the 7 SDS-PAGE group were placed in 5 sero-categories (A through E) according to the specific reaction and cross-reaction of their OMP

antisera to purified LOS from homologous and heterologous SDS-PAGE groups. In the panel of strains available for this evaluation, 85/91 (93%) were isolated during one period in one geographical area, and 85% of all strains were in one SDS-PAGE group. Within this large cluster, 3 of 7 SDS-PAGE groups, and 2 of 5 sero-categories were represented. Whether this represents a temporal and geographic clustering of LOS types, or whether this represents a predominance of LOS type in *H. ducreyi* may be resolved by evaluating LOS types of more diversely collected strains. Carbohydrate (CHO) and mass spectrometry analyses confirmed that all strains studied expressed many different LOS glycoforms at the cell surface. Strains belonging to serologic categories B and D expressed truncated LOS molecules and lacked the characteristic DD-heptose found only in *H. ducreyi* strains. Overall, these data show that structural and serologic heterogeneity among the *H. ducreyi* LOS may serve as the basis of a typing system.

The second goal of this thesis was to evaluate the immunogenicity and vaccine effect of the native (nHgbA) and recombinant (rHgbA) forms of the hemoglobin receptor of *H. ducreyi* as well as the recombinant form of the OMP D15 (rD15) in the temperature-dependent rabbit model (TDRM) of chancroid. The nHgbA protein was extracted from *H. ducreyi* strain 35000 grown in heme-depleted conditions to stimulate the expression of HgbA. Both rHgbA and rD15 were each extracted from separate strains of *Escherichia coli* expressing the genes cloned from strain 35000. Rabbits were immunized twice, 4 weeks apart, with 100 µg of the immunogens (nHgbA, rHgbA, rD15, PBS and rFetA) in Freund's complete adjuvant for the primary immunization and Freund's incomplete adjuvant for the booster immunization, and challenged 4 weeks after the booster with strains 35000

(homologous) and V1157 (heterologous) in doses ranging from 10^3 to 10^5 CFU. In this experimental infection model, control rabbits (n=14) immunized with PBS quickly developed ulcers at 100% of sites inoculated with 10^4 CFU of broth-grown *H. ducreyi* strain 35000. Ulcers persisted (mean $\pm$ SD) 11.5 ± 3.3 days and remained culture positive for 10.7 ± 2.8 days. With 10^3 CFU, 61% of the inoculations ulcerated, which persisted 4.7 ± 3.4 days and remained culture positive 9.1 ± 2.6 days. To evaluate immunization, measurement of lesions for comparison was carried out, with parallel control groups, at the inoculum titers expected to produce 100% and about 50% ulceration in a manner blinded to the vaccine status. Immunization with rFetA, the siderophore receptor of *Neisseria gonorrhoeae*, expressed and extracted exactly as rHgbA and rD15, did not modify disease compared with controls. Animals immunized with nHgbA had seropositivity in nHgbA- and OMP-based enzyme immunoassays (EIA), but sera reacted poorly to the corresponding 100-kDa band on Western blots of homologous OMP. nHgbA vaccination modified the course of a homologous challenge (n = 12) at an inoculation of 10^3 CFU: fewer rabbits developed any ulcers (4/12 for vaccinees vs. 11/14 for controls) and ulcers were culture positive a shorter time (5 ± 4.6 vs. 9.1 ± 2.6 days; $p=0.039$) than in controls. nHgbA-immunized rabbits inoculated with 10^4 CFU developed ulcers that lasted for 8.2 ± 3.7 days (vs. 11.5 ± 3.3 for controls, $p=0.028$). After heterologous infection (n = 3) with 10^4 CFU of broth-grown strain V1157, none of the lesions of nHgbA-vaccinated rabbits developed into ulcers and lesion aspirates were sterile, while 2 of 3 controls had ulcers, which were culture positive 6 ± 3.5 days ($p=0.04$).

Conversely, animals immunized with rHgbA had seropositivity to the 100-kDa band

on Western blots of homologous OMP and in rHgbA-based EIA, but not in an OMP-based EIA. The rHgbA vaccine also modified the course of an experimental homologous infection ($n = 15$), although results were very inconsistent from experiment to experiment. Indeed, only the duration of ulcers (7.6 ± 5.2 vs. 11.5 ± 3.3 days; $p=0.025$) was significantly reduced in rHgbA-vaccinated animals compared to controls at a 10^4 CFU inoculum. After a heterologous challenge ($n = 6$) with 10^4 CFU of broth-grown *H. ducreyi* strain V1157, no lesions ulcerated in rHgbA-vaccinated animals, while 2 of 3 controls had ulcers, which were culture positive 6 ± 3.5 ($p=0.04$) days. Overall, across vaccination groups and inoculum titers, there was statistically significant reduction in ulceration after infection compared with controls ($p=0.051$).

Results similar to the rHgbA vaccine were obtained when animals were immunized with the OMP rD15. The rD15 antisera had seropositivity to an 85-kDa band on Western blots of homologous OMP and in rD15-based EIA, but not in an OMP-based EIA. In terms of protection, there appeared to be different performances in different experiments: although rD15 provided partial protection against a homologous challenge infection in some experiments, it failed to protect animals against disease in others. After a homologous challenge ($n = 13$) with 10^4 CFU of *H. ducreyi* strain 35000, only the duration of ulcers (8.5 ± 4 vs. 11.5 ± 3.3 days; $p=0.047$) were reduced in rD15-vaccinated animals compared to controls. Reduced protection in some rD15-vaccinated animals was associated with a reduced humoral response. Indeed, rabbits in half of the experiments had lower antibody titers, which correlated with longer periods of culture positivity and ulceration ($p<0.05$).

In conclusion, these results suggest that nHgba may be a good vaccine candidate for

protection against chancroid since this vaccine reduced the number of ulcers and culture positivity in vaccinees compared to controls after both homologous and heterologous challenges. As for both recombinantly expressed proteins rHgbA and rD15, further experiments are needed to adequately determine their protective capabilities since there were many inconsistencies between experiments.

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LIST OF ABBREVIATIONS

3-AEC	3-amino-9-ethyl carbazole
ABTS	2,2'-azine-di-[-3-athyl-benzthiazolinsulfonat(6)]
ANOVA	Analysis of variance
BB	Binding buffer
CAP	Chocolate agar plates
CHO	Carbohydrate
CDC	Center for Disease Control
CDT	Cytolethal distending toxin
CE-ESMS	Capillary electrophoresis-electrospray mass spectrometry
CFU	Colony forming units
CHO	Carbohydrate
CPE	Cytopathic effect
CuZnSOD	Copper-zinc superoxide dismutase
ddH ₂ O	Double distilled water
DNA	Desoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ECM	Extracellular matrix
EDD	Estimated delivered dose
EDTA	Ethylene-diamine-tetraacetic acid
EIA	Enzyme-linked Immunosorbent Assay
EM	Electron microscopy

FCA	Freund's Complete Adjuvant
Fe	Iron
FIA	Freund's Incomplete Adjuvant
FBS	Fetal Bovine Serum
Gal	Galactose
Glc-Nac	N-acetyl-glucosamine
Glu	Glucose
GU	Genital Ulcers
GUD	Genital Ulcer Diseases
Hep	Heptose
HEPES	N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid
HFF	Human Foreskin Fibroblast
Hg	Hemoglobin
HIV	Human Immunodeficiency virus
HSP	Heat shock protein
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
Il	Interleukin
IPTG	Isopropyl-β-D-thiogalactoside
KDa	Kilodaltons
Kdo	3-deoxy-D-manno-octulosonic acid

LB	Luria-Bertani
LCDC	Laboratory Center for Disease Control and Prevention
LOS	Lipooligosaccharides
LPS	Lipopolysaccharides
MAb	Monoclonal antibody (ies)
M-H	Mueller-Hinton broth
M-PCR	Multiplex Polymerase Chain Reaction
MPL®	Monophosphoryl lipid A (SE = stable emulsion)
MUPI 100	Minimum ulcer-producing inoculum (100% of lesions develop into ulcers)
Neu5Ac	Sialic/neuraminic acid
nHgbA	Native HgbA
NRC	National Research Council
NZW	New Zealand White
O.D.	Optical density
OG	n-octyl-β-D-glucopyranoside
OMP	Outer membrane proteins
O/N	Overnight
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocytes

PMSF	Phenyl-methyl-sulfonyl fluoride
rD15	Recombinant D15
rFetA	Recombinant FetA
rHgbA	Recombinant HgbA
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STD	Sexually transmitted disease
TFA	Trifluoroacetic acid
Th	T helper
Tris-HCl	Hydroxymethyl methylammonium chloride
TDRM	Temperature-dependent rabbit model
WHO	World Health Organization
WT	Wild-type

CHAPTER 1 – INTRODUCTION

Also known as soft chancre, *chancre mou*, soft sore and *ulcus molle*, chancroid is a painful sexually transmitted genital ulcer disease (GUD) caused by the Gram-negative bacterium *Haemophilus ducreyi*. Although not life-threatening, chancroid is associated with important complications such as suppurative lymphadenitis, destructive phagedenic ulceration, and increased transmission of the human immunodeficiency virus (HIV). It is common in many parts of the world, antibiotic resistance is common among *H. ducreyi*, and natural chancroid does not provide protection against subsequent disease. The fastidious nature of *H. ducreyi* made it difficult to study the pathogenesis of chancroid, and the lack of a strain typing system has hindered the epidemiological analysis of this sexually transmitted disease (STD).

1.1 CHANCROID

1.1.1 Epidemiology of chancroid. The epidemiological study of chancroid has been hampered by the lack of knowledge of its natural history, its underreporting due to lack of surveillance, the self-limited nature of the disease, the diagnostic confusion with herpes or syphilis, and the lack of specific diagnostic tests and typing tools (Schulte *et al.* 1992; Trees and Morse 1995). For these reasons, no estimates for worldwide chancroid prevalence are available using the methodology developed for other STD such as syphilis and gonorrhea (www.who.int/dsa/cat98/std8.htm - STD fact sheet). Chancroid incidence varies greatly between countries and regions, as well as during time periods (www.who.int/dsa/cat98/std8.htm - STD fact sheet). For example, chancroid was endemic in many parts of Africa, Asia and Latin America (Mroczkowski and Martin 1994; Trees and Morse 1995) in the 1980s and early 1990s, but herpes genitalis is now

felt to be the most common cause of GUD in those regions (Rakwar *et al.* 1997). Chancroid is still common in parts of Africa like Senegal (Totten *et al.* 1999; Totten *et al.* 2000) and Madagascar (33% by Multiplex Polymerase Chain Reaction (M-PCR) (Behets *et al.* 1999a; Behets *et al.* 199b). In most industrialized countries, chancroid has become rare (www.who.int/dsa/cat98/std8.htm - STD fact sheet). Between 1975 and 1998, there were between 60 to 120 cases of chancroid reported annually in the United Kingdom, which were found mainly in the north of England (O'Farrell 2000). In Canada, there were only 3 cases reported in 1996 and none in 1997 (www.hc-sc.gc.ca/lcdc/hpb/publicat/ccdr/99vol25/25s1/25s1a-e.html). In the United States, the number of cases peaked in 1947 (9515 cases), then decreased over the next 30 years, with an average of 925 cases per year (Morse 1989; Trees and Morse 1995). In 1981, there was a dramatic increase in the number of chancroid cases, which reached a peak in 1987 with 4986 cases (Morse 1989; Schulte *et al.* 1992). The number of chancroid cases has declined steadily since then, with only 143 cases reported in 1999 (www.cdc.gov/nchstp/od/news/RevBrochure1pdfChancroid.htm). *H. ducreyi* was detected in ulcer specimens in Chicago (12% in 1996 by M-PCR) (Mertz *et al.* 1998a), Memphis (20% in 1996 by M-PCR) (Mertz *et al.* 1998a) and Mississippi (39% in 1994-95 by M-PCR) (Mertz *et al.* 1998b).

Prostitution contributes to the transmission of chancroid in both developing and developed countries (Schulte *et al.* 1992; Mroczkowski and Martin 1994; Trees and Morse 1995; Rakwar *et al.* 1997). The number of sexual partners and the pattern of sexual mixing seems to be the critical factor in the spread of chancroid, since the exchange of sex for drugs (crack cocaine) is associated with an increase in syphilis and

chancroid cases in the U.S (Schulte *et al.* 1992; Mroczkowski and Martin 1994; Trees and Morse 1995; DiCarlo *et al.* 1995).

It has been shown that chancroid is a promoting co-factor in the heterosexual transmission of HIV (Greenblatt *et al.* 1988; Simonsen *et al.* 1988; Cameron *et al.* 1989; Jessamine *et al.* 1990; Plummer *et al.* 1991; Wasserheit 1991; Behets *et al.* 1995; Tyndall *et al.* 1996; Magro *et al.* 1996; Cohen 1998). Three mechanisms have been proposed to explain how this infection enhances the transmission of HIV. First, increased shedding of the virus directly through the chancroidal ulcer of an HIV-infected person could facilitate its transmission to an uninfected partner (Cameron *et al.* 1989; Kreiss *et al.* 1989; Plummer *et al.* 1990; Trees and Morse 1995). Second, disruption of the epithelial barrier in chancroidal ulcers may represent a portal of entry for the virus, allowing it to infect CD4⁺ T-cells present at the ulcer site (Cunningham *et al.* 1985; Cameron *et al.* 1989; Engelkens *et al.* 1993). Third, genital secretions from HIV-positive patients coinfecting with chancroid contain higher levels of HIV than semen from HIV-positive patients without chancroid (Behets *et al.* 1995).

1.1.2 Clinical presentation. Multiple painful ulcers in the genital area characterize chancroid, for which the incubation period is between 4 and 7 days (Ronald and Plummer 1989; Morse 1989). Initially, a small inflammatory papule surrounded by a narrow erythematous zone will appear, before the emergence of a pustule 2 to 3 days later (Ronald and Plummer 1989; Morse 1989). The rupture of this pustule occurs rapidly, which results in a characteristic non-indurated, necrotic and sharply demarcated soft ulcer with ragged, undermined borders and an irregular, friable and painful base (Ronald and Plummer 1989; Mroczkowski and Martin 1994). The lesions are usually filled with a

gray to yellow purulent exudate with a foul odour and are rarely surrounded by inflammation (Freinkel 1987; Ronald and Plummer 1989; Morse 1989). Chancroidal ulcers may persist for 3 to 8 weeks (Ronald and Plummer 1989).

Many complications can arise from a chancroidal infection. Inguinal lymphadenopathy commonly accompanies chancroid in 30 to 50% of male patients, is usually unilateral and appears within 1 week of infection (Albritton 1989; Ronald and Plummer 1989; Morse 1989). In a proportion of cases, the inguinal node becomes fluctuant and ruptures, forming a draining abscess or a suppurative bubo characteristic of chancroid (Ronald and Plummer 1989; Morse 1989). A phagedenic ulcer, which is due to anaerobic superinfection of a chancroidal lesion, can also occur, leading to the extensive destruction of the external genitalia (Morse 1989). Concomitant HIV infection may also affect the appearance and the clinical course of chancroid. Indeed, a greater number of ulcers are often seen in HIV-infected patients (King *et al.* 1998), and the sometimes atypical lesions found in HIV+ patients do not seem to heal as fast and are more difficult to treat than those of HIV-uninfected patients (Trees and Morse 1995; King *et al.* 1998). The histological and immunohistochemical picture of chancroidal lesions for HIV+ and HIV- patients is identical (Magro *et al.* 1996; King *et al.* 1998), although more neutrophils are present in lesions of HIV- patients (Magro *et al.* 1996).

1.1.3 Histology and immunochemistry. Some reports described a 3-layer architecture in the natural chancroidal lesion (Heyman *et al.* 1945; Sheldon and Heyman 1946; Freinkel 1987; Abeck *et al.* 1997). Degenerate polymorphonuclear leukocytes (PMN), fibrin, red blood cells, debris as well as clumps of Gram-negative cocco-bacilli are present in the narrow necrotic surface layer (Freinkel 1987). The wide middle zone is

characterized by edema and lack of fibroblastic repair and shows endothelial proliferation of blood vessels infiltrated by neutrophils at the junction with the superficial zone (Freinkel 1987). A dense infiltration of plasma cells and lymphocytes was found in the deep zone, which merges with the middle zone (Freinkel 1987; Abeck et al. 1997).

However, more recent articles reported the absence of the trilaminar architecture and the plasma-rich infiltrate. Perivascular and interstitial mononuclear cell infiltrates containing macrophages and CD4/CD8 T lymphocytes were instead found in lesions (King *et al.* 1996; Magro *et al.* 1996), reminiscent of a delayed-type hypersensitivity (DTH) immune response. Similarly, PMNs, Langerhans cells, macrophages, CD4 T cells and interferon- γ , IL-8 and tumor necrosis factor- α mRNA were also found in cutaneous infiltrates (Spinola *et al.* 1994; Palmer *et al.* 1998b) of lesions in experimentally infected humans. Thus, the histology of chancroidal lesions may be dependent on the site and the timepoint at which the biopsy was taken. For example, biopsies taken soon after infection may show more of a cellular-based immune response, while those taken later after infection may show a different architecture and cell-types.

In experimental human chancroid, *H. ducreyi* was found in high number at the base of the pustule and sparsely distributed in the pustule itself and the subpustular dermis, in close association with PMN and macrophages (Bauer *et al.* 2000; Bauer *et al.* 2001). *H. ducreyi* did not associate with fibroblasts, keratinocytes, T cells, Langerhan's cells, laminin and fibronectin, but colocalized with collagen and fibrin, and was mostly found extracellularly, as previously reported (Marsch *et al.* 1978; Bauer *et al.* 2000; Bauer *et al.* 2001).

The histology of experimental rabbit infection has also been described (Desjardins *et al.* 1995; Desjardins *et al.* 1996). For the first 10 days after experimental infection, the rabbit lesions are characterized by necrotizing inflammation and abundant PMN. The zonal architecture of chancroid was visible at day 10. By day 15, cellular infiltrates consisted of acute inflammatory cells with lymphoid and plasma cells, while by day 21, the infiltrate was typical of a granulomatous reaction (histiocytes, lymphoid cells and lipid vacuoles).

1.1.4 Diagnosis of chancroid. The Ito-Reenstierna skin test, the Gram stain and the histological features of the chancroidal ulcer were formerly used as aids in the diagnosis of chancroid. None of them are used today since both false-negative and false-positive reactions were reported for the skin test (Greenblatt and Sanderson 1937; Greenblatt and Sanderson 1938; Heyman *et al.* 1945; Morse 1989), the Gram stain lacks adequate sensitivity (10 to 50%) for definitive diagnosis of chancroid (Coovadia *et al.* 1985; Sturm *et al.* 1987; Morse 1989; Chui *et al.* 1993; Joseph and Rosen 1994), and data concerning the unique 3-zone feature of the chancroidal ulcer is controversial.

1.1.4.1 Clinical diagnosis of chancroid. The diagnosis of chancroid is most often based upon the clinical appearance of the genital ulcer. A patient presenting with clinically compatible symptoms of chancroid such as one or more painful ulcers in the coronal sulcus or frenulum, no evidence of *Treponema pallidum* infection (by dark-field microscopy or serologic test), and presence of tender regional lymphadenopathy will be classified as a probable case of chancroid (Trees and Morse 1995). The accuracy of a clinical diagnosis of chancroid based solely on clinical appearance can be as low as 33% and as high as 89% (Sturm *et al.* 1987; Morse 1989; Dangor *et al.* 1990; Chui *et al.* 1993;

Roggen *et al.* 1993; Mroczkowski and Martin 1994; Orle *et al.* 1996; Behets *et al.* 1999a; Behets *et al.* 1999b), no doubt related both to clinical experience, and the actual absolute prevalence of chancroid versus other GUD.

1.1.4.2 Culture as a diagnostic tool of chancroid. A chancroid case is confirmed when *H. ducreyi* can be cultured from the clinical specimen (Trees and Morse 1995). Although culture of *H. ducreyi* is still the accepted standard for diagnosis, its sensitivity ranges between 35 % and 88% (Johnson *et al.* 1995; Orle *et al.* 1996; Morse 1989; Joseph and Rosen 1994), which is not enough to identify all cases of chancroid.

1.1.4.3 Biochemical diagnosis of chancroid. The rapid-ANA system from Innovative Diagnostics, which tests for 18 preformed enzymes, was studied as a means of diagnosis of chancroid (Shawar *et al.* 1990). Although the biochemical profiles of 19 fresh isolates of *H. ducreyi* were distinct (identification profile is 110500) from those of 66 strains representing 13 species, biochemical identification requires 2 days and results are affected by differences in growth (Shawar *et al.* 1990; Joseph and Rosen 1994).

1.1.4.4 Development of immunological diagnostic tools. Many groups have proposed the use of antibodies for the direct identification of *H. ducreyi* in clinical samples. A rabbit polyclonal antiserum raised against antigens isolated from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reacted with all *H. ducreyi* isolates tested (Roggen *et al.* 1993). Unfortunately, both sera had to be extensively adsorbed with other types of microorganisms to eliminate cross-reactivity. A polyclonal serum raised against live bacteria gave high immunofluorescence titers to *H. ducreyi* strains without prior adsorption (Finn *et al.* 1990).

A number of monoclonal antibodies (MAb) have been shown to react with *H. ducreyi*-specific surface-exposed antigens (Trees and Morse 1995). MAb 8H4 and 9D12 recognized all strains of *H. ducreyi* studied and detected the presence of this organism in experimental chancroidal lesions of an animal (Hansen and Loftus 1984). A MAb reactive with an outer membrane constituent of *H. ducreyi* had to be diluted to effectively reduce cross-reactivity and was not sensitive enough (50%) for clinical use (Schalla *et al.* 1986). A sensitivity of 93% and specificity of 63% was obtained when a mAb was evaluated for the detection of *H. ducreyi* in genital lesions smears using immunofluorescence (Karim *et al.* 1989).

1.1.4.5 Development of seroprevalence diagnostic tools. Immunoassays have been developed as seroprevalence tools for chancroid (Desjardins *et al.* 1992; Alfa *et al.* 1993b; Elkins *et al.* 2000a). An adsorption enzyme immunoassay (EIA) using a soluble bacterial antigen from *H. ducreyi* 35000 (Desjardins *et al.* 1992) developed in our laboratory had a sensitivity of 90% and a specificity of 64%. When this assay was evaluated in the field, its sensitivity was 53% and its specificity, 71% (Chen *et al.* 1997). Alfa and colleagues developed a LOS-based EIA with a sensitivity of 96% and a specificity of 97% (Alfa *et al.* 1993b), with 48% sensitivity and 89% specificity in the field (Chen *et al.* 1997). An OMP-based EIA was also developed (Elkins *et al.* 2000). Using three recombinantly expressed proteins (HgbA, TdhA and D15), this group obtained a sensitivity of 100% and a specificity of 62% in a panel of serum specimens collected from patients with chancroid, other GUD, and urethritis.

1.1.4.6 Development of molecular diagnostic tools. In DNA-DNA hybridization experiments, probes derived from DNA fragments encoding *H. ducreyi*-

specific proteins were unable to react with 10^3 CFU of *H. ducreyi*, and reacted weakly with 10^7 CFU of *Haemophilus* and *Pasteurella* species (Parsons *et al.* 1989). On the other hand, eight oligonucleotide-probes complementary to different regions in the 16S and 23S rRNA of *H. ducreyi* were 100% specific for strains of *H. ducreyi* (Rossau *et al.* 1991). None of these techniques are used since they are limited by the fact that bacteria need to be cultured from clinical specimens prior to the hybridization experiments.

PCR was also investigated as a means of diagnosis for chancroid (Chui *et al.* 1993; Johnson *et al.* 1994; West *et al.* 1995; Johnson *et al.* 1995; Orle *et al.* 1996; Gu *et al.* 1998; Totten *et al.* 1999). A unique multiplex PCR assay, which can simultaneously amplify DNA targets from *H. ducreyi*, *T. pallidum*, HSV-1 and HSV-2 had a sensitivity of 98.4 % for *H. ducreyi*, compared to 74.2 % for culture (Orle *et al.* 1996; Orle and Weiss 1999). This assay has been extensively used in the field (Morse *et al.* 1997; Mertz *et al.* 1998a; Mertz *et al.* 1998b; Behets *et al.* 1999a; Behets *et al.* 1999b; Risbud *et al.* 1999). A heminested PCR was 100 % sensitive and specific on cultured samples, while it had a sensitivity of 96% when it was performed directly on GU specimens (Gu *et al.* 1998). When the etiology of genital ulcers was compared using 2 different PCR and 2 different serologic assays, PCR was able to detect *H. ducreyi* in 83% of the patients (Totten *et al.* 2000).

1.1.5 Treatment. The Centers for Disease Control (CDC) and the World Health Organization (WHO) recommend a single 1g-dose of azithromycin taken orally, a single 250 mg-dose of ceftriaxone given intramuscularly (i.m.), a 500 mg-dose of erythromycin taken orally 4 times a day for 7 days, or a 500 mg-dose of ciprofloxacin taken orally twice a day for 3 days (www.cdc.gov/epo/mmwr/preview/mmwrhtml/00050909.htm)

www.who.int/HIV_AIDS/STIcasemanagement/STIManagemntguidelines/who_hiv_aids_2001.01/003htm#3.5). In human experimental infection, ciprofloxacin did not prevent disease while one dose of azithromycin could inhibit experimental disease for an average of 7 weeks (Thornton *et al.* 1998), suggesting that azithromycin may be a better choice to treat chancroid. In HIV+ patients, the WHO recommends ciprofloxacin as described above plus 100 mg of doxycycline twice daily for 14 days, or erythromycin as directed above but for a period of 14 days instead of 7 days (www.who.int/HIV_AIDS/STIcasemanagement/STIManagemntguidelines/who_hiv_aids_2001.01/002htm#2.2a).

1.1.6 Animal models of chancroid. Mice, rats, hamsters, guinea pigs, cats, dogs, goats, and sheep are refractory to *H. ducreyi* infection, while lesions can be produced in rabbits, monkeys and pigs following intraepithelial inoculation of *H. ducreyi* (Morse 1989; Purcell *et al.* 1991; Hobbs *et al.* 1995). Temperature sensitivity may have much to do with successful experimental infection.

1.1.6.1 Mice. When CBA mice were intradermally inoculated with 10^7 *H. ducreyi*, only half of the sites developed into ulcers that were macroscopically and histologically similar to natural chancroid and viable organisms were recovered from only 14% of the lesions (Tuffrey *et al.* 1988; Tuffrey *et al.* 1990). Inoculations with 10^6 and 10^5 CFU produced only small nodules that regressed without ulceration and no lesions were produced with an inoculum of 10^3 CFU (Tuffrey *et al.* 1988). Furthermore, killed *H. ducreyi* and *Neisseria gonorrhoeae* organisms as well as undiluted *H. ducreyi* and *N. gonorrhoeae* LOS could produce lesions similar to those produced by viable

organisms, clearly indicating that infection was not required to produce lesions in this mouse model (Tuffrey *et al.* 1990).

1.1.6.2 Rabbit. Reenstierna first introduced inoculation of *H. ducreyi* into the skin of rabbits as a model for chancroid in 1921 (Reenstierna 1921a; Reenstierna 1921b). Although used by many groups to measure virulence of *H. ducreyi* strains, large inocula (10^8 CFU) were needed to produce ulcerative necrotic dermal lesions (Feiner and Mortara 1945; Dienst 1948; Purcell *et al.* 1991). To improve this model, rabbits were housed in a temperature-controlled room to lower their skin temperature, simulating the lower temperature of the external human genitalia, thereby facilitating the replication of *H. ducreyi* and the development of lesions (Purcell *et al.* 1991). Necrotic skin lesions routinely developed in the skin of rabbits intradermally injected with 10^5 CFU (Purcell *et al.* 1991). Furthermore, lesions were macroscopically and histologically similar to that of natural chancroid and *H. ducreyi* was recovered from the lesions. Contrary to the mouse model, rabbits that received heat-killed *H. ducreyi* showed only minor inflammation at the site of inoculation, and those pretreated with ceftriaxone showed only transient erythema, confirming that viable organisms were required for the formation of lesions (Purcell *et al.* 1991).

The experimental rabbit model of chancroid is useful for many reasons. First, the lesions produced in this model are histologically similar to the ones in natural and experimental human chancroid (Desjardins *et al.* 1995; Desjardins *et al.* 1996; Purcell *et al.* 1991). Second, the cell-mediated immune response in rabbit ulcers (Desjardins *et al.* 1996) is similar to the one observed in humans (King *et al.* 1996; Magro *et al.* 1996; Spinola *et al.* 1996b; Palmer *et al.* 1998b). For example, the primary cell infiltrating the

lesion is heterophils, the rabbit equivalent of a PMN. Third, the incubation period and the gross anatomical appearance of lesions induced in the rabbit are similar to that of human chancroid, although the ulcers usually resolve faster (within 2 to 3 weeks) than in natural human chancroid (Ronald and Plummer 1989). Finally, lesions in rabbit are allowed to progress to the ulcerative stage and to resolve by themselves. The disadvantage of this model is that infection provides protection against subsequent re-infection (Purcell *et al.* 1991), which is not the case in natural and experimental human chancroid. Overall, although the temperature-dependent rabbit model of chancroid has some imperfections, it can fulfill the criteria of a “good” animal model (Smith 1989) for studies of virulence, infection and immunity and be used as a quantitative virulence assay. These criteria, as defined by Smith, include a titrable inoculum, a route of infection and a disease comparable to the natural infection, and the measurability of disease and immune response in the animal model.

1.1.6.3 Swine. Persistent ulcerative lesions histologically similar to human chancroidal ulcers also developed in pig ears after the inoculation of 10^7 CFU (estimated delivered dose (EDD) of 10^4 CFU) of *H. ducreyi* with an allergy-testing device (Hobbs *et al.* 1995). As in the rabbit model, viable *H. ducreyi* were responsible for ulcer formation and *H. ducreyi* organisms were recovered from the ulcer site up to 17 days after inoculation (Hobbs *et al.* 1995).

1.1.6.4 Primate. Two primate models for chancroid have been developed using pigtailed macaques (*Macaca nemestrina*) (Totten *et al.* 1994c; Sturm 1997). The clinical appearance and the progression of the lesions on the foreskin of male primates infected with 10^7 to 10^8 CFU of *H. ducreyi* were similar to natural chancroid, inguinal

lymphadenopathy was observed, and *H. ducreyi* was recovered up to 20 days after the initial inoculation. (Totten *et al.* 1994c). Viable *H. ducreyi* organisms capable of multiplication in the lesion were needed to produce ulcers (Totten *et al.* 1994c). Another group used *Macaca mulatta* to test the role of iron availability on virulence of *H. ducreyi* (Sturm 1997). The minimal ulcerative dose in this primate was 10^5 CFU for 3 strains, which was reduced to 10^4 when the animals were pretreated with i.m. iron (Sturm 1997). Despite appropriateness of this model in terms of ulcer formation and development of lymphadenopathy, the cost of procuring and housing these animals is prohibitive.

1.1.7 Human model of chancroid. In the experimental human model of chancroid, *H. ducreyi* is applied to the skin of the upper arm using an allergy-testing device (Spinola *et al.* 1994). For ethical reasons, the lesions are allowed to progress to the pustular stage, at which point volunteers are given antibiotics to clear the infection (Spinola *et al.* 1994). Early work revealed that the injection of 10^5 and 10^6 CFU (EDD of 10^2 - 10^3 CFU) of live, but not heat-killed *H. ducreyi* could produce mildly painful micropustules (Spinola *et al.* 1994). Although viable organisms were recovered from biopsy of the experimental lesions, they progressed too rapidly to mimic the natural infection (Spinola *et al.* 1994). The model was modified and standardized to reduce the rate of ulceration by reducing the size of the inoculum. An estimated inoculum of 30 CFU resulted in papule formation in 95% of the injection sites, while the pustule formation rate was 69% (Al-Tawfiq *et al.* 1998). Similar to the natural *H. ducreyi* infection, experimental human chancroid does not confer protection against subsequent exposure to the organism (Al-Tawfiq *et al.* 1999).

1.1.8 *In vitro* models of chancroid. The human skin can be divided into 2 layers: the stratified, squamous cell epithelium of the epidermis and the underlying connective tissue called the dermis (Ebling *et al.* 1992). Keratinocytes make up 95% of the epidermis, but melanocytes, Langerhans and Merkel cells are also found in this layer (Ebling *et al.* 1992). The dermis is comprised of collagen (75%), elastin and fibronectin and contains few cells (fibroblasts, mast cells, macrophages, lymphocytes and melanocytes) (Ebling *et al.* 1992).

Cell culture monolayers of primary keratinocytes (Brentjens *et al.* 1994; Gibson *et al.* 1997; Totten *et al.* 1994a) or human foreskin fibroblasts (HFF) (Alfa 1992; Alfa *et al.* 1993a; Lammel *et al.* 1993) were used as simple *in vitro* skin models for the study of the pathogenesis of chancroid. A coculture of the HaCaT keratinocyte cell line and foreskin fibroblasts was later developed since it more closely resembled human skin, retained its ability to express differentiation-specific markers and did not form tumors in nude mice (Zaretsky and Kawula 1999). A model of artificial skin composed of human foreskin keratinocytes and fibroblasts was also used to investigate the cytokines produced during the initial stages of an infection by *H. ducreyi* (Hobbs *et al.* 1998). Although these experimental systems have been particularly important in examining the attachment and entry of *H. ducreyi* into cells, as well as assessing cytopathic effect (CPE), they lack immune cells, which are important mediators of immunity and pathogenesis of inflammation in normal skin. Furthermore, recent reports have shown that *H. ducreyi* does not associate with fibroblasts or keratinocytes in the experimental human model of chancroid (Bauer *et al.* 2000; Bauer *et al.* 2001), casting doubt on the use of such *in vitro* assays in the study of the pathogenesis of *H. ducreyi*.

1.2 *Haemophilus ducreyi*

1.2.1 Classification and taxonomy. *H. ducreyi* was included in the genus *Haemophilus* because of its hemin requirement and its genomic G + C content (39%) (Lwoff and Pirotsky 1937; Killian and Biberstein 1984; Rossau *et al.* 1991). However, many reports have provided evidence against this classification. For instance, DNA hybridization and genetic transformation experiments demonstrate that a group of established or proposed *Haemophilus* species were 0 to 6% related to *H. ducreyi* (Casin *et al.* 1985; Albritton *et al.* 1986; Albritton 1989). Furthermore, this pathogen possesses isoprenoid quinone structures essentially different from that of other *Haemophilus* species (Carlone *et al.* 1988) and based on the 16S rRNA sequence, places *H. ducreyi* far away from *H. influenzae* in the phylogenetic tree of the family *Pasteurellaceae* (Dewhirst *et al.* 1992).

1.2.2 Colony morphology and appearance in Gram stain. *H. ducreyi* colonies are 0.5 to 1.0 mm in diameter, nonmucoid, dome-shaped, granular and yellow-gray in color (Albritton 1989; Ronald and Plummer 1989; Morse 1989). When this microorganism is grown under aerobic conditions, colonies of different size and opacity may appear, giving the impression that the culture contains a mixed flora (Ronald and Plummer 1989; Albritton 1989; Morse 1989). The minimal doubling time of *H. ducreyi in vivo* is 16.5 hours (Throm *et al.* 2001). Considerable intercellular adherence between *H. ducreyi* cells explains the observations that *H. ducreyi* colonies can be pushed intact across the agar surface and that substantial autoagglutination occurs when the infectious agent is grown in liquid culture (Albritton 1989; Ronald and Plummer 1989; Morse 1989).

Cultured *H. ducreyi* appear as pleomorphic Gram-negative non-spore-forming, nonmotile bacillus of an average length of 1.2 to 1.5 μm and an average width of 0.5 μm

(Albritton 1989). The characteristic arrangement of parallel chains described as “railroad tracks” is associated with liquid culture, while the unique complex whorls described as “school of fish” or “fingerprints” are more common when *H. ducreyi* is grown on solid media and is rarely seen in smears from clinical specimens (Albritton 1989; Morse 1989).

1.2.3 Nutritional requirements. *H. ducreyi* has a strict heme requirement, confirmed by the inability to synthesize porphyrins or porphobilinogen from δ -aminolevulinic acid (Hammond *et al.* 1978; Albritton *et al.* 1981) and the fact that iron increases virulence of *H. ducreyi* in a primate model of chancroid (Sturm 1997). Glucose and glutamine are also required for the growth of *H. ducreyi*, and albumin has been shown to decrease the doubling time of this organism by absorbing toxic metabolic products (Dziuba *et al.* 1993).

1.2.4 Biochemistry and cell wall composition. No unique biochemical characteristics are known for *H. ducreyi* and its fastidious nature results in a very limited biochemical activity profile. *H. ducreyi* tests positive for alkaline phosphatase, β -lactamase, aminopeptidase and nitrate reductase (Albritton 1989; Morse 1989; Joseph and Rosen 1994). This microorganism is asaccharolytic, the absence of glycosidase activity limiting its ability to catabolize polysaccharides (Morse 1989). Although *H. ducreyi* is usually considered catalase negative and oxidase positive, conflicting results have been reported, depending on the type of test and the substrate used during the experiment (Albritton 1989; Morse 1989). Weak urease activity has been found in some strains of *H. ducreyi* (Albritton 1989). C_{14} , C_{16} , C_{18} and C_{20} fatty acids were found in all *H. ducreyi* strains studied (Albritton 1989). However, the cell wall of *H. ducreyi* is slightly different from

that of other *Haemophilus* species in that it has a higher concentration of C₁₄ and a lower concentration of C₁₆ and C₂₀ fatty acids (Odumeru *et al.* 1987).

1.2.5 Iron (Fe) acquisition. Hemoglobins (Hg) from various animal origins (Elkins 1995), catalase, hemin, heme-albumin and Hg-haptoglobin complexes can supply Fe to *H. ducreyi* (Lee 1991). Lactoferrin, transferrin, FeCl₃ do not support the growth of *H. ducreyi* since they do not contain heme, an essential nutrient for the growth of this bacterium (Lee 1991). Although cytochrome C₁₁₁ contains heme, a covalent bond prevents its release and use by *H. ducreyi* (Lee 1991).

A 100-kDa surface-exposed outer membrane protein that bound Hg was identified in *H. ducreyi* (Elkins 1995; Stevens *et al.* 1996). Termed HgbA (Elkins 1995) or HupA (Stevens *et al.* 1996), this protein was immunologically and functionally conserved in all strains studied, was regulated by the level of heme in the medium and bound to Hg from human and animal sources (Elkins 1995). Directed mutations of the *hgbA* gene abolished the expression of HgbA (Elkins *et al.* 1995; Stevens *et al.* 1996) as well as the binding and the use of Hg as a source of heme in *E. coli* (Elkins *et al.* 1995). Hg uptake of *H. ducreyi* via HgbA is dependent upon the TonB system, which is comprised of 3 proteins, TonB, ExbB and ExbD that mediate the transfer of energy from the cytoplasmic to the outer membrane (Elkins *et al.* 1998). An isogenic *hupA hgbA* mutant was less virulent than the wild-type (WT) parent strain in the temperature-dependent rabbit model of chancroid (TDRM) (Stevens *et al.* 1996) and was unable to progress to the pustular stage in the human model of chancroid (Al-Tawfiq *et al.* 2000b). These results suggest a role for the Hg receptor in virulence of *H. ducreyi* and suggest that Hg is the most important source of heme/Fe in the initial stages of experimental human infection.

TABLE 1

Potential virulence factors of *H. ducreyi*. Abbreviations: **A** = reduced virulence, **act.** = activity, **assoc.** = associated, **biol.** = biological, **CMP-NeuAc** = N-acetylneuraminic acid cytidyltransferase, **CPE** = cytopathic effect, **CuZnSOD** = copper-zinc superoxide dismutase, **dep.** = dependent, **exp.** = experimental, **eukar.** = eukaryotic, **fibron.** = fibronectin, **HFE** = human foreskin epithelial cells, **HFF** = human foreskin fibroblasts, **huM** = human model of chancroid, **I** = induced, **ind.** = independent, **inf.** = infection, **memb.** = membrane, **mo** = macrophages, **Neg** = negative, **neut.** = neutrophils, **oxidati.** = oxidative, **prolif.** = proliferation, **raM** = rabbit model of chancroid, **stat.** = stationary, **trans.** = transferase, **V** = virulent

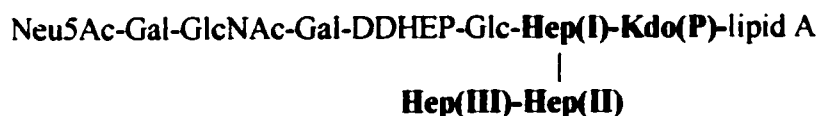
M.W.	Gene	Protein	Role in pathogenesis	Virulence of mutant	Reference
260	<i>lspA1</i> <i>lspA2</i>	Filamentous hemagglutinin-like protein	- Transcripts expressed <i>in vivo</i>		Ward, 98 Throm, 01
125	<i>hhdA</i>	Hemolysin structural prot. (Cell-assoc. + produced in log phase) (Conserved)	- CPE on HFF & keratinocytes - Lysis of HFF, HFE, mo, T + B lymphocytes - Evasion Hep-2 - Vaccine = NO protection - Transcripts expressed <i>in vivo</i>	- V in raM - No CPE on HFF - Less CPE against HaCaT	Palmer, 94 Totten, 95 Palmer, 95 Alfa, 96 Palmer, 96 Palmer, 98 Wood, 99 Dutro, 99 Young, 01 Throm, 01
100	<i>hgbA/hupA</i>	Hg receptor (Conserved)	- Surface-exp. - TonB-dep.	- A in hu+raMs	Elkins, 95 Elkins <i>et al.</i> 95 Elkins, 98 Stevens, 96 Al-Tawfiq, 00
85	<i>d15</i>	OMP (Conserved)			Thomas, 01
81	<i>ldhA</i>	Heme receptor (Conserved)	- TonB-dep. - Transcripts expressed <i>in vivo</i>		Thomas, 98 Throm, 01
70	<i>dnaK</i>	HSP	- Neg. modulator of <i>groEL</i>		Parsons, 92 Brown, 93 Parson, 99
61	<i>hhdB</i>	OMP	- Secretion of hemolysin	- V in huM	Palmer, 95 Palmer, 98 Young, 01
60	<i>groEL</i>	HSP (↑ basal levels) (I in stat. phase, H ₂ O ₂ and adh.) (C-surface ass.)	- Binding to fibron. + eukar. /Hep-2 cells - 5x Ec GroEL - 4x Hd DnaK		Parsons, 92 Brown, 93 Parsons, 97 Frisk, 98
55	<i>nadV</i>		- NAD ind. growth		Martin, 01
43		MOMP			Spinola, 93
40	<i>dnaJ</i>	HSP			Parsons, 97 Parsons, 99
40	<i>sodC</i>	CuZnSOD (Conserved periplasmic prot.)	- Role - survival to oxidati. burst by neut. + mo		Langford, 97 San Mateo, 98 San Mateo, 99
40	<i>LosB</i>	DDHep trans.	- Transcripts expressed <i>in vivo</i>	- Strain 1381 = ↓ adher. - V in huM	Stevens, 97 Gibson, 97 Young, 99

40	<i>losB</i>			- Serum resistant	Hiltke, 99 Throm, 01
40	<i>dnaJ</i>	HSP			Parsons, 99
38.5	<i>waaQ</i>	Heptosyltransferase III	- LOS biosynthesis		Filiatrault, 00
37-39	<i>momp ompA2</i>	MOMP OmpA2	- Contribution to serum resistance - Transcripts expressed <i>in vivo</i>	- Sensitive to NHS (<i>momp</i>) - V in huM	Spinola, 93 Klesney-Tait, 97 Hiltke, 99 Bauer 00 Throm, 00 Throm, 01
35	<i>lst</i>	Sialyltransferase	- LOS biosynthesis - Transcripts expressed <i>in vivo</i>	- V in huM	Bozue, 99 Young, 99 Throm, 01
33	<i>lgtB</i>	Galactosyltransferase II	- LOS biosynthesis		Sun, 00
32	<i>znuA</i>	ZnuA (Periplasmic)	- Zinc transporter	- A in raM	Lewis, 99
30	<i>dsrA</i>	DsrA (Conserved)	- Serum resistance	- A in huM	Elkins, 00 Bong, 01
28		Lipoprot. (Conserved)			Hiltke, 96
26	<i>lbgA</i>		- LOS expression	- V in raM	Stevens, 97
25	<i>neuA</i>	CMP-NeuAc synthetase	- LOS biosynthesis		Tullius, 96
24	<i>fipA</i>	Pilin monomer (Conserved + surface-exposed)	- Possible role in binding to laminin - cDNA + protein expressed <i>in vivo</i>	- V in hu+ra Ms	Spinola, 90 Castellazzo, 92 Frisk, 95 Brentjens, 96 Al-Tawfiq, 00 Bauer, 00 Throm, 01
21	<i>gmhA</i>	Pphep isomerase	- LOS biosynthesis	- A in raM	Bauer, 98
25 30 20	<i>cdtA</i> <i>cdtB</i> <i>cdtC</i>	Cytolethal Distending Toxin (CDT) (Presence in cell-free culture supernatant)	- Cytotoxic effect on HeLa, Hep-2, HaCat keratinocytes + fibroblasts - Induces apoptosis of Jurkat T + B cells	- V in hu+ra Ms (<i>cdtC</i>)	Purvén, 92 Lagergård, 93 Purvén, 95 Cope, 97 Purvén, 97 Stevens, 99 Gelfanova, 99 Cortes-Bratti,

25 30 20	<i>cdtA</i> <i>cdtB</i> <i>cdtC</i>		- Induces cell-cycle arrest in late G2 phase (Hep-2 + keratinocytes) - Fibroblasts: arrest in G1 + G2 - Transcripts expressed <i>in vivo</i> (<i>cdtB</i>) - Affects prolif. / biol. act. of stimulated T & B cells		99 Young, 01 Throm, 01 Svensson, 01 Cortes-Bratti, 01
18	<i>pal</i>	Lipoprot. (Conserved)	- Role in maintaining memb. Structure - Expressed during human exp. inf.	- A in huM	Spinola, 92 Spinola, 96 Fortney, 00 Bauer, 00
17	<i>fur</i>	Ferric uptake regulation			Carson, 96
14	<i>groES</i>	Heat shock prot. (HSP)			Parson, 92
??	<i>lspA2</i>				Ward, 98
??	<i>waaf</i> homolog	Heptosyltransferase II	- LOS biosynthesis	- A in raM	Bauer, 99
??	<i>lgtI</i>	Glucosyltransferase	- LOS biosynthesis		Filiatrault, 00
??	<i>losB</i>			- V in huM	Young, 99
??	<i>tolB</i>		- Upstream of <i>pal</i>		Fortney, 00
??	<i>djlA</i>		- Downstream of <i>pal</i>		Fortney, 00
??	<i>exbB</i> <i>exbD</i> <i>tonB</i>	Ton system	- Transfer energy from inner to outer memb.		Elkins, 98

Under low heme conditions, *H. ducreyi* also expresses a TonB-dependent heme receptor, TdhA (Thomas *et al.* 1998). Transcripts of *tdhA* were detected in biopsies of human volunteers infected experimentally with *H. ducreyi* (Throm and Spinola 2001).

1.2.6 Lipopolysaccharides. The *H. ducreyi* lipopolysaccharides (LPS) contain heptose, hexose, hexosamines and 3-deoxy-D-manno-octulosonic acid (Kdo), but not mannose and rhamnose, sugars found in the enterobacterial O-antigen (Odumeru *et al.* 1987). They are more correctly referred to as lipooligosaccharides (LOS) since they do not possess the repeating O-antigen characteristic of enteric bacteria and contain less than 10 sugars, closely resembling the LOS of *H. influenzae* and *N. gonorrhoeae* (Melaugh *et al.* 1994). Situated in the outer membrane, the LOS of *H. ducreyi* can be divided into 3 sections: the lipid A, the inner core, and the outer core, as shown in the structure of the main LOS glycoform of *H. ducreyi* strain 35000 (bold denotes the core oligosaccharide; DDHep = D-glycero-D-manno-heptose; Gal = galactose; Glc = glucose; GlcNAc = N-acetylglucosamine; Hep = L-glycero-D-manno-heptose; Neu5Ac = sialic acid):



The lipid A moiety, which anchors the LOS into the bacterial cell membrane via the Kdo molecule, has been linked with mitogenic, pyrogenic and adjuvant activities in the LPS of enteric bacteria (Mandrell *et al.* 1993). The core of *H. ducreyi* LOS is made up of 3 α -linked L-glycero-D-manno-heptoses and a phosphorylated Kdo molecule and is identical to the inner core of the LOS molecule of *H. influenzae* strain 2019 (Melaugh *et al.* 1994). The outer core, the site where most of the variability in the LOS structure of *H. ducreyi*

occurs, usually contains one or more branches containing glucose, galactose and glucosamine (Melaugh *et al.* 1992). Some strains of *H. ducreyi*, including 35000, harbor an atypical 1,4-linked D-glycero-D-manno-heptose not found in any other Gram-negative bacteria (Melaugh *et al.* 1992; Melaugh *et al.* 1994; Schweda *et al.* 1994). LOS species from a single organism may differ in their phosphorylation and sialylation states and the terminal sugars of their branched region (Melaugh *et al.* 1994; Schweda *et al.* 1995).

Many enzymes involved in the biosynthesis of the *H. ducreyi* LOS have been identified (Table 1). An N-acetylneuraminic acid cytidyltransferase (*neuA*) and a sialyltransferase (*lst*) are involved in sialylation of the *H. ducreyi* LOS (Tullius *et al.* 1996; Bozue *et al.* 1999). Products from the *lgbA* and *lgbB* genes are 48 to 52% similar to the product of the *H. influenzae* type b *lex-1 lic2A* gene and 52% similar to the RfaK protein of *E. coli*, respectively (Stevens *et al.* 1997). However, the tandem repeats of the nucleotide tetramer CAAT present in the *lex-1 lic2A* gene, which are involved in LOS phase variation of *H. influenzae*, are not present in *H. ducreyi lgbA* (Stevens *et al.* 1997). The *lgbB rfaK* gene of *H. ducreyi* likely encodes a D-glycero-D-manno-heptosyltransferase, since the LOS of a mutant of this gene terminates prior to the addition of the DD-heptose (Gibson *et al.* 1997). The GmhA protein of *H. ducreyi* is 87% identical to the *H. influenzae* GmhA and 73% identical to the *E. coli* GmhA, which encodes a phosphoheptose isomerase, an enzyme essential for the synthesis of the ADP-L-glycero-D-manno-heptose precursor (Bauer *et al.* 1998). Heptosyltransferase II and III, respectively, responsible for the addition of heptose II and III on the LOS molecule, are encoded by the *waaf* and *waaQ* homologs of *H. ducreyi* (Bauer *et al.* 1999; Filiatrault

et al. 2000). The *lgtB* gene of *H. ducreyi* encodes a galactosyltransferase II, while the *lgtF* gene encodes a glucosyltransferase (Sun *et al.* 2000; Filiatrault *et al.* 2000).

It has been speculated that the LOS molecule of *H. ducreyi* may be involved in the evasion of the immune system because of the presence of LOS epitopes mimicking human glycosphingolipids, helping *H. ducreyi* escape detection by the host immune response (Campagnari *et al.* 1990; Mandrell *et al.* 1992; Mandrell *et al.* 1993), and the presence of sialic acid, having a role in preventing activation of the alternative complement pathway (Edwards *et al.* 1982; Schweda *et al.* 1995; Melaugh *et al.* 1996). However, it has been shown that sialic acid does not affect virulence in the experimental human model of chancroid (Young *et al.* 1999), and in fact, nothing beyond the Kdo-3 heptose-glucose is needed for pustule formation (Young *et al.* 2001), as determined in the experimental human model of chancroid.

1.2.7 Outer membrane proteins (OMP). A pilin protein, a major OMP (MOMP), a D15 homolog and a protein involved in serum resistance have been identified in the outer membrane of *H. ducreyi*. A high-density fibrilla-like material was detected in many *H. ducreyi* strains examined by electron microscopy (EM) (Spinola *et al.* 1990; Frisk *et al.* 1995). A polypeptide encoded by a 570-bp open reading frame (ORF) termed *ftpA* was identical to the purified 24-kDa-protein monomer extracted from piliated cells (Spinola *et al.* 1990; Castellazzo *et al.* 1992; Brentjens *et al.* 1996). Although it has physical characteristics similar to those of other pili, FtpA lacks homology to any known pilin sequences and a cleavable signal sequence. However, it has homology to the Dps protein from *E. coli* and the TpF1/4D antigen from *Treponema pallidum*, which associate to form ordered rings and are similar to known or putative prokaryotic bacterioferritins (Brentjens

et al. 1996). While FtpA is expressed *in vivo* by *H. ducreyi*, a pilin inactivated mutant of *H. ducreyi* was as virulent as its parent in the human model of chancroid (Al-Tawfiq *et al.* 2000a; Bauer and Spinola 2000; Throm and Spinola 2001).

The most abundant protein of the *H. ducreyi* outer membrane is comprised of two proteins (37-39 kDa), members of the OmpA family of Gram-negative OMP encoded by the independently transcribed tandem genes *momp* and *ompA2* (Klesney-Tait *et al.* 1997). Although MOMP is 4 to 5 times more abundant than OmpA2 in the outer membrane of strain 35000, it is not required for pustule formation in human volunteers since a MOMP-deficient mutant formed pustules and was recovered from lesions at a rate similar to that of its parent (Spinola *et al.* 1993; Klesney-Tait *et al.* 1997; Throm *et al.* 2000). Transcripts for *ompA2* and *momp* were found to be expressed *in vivo* during infection of human volunteers (Bauer and Spinola 2000; Throm and Spinola 2001).

D15 was first identified as an immunogenic, surface-exposed and conserved 80-kDa OMP of *H. influenzae* (Thomas *et al.* 1990; Loosmore *et al.* 1997). Passive transfer of anti-rD15 antibodies protected infant rats from challenge with *H. influenzae* type b and type a in infant rat models of bacteremia (Thomas *et al.* 1990; Loosmore *et al.* 1997). An 87-kDa surface-exposed outer membrane antigen was later identified in *Pasteurella multocida*. Oma87 is encoded by a 2,372 nucleotide ORF and has 75% identity with *H. influenzae* type b D15 (Ruffolo and Adler 1996). In passive immunization experiments, an Oma87-specific antiserum protected mice against a homologous, lethal *P. multocida* challenge (Ruffolo and Adler 1996). *H. ducreyi* also expresses a conserved 85-kDa OMP, encoded by a 2,379-bp ORF, which is 65% and 62% similar at the amino acid

level to the D15 protein from *P. multocida* and *H. influenzae* type b, respectively (Thomas *et al.* 2001).

DsrA is a 30-kDa OMP required for expression of serum resistance in *H. ducreyi* since 3 naturally occurring, avirulent and serum-sensitive DsrA- strains were converted to a serum-resistance phenotype when complemented in *trans* with a plasmid expressing *dsrA* (Elkins *et al.* 2000b). Although it has previously been reported that truncation of LOS was responsible for serum susceptibility (Odumeru *et al.* 1985; Odumeru *et al.* 1987), expression of *dsrA* in serum-susceptible *H. ducreyi* strains conferred serum resistance without changing LOS composition, suggesting that LOS is not exclusively involved in this process (Elkins *et al.* 2000b; Sun *et al.* 2000). Papules caused by a DsrA-deficient mutant were significantly smaller than the parent strain, and did not progress to the pustular stage of disease (Bong *et al.* 2001)

1.2.8 Lipoproteins. Two lipoproteins have been identified in *H. ducreyi*. The 18-kDa surface-exposed, non-heat-modifiable conserved OMP of *H. ducreyi* is encoded by a 471-bp ORF designated *pal* (Spinola *et al.* 1992). PAL possesses a signal peptide characteristic of lipoproteins and has extensive homology to 2 peptidoglycan-associated proteins: *H. influenzae* P6 (62% identity and 71% similarity) and *E. coli* PAL (57% identity and 66% similarity), which is thought to have a role in stabilizing the outer membrane through its association to peptidoglycan (Mizuno 1979; Woodruff and Hancock 1989; Lazzaroni and Portalier 1992; De Mot and Vanderleyden 1994; Spinola *et al.* 1996a). Although PAL does not copurify with peptidoglycan in *H. ducreyi* (Spinola *et al.* 1992), suggesting an alternate function in bacterial survival, it is expressed *in vivo* in the human experimental model of chancroid (Bauer and Spinola 2000). PAL

facilitates the ability of *H. ducreyi* to progress to the pustular stage in the human experimental model of chancroid since the pustule formation rate, the rate of recovery and the papules of a PAL-negative mutant were reduced compared to those of the parent strain (Fortney *et al.* 2000). Additionally, the 28-kDa sarkosyl-insoluble lipoprotein is encoded by a 626-bp ORF designated *hlp* and is expressed in biopsies from human volunteers infected with *H. ducreyi* (Hiltke *et al.* 1996; Bauer and Spinola 2000).

1.2.9 Periplasmic proteins. A functional copper-zinc superoxide dismutase (CuZnSOD) and a zinc-binding homolog were identified in the periplasmic space of *H. ducreyi*. The CuZnSOD has 64% sequence identity to the SodC protein from *H. parainfluenzae* and is encoded by a 597-bp ORF (Langford *et al.* 1997; San Mateo *et al.* 1998). It may play a role in detoxifying superoxide from an exogenous source since the CuZnSOD null mutant strain was significantly more susceptible to killing by extracellular superoxide than the WT strain (San Mateo *et al.* 1998). This mutant was also recovered in fewer numbers from fewer ulcers than the WT parent strain in immunocompetent pigs, while both strains survived well in the skin of neutropenic animals, suggesting a potential role for this enzyme in the survival of *H. ducreyi* among neutrophils in chancroidal ulcers (San Mateo *et al.* 1999b).

Possibly functioning as the binding component of a zinc transporter system, the 32-kDa-protein ZnuA of *H. ducreyi* is 43% identical to the *E. coli* ZnuA/YebL zinc-binding protein and 54% identical to the *H. influenzae* (NTHI 6564) periplasmic-zinc-binding protein Pzpl (Lewis *et al.* 1999). A *H. ducreyi znuA* negative mutant grew more slowly than the WT parent strain *in vitro*, and exhibited significantly decreased virulence in the TDRM of chancroid (Lewis *et al.* 1999), compared to the WT strain.

1.2.10 Toxins. Both hemolytic and cytotoxic activities are elaborated by *H. ducreyi*. The heat- and protease-labile hemolytic activity is active against erythrocytes of horse origin and human foreskin fibroblasts (HFF) (Palmer *et al.* 1994). Its production is greatest in the late log phase of growth and at a pH of 7.0 and is enhanced by the addition of calcium (Totten *et al.* 1995). Highly conserved among *H. ducreyi* strains, hemolysin is encoded by 2 genes in *H. ducreyi*, *hhdA* and *hhdB*, which are expressed as a 125-kDa hemolysin and a 61-kDa protein, respectively (Palmer and Munson 1995). A *H. ducreyi* hemolysin mutant was unable to damage HFF, implying that hemolysin is responsible for the CPE observed on cultured HFF (Palmer *et al.* 1996; Alfa *et al.* 1996). However, there was no difference in the pustule formation rate and the cellular infiltrates of lesions between the hemolysin negative mutant and its parent in the human model of chancroid (Palmer *et al.* 1998a; Young *et al.* 2001). The *H. ducreyi* hemolysin was shown to lyse HFF, human foreskin epithelial cells, human blood mononuclear cells, macrophage-like cells, as well as T and B lymphocytes, but did not affect PMN (Wood *et al.* 1999). The hemolysin is expressed *in vivo* and is immunogenic since antibodies to this protein were found in animals experimentally infected with *H. ducreyi* and in patients with natural chancroid (Dutro *et al.* 1999; Throm and Spinola 2001). Immunization of rabbits with hemolysin did not affect development and severity of lesions in the TDRM, but did reduce the recovery of the WT organism (Dutro *et al.* 1999), but not hemolysin-negative mutants, from lesions.

Interestingly, products from 2 large *H. ducreyi* conserved ORFs, *lspA1* and *lspA2* (12,500 and 14,800 nt, respectively), had significant similarity to the N-terminal 1000 amino acids of HhdA and to other proteins (*Proteus Mirabilis* Hpma, HMW1A and

HMW2A adhesins produced by nontypeable *H. influenzae*), and were 43% similar to the N-terminal half of *Bordetella pertussis* FhaB (Ward *et al.* 1998). Transcripts of both *lspA1* and *lspA2* were detected in biopsies of human volunteers experimentally infected with *H. ducreyi* (Throm and Spinola 2001).

Cell injury and death was observed in HaCaT keratinocytes, Hep-2 and HeLa cells within 24 hours of incubation with *H. ducreyi*, (Abeck and Korting 1991; Abeck *et al.* 1991; Purvén and Lagergård 1992; Stevens *et al.* 1999). This heat- and pronase-cytotoxic activity was detected in approximately 89% of *H. ducreyi* strains from diverse geographical origins, is detected in culture supernatants of exponential growth of *H. ducreyi* in liquid culture, and is accumulated in the cytoplasm or the periplasmic space when the organism is grown on solid media (Purvén and Lagergård 1992; Purvén *et al.* 1995). Immunization of rabbits with live *H. ducreyi* or bacterial sonicates elicits toxin-neutralizing serum antibodies (Lagergård and Purvén 1993). This cytotoxin is encoded by the *cdtABC* gene cluster (Cope *et al.* 1997). CdtC has homology to the cytolethal distending toxin (CDT) expressed by a number of enteric pathogens, including *E. coli*, *Shigella* and *Campylobacter* species (Purvén *et al.* 1997; Cope *et al.* 1997). Although inactivation of the *cdtC* gene in *E. coli* eliminated its ability to kill HeLa cells and HaCaT keratinocytes, mutation of the gene in *H. ducreyi* did not affect its virulence in the TDRM (Stevens *et al.* 1999; Young *et al.* 2001). CDT inhibits the proliferation of peripheral blood mononuclear cells (PBMC), Jurkat T-cell and primary human T-cell lines, induces apoptosis in Jurkat T- and B-cell lines (Gelfanova *et al.* 1999), and affects the proliferation and biological activities of stimulated human T- and B-cells (Svensson *et al.* 2001). CDT may have a role in ulcer development by interfering with T-cell responses

and inducing cell cycle arrest and apoptosis via the DNA damage checkpoint pathways (Cortes-Bratti *et al.* 1999; Cortes-Bratti *et al.* 2001). Transcripts of *cdtB* were expressed *in vivo* (Throm and Spinola 2001).

1.2.11 Heat shock proteins (HSP). Newly synthesized proteins of approximately 14-, 58.5-, 74- and 78-kDa were identified in *H. ducreyi* during heat shock (Brown *et al.* 1993). The 58.5-kDa protein, the most abundant immunogenic protein produced during heat-shock, is encoded by a 1641-bp ORF and is a homolog of the GroEL HSP (Parsons *et al.* 1992). Lower levels of GroEL translate into a diminished ability to survive heat and oxidative stresses and a reduction in the number of *H. ducreyi* cells adherent to human cells (Parsons *et al.* 1997). This HSP may be associated with the bacterial cell surface and partially influence *H. ducreyi* adherence to Hep-2 cells (Frisk *et al.* 1998b). The 78-kDa DnaK homolog is expressed at temperatures of 30-35 °C (Parsons *et al.* 1992; Brown *et al.* 1993; Parsons *et al.* 1997), and the 14-kDa GroES homolog is encoded by a 288-bp ORF (Parsons *et al.* 1992). Stationary phase *H. ducreyi* highly express GroEL and GroES, averaging 5-fold greater than in *E. coli* (Parsons *et al.* 1997).

1.2.12 Pathogenesis. Although chancroid is invasive locally, systemic spread does not occur, no fatalities have been reported and extragenital lesions are uncommon (Albritton 1989; Trees and Morse 1995). *H. ducreyi* is a strict human pathogen thought to enter the skin of the genitalia through an abrasion in the mucosal epithelium during sexual intercourse (Morse 1989; Abeck and Korting 1991; Hobbs *et al.* 1998). However, the precise sequence of events leading to lesion formation is largely unknown, though several observations appear relevant. For instance, *H. ducreyi* can bind components of the extracellular matrix (ECM), such as fibrinogen, fibronectin, collagen, gelatin and laminin

(Abeck *et al.* 1992; Bauer and Spinola 1999). However, *H. ducreyi* was found to colocalize only with collagen and fibrin, and not laminin or fibronectin, in pustules from an experimental human infection (Bauer *et al.* 2001). Pili and full-length LOS do not seem to be required for this activity, since *fipA* and *losB* mutants bound to the ECM as well as the parent strains (Alfa and DeGagne 1997; Frisk *et al.* 1998b; Bauer and Spinola 1999). Purified *H. ducreyi* LOS, but not LPS from *E. coli* and *H. influenzae* LOS, caused abscesses similar to those caused by live and heat-killed organisms when injected intradermally into the skin of a rabbit (Campagnari *et al.* 1991). Furthermore, purified *H. ducreyi* LOS was a potent inducer of IL-8 in a coculture of HaCat keratinocytes and foreskin fibroblasts (Zaretsky and Kawula 1999). Taken together, these observations suggest that LOS may be involved in ulcer formation and in the migration of neutrophils to the ulcer site. *H. ducreyi* may also be transmissible before ulceration since it was recovered from papular and pustular lesions, and may replicate between papular and pustular stages of diseases in the human experimental model of chancroid (Spinola *et al.* 1996b; Al-Tawfiq *et al.* 2000b).

H. ducreyi is seen extracellularly more often than intracellularly, in proximity to PMN (Joseph and Rosen 1994; Bauer and Spinola 2000; Bauer *et al.* 2001). *In vitro*, *H. ducreyi* organisms attach to cultured HFF and keratinocytes, but are rarely internalized by these cells (Alfa 1992; Alfa *et al.* 1993; Brentjens *et al.* 1994; Alfa *et al.* 1995). *In vivo*, *H. ducreyi* was visible in the dermis amidst necrotic host cells and debris, but was not associated with keratinocytes, fibroblasts, or T and Langerhans cells (San Mateo *et al.* 1999a; Bauer and Spinola 2000; Bauer *et al.* 2001). In fact, only cells of epithelial origin

internalized cell-associated heat-killed and live *H. ducreyi* organisms in *in vitro* experiments (Shah *et al.* 1992; Totten *et al.* 1994a).

1.2.13 Immune response to *H. ducreyi* infection. Although there is evidence of both a humoral and a cell-mediated response mounted against a *H. ducreyi* infection, it is non-protective since reinfection is common, and infection following repeated or serial autoinoculations were observed in early human studies (Albritton 1989; Trees and Morse 1995; Al-Tawfiq *et al.* 1999). However, there may be control of bacterial replication by the host immune system since there are a greater number of organisms in neutropenic pigs than in immunocompetent swine (San Mateo *et al.* 1999b). However, the immune response to *H. ducreyi* may play a role in ulcer development since pustules did not form and surface epithelia remained intact in neutropenic animals (San Mateo *et al.* 1999b), although histopathology of chancroidal lesions is identical between HIV+ and HIV- patients (King *et al.* 1998).

1.2.13.1 Cell-mediated response. *H. ducreyi* infection is characterized by the presence of CD4+ and CD8+ T lymphocytes, macrophages and neutrophils at the ulcer site, in natural and in all experimental models of chancroid (Spinola *et al.* 1994; Hobbs *et al.* 1995; Desjardins *et al.* 1995; Desjardins *et al.* 1996; Magro *et al.* 1996; King *et al.* 1996; King *et al.* 1998; Palmer *et al.* 1998b; Spinola *et al.* 1996b). A preparation of *H. ducreyi* antigens induced the production of IL-2 in cultured lymphocytes (Abeck and Korting 1991). Infection with live *H. ducreyi* and LOS elicited an increased secretion of IL-6 and IL-8 in a model of artificial skin composed of foreskin fibroblasts and keratinocytes as well as in a coculture of HaCaT keratinocytes and foreskin fibroblasts (Hobbs *et al.* 1998; Zaretsky and Kawula 1999). Since both IL-8 and IL-6 are involved

in chemoattraction and mobilization of neutrophils, these results are consistent with the presence of neutrophils in chancroidal ulcers.

1.2.13.2 Humoral response. Passive immunization with polyclonal IgG purified from immune sera to *H. ducreyi* 35000 whole-cell lysate or to a pilus preparation did not protect animals in the TDRM of chancroid (Desjardins *et al.* 1996). High titers of IgG antibodies to the 24-kDa protein, the fine-tangled pili of *H. ducreyi*, and cytotoxin neutralizing antibodies were found in 66% of patients with chancroid, compared to only 4% of uninfected donors (Frisk *et al.* 1995; Purvén *et al.* 1997). Rabbit and human antisera containing antibodies specific to the pili and LOS did not enhance killing of *H. ducreyi* (Frisk *et al.* 1998a). Taken together, these data suggest that although a humoral response is readily obtained after *H. ducreyi* infection, it is not protective.

1.2.14 Typing systems. Although several attempts have been made at developing typing methods to classify and organize strains of *H. ducreyi*, none have yet been adopted. Bacterial components such as OMP, enzymes and lectins, *H. ducreyi*-specific antibodies and molecular-based methods have been investigated as potential typing systems.

1.2.14.1 Structural methods. Within a molecular range of 25 to 50 kDa, 7 OMP patterns were found among 105 *H. ducreyi* isolates (Odumeru *et al.* 1983). Although storage, age of culture and serial passage did not affect OMP patterns, differences between patterns were difficult to distinguish and not very discriminative since half of the isolates belonged to a single subtype (Odumeru *et al.* 1983). Bacterial enzymes were also investigated as the basis of a typing system (Van Dyck and Piot 1987). Unfortunately, *H. ducreyi* expresses few enzymes and their expression may be dependent on the type of media used in culture. Twenty different lectin patterns were found among 43 isolates

(Korting *et al.* 1988). However, lectin agglutination may be misleading since *H. ducreyi* naturally tends to form clumps when grown in broth cultures and on solid media.

1.2.14.2.Immunological methods. Another means of categorizing *H. ducreyi* strains are systems that use *H. ducreyi*-specific antibodies (Finn *et al.* 1990; Roggen *et al.* 1992; Roggen *et al.* 1993). One of the drawbacks of using immunological methods for classification purposes is the extensive cross-reactivity exhibited by *H. ducreyi* antisera to many other gram-negative organisms, especially those in the family *Pasteurellaceae* (Denys *et al.* 1978; Roggen *et al.* 1993). Seven different immunopatterns were identified in 63 *H. ducreyi* isolates using a pool of 238 well-characterized human antisera, and 8 different immunotypes with a rabbit polyclonal antiserum to antigens isolated in SDS-PAGE (Roggen *et al.* 1992; Roggen *et al.* 1993). Although the rabbit antisera had a sensitivity and specificity of 100% in an immunosorbent assay (EIA), both antisera had to be extensively adsorbed with *Haemophilus* species, *E. coli*, *Actinobacillus* and *Pasteurella* species.

1.2.14.3.Molecular methods. DNA-based typing methods have become popular for classifying bacterial cells (Sarafian *et al.* 1991a; Sarafian *et al.* 1991b; Brown and Ison 1993; Pillay *et al.* 1996). Plasmid analysis may be discriminative enough (7 different plasmids were found in 29 isolates) to serve as the basis of a classification system for *H. ducreyi* strains, but mobility between strains makes them unstable characteristics (Sarafian *et al.* 1991a). Ribotyping was also proposed as a typing system. Although 4 Hinc II and 8 Hind II ribotypes were found among 44 *H. ducreyi* isolates, and 9 BglII, 8 Hind III and 5 BstE II were found in 30 strains, the majority of isolates

clustered into one pattern (Sarafian *et al.* 1991b; Pillay *et al.* 1996). This may be linked to the clonal nature of *H. ducreyi*.

1.3 VACCINES

1.3.1 Types of vaccines. A vaccine is any preparation intended for active immunological prophylaxis (Powell and Newman 1995). Live attenuated, inactivated and subunit are types of vaccines currently used to protect against infectious diseases. The live attenuated vaccine contains infectious agents whose virulence is attenuated either due to gene mutation or passage of the microorganism in an animal or in cell culture. Although active replication of the organism permits the stimulation of an immune response closely resembling that of the illness without causing a full-blown disease, potentially harmful infection may be established in immunocompromised individuals receiving the vaccine or with reversion of a mutant to a virulent form. The advent of inactivated vaccines tried to solve this problem by using organisms unable to replicate. However, heat or chemical treatments used to kill the organisms may result in antigens with three-dimensional conformation different than that of the live organism in natural infection, which may not allow the protective immune response to develop. A drawback of both live attenuated and inactivated vaccines is that they may contain antigens, such as LPS, which may non-specifically activate the immune system and be harmful to the vaccinee. Subunit vaccines contain a specific element of the disease-causing agent, like a protein, which does not produce such activation. Since this type of vaccine targets a specific immune response, it may reduce side effects seen with other “whole” vaccines.

Novel types of vaccines, such as plasmid delivery vehicles and protein-expressing bacteria and viruses, are currently being examined to develop specific immune responses.

The DNA vaccine, a plasmid that encodes the gene of a virulence factor, can be injected in the muscles of the patient, where it replicates for an extended period of time and stimulates the immune system. An avirulent infectious agent expressing a foreign protein can also be used as a live carrier vaccine to target a specific organ and to stimulate a specific immune response. This protein-expressing agent replicates at the site of infection, but does not cause disease. Although this system is similar to live attenuated vaccines, the threat of disease development is theoretically low since the replicating organism is avirulent.

1.3.2 Adjuvants. An adjuvant is a substance added to a vaccine to enhance its antigenicity (Powell and Newman 1995). Adjuvants are becoming more important in vaccine development, since they may help develop a particular immune response. First described in 1926, alum is the only adjuvant used in human vaccines, although many others are being developed and tested in human clinical trials (reviewed in Powell and Newman 1995). Alum refers to the aluminium phosphate salts onto which the vaccine component is adsorbed. This adjuvant stimulates the production of IL-4 from T-helper-2 (Th2) subsets in mice, and enhances the production of IgG1 and IgE (Powell and Newman 1995).

In this study, 2 different adjuvants were used: Freund's adjuvant and Monophosphoryl lipid A (MPL®) produced by Ribi Immunochem. Freund's is a mixture of an emulsifier (Arlacel A – 15%) and mineral oil (85%) in its incomplete form (FIA), while the complete formulation (FCA) also contains 500 µg of heat-killed and dried *Mycobacterium tuberculosis* per ml of emulsifier mixture. Although Freund's adjuvant stimulates a strong cell-based immunity, formation of granulomas and abscesses at the

site of injection, and the development of arthritis, amyloidosis, and allergic reactions precludes its use in humans. On the other hand, MPL® is an adjuvant derived from the LPS of *Salmonella minnesota* strain R595 and is composed of a series of 4'-monophosphoryl lipid A species that vary in the extent and position of fatty acid substitution (Powell and Newman 1995). MPL® has been shown to stimulate the production of interferon- γ (IFN- γ) and IL-2 in cultured monocytes and macrophages and was shown to be safe, well tolerated and to provide a heightened immune response to coadministered antigens in human clinical trials (Powell and Newman 1995).

1.3.3 A vaccine against chancroid. Cell envelopes, pili, LOS, and HhdA have been investigated as vaccine candidates in the TDRM of chancroid (Hansen *et al.* 1994; Desjardins *et al.* 1995; Dutro *et al.* 1999). Immunization of rabbits with strain 35000 cell envelopes provided protection only against infection with a homologous strain, while vaccination of cell envelope from strain Cha-1 provided homologous as well as heterologous protection (Hansen *et al.* 1994). Immunized rabbits had high titers of antibodies, a DTH reaction 24 to 48 hours after infectious challenge, and the cellular infiltrates of lesions from immunized rabbits experimentally infected consisted mainly of lymphocytes and histiocytes (Hansen *et al.* 1994). The pilus preparation from strain 35000 induced homologous and heterologous strain protection in the animal model and an immune response similar to that obtained with cell envelopes. Although the LOS vaccine provided no protection against experimental animal chancroid, HhdA succeeded in reducing the recovery of organisms (Desjardins *et al.* 1995; Dutro *et al.* 1999).

1.4 Rationale and hypotheses:

1.4.1 Electrophoretic, serologic and structural heterogeneity of the *H. ducreyi* LOS.

Although many kinds of typing schemes have been investigated as the basis of a classification system for *H. ducreyi* strains, one has yet to be adopted as a standard. Many reports have suggested that there is structural heterogeneity among the LOS molecule of *H. ducreyi*. However, it has not been systematically studied among a diverse group of strains. Therefore, I hypothesized that *H. ducreyi* LOS has heterogeneity based on structural differences, which may be resolved in specific electrophoretic profiles and serologic reactions. This measured variation may lead to a basis for epidemiological application of a typing system.

1.4.2 Vaccine development against chancroid. Since chancroid is a risk factor in the heterosexual transmission of HIV, a vaccine against chancroid may be a way of reducing the transmission of HIV in endemic areas by reducing the incidence and prevalence of this STD. Two outer membrane proteins were chosen as vaccine candidates: the hemoglobin receptor of *H. ducreyi*, HgbA, and D15. HgbA was chosen since it is conserved among strains of *H. ducreyi*, and an Hg receptor mutant had reduced virulence in the TDRM and the human model of chancroid, suggesting that this antigen may be involved as a virulence factor in ulcer formation. Although not much is known about the function that the D15 antigen plays in the pathogenesis of chancroid, passive transfer of anti-rD15/anti-Oma87 antibodies protected animals against a lethal challenge. I hypothesized that HgbA and D15, are potential vaccine candidates against chancroid and this would be demonstrable in the TDRM.

1.5 Objectives.

1.5.1 Electrophoretic, serologic and antigenic heterogeneity of *H. ducreyi* LOS.

- 1. Investigate the SDS-PAGE heterogeneity of LOS in the OMP preparations of 91 clinical isolates.**
- 2. Investigate and correlate the serologic diversity of the LOS of *H. ducreyi* type-strains using rabbit antisera raised against OMP, in Western blotting of purified LOS and in purified LOS-based EIA.**
- 3. Investigate the structural heterogeneity of purified LOS from chosen *H. ducreyi* strains, representative of SDS-PAGE groups, using carbohydrate (CHO) and mass spectrometry analyses.**

1.5.2 Vaccine development against chancroid.

- 1. Purify nHgbA, rHgbA and rD15 by affinity chromatography.**
- 2. Investigate the humoral response in HgbA- and rD15- vaccinated animals using OMP- and purified protein-based EIA and screening Western blots.**
- 3. Investigate the vaccine potential of nHgbA, rHgbA and rD15 against homologous and heterologous challenges in the TDRM of chancroid.**

CHAPTER 2 - MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions. The *H. ducreyi* strains studied (table 2) are clinical isolates (total = 91) of ulcer and bubo origin stored in 2% skim milk and 20% glycerol (EM Science, Gibbstwon, NJ) at – 80 °C. They were grown on enriched chocolate agar plates (CAP) composed of GC agar base (Difco, Sparks, MD), 1% (w/v) bovine hemoglobin (Hg) (BBL/Becton Dickinson Microbiology Systems, Cockeysville, MD), and 1% Isovitalex (BBL/Becton Dickinson Microbiology Systems, Cockeysville, MD). All *H. ducreyi* isolates grown on CAP were incubated for 24 to 96 hours at a temperature of 33 °C in a high humidity environment supplied with 5% CO₂. For native HgbA (nHgbA) extraction, strain 35000 was grown in low-heme GC broth (15 mg/l of Hg), which consisted of GC broth (15 g/l of proteose peptone (Difco, Sparks, MD), 23 mM potassium phosphate dibasic (Fisher Scientific, Fair Lawn, NJ), 7.3 mM potassium phosphate monobasic (Fisher Scientific, Fair Lawn, NJ), and 86 mM sodium chloride (BDH, Toronto, ON)), 1.5% low-Hg Fetal Bovine Serum (FBS) (Wisent, St-Bruno, QC – hemoglobin concentration = 8 to 11 mg/dl), 1% Isovitalex, and 2 mg/l of Vancomycin (Sigma, St-Louis, MI). Isolates cultured in low-heme GC broth were incubated 22 hours at 33.5 °C in 5% CO₂ in 1 liter Fernback flasks (Fisher Scientific, Fair Lawn, NJ). Strains used in the TDRM of chancroid used isolates grown in Mueller-Hinton (M-H) + FBS (M-H+FBS) broth, consisting of 42% M-H broth (BBL/Becton Dickinson & Co., Cockeysville, MD), 42% α -MEM (Gibco, Grand Island, NY) and 16% FBS (Gibco, Grand Island, NY) at a temperature of 33 °C for 12.5 hours. Strain AX557 was grown in GC broth supplemented with 1% Isovitalex and 5% FBS (Gibco, Grand Island, NY).

TABLE 2. List of *H. ducreyi* strains studied in this thesis. The strains are grouped according to their place and time of isolation.

<i>Country of isolation</i>	<i>STRAIN =</i>
Winnipeg, Canada	35000
Nairobi, Kenya (1981)	AX557, C148, BG411
Nairobi, Kenya (1986-1987)	G24896, G29677, G29761, H40529, HG0890, HG0894, J910, J1158, J1159 , J1321, K1143, K3367, K3588, K4163, K4167, K4894, K4913, K6456, K6757, K6856, K10124, K10159, K10161, KID661, KF128, KF133, KF140, KF142, KF143, PPC184, PPC193, PPC221, PPC236, PPC248/ulcer, PPC248/bubo, PPC263/1293 , PPC267, PPC275, PPC281, PPC285, PPC287, PPC295/1300, PPC307, PPC312, PPC316, PPC318, PPC322, PPC323, PPC333, PPC335, PPC337, PPC338/1312, PPC338/1313, PPC342, PPC352, PPC358/1315 , PPC415, PPC418, RO-01, RO-02, RO-03, RO-08, RO-12, RO-20/1345, RO-21, RO-22, RO-23, RO-24, RO-27a, RO-27b, RO-30, RO-34, RO-35, RO-38, RO-40, RO-42, RO-45, RO-48, RO-49, RO-51, RO-53/1367, RO-53/1368, V-J4658, VG770, Z115
Seattle, U.S.A.	V1157
Pasteur Institute Paris, France	36-F-2

Escherichia coli strain BL21 (DE3), pUNCH 672, pLysS, (A672-10) containing the plasmid pUNCH 672 encoding *H. ducreyi* HgbA, kindly donated by Christopher Elkins of the University of North Carolina in Chapel Hill (Elkins *et al.* 2000a), was grown in Luria-Bertani (LB) broth (Difco, Sparks, MD) supplemented with the following antibiotics: 20 µg/ml of chloramphenicol and 30 µg/ml of kanamycin (both from Sigma, St-Louis, MI).

2.2 Animals. A total of 112 male New Zealand White (NZW) rabbits purchased from Charles River Canada (St-Constant, QC) were housed in an 11.7-m² room in individual cages at the animal care facility of the University of Ottawa. Animals used for the production of antiserum were housed at ambient temperature (23 °C), while those used for the TDRM of chancroid (protocol number MI-89) were kept at an ambient temperature of 15 ± 1°C with a Thermo Air Plus conditioning unit and forced air circulation with a ducted fan. Rabbits for all experiments were age and weight matched from acquisition and housed under identical conditions for the duration of experiments.

2.3 Outer membrane proteins (OMP) preparations. Using a sterile wooden stick, 10 small (15mm x 100mm) CAP were inoculated with bacteria from frozen stocks and left to grow for 48 hours. The bacterial lawns were harvested with a cotton swab in 0.01 M hydroxymethyl methylammonium chloride (Tris-HCl) (BDH, Toronto, ON) pH 7.0, centrifuged at 3000 X g for 10 minutes, and the pellet suspended in 10 mM N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0 (Sigma, St-Louis, MI). The suspension was thereafter sonicated on ice (6 rounds: 30 seconds sonication followed by a 15 second break) using a sonifier/cell disrupter (Branson Sonic Power Co.). The sonicated product was centrifuged at 10 000 X g (RC-70 centrifuge, Sorval,

DuPont, Mississauga, ON) for 20 minutes. The pellet was discarded and the supernatant was centrifuged a second time at 100 000 X g for one hour at 4 °C. The resulting pellet was suspended in 1% N-lauroyl-sarcosine (Sarkosyl) (Sigma, St-Louis, MI) in 10 mM HEPES, and was incubated at room temperature (RT) for 45 minutes before being centrifuged at 100 000 X g for 1 hour at 4°C. The pellet, which contains the OMP, was suspended in 100 µl of phosphate-buffered saline (PBS) (10 mM Na₂HPO₄ (BDH, Toronto, ON), 10 mM NaH₂PO₄ (BDH, Toronto, ON) and 0.15 M NaCl (BDH, Toronto, ON)). Protein concentration of OMP preparations was determined using either the BIO-RAD protein assay kit (BIO-RAD, Hercules, CA) or the BCA protein assay reagent (Pierce, Rockford, IL).

2.4 Lipooligosaccharide (LOS) extraction. LOS from 48-hour plate cultures were isolated by the hot phenol extraction method of Westphal and Jann (Westphal and Jann 1965). The crude extract contained in the water phase of the hot phenol extraction, which contains LOS as well as carbohydrates (CHO), was dialyzed against distilled water and lyophilized. The lyophilized product was suspended in 20 ml of ddH₂O and washed 3 times (105 000 X g ultracentrifugation for 3 hours at 4 °C). The purified extract was lyophilized and kept at RT. The samples were visualized on a gel (SDS-PAGE) containing 17 % acrylamide and 2.5 % urea (BDH, Toronto, ON) and stained with the BIO-RAD silver staining kit (BIO-RAD, Hercules, CA) or the Tsai and Frasch silver staining method (Tsai and Frasch 1982) to assess purity.

2.5 Neuraminidase treatment of *H. ducreyi* LOS. Purified LOS were treated with neuraminidase as previously described (Mandrell *et al.* 1992). Briefly, purified LOS were suspended in 50 µl of sterile ddH₂O at a concentration of 2 mg/ml. Neuraminidase

type V from *Clostridium perfringens* (Sigma, St-Louis, MI) was diluted at a concentration of 2 mg/ml in sterile ddH₂O. Twenty-five microliters of the LOS suspension were mixed with 25 µl of the neuraminidase suspension and incubated at 37 °C for 2 hours. The reaction was stopped with 50 µl of Laemmli sample buffer (10% glycerol, 5% 2-βMercaptoethanol (BDH, Toronto, ON), sodium dodecyl sulfate (SDS) (ICN, Aurora, OH), hydroxymethyl methylammonium (Tris) (BDH, Toronto, ON), 0.25% bromophenol blue (Fisher Scientific, Fair Lawn, NJ)). The neuraminidase-treated LOS were stored at – 70 °C.

2. 6 Native HgbA (nHgbA) – *H. ducreyi* Hg receptor - extraction. One-liter of low heme-GC broth was inoculated with 2.4×10^6 CFU of *H. ducreyi* strain 35000, and incubated 22 hours at 33 °C in 5% CO₂. The bacterial cells were centrifuged at 10 000 X g for 20 minutes and the pellets were suspended in 10 mM HEPES before being frozen overnight (O/N) at – 20 °C with 1 mg/ml of lysozyme (Sigma, St-Louis, MI). Each nHgbA extraction was performed on a pool of 16 individually frozen pellets.

Pellets were thawed, 2 at a time, at RT and subjected to 2 rounds of French press (French Pressure cell press, American Instrument Co.) before being centrifuged at 10 000 X g for 20 minutes at 4 °C. The pellet was frozen and identified as “1st pellet”, while the supernatant was centrifuged at 100 000 X g for 1 hour at 4 °C. This second pellet was frozen and labeled “crude membrane”. Either pooled pellet may be used as starting material for the nHgbA extraction. However, cleaner extraction products have always been obtained using the “1st pellets”. Thus all of the nHgbA extracts used for vaccine purposes were obtained from the “1st pellet” preparation.

The pooled pellets were suspended in ice cold 1% Sarkosyl in 10 mM HEPES using a 10 ml syringe fitted with a blunted needle and rocked at 37 °C for 30 minutes. The suspension was centrifuged at 100 000 X g for 1 hour at 4 °C. The supernatant was discarded and the pellet re-suspended and re-extracted for 30 minutes at 37 °C with ice cold 1% Sarkosyl in 10 mM HEPES, then centrifuged at 100 000 X g for 1 hour. The pellet was diluted to approximately 1 mg/ml in sterile ddH₂O. An equal volume of 2% Zwittergent 3,14 (Calbiochem, San Diego, CA) in Tris-based saline ethylene-diamine-tetraacetic Acid (TBS-EDTA) (50 mM Tris, 5 mM EDTA (BDH, Toronto, ON) and 150 mM NaCl) was added to the pellet and the suspension was incubated at 37 °C for an hour. After the incubation, an equal volume of TBS-EDTA was added to the extract, which was transferred to cool Oak Ridge tubes (Nalgene, Rochester, NY) and centrifuged for 15 minutes at 8000 X g. The supernatant was mixed, at a ratio of 0.5 ml of Hg-agarose slurry per liter of culture, with the pre-washed agarose-beads (washed twice in sterile PBS (Sigma, St-Louis, MI), and rocked O/N at 4 °C. The slurry was pelleted at 1500 X g for 5 minutes, the supernatant discarded and the beads transferred to a column (Econo columns, BIO-RAD, Hercules, CA). The agarose containing the ligand-receptor complex was washed with 20 bed-volumes of chilled 0.5% Zwittergent 3,14 in PBS followed by 10 bed-volumes of chilled 1% n-octyl- β -D-glucopyranoside (OG) (Calbiochem, San Diego, CA) in PBS. Purified HgbA was eluted with 3 bed volumes of chilled 1% OG in 1.5% (0.1 M) glycine (ICN, Aurora, OH) pH 2.3, and immediately neutralized with 2 M Tris pH 8.0. The eluant was concentrated at 4 °C to a volume of 1 ml in a Centriprep 50 (Amicon, Beverly, Massachusetts). HgbA preparation was suspended in 1% OG in PBS and concentrated a second time to exchange buffers. To recover residual HgbA bound to

the membrane, 500 μ l of 1%OG in PBS was added, vortexed and the contents were pooled with previous material. The Centricon was then vortexed to free the protein from the membrane and the content was pooled with the purified protein. The protein content of the extract was determined using the BCA protein assay kit.

2.7 Recombinant HgbA (rHgbA) extraction. rHgbA, which is expressed as a fusion protein containing a hexahistidine leader, was purified under denaturing conditions and re-natured by dialysis as previously described (Elkins *et al.* 2000a). The *E. coli* strain A672-10 was grown in 2 liters of LB broth supplemented with 20 μ g/ml of chloramphenicol and 30 μ g/ml of kanamycin at 37 °C until the optical density (OD) at 600 nm of the culture reached 0.4 to 0.6. Two millimolar of isopropyl- β -D-thiogalactoside (IPTG) (Sigma, St-Louis, MI) was added to the culture and incubated for 30 minutes. Then, 0.2 mM of Rifampicin (Sigma, St-Louis, MI) was added to the culture and incubated another 2 hours at 37 °C. The bacterial cells were harvested by centrifugation (10 000 X g for 20 minutes) and collected in a pre-weighed 50 ml centrifuge tube, suspended in cell disruption buffer (3 ml Tris-EDTA-NaCl (TEN) buffer (5 mM Tris, 1mM EDTA and 100 mM NaCl) per gram of pellet weight, 0.8mg/gram of pellet weight of lysozyme stock, 4 mg of deoxycholate (Sigma, St-Louis, Missouri) per gram of pellet weight and 8 μ l of 0.1 M Phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma, St-Louis, MI)) and frozen O/N at – 20 °C. The bacterial pellet was thawed at RT, French pressed 3 times, and then centrifuged for 20 minutes at 10 000 X g. The pellet was rinsed twice and washed 4 times in binding buffer (BB) without urea (Novagen, Madison, WI – 40 mM Imidazole, 4 M NaCl and 160 mM Tris-HCl, pH 7.9). The inclusion bodies were suspended in 6 M-urea-BB, incubated at 4 °C for 60 minutes to solubilize the contents of

the inclusion bodies, then centrifuged for 30 minutes at 50 000 X g. The supernatant was loaded onto a nickel column (Novagen, Madison, WI), washed with 100 ml of 6M-urea-BB, eluted with elution buffer (Novagen, Madison, WI – 4 M imidazole, 2 M NaCl and 80 mM Tris-HCl, pH 7.9), collected in dialysis tubes (Tube-o-Dialyzers, MWCO 50 000 kDa, RPI, Mount Prospect, IL) and dialyzed O/N against 4M urea in PBS. Zwittergent 3,14 was added to the content of the dialysis tubes (0.35g of Zwittergent 3,14 per 7 ml of eluant) and dialysed over several days against PBS-EDTA containing decreasing amount of urea. Finally, the tubes were left to dialyze O/N against PBS-EDTA and stored in microtubes at – 20 °C.

2.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

OMP, purified LOS and protein preparations (nHgbA, rHgbA and rD15 preparations) were analyzed by SDS-PAGE using the BIO-RAD mini-protean II system (BIO-RAD, Hercules, CA). Ten micrograms (per lane) of OMP and five micrograms (per lane) of protein preparations were subjected to electrophoresis on 10% acrylamide gels at 200 volts. Purified LOS (1µg/lane – dry weight) were visualized on a 17% acrylamide/2.5 % urea SDS-PAGE at 15 mA per gel.

2.9 Gel staining. The OMP and proteins preparations were stained with a modified Coomassie Blue and Bismarck Brown stain (Choi *et al.* 1996). The LOS (figures 2A, 2B, 3, 5 and 14C) were silver stained using either the BIO-RAD silver staining kit according to the manufacturer's procedure or the more sensitive method of Tsai and Frasch (Tsai and Frasch 1982).

2.10 Western Blot. Purified LOS preparations were subjected to electrophoresis and transferred at 100 volts for 1 hour onto a nitrocellulose membrane (Amersham,

Buckinghamshire, England) using the Protean II blotting apparatus from BIO-RAD (BIO-RAD, Hercules, CA). The membrane was blocked with 2% skim milk in PBS for 1 hour at 37 °C and incubated at 37 °C for 2 hours with an anti-OMP antiserum (described below) at a dilution of 1:800. The membrane was washed 5 times for 5 minutes in 0.1% Tween 20 (BIO-RAD, Hercules, California) in PBS then incubated for 1 hour at 37 °C with a goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (BIOsource, Camarillo, CA) at a dilution of 1:2000. The membrane was washed 5 times for 5 minutes in PBS alone. Substrate for the horseradish peroxidase enzyme is 3-AEC (5.7 mM 3-amino-9-ethyl carbazole (3-AEC) (Sigma, St-Louis, MI), 0.035 M sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2$) (BDH, Toronto, ON), 30% N,N-dimethylformamide (BDH, Toronto, ON), pH 5.0). The membrane was washed 3 times for 5 minutes in ddH₂O to stop the reaction.

2.11 Screening Western blot. OMP preparations were electrophoresed and transferred onto a nitrocellulose membrane at 200 volts for 2 hours. The membrane was blocked with 2% skim milk in PBS for 1 hour at 37 °C, and placed into a Multi-screen apparatus (BIO-RAD, Hercules, CA). Two hundred microliters of an rHgbA, nHgbA or rD15 antiserum, dilution at 1:1000 in 1% skim milk in PBS, was placed into each slot and the membrane was rocked at RT for 1 hour. The membrane was washed twice with 400 µl of 0.2% Tween 20 in PBS directly in the slots of the Multi-screen apparatus, then incubated at RT for 1 hour with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody at a dilution of 1:2000. The membrane was removed from the multi-screen apparatus, washed twice in PBS alone and stained with 3AEC as described above.

2.12 LOS-based EIA. Serologic response to immunization with OMP was measured using an Enzyme-linked Immunosorbent Assay (EIA) as previously described (Desjardins *et al.* 1995).

2.13 Protein preparations (nHgbA, rHgbA, rD15)- and OMP-based EIA. Antigens (protein preparations or OMP preparations) diluted in 0.1 M carbonate buffer pH 9.5 (3.75 mM Na₂CO₃ (BDH, Toronto, ON) and 8.72 mM of NaHCO₃ (BDH, Toronto, ON)) at a concentration of 2 µg/ml were added, 100 µl per well, to ProBind 96 well plates (Falcon, Franklin Lakes, NJ). The plates were incubated O/N at RT, then washed with PBS with a plate washer (BIO-RAD, Hercules, CA) and blocked O/N with 2% BSA in PBS. Serum samples were added to the plates in serial twofold dilutions starting from 1:250 and incubated 1 hour at RT. The plates were washed 3 times in 0.05% Tween 80 in PBS. Horseradish peroxidase conjugated goat anti-rabbit secondary antibody was added to the plates at a 1:2000 dilution, incubated for 1 hour at RT, and then washed 5 times. One hundred microliter of the developing buffer (0.36 mM 2,2'-azino-di-[3-ethyl-benzthiazolinsulfonat(6)] (ABTS, Boehringer Mannheim, West Germany) and 0.03% H₂O₂ (Sigma, St-Louis, MI) dissolved in citrate buffer (0.1 M citric acid (BDH, Toronto, Ontario) and 0.02 M sodium phosphate (Na₂HPO₄)) was added to each well, the plates were incubated for 25 minutes at RT and read at 405 nm using the DMS 200 spectrophotometer (Varian).

2.14 Sugar analysis of LOS. Hydrolysis of LOS with trifluoroacetic acid as well as the sugar analysis of the hydrolyzed product was done by Adèle Martin of the National Research Council of Canada (NRC) in Ottawa, Canada. Two hundred micrograms of purified LOS was hydrolyzed in 0.3 to 0.5 ml of 2 M TFA (BDH, Toronto, ON) at 125 °C

for 1 hour. The solution was cooled at RT (final temperature of 23°C) and then evaporated with a stream of compressed nitrogen (Air Products, Brampton, ON) at a temperature of 50 °C for 30 to 45 minutes. One milliliter of isopropanol (BDH, Toronto, ON) was added to the tubes and left to dry at RT for 5 minutes. The reduction and acetylation of the glycoside mixtures was carried out as previously described (Sawardeker *et al.* 1965). The oligosaccharide portion of the LOS was purified using a chloroform extraction. Briefly, 0.3 ml of chloroform (BDH, Toronto, ON) and 0.3 ml of water was added to the dried product, the mixture was stirred and the aqueous phase removed. This was repeated, the chloroform phase was dried and the product was suspended in 30 µl of chloroform. Sugar analysis on the samples was carried out by gas phase chromatography (HP5890 Series II Gas Chromatograph).

2.15 Deacylation of *H. ducreyi* LOS (for mass spectrometry analysis). Adèle Martin performed the hydrazinolysis of LOS at the NRC in Ottawa, Canada according to the procedure of Holst (Holst *et al.* 1991; Masoud *et al.* 1994). Briefly, 100 µg of purified LOS was hydrolyzed in 0.2 ml of 98% anhydrous hydrazine (Aldrich Chemical Co., Milwaukee, WI) for 1 hour at 37 °C with constant stirring to release O-linked fatty acids. The solution was cooled on ice and 0.6 ml of acetone was added to stop the reaction, then centrifuged for 25 minutes at 13 000 X g. The pellet was washed 3 times in 0.6 ml of acetone, then suspended in 80% acetone, and centrifuged a last time for 45 minutes at 13 000 X g. The pellet was lyophilized O/N and suspended in 100 µl of water for mass spectrometry analysis. Pierre Thibault performed and analysed the results from the capillary electrophoresis-electrospray mass spectrometry (CE-ESMS) at the NRC in

Ottawa, Canada as previously described (Auriola *et al.* 1996; Thibault *et al.* 1999) using a triple quadrupole PE/Sciex API 3000 mass spectrometer (PE/Sciex, Concord, ON).

A crystal model 310 CE instrument (AYI Unicam, Boston, MA, USA) was coupled to an API 3000 mass spectrometer (Perkin-Elmer\Sciex, Concord, Canada) via a microionspray interface. A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 $\mu\text{l}/\text{min}$ to a low dead volume tee (250 μm i.d., Chromatographic Specialities, Brockville, Canada). Fused-silica capillaries with 192 μm o.d. X 50 μm i.d. were obtained from Polymicro Technologies (Phoenix, AZ, USA). All aqueous solutions were filtered through a 0.45- μm filter (Millipore, Bedford, MA, USA) before use. An electrospray stainless steel needle (27 gauge) was butted against the low dead volume tee and enabled the delivery of the sheath solution to the end of the capillary column. The separation was obtained on about 90 cm length bare fused-silica capillary using 30 mM morpholine/formic acid in deionized water, pH 9.0, containing 5% methanol. A voltage of 30 kV was typically applied at the injection. The outlet of the capillary was tapered to ca. 15 μm i.d. using a laser puller (Sutter Instruments, Novato, CA, USA). Mass spectra were acquired with dwell times of 3.0 ms per step of 1 m/z unit in full-mass scan mode.

2.16 Production of anti-LOS antibodies. Immunization with purified LOS did not result in the production of IgG antibodies against LOS (Desjardins *et al.* 1995), but rather in low titer IgM antibodies. To obtain high titer IgG antibodies to LOS, NZW rabbits (2.5 kg) were i.m. immunized with 100 μg of OMP in Freund's complete adjuvant (FCA) (Gibco, Grand Island, NY), and boosted 4 weeks later with 100 μg of the same OMP preparation in Freund's incomplete adjuvant (FIA) (Gibco, Grand Island, NY). The rabbits were bled through cardiac puncture 4 weeks after the booster. The collected

blood was coagulated at RT for 2 to 3 hours and centrifuged at 2 000 X g for 5 minutes. The serum was collected in 500 µl aliquots and stored at – 70 °C.

2.17 Adsorption of anti-LOS serum using whole cells. The adsorption experiment previously described by Campagnari and colleagues (Campagnari *et al.* 1987) was modified as follows: strains J1159 (LOS group 6) and 1293 (LOS group 3) were cultured for 16 hours in M-H + FBS broth and the cells were heat-killed in a water bath at 60 °C for 1 hour. To adsorb the anti-OMP sera, 500-µl antiserum samples were incubated with 2.5×10^{10} J1159 *H. ducreyi* cells and 2.5×10^{10} of strain 1293 O/N with gentle agitation at 4 °C to minimize non-specific binding. The anti-1293 serum was adsorbed with 5×10^{10} cells of strain J1159, and the anti-J1159 serum was adsorbed with 5×10^{10} cells of strain 1293. The suspensions were centrifuged at 1 500 X g for 10 minutes, the supernatant (anti-OMP serum) recovered and frozen at – 70°C.

2.18 Immunization with rHgbA, nHgbA, rD15 and rFetA. Eight-week old NZW rabbits (approximately 1.5 kg in weight when first vaccinated) were immunized as described by Desjardins and colleagues (Desjardins *et al.* 1995). Briefly, animals were immunized with 100 µg of purified protein (n = 12 for nHgbA, n = 15 for rHgbA, n = 13 for rD15 in homologous challenge; n = 3 for nHgbA and n = 6 for rHgbA in heterologous challenge) in 350 µl of FCA, and boosted 4 weeks later with 100 µg of the same protein in 350 µl of FIA. Half of the dose was given i.m., while the other half was given subcutaneously. Controls included rabbits similarly immunized with 100 µg of rFetA (n = 8) in FCA/FIA, and rabbits sham-vaccinated with 350 µl of PBS in FCA/FIA (n = 14 for homologous challenge; n = 3 for heterologous challenge). FetA is a enterobactin siderophore receptor from *Neisseria gonorrhoeae* (Carson *et al.* 1999) used as an

unrelated protein control in vaccine experiments. rFetA was extracted exactly as rHgbA in Christopher Elkins' laboratory at the University of Chapel Hill.

Three additional rabbits (#26, 27 and 28) were immunized with rHgbA in MPL-SE®, which was kindly supplied by Ribi Immunochem, for evaluation of immunogenicity. MPL® is an adjuvant derived from the LPS of *Salmonella minnesota* strain R595 composed of a series of 4'-monophosphoryl lipid A species that vary in the extent and position of fatty acid substitution (Powell and Newman, 1995). In human clinical trials, MPL® was safe, well tolerated and provided a heightened immune response to coadministered antigens, although it proved to be pyrogenic at high doses (Powell and Newman, 1995). Rabbits were bled at 2-week intervals after the initial immunization (weeks 0, 2, 4, 6, 8) and 20 days post-challenge by the staff of the animal care service of the Faculty of Medicine of the University of Ottawa. The schedule of vaccination is shown in Table 4.

2.19 Temperature-dependent rabbit model (TDRM) of chancroid (vaccine model).

The challenge experiment following vaccination of rabbits was carried out as previously described by Desjardins and colleagues (Desjardins *et al.* 1995). Briefly, vaccine protection against infection with homologous (35000) and heterologous (V1157) strains was assayed by challenging the animals with 3 triplicate 100- μ l doses of 10^5 to 10^3 CFU of mid-log phase, broth-grown live *H. ducreyi* 4 weeks after the second immunization. CFU counts for each inoculum in each rabbit were directly determined in duplicate by plating appropriate dilutions of each inoculum from the injecting syringe onto CAP for colony counts. With the operator blinded to vaccine status of each animal, 2 out of the 3 lesions were scored (0, nil; 1, redness; 2, induration; 3, suppuration; and 4, ulceration)

and measured (transverse lesion diameter) every 48 hours for a period of 20 days. The third lesion was sampled by sidewise injection of 100 µl of PBS and back-aspirated, and cultured for viable *H. ducreyi* on CAP. Identification was based on characteristic colonial morphology, the push test, and microscopic examination of isolates for the unique characteristic appearance of *H. ducreyi* on Gram's stain (D'Costa *et al.* 1986). Strain V1157, a clinical isolate from Seattle, was chosen as the challenge strain in a heterologous protection study, on account of its distinct LOS SDS-PAGE band profile compared to strain 35000 (figure 1).

2.20 Comparative statistical analysis. Since 2 out of the 3 inoculation sites were measured and scored on each rabbit, lesion size and lesion score results were obtained by averaging the data of these 2 non-manipulated lesions to generate a single mean lesion size and mean lesion score measurement for each inoculum size, in each rabbit on each day of observation. Comparative statistical analysis of these data over 10 days of observation was performed with the one-way repeated measures analysis of variance (ANOVA), with Bonferri t test for pairwise comparisons. Comparative evaluation of inoculum size, culture positivity, cumulative lesion size, peak lesion score and duration of ulcer (results in the table) was performed with the Student's t test. The proportion of lesions that ulcerated tallied in lesion pairs were compared between groups using Fisher's exact test. A p value ≤ 0.05 was accepted as the level of significance. All data was compared using the Sigma Stat (version 2.0) software from Jandell Scientific (San Rafael, California). Jennifer Clinch performed Fisher's exact test (FET).

TABLE 3 - Schedule of vaccination

Immunogen Experiment	nHgbA (2 X 100 µg)	rHgbA (2 X 100 µg)	rD15 (2 X 100 µg)	rFetA (2 X 100 µg) CONTROL
#0 (Preliminary)	NO immunization	1 st & 2 nd . extract from CE's lab (Ra to Rc)	NO immunization	NO immunization
#1	1 st & 2 nd . E1 of 19/01/99 (R1 to R3)	1 st & 2 nd . E1 of 2/11/99 (R4 to R6)	1 st & 2 nd . 2.7 µg/µl (R7 to R9)	1 st and 2 nd . 0.53 µg/µl (R10 to R12)
#2	1 st & 2 nd . E1 of 29/01/99 (R16 to R18)	1 st & 2 nd . E1 of 2/11/99 (R19 to R22) (R26 to R28/ MPL-SE)	1 st & 2 nd . 1.7 µg/µl (R23 to R25)	1 st & 2 nd . 0.43 µg/µl (R29 and R30)
#3	1 st & 2 nd . E1 of 09/02/99 (R34 to R37)	1 st & 2 nd . E1 of 14/06/99 (R38 to R41)	1 st & 2 nd . 1.7 µg/µl (R42 to R45)	1 st & 2 nd . 0.43 µg/µl (R46 to R48)
#4 (Cross-strain protection assay)	NO immunization	1 st . E1 of 22/07/99 2 nd . E3 of 22/07/99 (R52 to R65)	NO immunization	NO immunization
#5	1 st & 2 nd . E1 of 22/07/99 (R70 to R72)	1 st & 2 nd . E1 of 21/11/99 (R73 to R75)	1 st & 2 nd . 2.7 µg/µl (R76 to R78)	NO immunization
#6 (Cross-strain protection assay)	1 st . E1 of 26/01/00 2 nd . E1 of 16/03/00 (R82 to R89)	1 st & 2 nd . E1 of 21/11/99 (R90 to R95)	NO immunization	NO immunization

“E” refers to eluate and “R” refers to rabbit

“1st” refers to the first immunization and “2nd” refers to the second immunization

CHAPTER 3 – RESULTS

3.1 Study of the electrophoretic, serologic and structural heterogeneity of the *H. ducreyi* LOS.

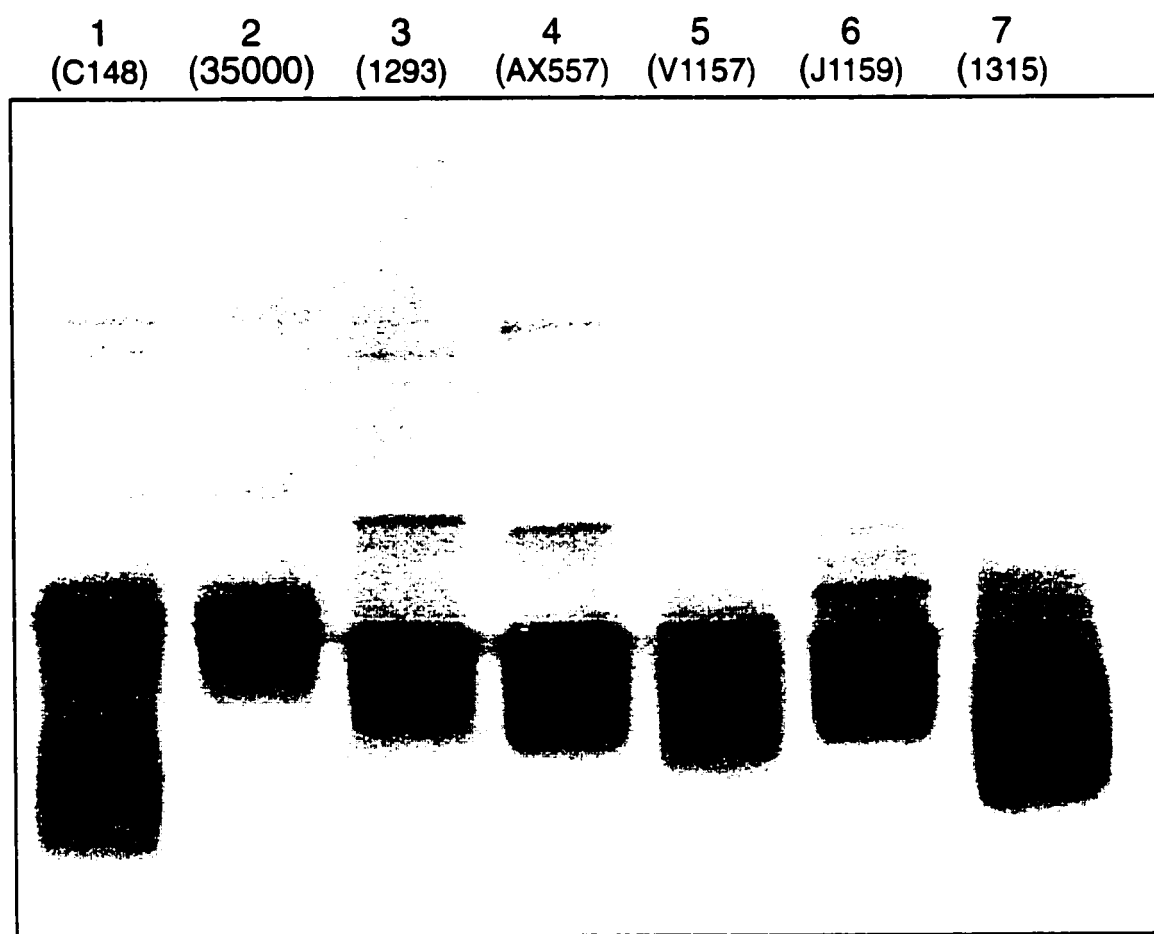
3.1.1 Electrophoretic heterogeneity of the *H. ducreyi* LOS. To determine if LOS structure can be used to categorize *H. ducreyi* strains into related subgroups, the banding pattern of the LOS molecules from 91 clinical isolates (arranged by origin in table 2) was evaluated using silver-stained SDS-PAGE. The *H. ducreyi* strains were placed in groups according to the number and the intensity of each of the band present (figure 1 and table 4). Strains from group 1 have a SDS-PAGE pattern that consists of 6 bands, which includes an intense doublet, 2 lighter bands and two diffuse bands (figure 1 - lane 1). The band pattern of strains from group 2 is identical to that of group 1, except for the absence of the diffuse band of lower molecular weight (figure 1 - lane 2). Two bands represent the LOS of strains from group 3 and group 4 (figure 1 - lanes 3 and 4, respectively), but the bands from SDS-PAGE group 4 are of slightly lower molecular weight. The LOS molecule from the strains belonging to group 5 has a band pattern consisting of 3 bands: 2 dark bands and 1 light band (figure 1 - lane 5). The band pattern of strains from group 6 have consists of 5 bands: 2 very dark bands and 3 light bands (figure 1 - lane 6). Finally, the banding pattern of strains from group 7 has several light bands, a darker band and a diffuse band, which looks similar to the diffuse band present in the band pattern of LOS from strains belonging to group 1 (figure 1 - lane 7).

Preliminary analysis of LOS heterogeneity by silver stained SDS-PAGE was performed on OMP preparations. All other experiments (mass spectrometry, EIA and

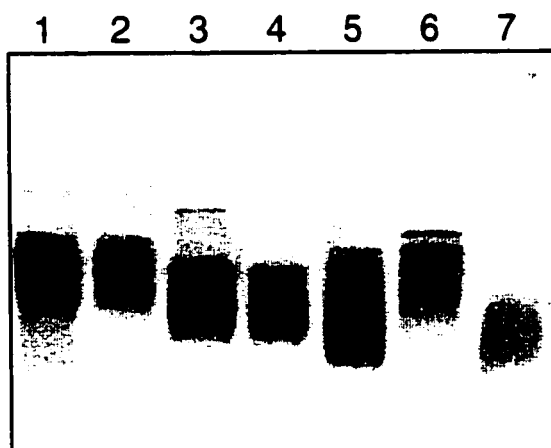
FIGURE 1

SDS-PAGE of OMP preparations (A and C) and purified LOS (B). A - OMP preparations were electrophoresed and silver stained. B - Purified LOS were electrophoresed and silver stained. C - OMP preparations were electrophoresed and stained with a modified Coomassie blue stain.

A



B



C

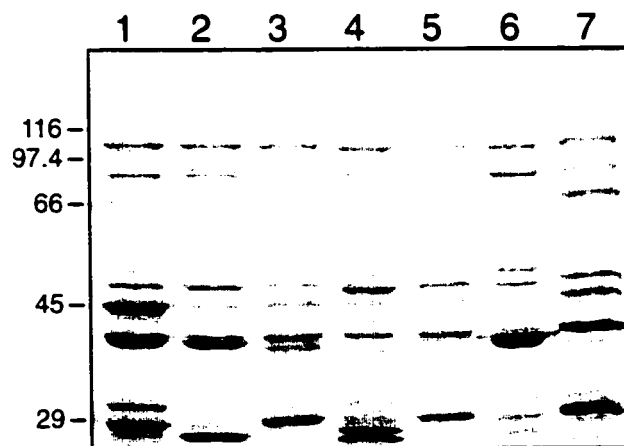


TABLE 4

Presumptive grouping of *H. ducreyi* strains according to SDS-PAGE band patterns

<u>SDS-PAGE GROUP</u>	<u>H. DUCREYI STRAINS</u>
1	C148, K10159, RO-30
2:	35000, VG770, RO-34, RO-40, J1321, K4912, K4163, PPC236, RO-08, PPC338, KID661, HG0894, K10161, KF133, RO-27, PPC323, RO-45, RO-53, K6456, KF142, H40529, K3588, K6757, PPC221, PPC275, RO-40, K4894, PPC248, PPC285, RO-35, RO-48, RO-53, RO-38, RO-49, PPC322, RO-01, RO-42, PPC333, PPC335, PPC352, RO-O3, RO-12, RO-21, RO-24, RO-20, Z115, J910, HG0890, KF140, PPC184, PPC281, PPC295, PPC316, K3367, K6856, KF128, KF143, PPC287, G24896, PPC418, RO-22, G29761, J1158, K1143, PPC267, K4167, K10124, PPC248, PPC307, PPC338, RO-51, PPC312, PPC342, RO-02, PPC318, PPC337, RO-23, RO-27
3:	PPC263/1293, 36-F-2, PPC415
4:	AX557, V-J4658, G29677, PPC193
5:	V1157
6:	J1159
7:	PPC358/1315, BG411

Western blots) used purified LOS. One strain was chosen from each LOS group (identified in bold in tables 2 and 4) to further study heterogeneity of the *H. ducreyi* LOS between strains of different SDS-PAGE groups. To determine if LOS present in OMP preparations matched purified LOS, we compared the SDS-PAGE banding patterns of both extracts. The patterns were identical for all strains studied, except for that of SDS-PAGE group 6 (strain J1159) which varied only in the relative intensity of the different bands (compare figure 1A to 1B). Except in SDS-PAGE group 2 for strain 35000, which is a well-characterized laboratory strain first reported as a clinical isolate (Hammond *et al.* 1978), strains were chosen arbitrarily: C148 was chosen to represent group 1, PPC263/1293 for group 3, AX557 for group 4, V1157 for group 5, J1159 for group 6 and PPC358/1315 for group 7. SDS-PAGE LOS group number (1 to 7) is referred to for structural and serologic grouping, instead of the strain used in the experiments.

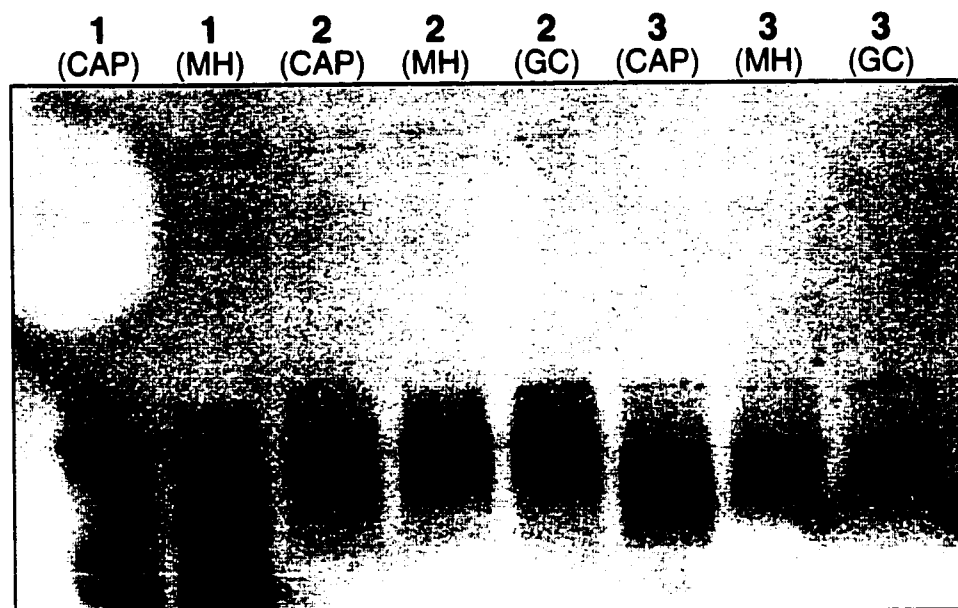
The SDS-PAGE heterogeneity of OMP from each LOS group was also studied. As seen in figure 1C, the OMP banding patterns from the 7 different LOS SDS-PAGE groups are very different from one another. Furthermore, they are more complex to analyze than the banding patterns of LOS since many more bands of different intensity are present.

3.1.2 Variation of the *H. ducreyi* LOS in different culture conditions. It had been reported that LOS profiles varied under different culture conditions (Abeck *et al.* 1987). To verify this observation, the LOS banding patterns of strains representing each SDS-PAGE group grown in 2 different broths, M-H and GC, were compared to the LOS banding pattern of organisms grown on CAP. Except for SDS-PAGE group 6, the LOS banding patterns did not change under different culture conditions (figure 2). As for the

FIGURE 2

Electrophoretic patterns of LOS from *H. ducreyi* strains grown in different culture conditions. Six different *H. ducreyi* strains were grown in M-H and GC broths and on CAP. Bacterial cells were collected, their OMP prepared, subjected to electrophoresis and silver stained.

A



B

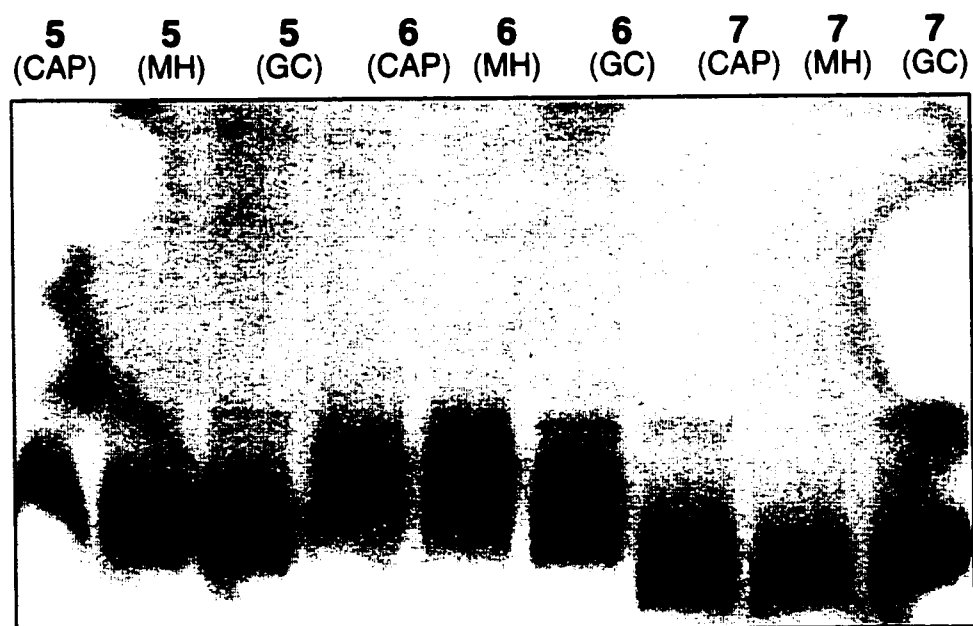
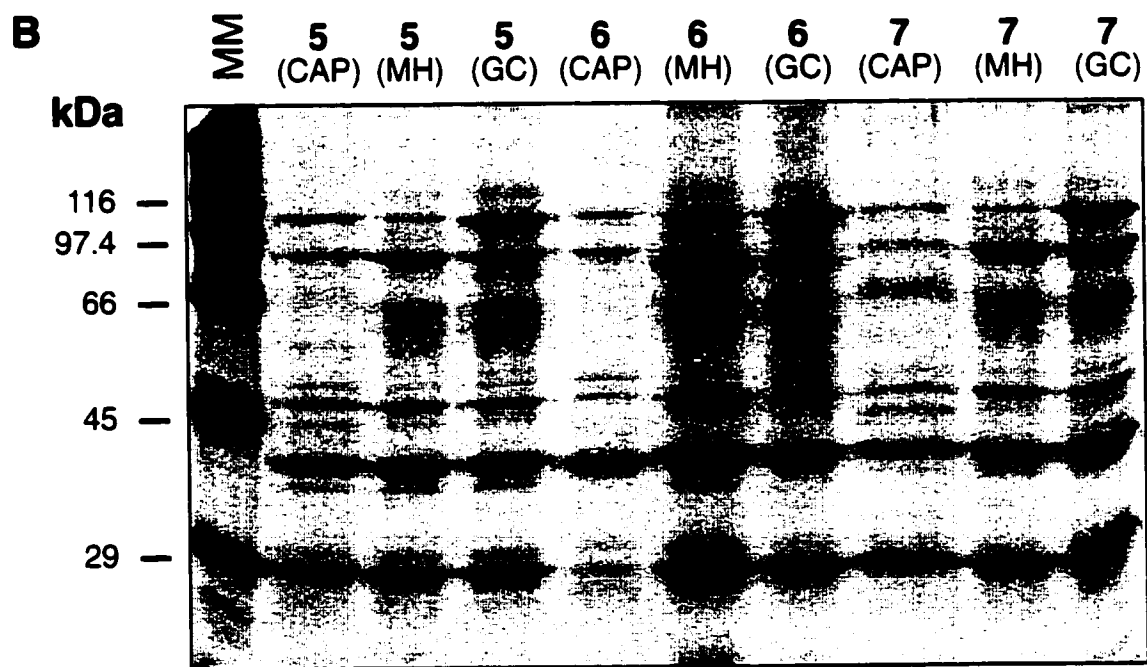
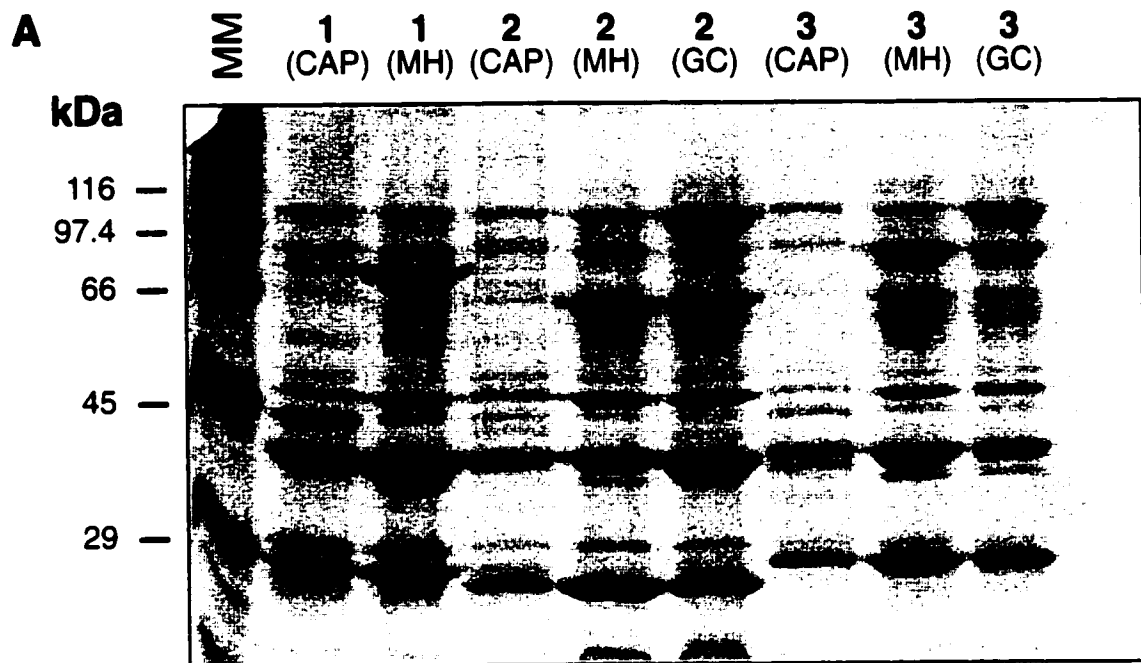


FIGURE 3

Electrophoretic patterns of OMP from *H. ducreyi* strains grown in different culture conditions. Six different *H. ducreyi* strains were grown in M-H and GC broths and on CAP. Bacterial cells were collected, their OMP prepared, subjected to electrophoresis and stained.



LOS from group 6, only the intensity of the bands, and not the number or the molecular weight of the bands, differed between J1159 grown in GC broth versus M-H broth and CAP (figure 2B – compare lanes 4, 5 and 6). Conversely, the type and concentration of outer membrane proteins varied greatly from one culture medium to the other (figures 3A and 3B).

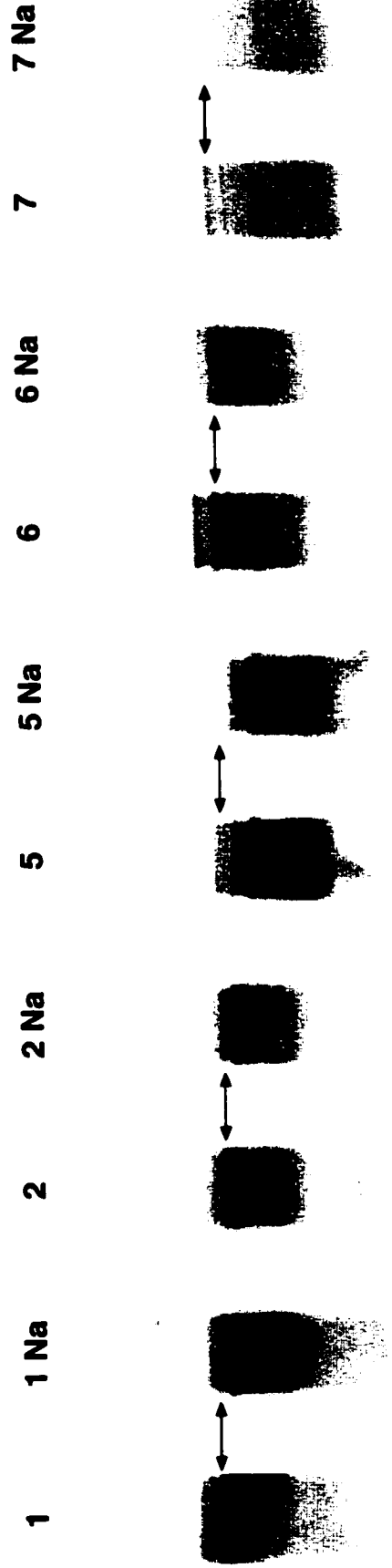
3.1.3 Sialylation state of the *H. ducreyi* LOS. Some *H. ducreyi* strains, including 35000, express LOS glycoforms with a terminal sialic acid molecule (Melaugh *et al.* 1996). To determine whether this was a unique feature of this strain or it was a general property of all *H. ducreyi* strains studied, purified LOS extracts were treated with *C. perfringens* neuraminidase. Figure 4 compares the SDS-PAGE banding patterns of LOS before and after neuraminidase treatment (Na). The arrow in figure 4A indicates the 5.1-kDa band that is likely affected by the neuraminidase treatment (Bozue *et al.* 1999; Melaugh *et al.* 1996). In LOS from SDS-PAGE groups 1, 2, 5, 6 and 7, there is either reduction in intensity of the 5.1 kDa, as for group 1 and 2, or a complete disappearance of this band, like in groups 5 to 7 (figure 4A). Although the band patterns of LOS from SDS-PAGE groups 3 and 4 do not have a 5.1-kDa band, there was the disappearance of the higher molecular weight band. However, the intensity of the lower molecular band was not affected (figure 4B).

3.1.4 Serologic diversity of *H. ducreyi* LOS. The SDS-PAGE LOS profiles shown in figure 1 suggest that common structures may be found between the LOS of different SDS-PAGE groups. To assess the extent to which different strain types share antigenicity, OMP antiserum from each SDS-PAGE group was assessed for homologous reaction and cross-reaction by both Western blot and EIA using LOS from each SDS-

FIGURE 4

Neuraminidase treatment of *H. ducreyi* LOS. Purified LOS were treated with *C. perfringens* neuraminidase as described in the materials and methods, electrophoresed and silver stained. Na refers to neuraminidase-treated LOS.

A



B

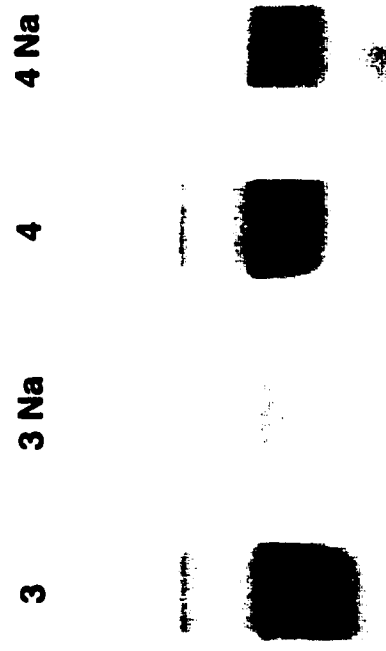


FIGURE 5

Serological reactivity of the antiserum to OMP from SDS-PAGE group 1. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 1 (C148) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (A and B) and in Western blots (C and D). In B and D, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

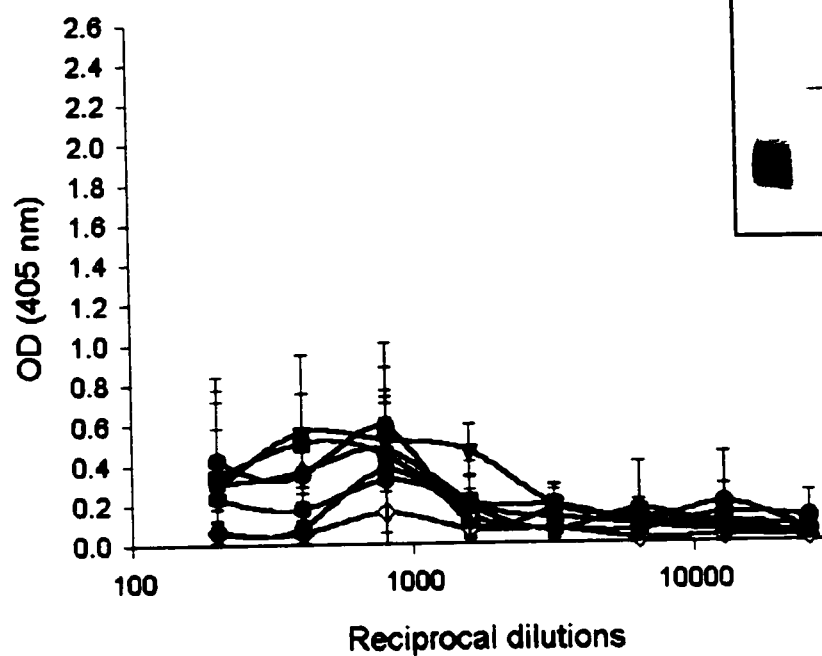
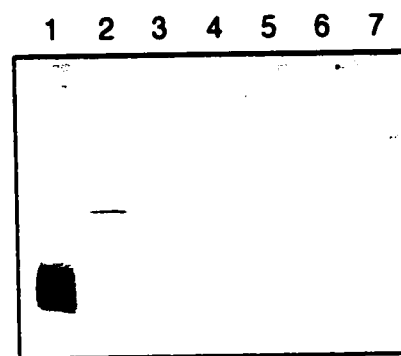
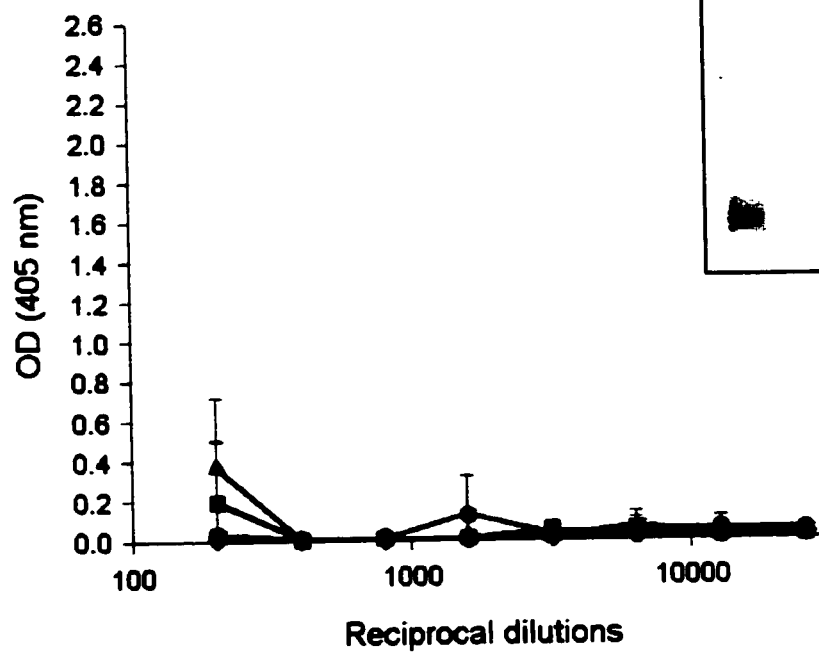
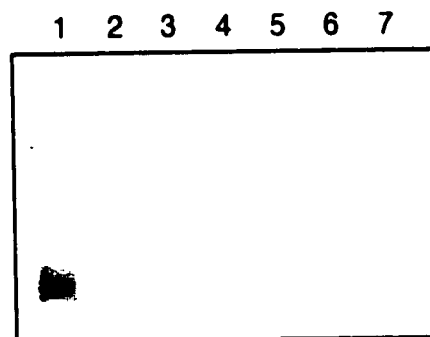
A**C****B****D**

FIGURE 6

Serological reactivity of the antiserum to OMP from SDS-PAGE group 2. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 2 (35000) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (**A** and **B**) and in Western blots (**C** and **D**). In **B** and **D**, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

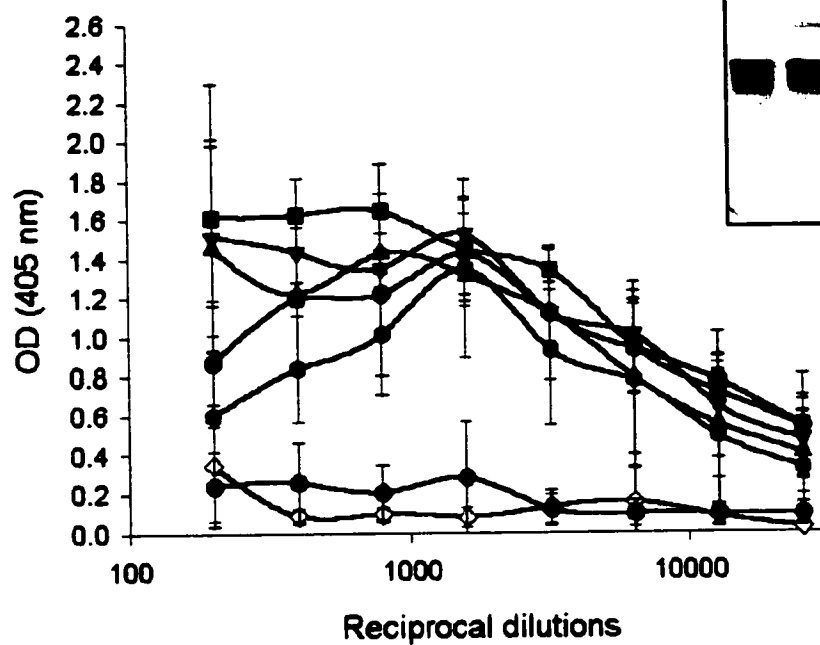
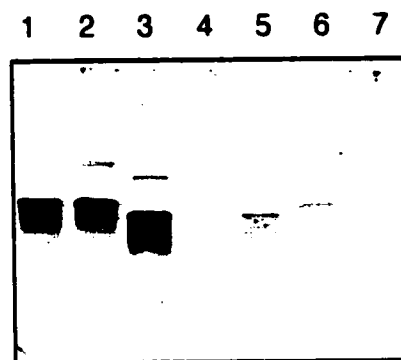
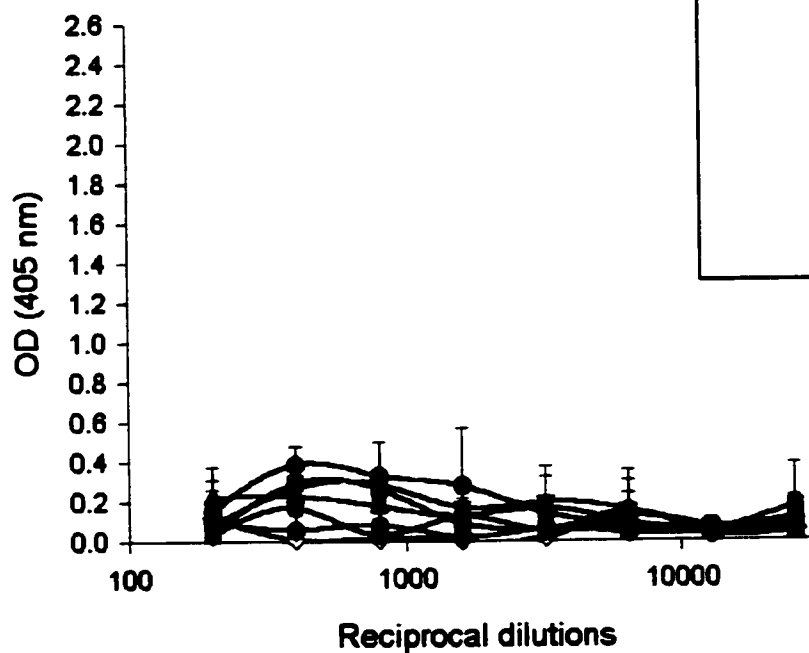
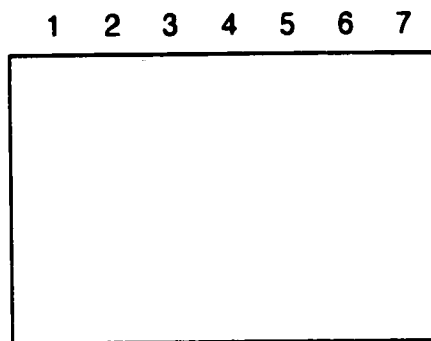
A**C****B****D**

FIGURE 7

Serological reactivity of the antiserum to OMP from SDS-PAGE group 3. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 3 (1293) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (**A** and **B**) and in Western blots (**C** and **D**). In **B** and **D**, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE group 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

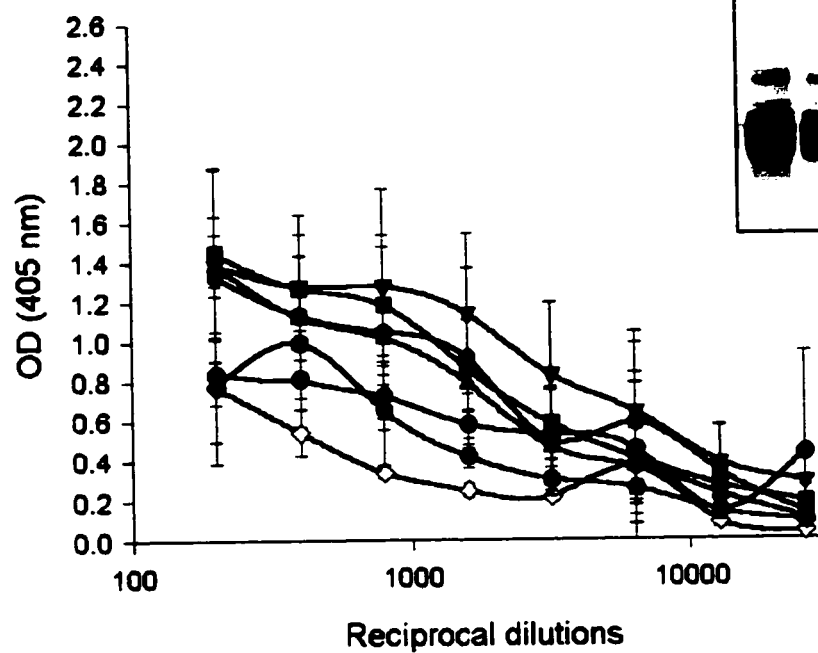
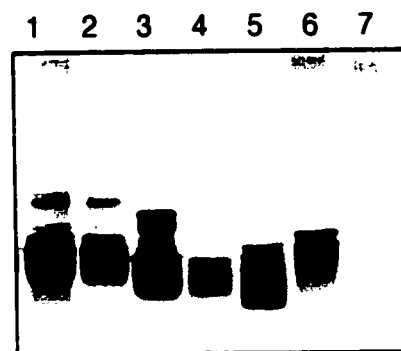
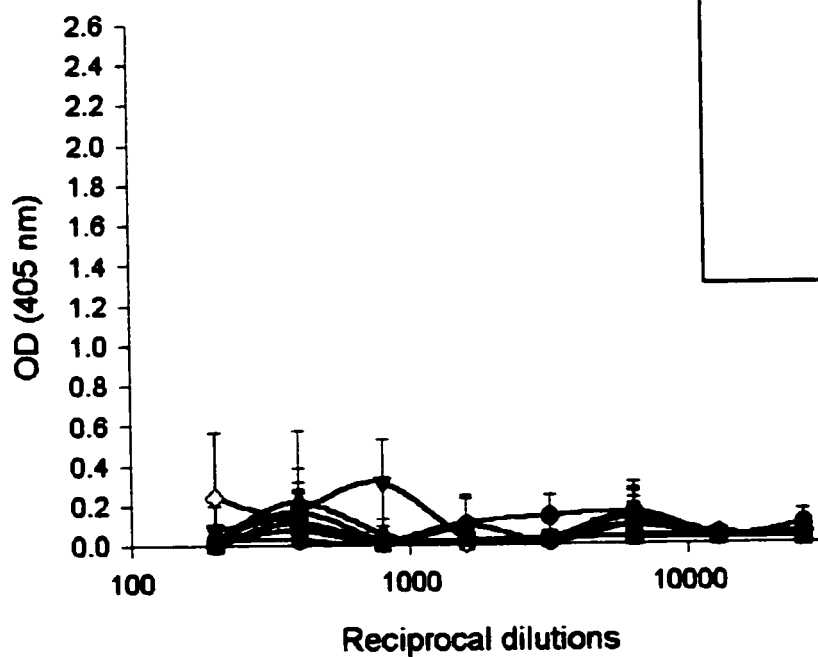
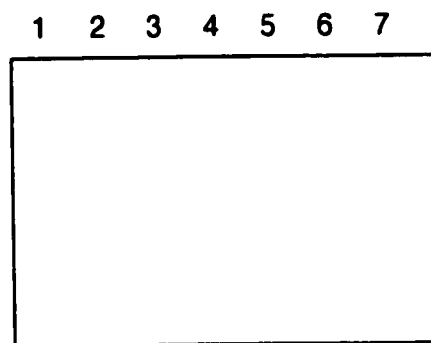
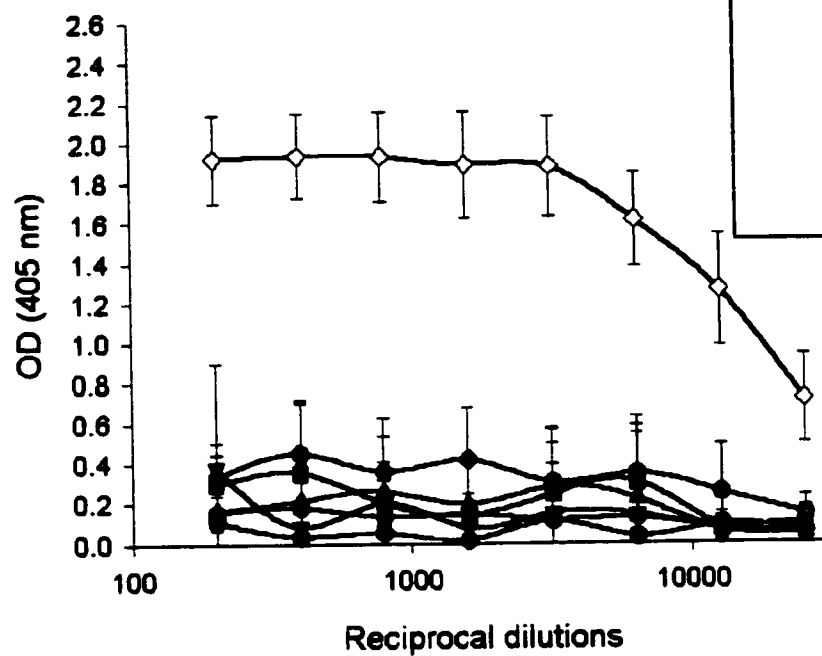
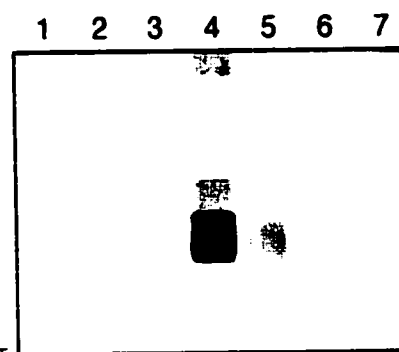
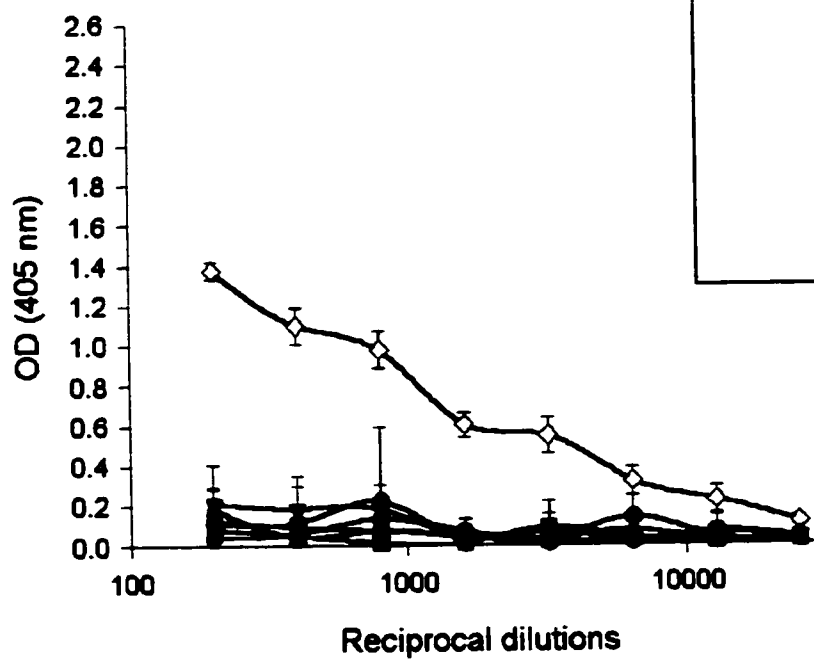
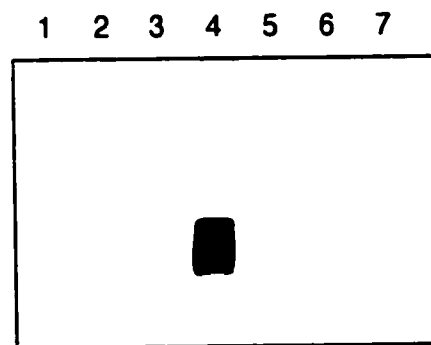
A**C****B****D**

FIGURE 8

Serological reactivity of the antiserum to OMP from SDS-PAGE group 4. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 4 (AX557) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (**A** and **B**) and in Western blots (**C** and **D**). In **B** and **D**, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

A**C****B****D**

PAGE group as antigen. Although data using the un-adsorbed OMP antisera suggests that the LOS from the 7 SDS-PAGE groups share some immunological determinants, the extensive cross-reactivity detected in both EIA and Western blots precluded further conclusions to be made (figures 5 to 11 – panels A (EIA) and C (Western blots)).

Antiserum from each SDS-PAGE group was adsorbed with whole cells from SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) since the sera raised to OMP from these strains were between themselves reactive across all 7 groups (figures 7C and 10C). Antiserum to OMP from SDS-PAGE group 3 was adsorbed with whole cells from SDS-PAGE group 6, and vice versa. In an EIA, it was found that only the adsorbed antisera to OMP from SDS-PAGE groups 4 and 5 were able to recognize their homologous LOS (figures 8B and 9B). In Western blot, the adsorbed OMP antiserum from SDS-PAGE groups 1, 4, 5 and 7 detected only their homologous purified LOS (figures 5D, 8D, 9D and 11D). However, the adsorbed OMP antiserum to SDS-PAGE groups 2, 3 and 6 did not detect any LOS specimens, not even of their homologous strain (figures 6D, 7D and 10D). Thus, 4 groups showed specific homologous reaction and extinction of heterologous reaction, while 3 remaining groups showed cross-extinction of homologous and heterologous reaction, suggesting 5 serologic categories (table 5).

3.1.5 Sugar analysis of the *H. ducreyi* LOS. The carbohydrate (CHO) content of purified LOS from each of the 7 SDS-PAGE groups was investigated by gas chromatography. All LOS molecules studied possessed glucose, N-acetyl-glucosamine and LD-Heptose. However, the characteristic DD-heptose was not found in the LOS of SDS-PAGE groups 4 and 7. Furthermore, the LOS from SDS-PAGE group 7 did not possess any galactose. These results suggest that strains from SDS-PAGE groups 4 and 7

FIGURE 9

Serological reactivity of the antiserum to OMP from SDS-PAGE group 5. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 5 (V1157) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (A and B) and in Western blots (C and D). In B and D, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

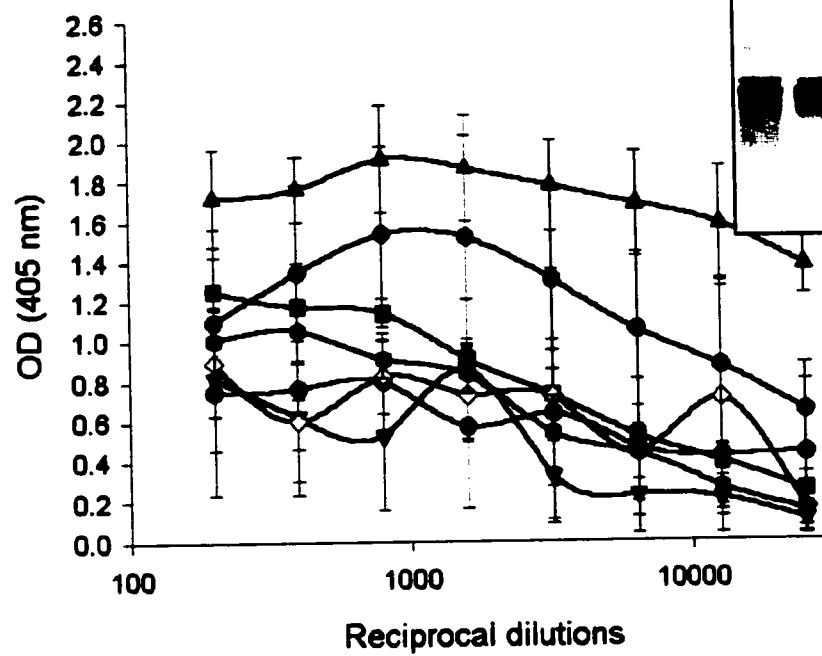
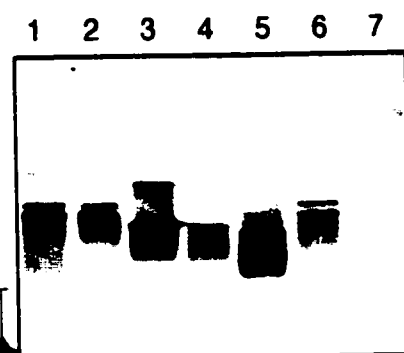
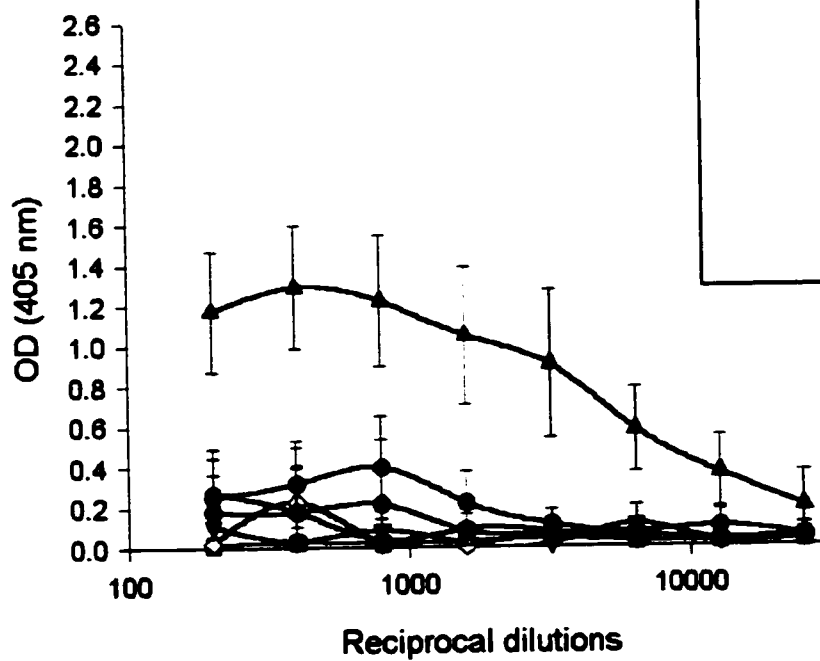
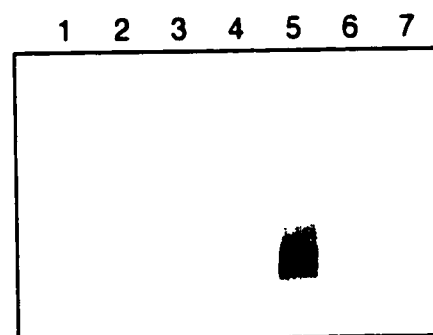
A**C****B****D**

FIGURE 10

Serological reactivity of the antiserum to OMP from SDS-PAGE group 6. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 6 (J1159) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (**A** and **B**) and in Western blots (**C** and **D**). In **B** and **D**, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE group 3 (strain 1293) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

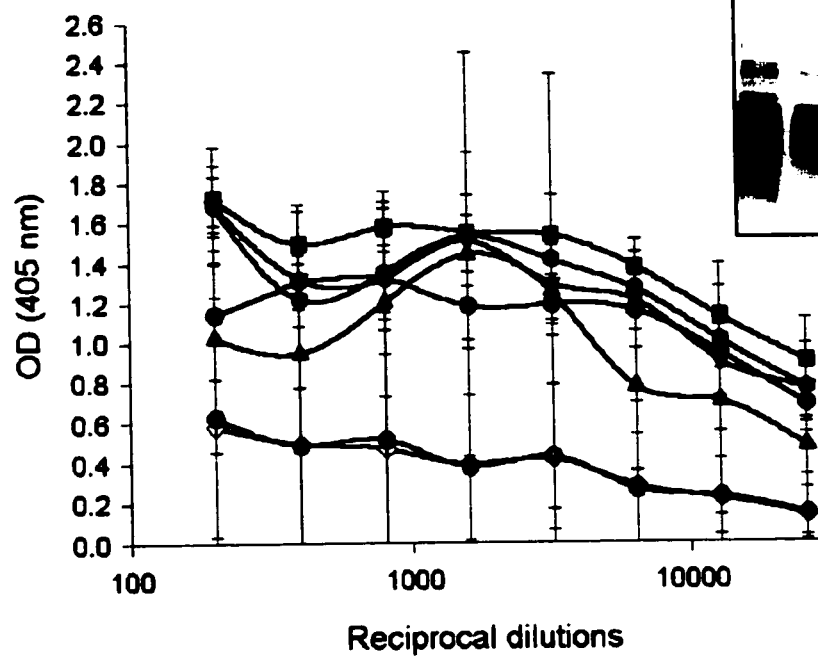
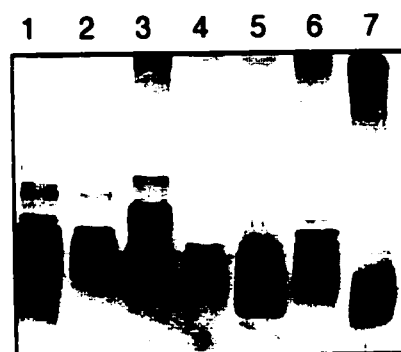
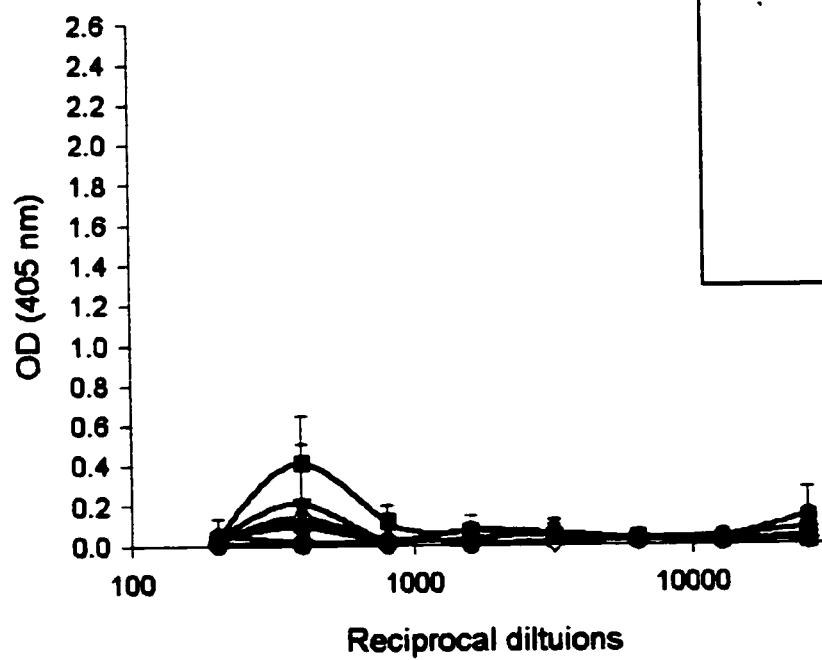
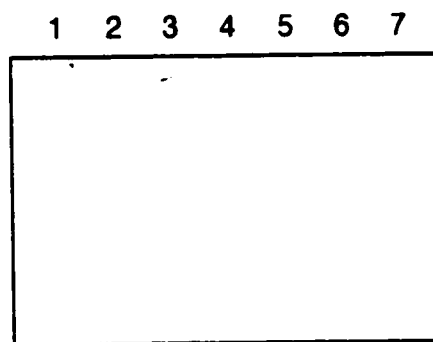
A**C****B****D**

FIGURE 11

Serological reactivity of the antiserum to OMP from SDS-PAGE group 7. Rabbits were vaccinated and boosted 4 weeks later with 100 µg of OMP from SDS-PAGE group 7 (1315) in Freund's adjuvant. The anti-OMP serum was assayed against purified LOS in an LOS-based EIA (**A** and **B**) and in Western blots (**C** and **D**). In **B** and **D**, the anti-OMP serum was adsorbed with whole cells of SDS-PAGE groups 3 (strain 1293) and 6 (strain J1159) as described in materials and methods.

LEGEND: black circle = purified LOS from SDS-PAGE group 1, red inverted triangle = purified LOS from SDS-PAGE group 2, green square = purified LOS from SDS-PAGE group 3, yellow diamond = purified LOS from SDS-PAGE group 4, dark blue triangle = purified LOS from SDS-PAGE group 5, mauve hexagon = purified LOS from SDS-PAGE group 6, light blue circle = purified LOS from SDS-PAGE group 7.

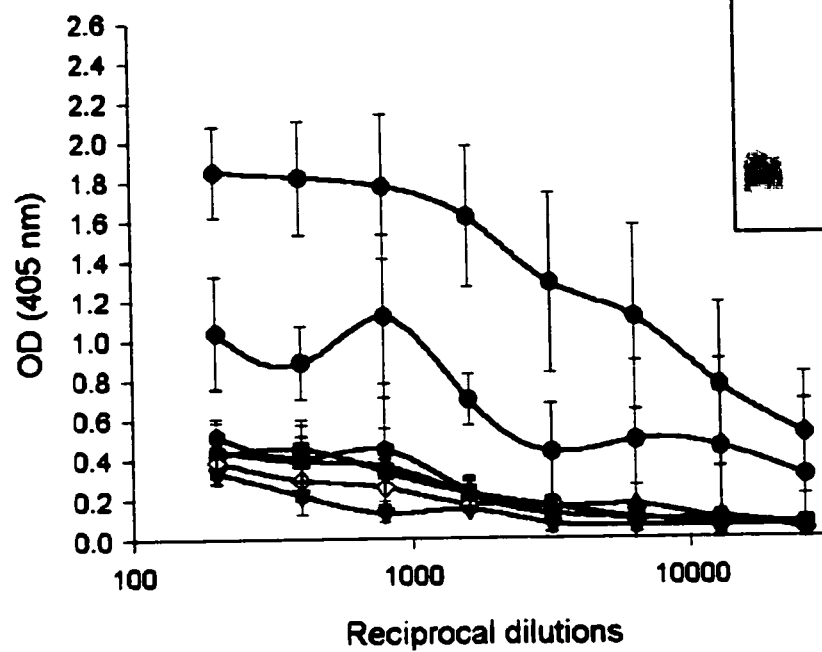
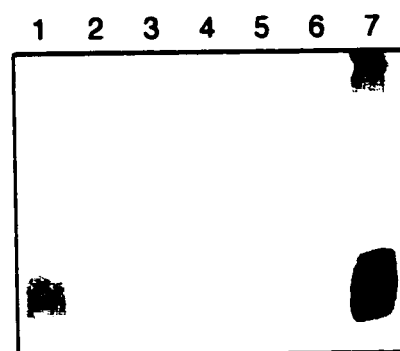
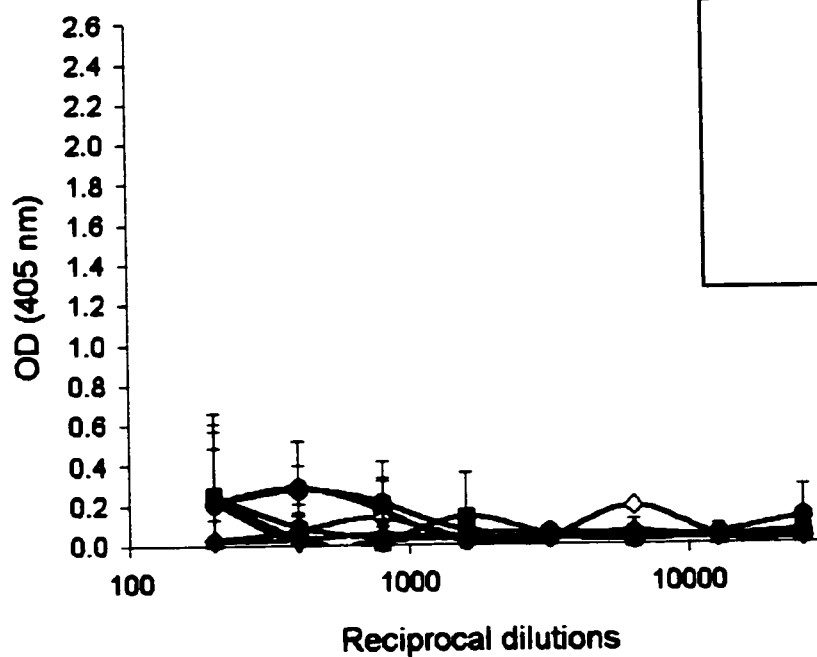
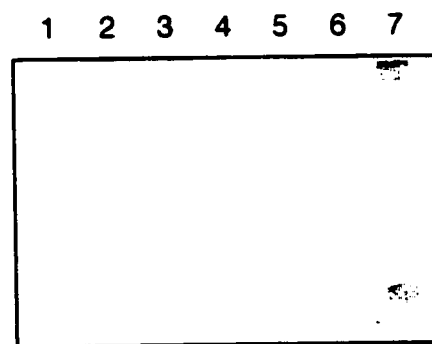
A**C****B****D**

TABLE 5**Grouping of *H. ducreyi* strains based on serological profiles**

<u>SEROLOGICAL CATEGORY</u>	<u>TYPE-STRAIN</u> (SDS-PAGE GROUP)
A^a	C148 (1)
B^a	AX557 (4)
C^a	V1157 (5)
D^a	PPC358/1315 (7)
E^b	35000 (2) PPC263/1293 (3) J1159 (6)

^a by specific homologous serologic reaction to LOS after adsorption**^b by cross-extinction of homologous reaction after adsorption**

may express LOS molecules that are structurally different than those found in the other LOS groups.

3.1.6 Mass spectrometry analysis of the *H. ducreyi* LOS. Purified LOS from all 7 SDS-PAGE groups were subjected to capillary electrophoresis-electrospray mass spectrometry (CE-ESMS). The structures of the LOS molecules were proposed based on molecular mass (table 6). All the strains studied express many different LOS glycoforms at their cell surface. Strains from SDS-PAGE groups 2 and 6 (serologic category E) expressed the greatest number of different LOS glycoforms at their surface with 8 and 9, respectively (table 6). Four different LOS glycoforms are expressed at the surface of strains belonging to SDS-PAGE groups 3 and 5, while groups 4 and 7 both express 3 different LOS glycoforms. Strains from SDS-PAGE group 1 expressed only 2 glycoforms at their surface. Mass spectrometry results also reveal that only strains from SDS-PAGE groups 1, 2, 3, and 6 express sialylated LOS (table 6).

TABLE 6**Mass spectrometry of purified LOS from SDS-PAGE groups 1 to 7**

PAGE group # (strain)	Serologic category	Glyco- form	Relative intensity (%)	Assignment
1 (C148)	A	A B	81.9 100	Hex ₃ Hep ₄ HexNAc ₁ KDO (P) Hex ₃ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (P)
2 (35000)	E	A B C D E F G H	29.3 16.9 14.0 26.3 100 20.3 29.6 70.2	Hex ₃ Hep ₄ HexNAc ₁ KDO ₁ (PPE) Hex ₁ Hep ₄ HexNAc ₁ Neu5Ac₁ PE ₁ KDO Hex ₂ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO Hex ₄ Hep ₄ HexNAc ₁ KDO (P) Hex ₃ Hep ₄ HexNAc ₁ KDO (P) Hex ₃ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (PPE) Hex ₂ Hep ₄ HexNAc ₁ KDO (P) Hex ₃ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (P)
3 (1293)	E	A B C D	90.9 100 57.3 35.6	Hex ₃ Hep ₃ Neu5Ac₁ KDO (PE) Hex ₂ Hep ₄ HexNAc ₁ KDO (PPE) Hex ₃ Hep ₄ HexNAc ₁ KDO (P) Hex ₂ Hep ₄ HexNAc ₁ KDO (P)
4 (AX557)	B	A B C	19.1 60.3 100	Hex ₃ Hep ₃ HexNAc ₁ KDO (P) Hex ₂ Hep ₃ HexNAc ₁ KDO (PPE) Hex ₂ Hep ₃ HexNAc ₁ KDO (P)
5 (V1157)	C	A B C D E	47.0 47.0 61.8 100 72.0	Hex ₁ Hep ₃ HexNAc ₁ KDO (PPE) Hex ₁ Hep ₃ KDO (P) Hex ₂ Hep ₂ HexNAc ₁ KDO (PPE) Hex ₁ Hep ₄ KDO (PPE) Hex ₁ Hep ₄ KDO (P)
6 (J1159)	E	A B C D E F G H I	18.2 21.7 13.5 75.5 24.4 48.1 31.6 100 33.6	Hex ₃ Hep ₄ HexNAc ₁ KDO (PPE, PPE) Hex ₂ Hep ₃ HexNAc ₁ Neu5Ac₁ KDO (PE ₂) Hex ₄ Hep ₄ HexNAc ₁ KDO (P, PPE) Hex ₃ Hep ₄ HexNAc ₁ KDO (PPE) Hex ₄ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (P, PPE) Hex ₂ Hep ₄ HexNAc ₁ KDO (PPE) Hex ₃ Hep ₄ HexNAc ₁ KDO (P) Hex ₃ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (PPE) Hex ₃ Hep ₄ HexNAc ₁ Neu5Ac₁ KDO (P)
7 (1315)	D	A B C	17.8 34.8 100	Hex ₁ Hep ₄ KDO (P) Hep ₃ KDO (PE) Hep ₃ KDO (P)

3.2 Vaccine development against chancroid.

3.2.1 nHgbA as a vaccine candidate in the TDRM of chancroid.

3.2.1.1 Purity of nHgbA extracts. nHgbA preparation contained some extraneous proteins as shown by faint but detectable bands when large amounts of the eluted protein were subjected to SDS-PAGE and stained with Coomassie (figures 12A (lane E1) and 13A). To determine whether the additional bands present in nHgbA preparation were degraded products of nHgbA or unrelated proteins obtained during the extraction process, nHgbA preparation was subjected to Western blotting with an rHgbA antiserum. This antiserum recognized most of the extraneous bands (figure 13B).

Since nHgbA was extracted from whole cells, LOS could have contaminated the purified product. To determine the presence of such contaminants, nHgbA preparation was subjected to SDS-PAGE and silver stained. A low molecular weight band characteristic of LOS was found in all lanes containing purified nHgbA, suggesting that LOS may be present in nHgbA preparation used for vaccine purposes (figure 13C). For this reason, vaccinogens are referred to as preparations rather than purified substances.

3.2.1.2 Immune response to nHgbA immunization in rabbits challenged with a homologous strain. Western blots and EIA were used to qualify and quantify, respectively, the humoral immune response of rabbits immunized with vaccine antigens. In Western blots (figure 14), sera from most of the nHgbA-immunized rabbits reacted to the 100-kDa band of HgbA from OMP. However, most also recognized 2 other proteins with molecular masses between 37 and 43 kDa, which may be MOMP (37-39 kDa) and OmpA2 (43 kDa), two major OMP from *H. ducreyi* (Klesney-Tait *et al.* 1997; Spinola *et al.* 1993). These Western blots do not assess reactivity to LOS since they only show

FIGURE 12

SDS-PAGE of the nHgbA extraction. Each step of the nHgbA extraction process from 16 liters of broth-grown *H. ducreyi* in Hg-reduced conditions was monitored by SDS-PAGE (A) and (B). (A): SM = Starting material (15 μ l), ZwI = Zwittergent-insoluble (15 μ l), ZwS = Zwittergent-soluble (15 μ l), Unb = unbound (15 μ l), MM = molecular markers (10 μ l), BB = boiled beads (15 μ l), E1 = eluate 1 (15 μ l), E2 = eluate 2 (15 μ l). (B): TM = total membranes (5 μ l), SS1 = first sarkosyl-soluble fraction (5 μ l), SS2 = second sarkosyl-soluble fraction (5 μ l), OMP = outer membrane proteins (5 μ l), Sw = sample wash (15 μ l), Zww = Zwittergent wash (15 μ l), Ogw = octoglucoside wash (15 μ l), and dia = dialysate (15 μ l). (C): SDS-PAGE of the nHgbA protein preparation (5 μ l) stained with Coomassie blue.

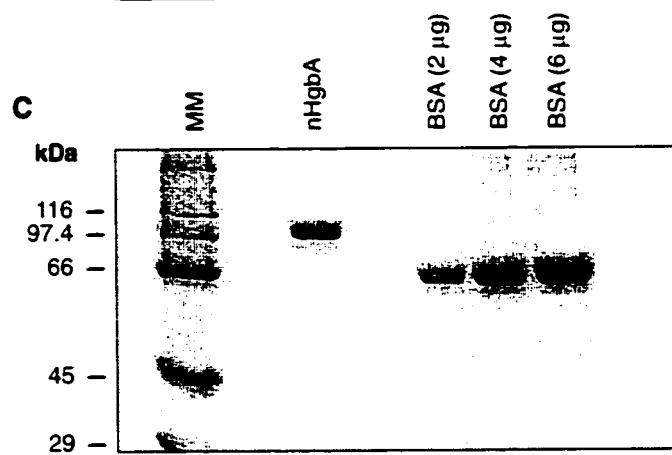
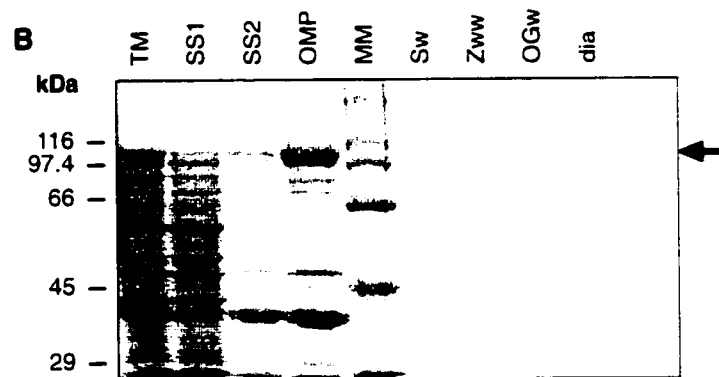
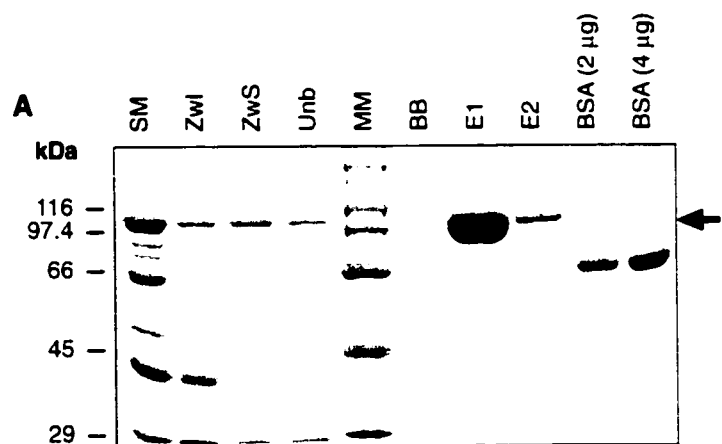


FIGURE 13

SDS-PAGE of nHgbA extracts. **A** - Purified nHgbA was electrophoresed and stained with Coomassie blue. **B** - Purified nHgbA was transferred onto a nitrocellulose membrane and blotted with an rHgbA rabbit antiserum (1:1000). **C** - Purified nHgbA was subjected to electrophoresis and silver stained. The arrow indicates the 100-kDa band of HgbA.

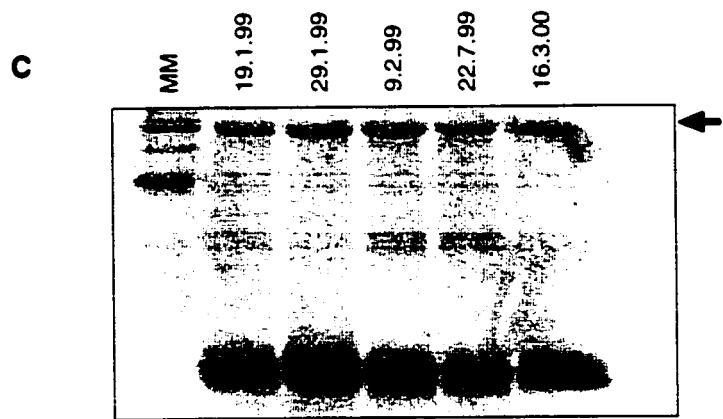
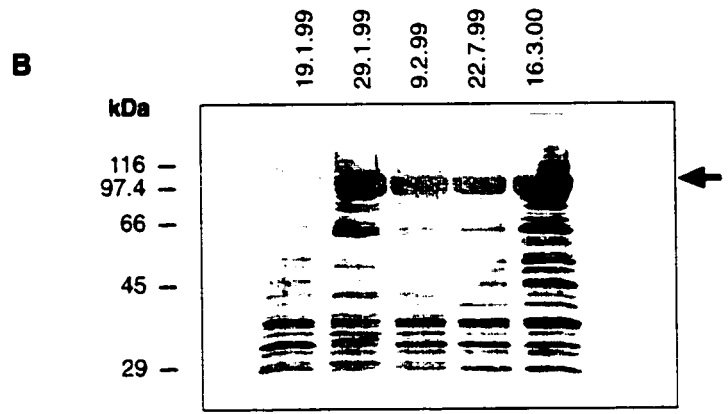
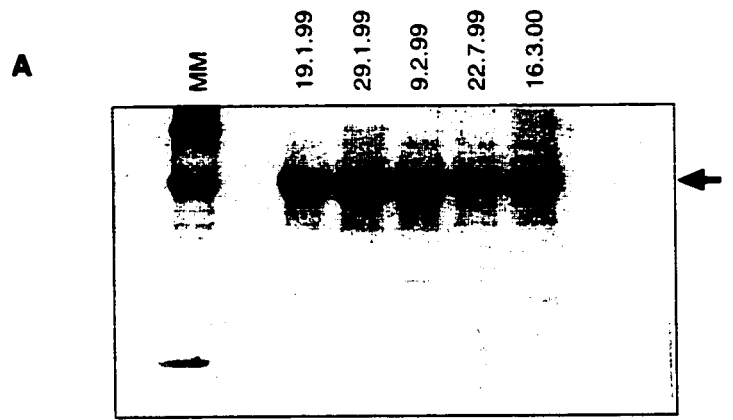


FIGURE 14

Immune response of rabbits immunized with the nHgbA vaccine. Screening Western blots of nHgbA antisera to *H. ducreyi* OMP (2µg/lane) from strain 35000 (homologous). The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 100-kDa band of HgbA.

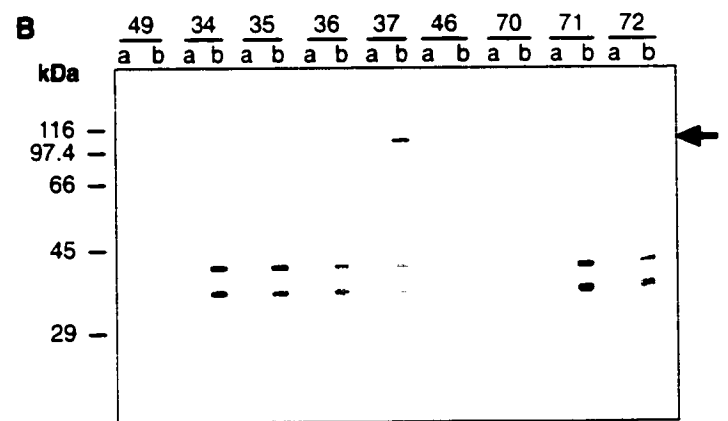
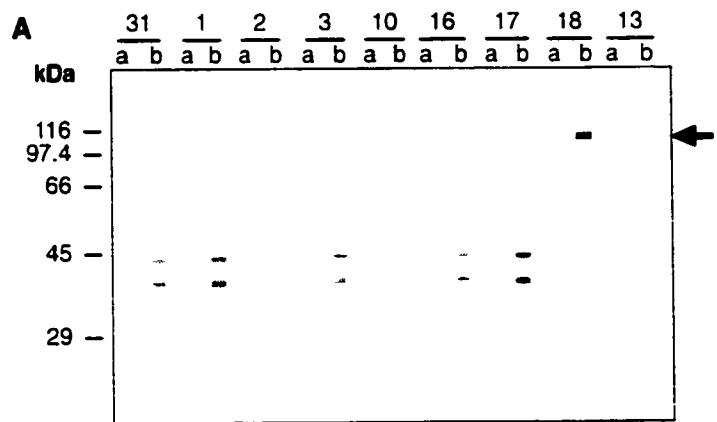
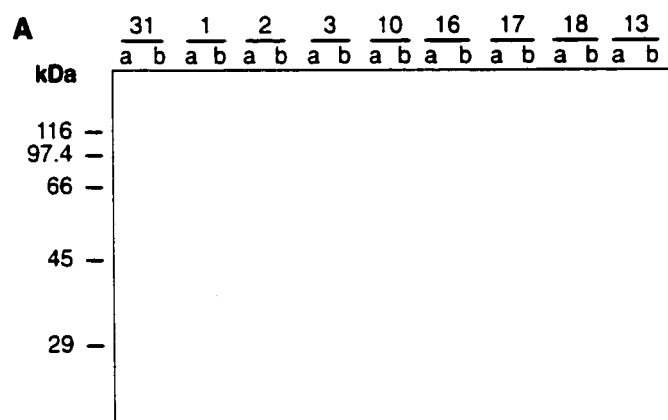


FIGURE 15

Immune response of rabbits immunized with the nHgbA vaccine. Screening Western blots of nHgbA antisera to *H. ducreyi* OMP (2µg/lane) from strain FX504 (HgbA mutant). The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 100-kDa band of HgbA.



proteins of 29 kDa and higher molecular weight.

To determine if these additional bands represented degradation products of HgbA or were unrelated proteins, the antisera was blotted against OMP extracted from strain FX504 (Elkins *et al.* 1995), which is an isogenic *hgbA* mutant of strain 35000 that does not express the Hg receptor. In a screening Western blot using OMP from strain FX504, the bands corresponding to the 2 unknown proteins were retained while the HgbA was absent (figure 15). Antisera from some PBS- (figure 15A – rabbit 31/lane b) and rFetA-immunized (data not shown) animals also reacted to these OMP, suggesting that they were not the result of specific immunization.

In nHgbA-based EIA, immunization with nHgbA preparations produces a serologic response to nHgbA (figures 16B and 17B). Optical density increased rapidly 2 and 4 weeks after the first vaccination. This was followed by further rise at week 6. The response then plateaued at week 8 to the end of the experimental infection model, 3 weeks after the challenge infection. Sera reacted similarly in an OMP-based EIA (figures 16A and 17A). Figure 18 shows the immune response of each individual rabbit to OMP (figure 17A) and nHgbA preparations (figure 17B).

3.2.1.3 Vaccine effect of nHgbA immunization on homologous *H. ducreyi* challenge.

Although PBS was used as a standard control in all vaccination experiments, we used rFetA, an unrelated TonB-dependent receptor expressed by *Neisseria* species (Carson *et al.* 1999) as a protein antigen control. This protein was expressed and purified in the same manner as rHgbA in the laboratory of Christopher Elkins of the University of North Carolina in Chapel Hill (figure 18). Figure 19 shows the effect of immunization of rabbits grown strain 35000. The lesion score (figures 19A, $p=0.068$) and lesion size

FIGURE 16

Humoral immune response of nHgbA-immunized rabbits. A - Anti-nHgbA assayed in an OMP-based EIA. B - The same serum was also assayed in an nHgbA-based EIA. The mean $\pm$ 1 SE of optical densities is shown.

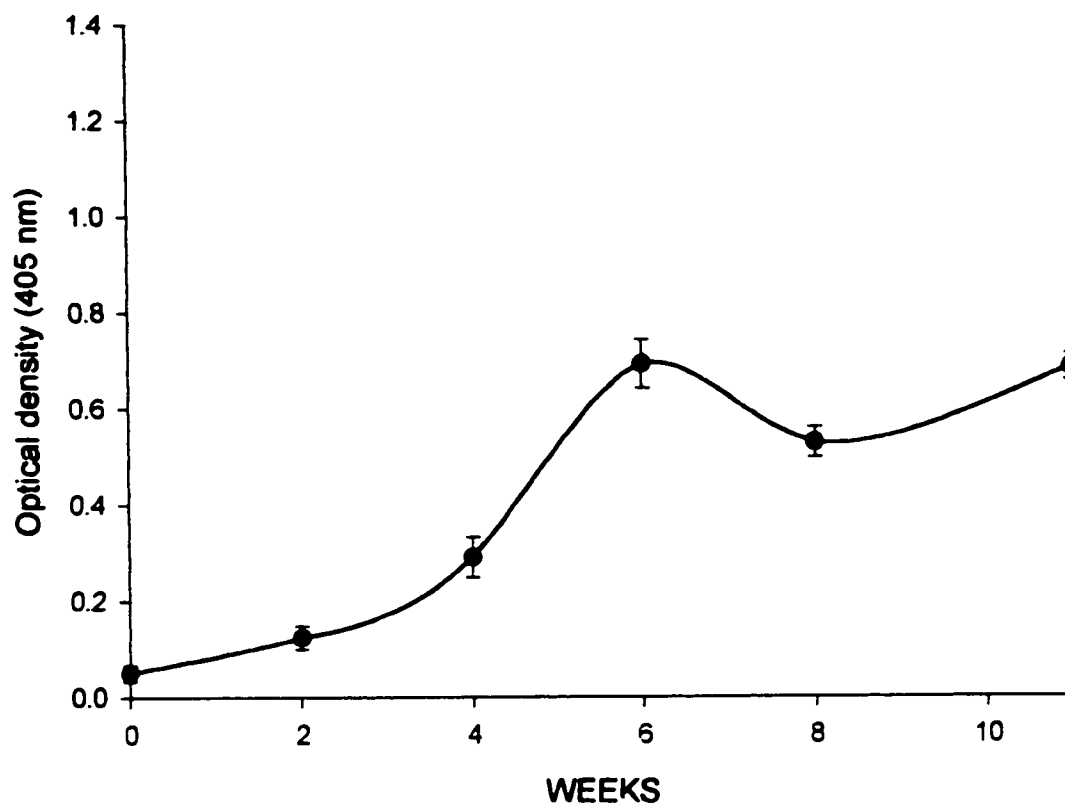
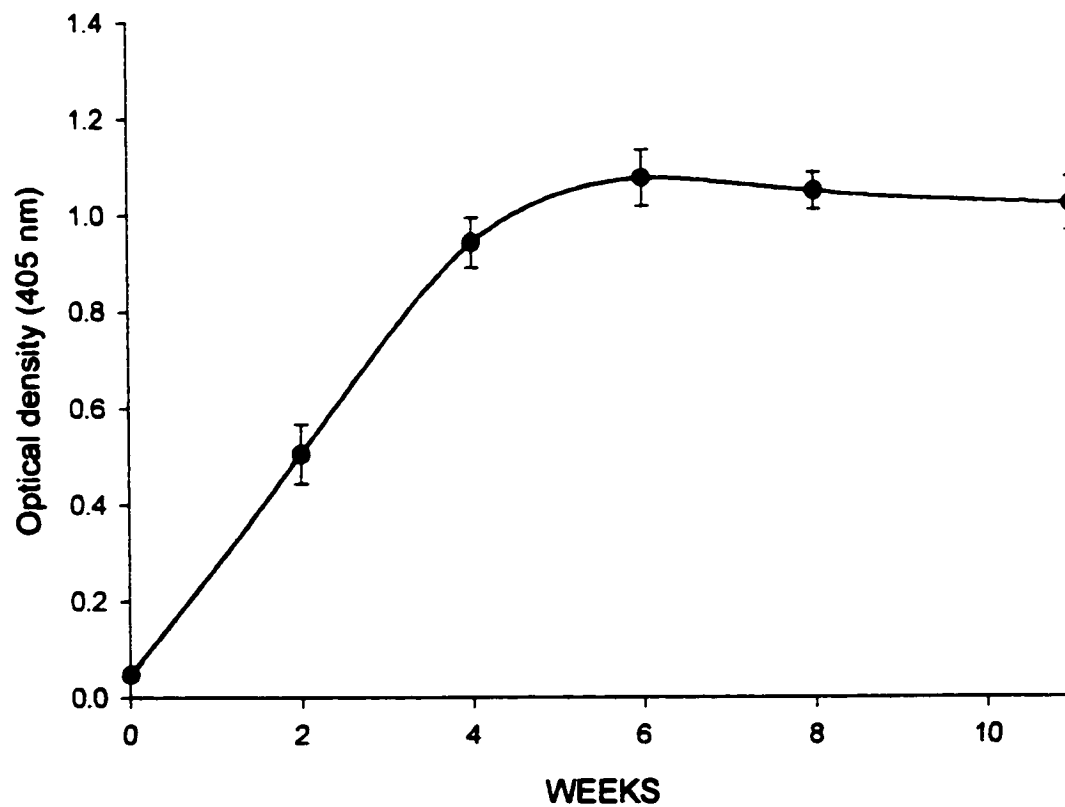
A**B**

FIGURE 17

**Humoral immune response of nHgbA-immunized (per rabbit). A – OMP-based EIA.
B – nHgbA-based EIA.**

LEGEND: rabbit 1 (black circles), 2 (inverted red triangle), 3 (light green squares), 16 (yellow diamonds), 17 (blue triangles) and 18 (pink hexagons), rabbit 34 (light blue circle), 35 (grey inverted triangle), 36 (burgundy square), 37 (dark green diamond), 70 (brown triangle), 71 (dark blue circle) and 72 (purple circle).

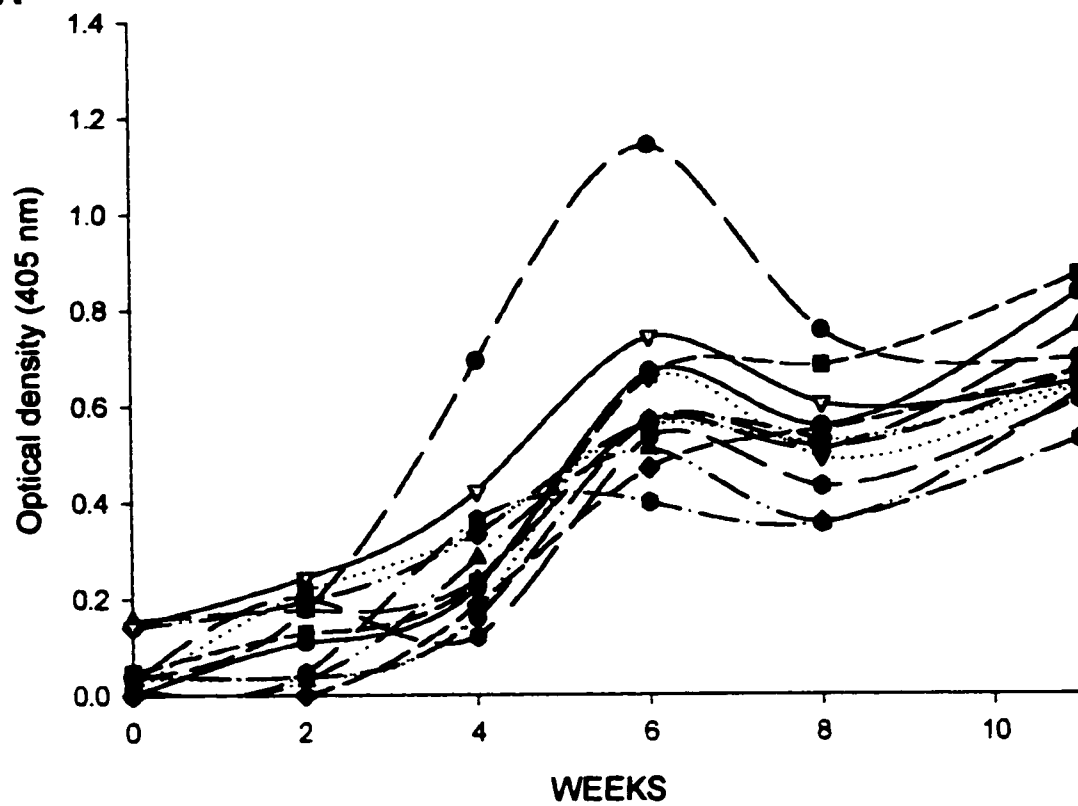
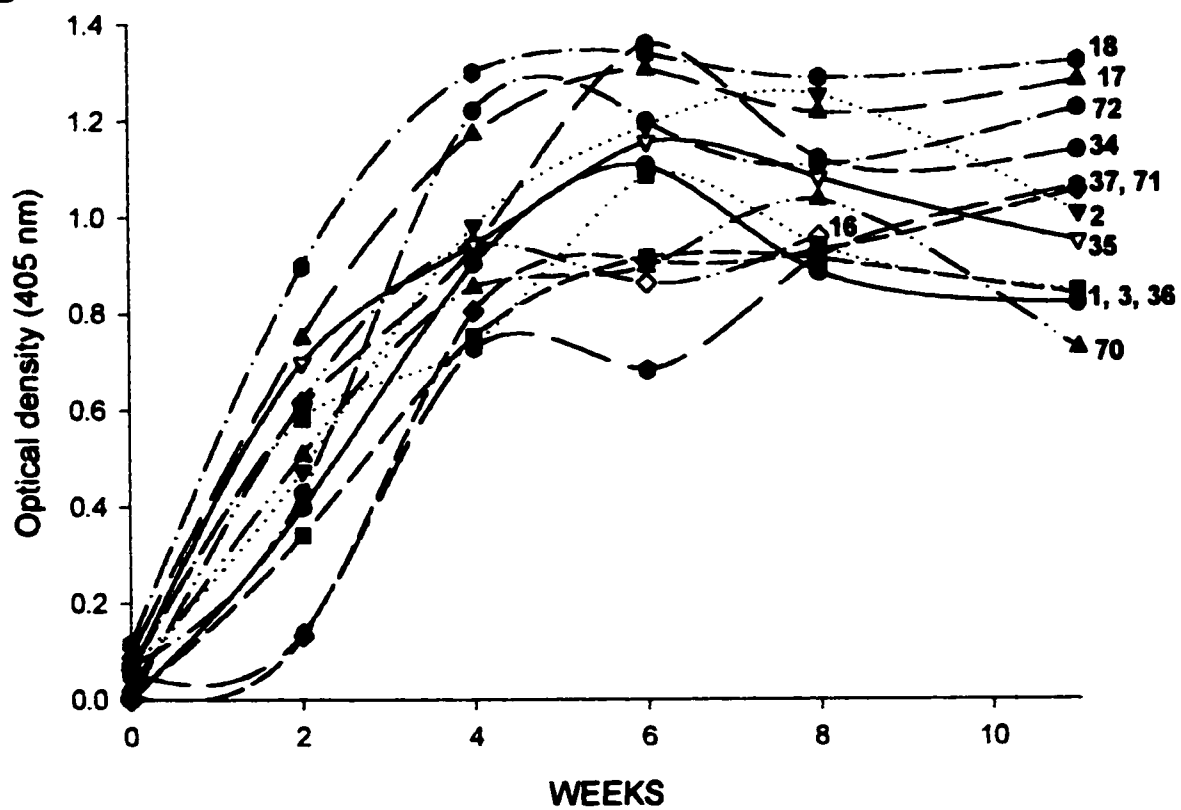
A**B**

FIGURE 18

SDS-PAGE of the rFetA protein. Five microliters of 2 different preparations of the rFetA protein (**A** – 0.43 µg/ml and **B** – 0.53 µg/ml) were subjected to SDS-PAGE at 200 volts for 45 minutes and stained with a Coomassie/Bismarck Brown stain. Lanes: MM, molecular weight markers; BSA, Bovine serum albumin

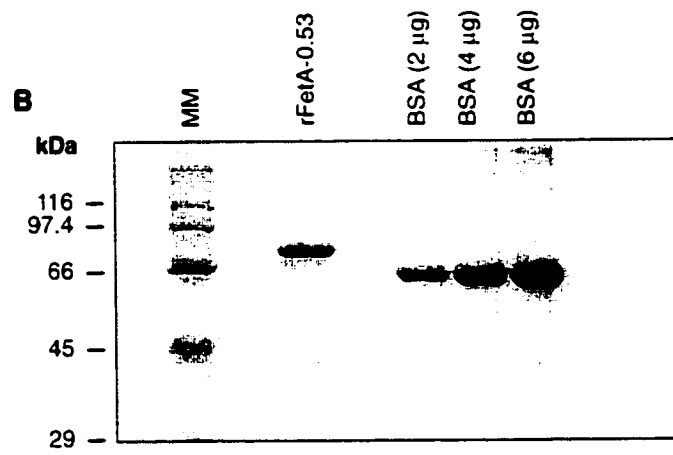
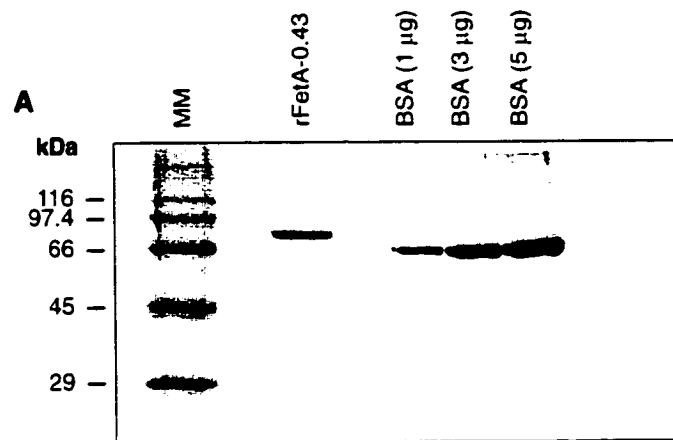


FIGURE 19

The course of an experimental homologous infection with 10^3 CFU of *H. ducreyi* strain 35000 after immunization with nHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of nHgbA ($n = 12$), 350 μ l of PBS ($n = 14$) or 100 μ g of rFetA ($n = 8$) in Freund's adjuvant and challenged with 10^3 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with nHgbA (red squares) ($p=0.068$) and rFetA (white inverted triangles) ($p=0.965$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with nHgbA (red bars) ($p=0.061$) and rFetA (white bars) ($p=0.862$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

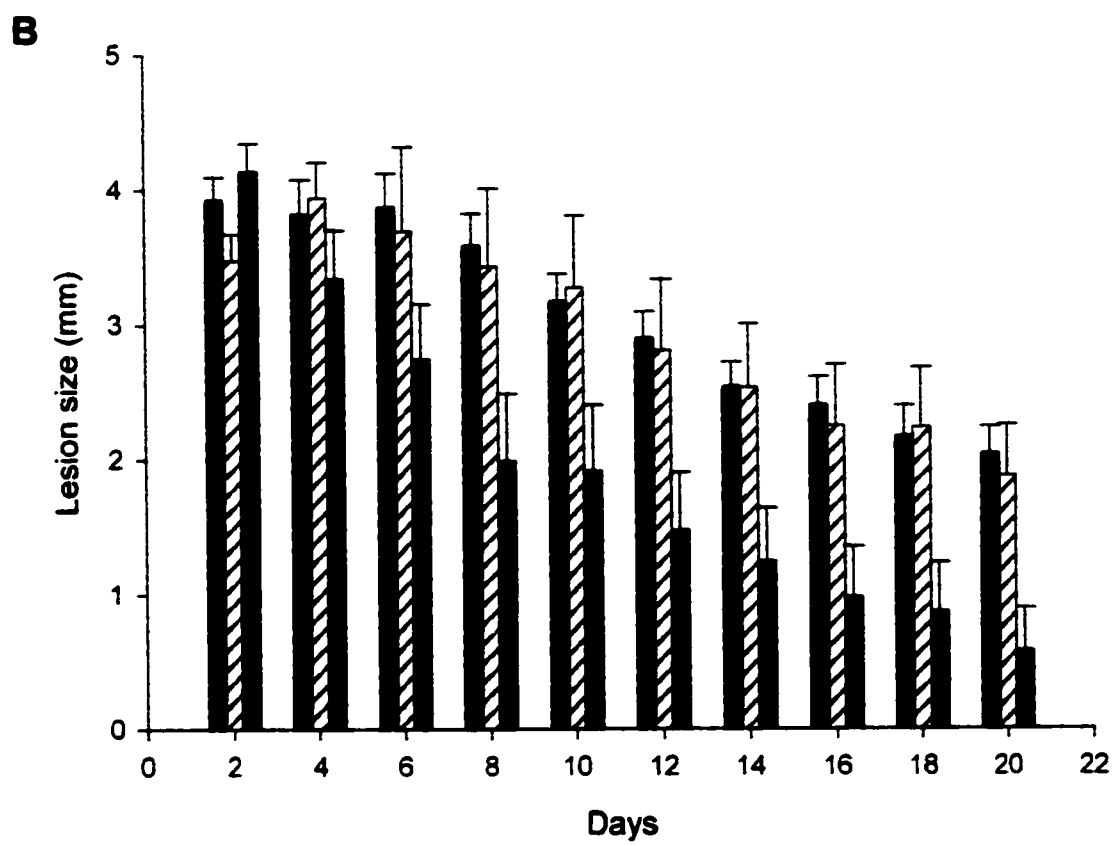
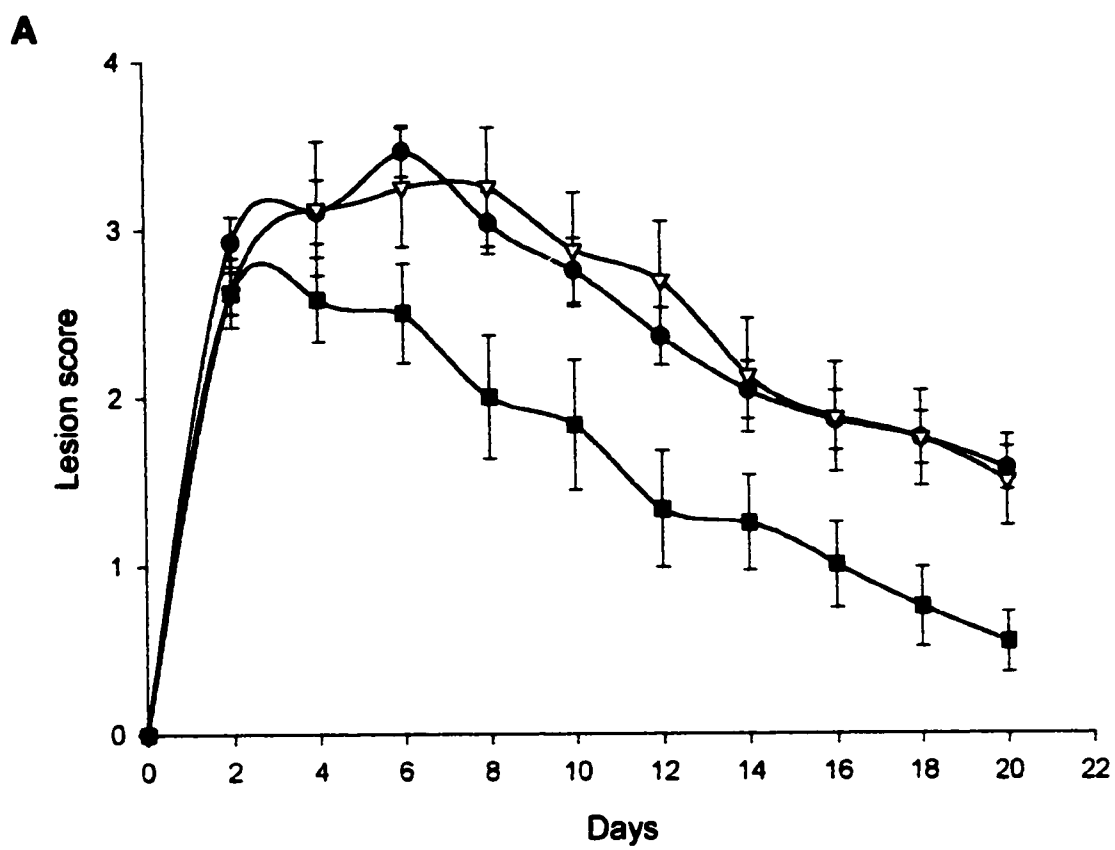
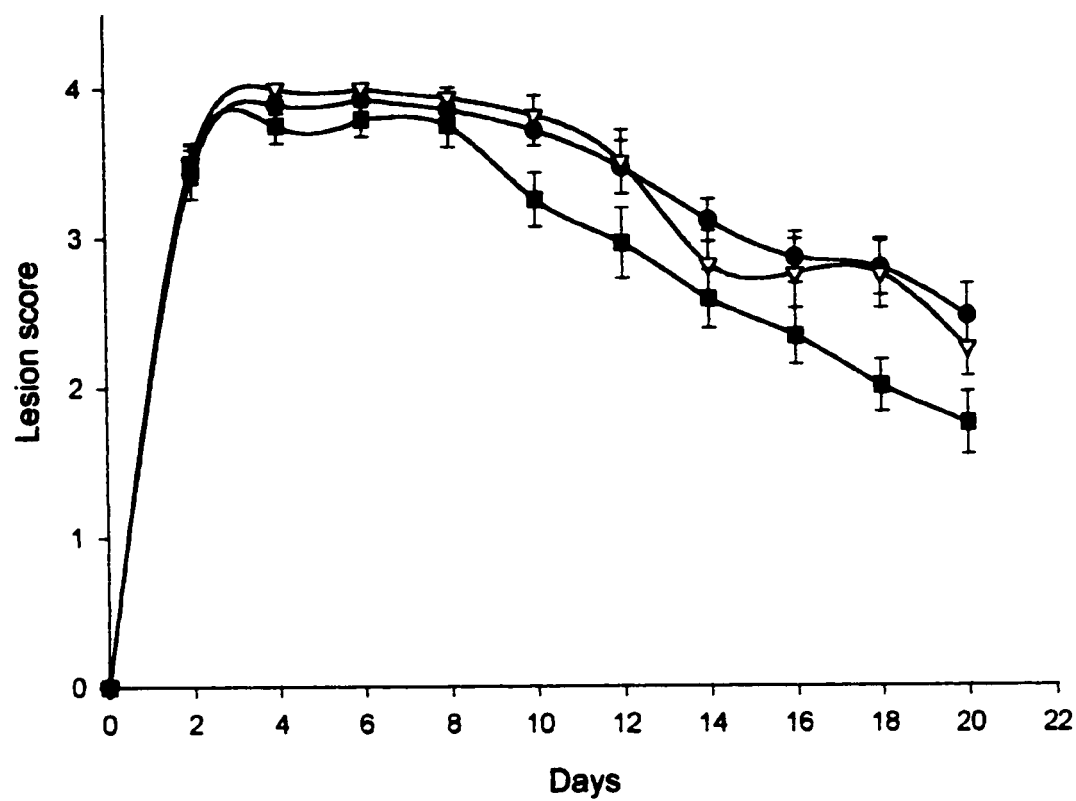
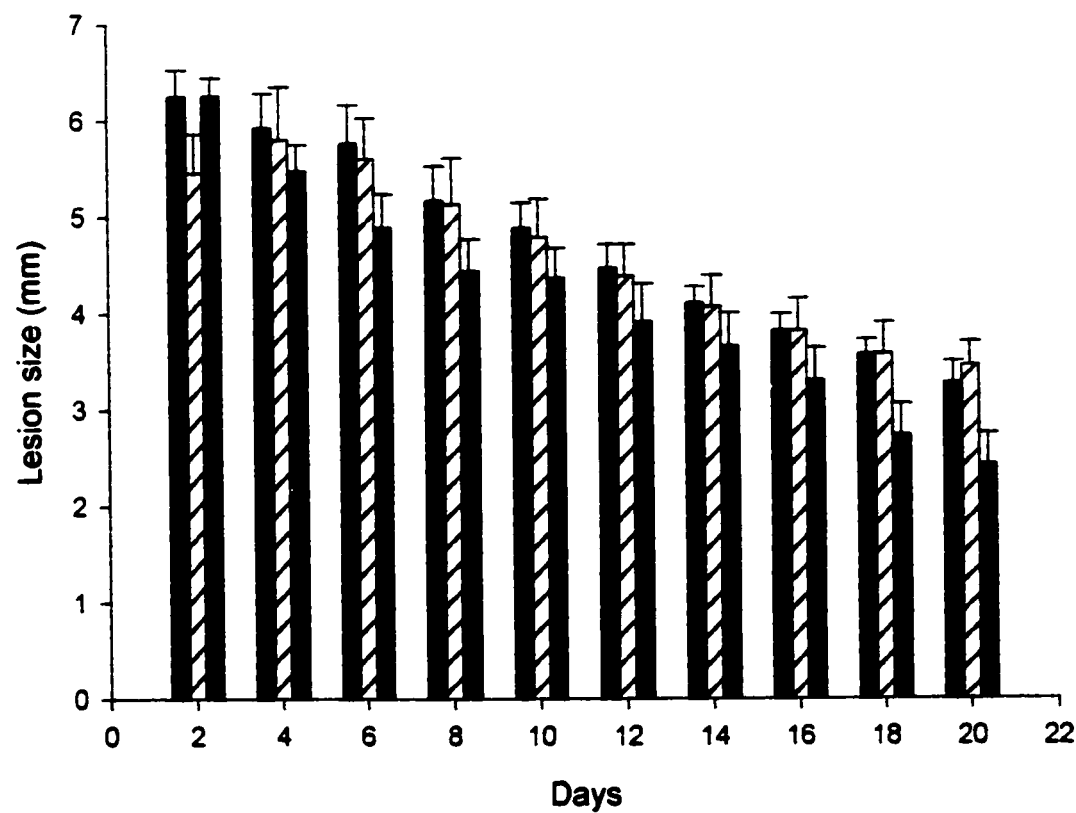


FIGURE 20

The course of an experimental homologous infection with 10^4 CFU of *H. ducreyi* strain 35000 after immunization with nHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of nHgbA ($n = 12$), 350 μ l of PBS ($n = 14$) or 100 μ g of rFetA ($n = 8$) in Freund's adjuvant and challenged with 10^4 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with nHgbA (red squares) ($p=0.481$) and rFetA (white inverted triangles) ($p=0.844$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with nHgbA (red bars) ($p=0.481$) and rFetA (white bars) ($p=0.885$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

A**B**

(figures 19B, $p=0.061$) of nHgbA-immunized rabbits were reduced compared to sham-immunized rabbits, although not to a statistically significant level. This effect was not seen in nHgbA-immunized rabbits challenged with either 10^4 or 10^5 CFU (figures 20 and 21). Rabbits immunized with rFetA did not differ from controls (white inverted triangles and white bars in figures 19 to 21 – see also table 7). Table 7 compares the characteristics of the lesions produced after a homologous experimental challenge in nHgbA-immunized and control animals. After a challenge with 10^3 CFU of *H. ducreyi* strain 35000, 4 out of 12 rHgbA-immunized rabbits developed ulcers, versus 11 of 14 control rabbits ($p\leq 0.05$), and only 25% (6/24) of the lesions of nHgbA-immunized rabbits developed ulcers, compared to 61% (17/28) in sham-immunized rabbits ($p=0.013$). The mean number of culture positive days (5 ± 4.6 versus 9.1 ± 2.6 days, $p=0.039$) was reduced in nHgbA-immunized rabbits compared to sham-immunized rabbits. For nHgbA vaccination, challenge with 10^4 CFU revealed no decrease in the number of rabbits developing any ulcer (table 7), although ulcers developed at 20 (83%) of 24 sites, versus 28 (100%) in controls ($p=0.039$). nHgbA vaccination also reduced the duration of ulcers (8.2 ± 3.7 versus 11.5 ± 3.3 days – $p=0.028$) after an infection with 10^4 CFU.

3.2.1.4 Vaccine effect of nHgbA immunization on heterologous *H. ducreyi* challenge.

Inoculation of 10^3 CFU *H. ducreyi* strain V1157 did not produce ulcers in controls or in vaccinated rabbits (data not shown). At 10^4 CFU, none of the vaccinees developed ulcers, versus 2 of 3 control rabbits developed 3 ulcers (table 8). Immunization of rabbits with nHgbA reduced both lesion score (figure 22A, $p=0.21$) and size (figure 22B, $p=0.019$) compared to sham-immunized rabbits. No organisms could be cultured from lesions of nHgbA-vaccinated rabbits challenged with strain V1157, though lesions were

FIGURE 21

The course of an experimental homologous infection with 10^5 CFU of *H. ducreyi* strain 35000 after immunization with nHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of nHgbA ($n = 12$), 350 μ l of PBS ($n = 14$) or 100 μ g of rFetA ($n = 8$) in Freund's adjuvant and challenged with 10^5 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with nHgbA (red squares) ($p=0.515$) and rFetA (white inverted triangles) ($p=0.974$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with nHgbA (red bars) ($p=0.478$) and rFetA (white bars) ($p=0.903$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

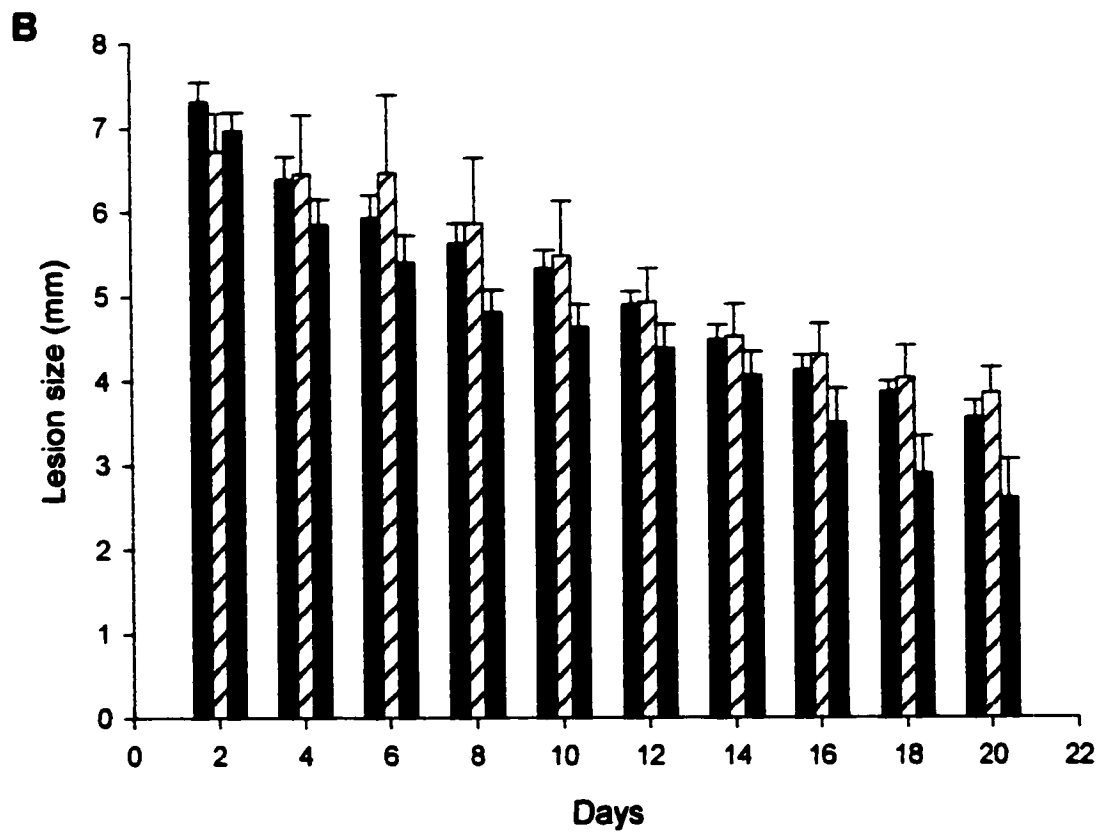
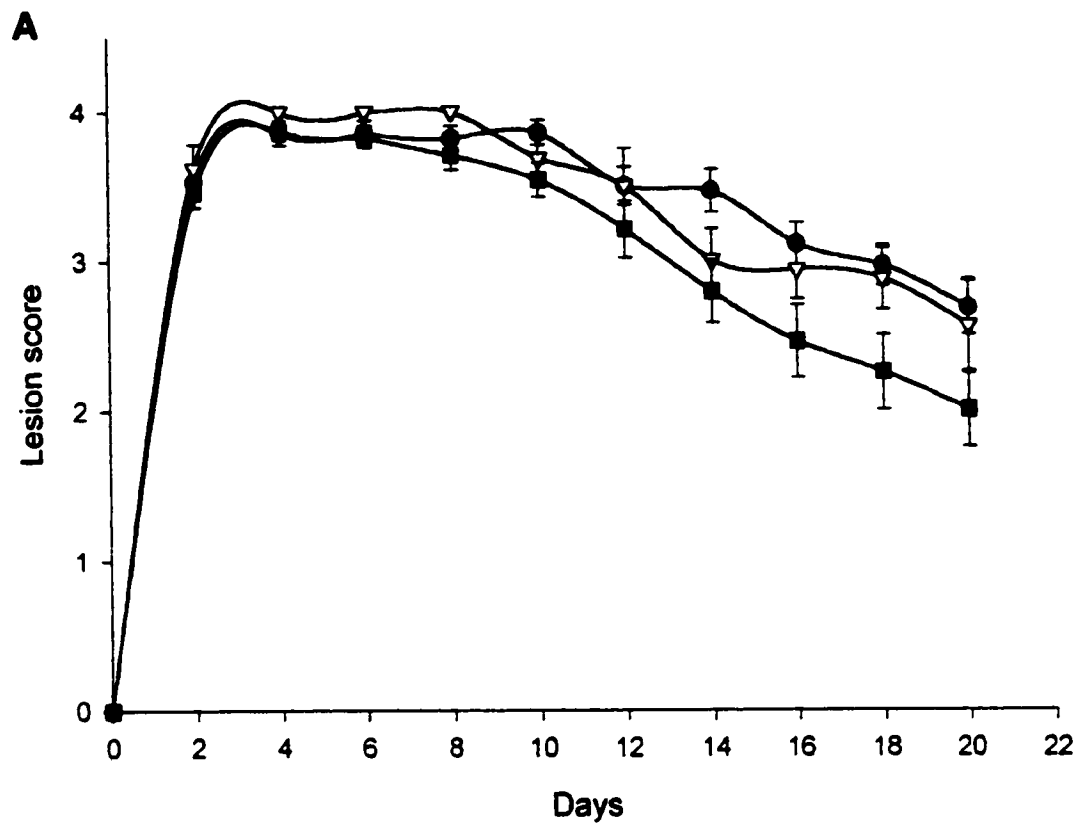


TABLE 7

Virulence of *H. ducreyi* 35000 (homologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs ^c			Mean duration of ulcers (days) ±SD
						N	E	B [*]	
Sham (n=14)	10 ⁵	5.0±1.9	10.1±4.2	51.4±6.3	4±0	0	1	13	12.0±3.2
	10 ⁴	4.2±2.1	10.7±2.8	47.2±8.8	4±0	0	0	14	11.5±3.3
	10 ³	3.7±2.0	9.1±2.6	30.2±6.9	3.5±0.5	3	5	6	4.7±3.4
nHgbA (n=12)	10 ⁵	8.1±3.2 ^a	8.2±3.2	45.0±11	4±0	0	2	10	9.2±3.6 ^a
	10 ⁴	6.7±2.3 ^a	7.8±4.9	43.6±12	3.8±0.3	1	2	9	8.2±3.7 ^a
	10 ³	6.8±2.3 ^a	5.0±4.6 ^a	19.2±12 ^a	3.0±0.7 ^a	8	2	2 ^b	2.3±3.8
rFetA (n=8)	10 ⁵	7.1±4.0	9.0±4.1	52.6±14	4±0	0	0	8	11.8±3.7
	10 ⁴	5.7±2.0	6.8±3.2 ^a	46.0±10	4±0	0	0	8	11.4±3.2
	10 ³	6.0±2.7 ^a	7.8±4.1	29.5±12	3.5±0.7	2	2	4	5.9±4.7

^{*} N = none (0/2); E = either (1/2); B = both (2/2)

^a p≤0.05, Student's t test versus sham

^b p=0.023, FET for any ulceration versus sham

^c p=0.05, FET for proportional ulceration across vaccination groups and inoculum titers

FIGURE 22

The course of an experimental heterologous infection with 10^4 CFU *H. ducreyi* strain V1157 after immunization with nHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of nHgbA ($n = 3$) and 350 μ l of PBS ($n = 3$) in Freund's adjuvant and challenged with 10^4 CFU *H. ducreyi* strain V1157 4 weeks later. Lesions were monitored 14 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with nHgbA (inverted red triangles) ($p=0.021$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with nHgbA (red bars) ($p=0.019$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

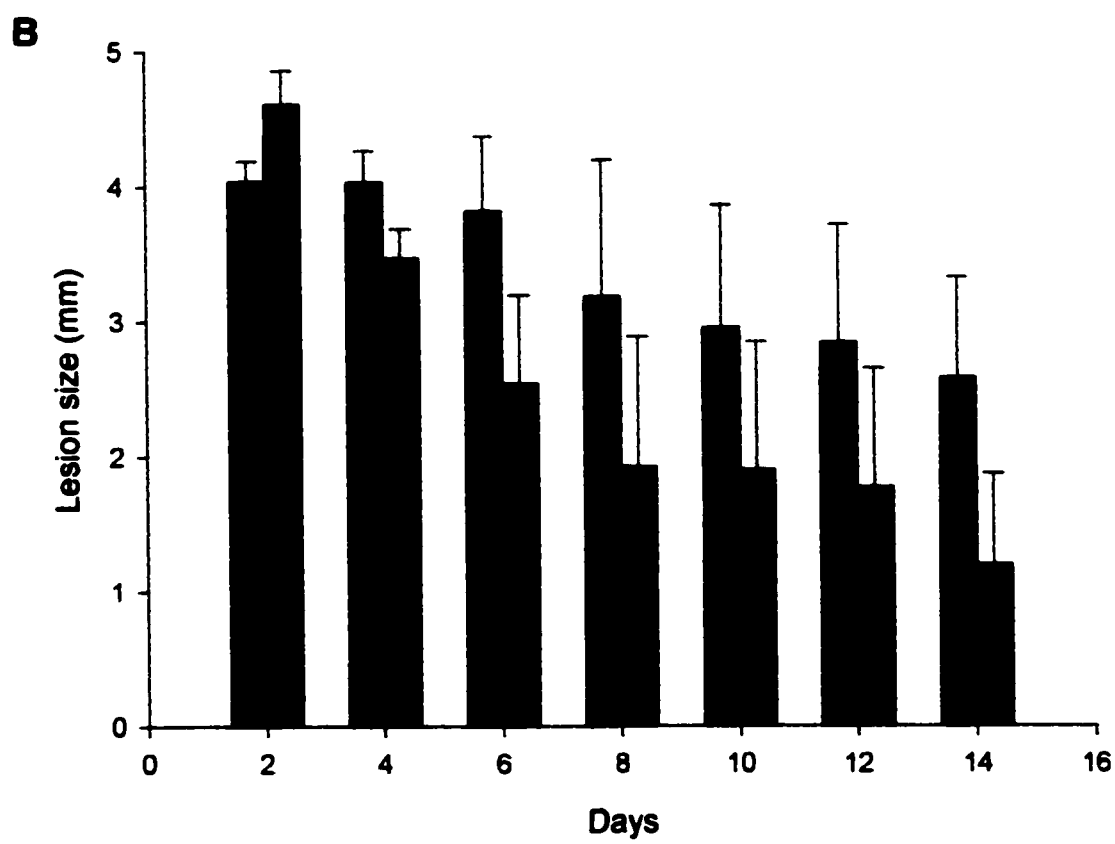
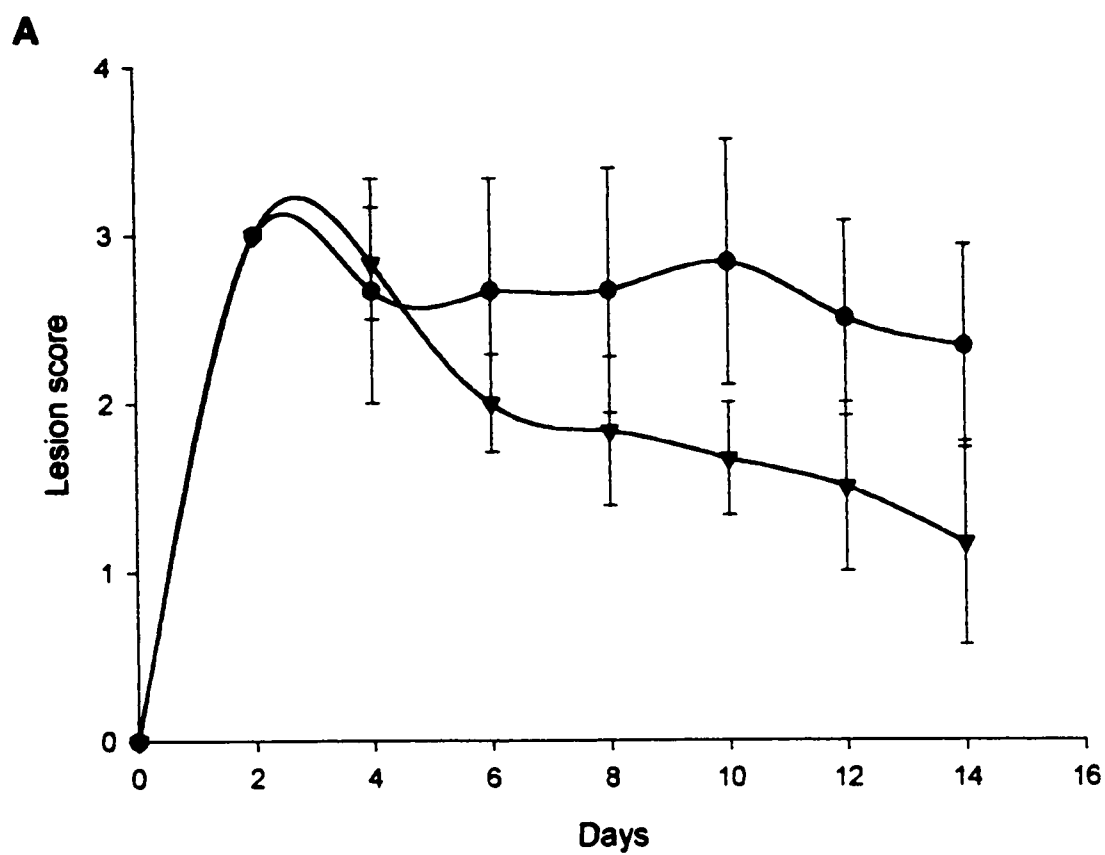


FIGURE 23

The course of an experimental heterologous infection with 10^5 CFU *H. ducreyi* strain V1157 after immunization with nHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of nHgbA ($n = 3$) and 350 μ l of PBS ($n = 3$) in Freund's adjuvant and challenged with 10^5 CFU *H. ducreyi* strain V1157 4 weeks later. Lesions were monitored 14 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with nHgbA (inverted red triangles) ($p=0.220$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with nHgbA (red bars) ($p=0.796$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

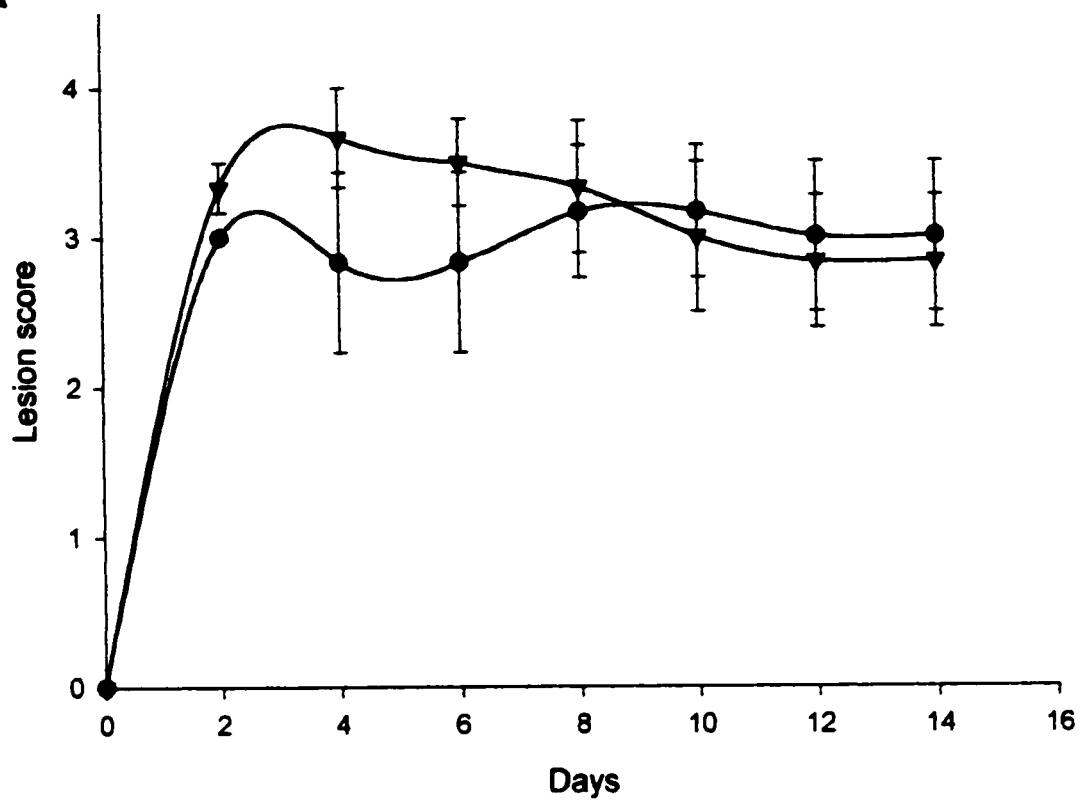
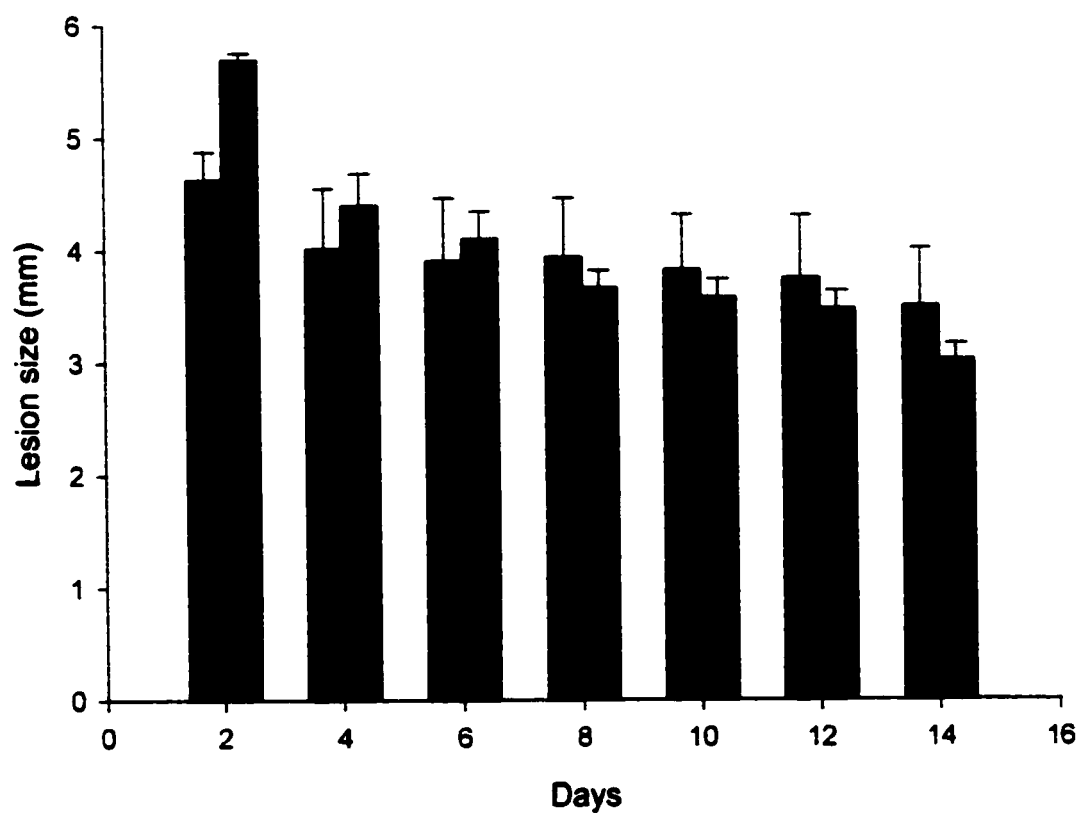
A**B**

TABLE 8

Virulence of *H. ducreyi* V1157 (heterologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean Cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs			Mean duration of ulcers (days) ±SD
						N	E	B*	
Sham (n=3)	10 ⁵	2.0±0.6	6.0±5.3	31.5±12.8	3.3±0.6	2	0	1	5.3±9.2
	10 ⁴	1.2±0.2	6.0±3.5	26.7±12.7	3.7±0.6	1	1	1	6.0±6.6
nHgbA (n=3)	10 ⁵	1.9±0.7	0±0	27.9±2.0	3.7±0.6	1	0	2	6.0±5.6
	10 ⁴	1.0±0.2	0±0 ^a	17.4±7.8	3.0±0	3	0	0	0±0

* N = none (0/2); E = either (1/2); B = both (2/2)

^a p≤0.05, Student's t test versus sham

culture positive for 6 ± 3.5 days ($p=0.04$) in sham-immunized animals (table 8). After a challenge with 10^5 CFU of strain V1157, mean cumulative lesion size and mean duration of ulcers remained similar to sham-vaccinated animals (figure 23). Lesions were culture-negative in nHgbA-immunized rabbits, while they remained culture positive for 6 ± 5.3 days in controls ($p=0.121$). Thus, these results suggest that nHgbA vaccination provides partial protection against an experimental *H. ducreyi* infection with 10^4 CFU only.

3.2.1.5 Immune response to nHgbA immunization in rabbits challenged with a heterologous strain. Figure 24 shows the reactivity of nHgbA antisera against OMP extracted from strains 35000 (figure 24A) and V1157 (figure 24B). None of the nHgbA antisera tested reacted with 100-kDa band in OMP from either strain on Western blot. However, some nHgbA antisera reacted to the two unknown proteins of molecular weight 37 to 43 kDa in the OMP preparation from strain 35000 and V1157 (figure 24).

Antisera from nHgbA-vaccinated rabbits behaved similarly in OMP- (figure 25A) and nHgbA-based (figure 25B) EIA. Antibody titers increased steadily from weeks 0 to week 4. There was a moderate increase at week 6, which stabilized at week 8 before increasing again in antisera taken at the end of the experimental model. Figure 26 examines the humoral response of individual rabbits in OMP- (figure 26A) and nHgbA-based (figure 26B) EIA.

3.2.2 rHgbA as a vaccine candidate in the TDRM of chancroid.

Although vaccination of rabbits with nHgbA, the native form of the Hg receptor of *H. ducreyi*, showed promise in providing partial protection against chancroid in an experimental animal model, extraction of this protein is quite laborious and time consuming. In fact, 16 liters of *H. ducreyi* culture were required to extract 1 to 2 mg of

FIGURE 24

Immune response of rabbits immunized with the nHgbA vaccine. Screening Western blots of nHgbA antisera to *H. ducreyi* OMP (2µg/lane) from strains 35000 (**A**) and V1157 (**B**). The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 100-kDa band of HgbA.

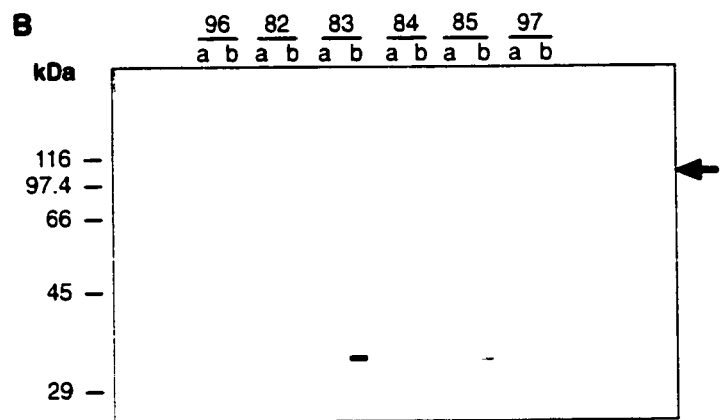
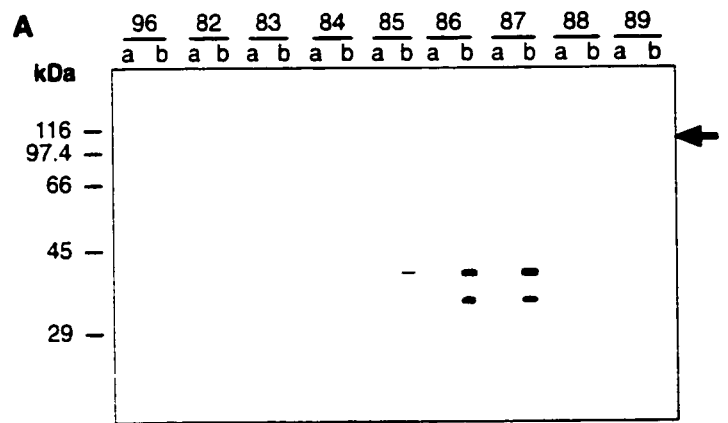


FIGURE 25

Humoral immune response of nHgbA-immunized rabbits. A - Anti-nHgbA assayed in an OMP-based EIA. B - The same serum was also assayed in an nHgbA-based EIA. The mean $\pm$ SE of optical densities is shown.

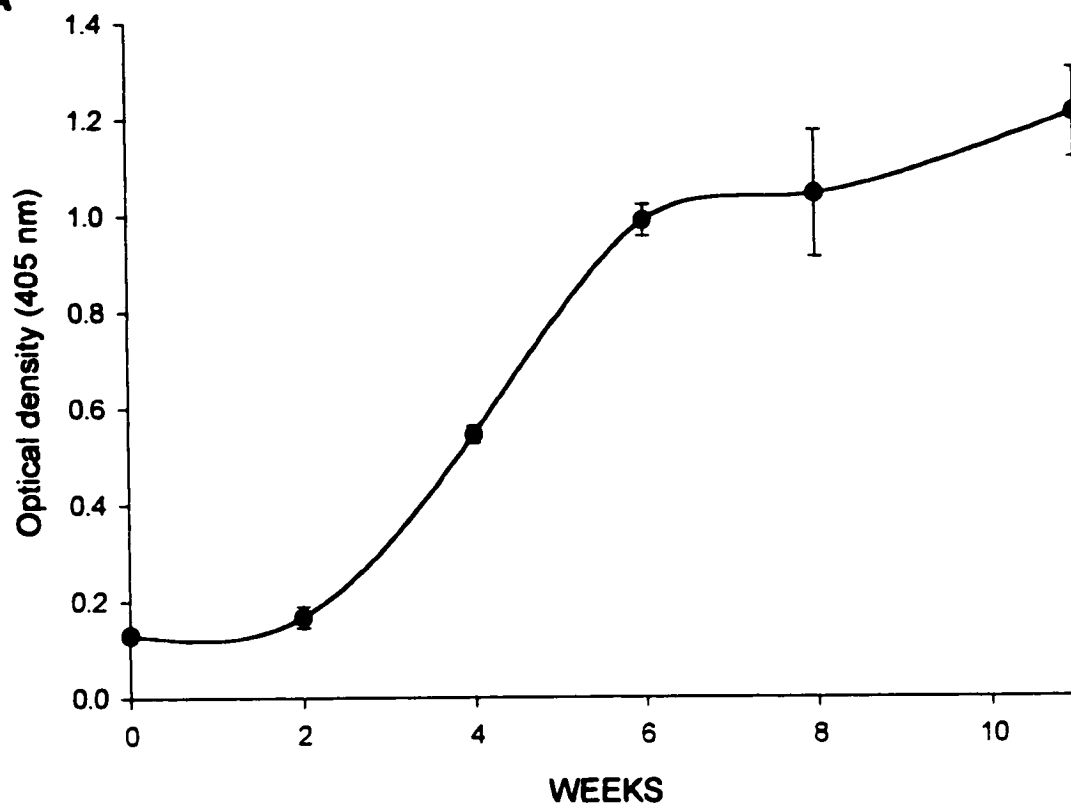
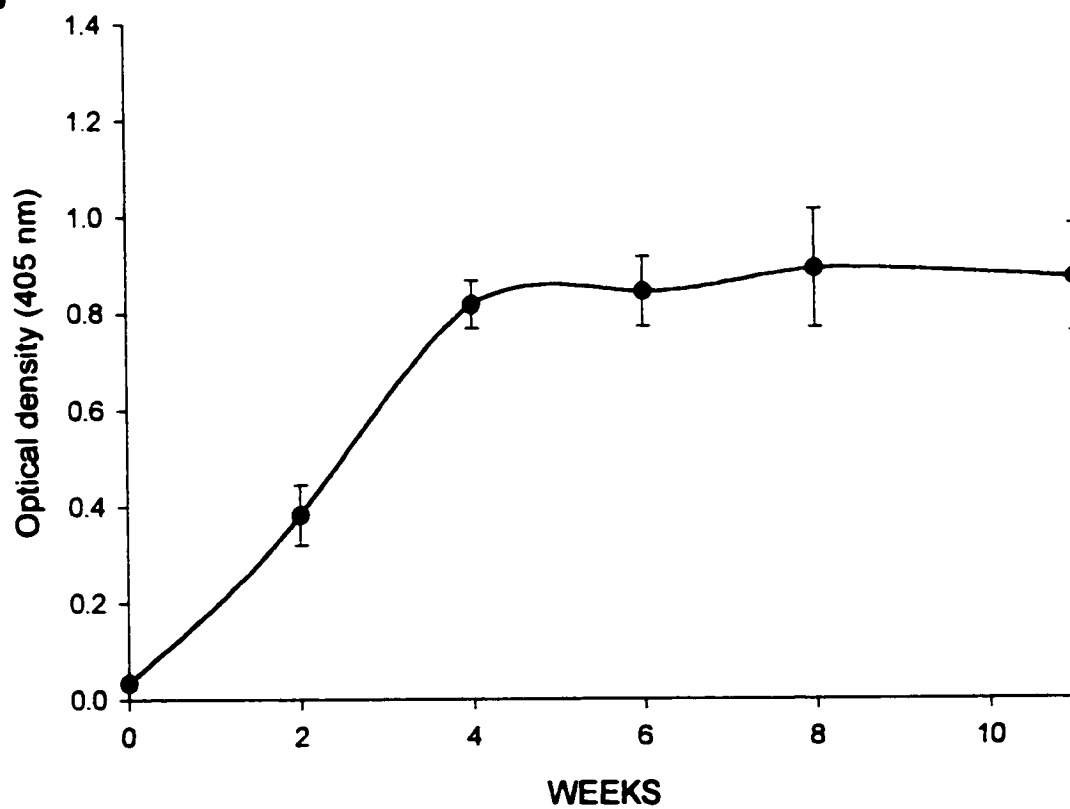
A**B**

FIGURE 26

Humoral immune response of nHgbA-immunized rabbits (per rabbit). A - Anti-nHgbA assayed in an OMP-based EIA. B - The same serum was also assayed in an nHgbA-based EIA.

LEGEND: Rabbit 82 (black circle), 83 (red inverted triangle), 84 (green square) and 85 (yellow diamond).

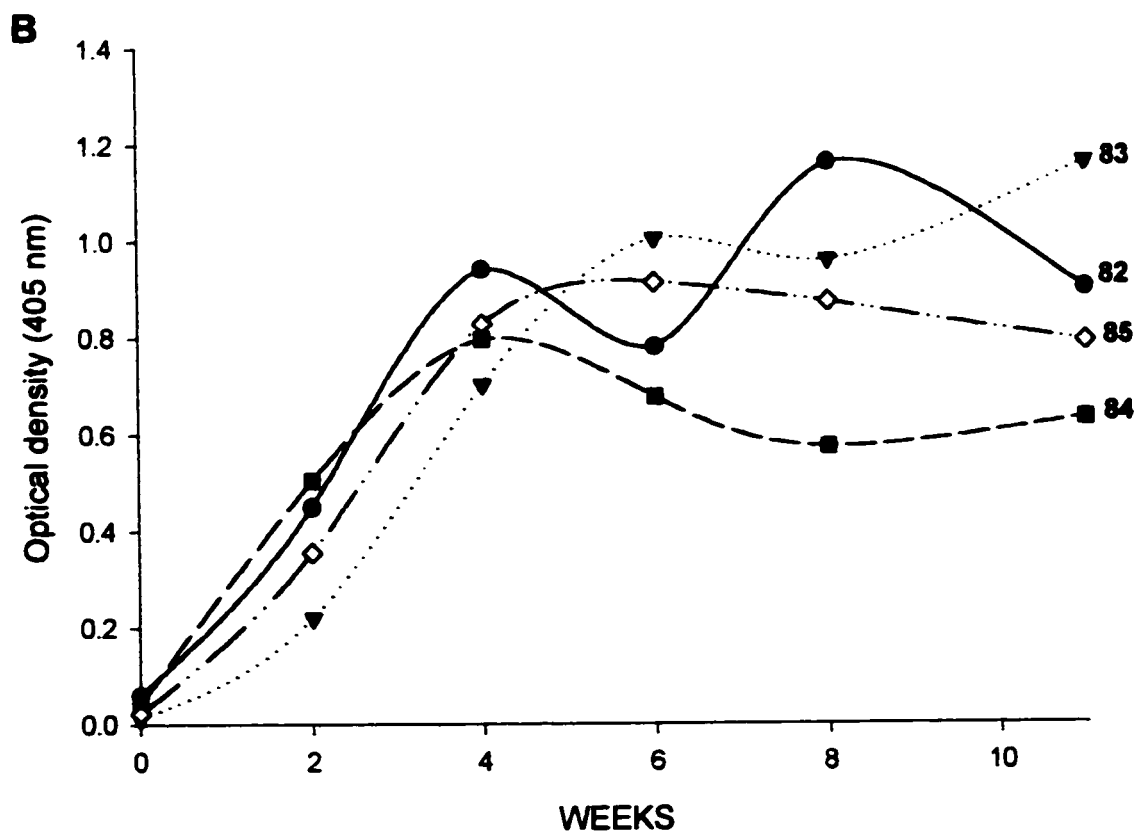
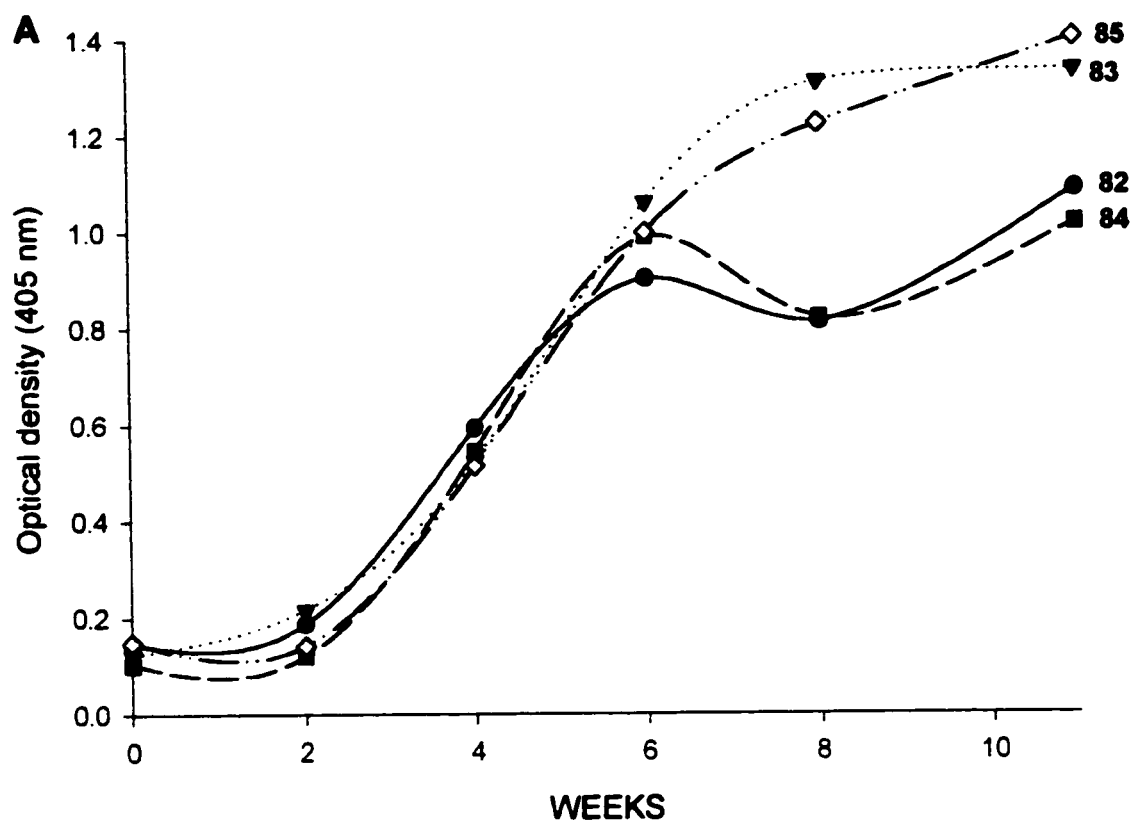


FIGURE 27

SDS-PAGE of the rHgbA extraction. Each step of the rHgbA extraction process from 2 liters of broth-grown *E. coli* in inducing conditions was monitored by SDS-PAGE (A through C). (A) pIPTG = cells before the addition of IPTG (6×10^7 cells), pRIF = cells before the addition of Rifampicin (6×10^7 cells) (30 minutes in IPTG), RIF = cells two hours after the addition of Rifampicin (6×10^7 cells). (B): SM = Starting material (15 μ l) – whole *E. coli* cells, IB = Inclusion bodies (15 μ l), US = urea soluble fraction (15 μ l), UnS = urea non-soluble fraction (15 μ l), FT = flow through (15 μ l) from nickel column, BBw = binding buffer wash (15 μ l), and MM = molecular markers (10 μ l) (C): E = Eluate (10 μ l). (D): SDS-PAGE of the rHgbA preparation (10 μ l) stained with coomassie blue.

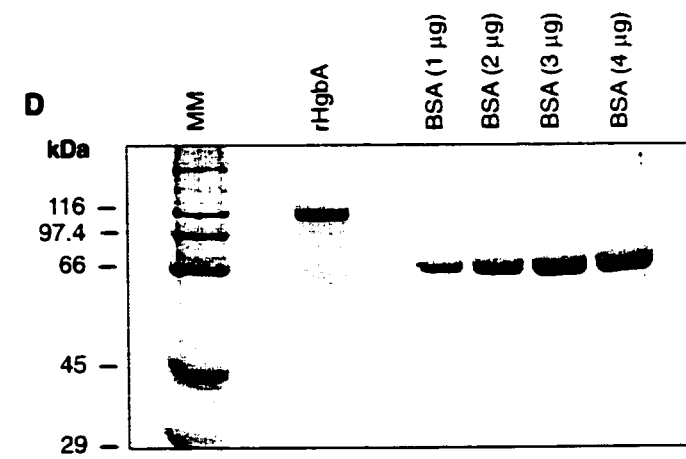
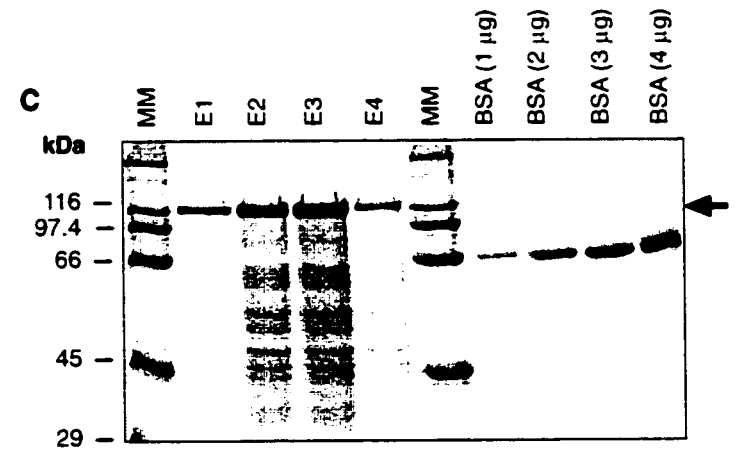
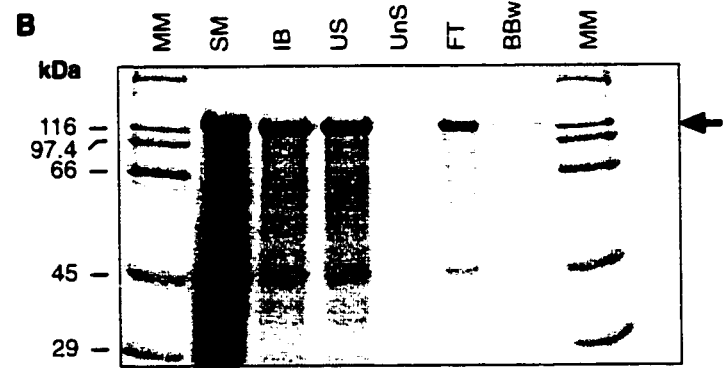
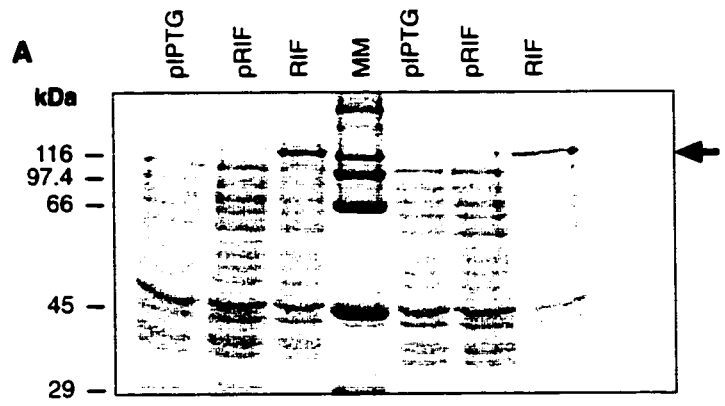


FIGURE 28

The course of an experimental homologous infection with 10^3 CFU of *H. ducreyi* strain 35000 after immunization with rHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rHgbA ($n = 15$), 350 μ l of PBS ($n = 14$) or 100 μ g of rFetA ($n = 8$) in Freund's adjuvant and challenged with 10^3 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rHgbA (red squares) ($p=0.254$) and rFetA (white inverted triangles) ($p=0.965$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = null, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rHgbA (red bars) ($p=0.340$) and rFetA (white bars) ($p=0.862$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

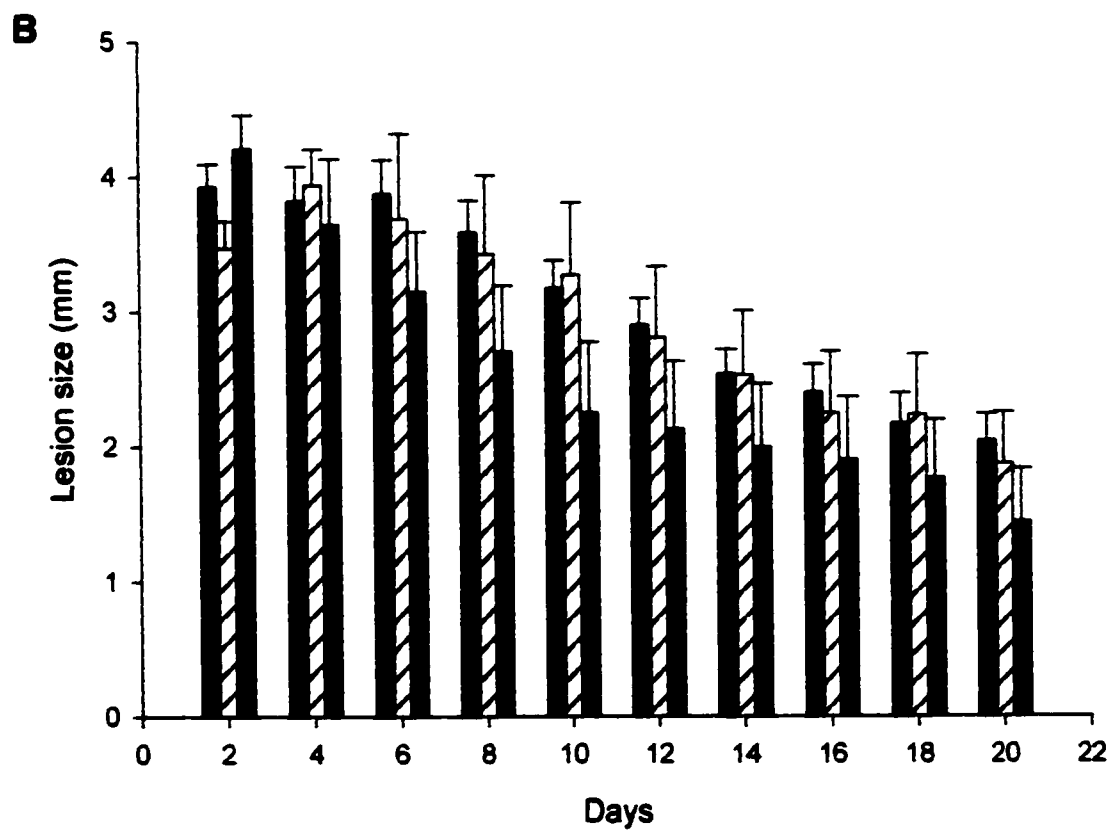
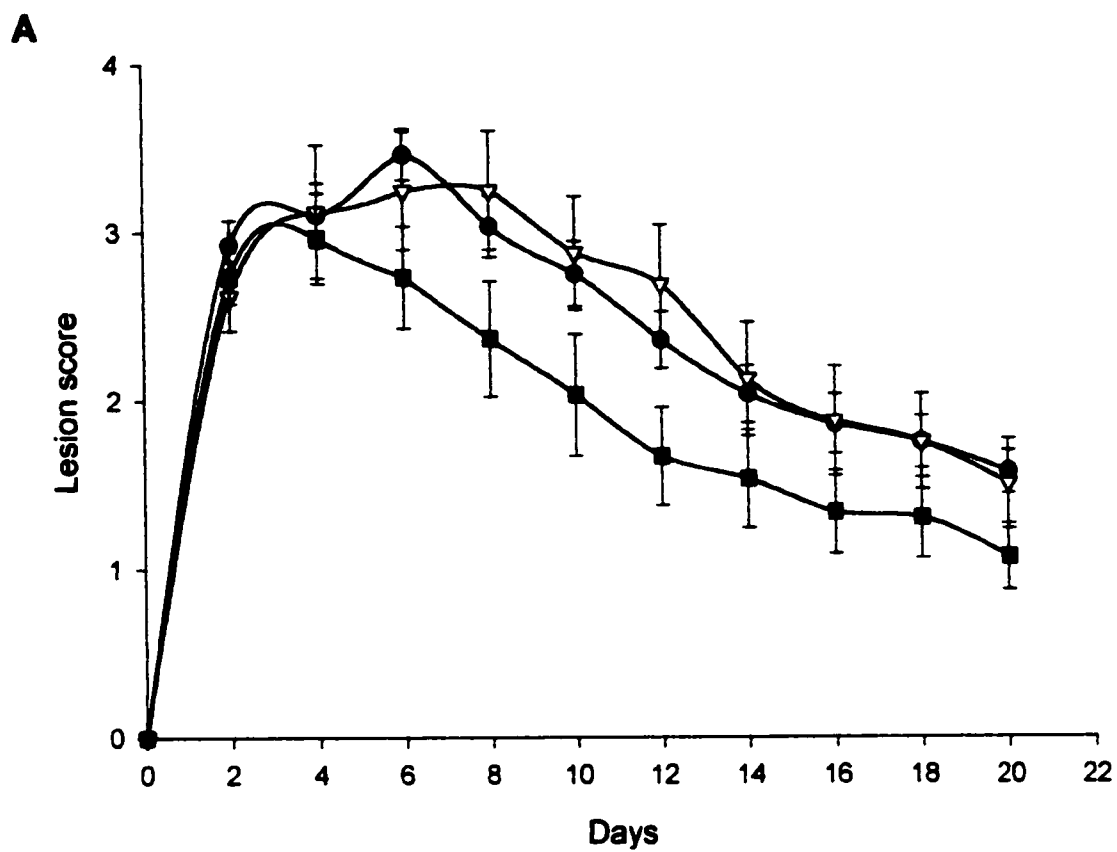


FIGURE 29

The course of an experimental homologous infection with 10^4 CFU of *H. ducreyi* strain 35000 after immunization with rHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rHgbA (n = 15), 350 μ l of PBS (n = 14) or 100 μ g of rFetA (n = 8) in Freund's adjuvant and challenged with 10^4 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesions score ($\pm$ SE) of rabbits vaccinated with rHgbA (red squares) (p=0.212) and rFetA (white inverted triangles) (p=0.844) is compared to sham-immunized rabbits (black circles). **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rHgbA (red bars) (p=0.580) and rFetA (white bars) (p=0.885) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

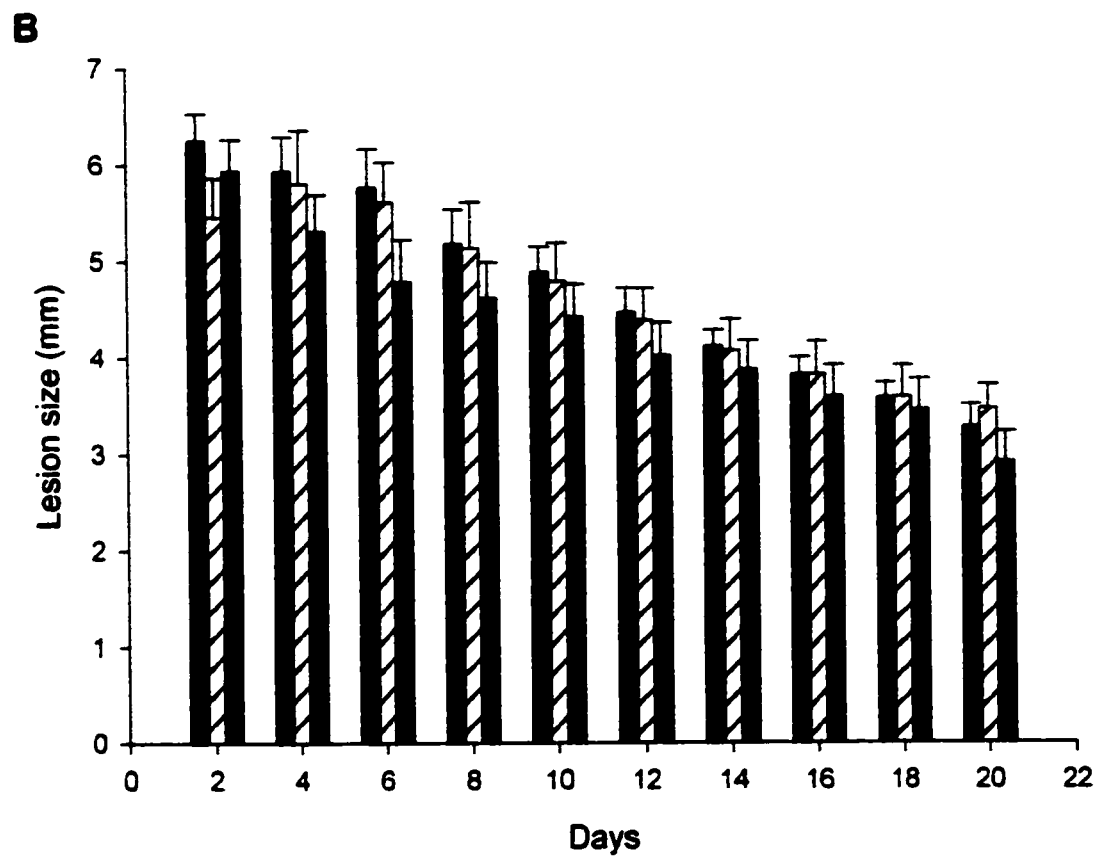
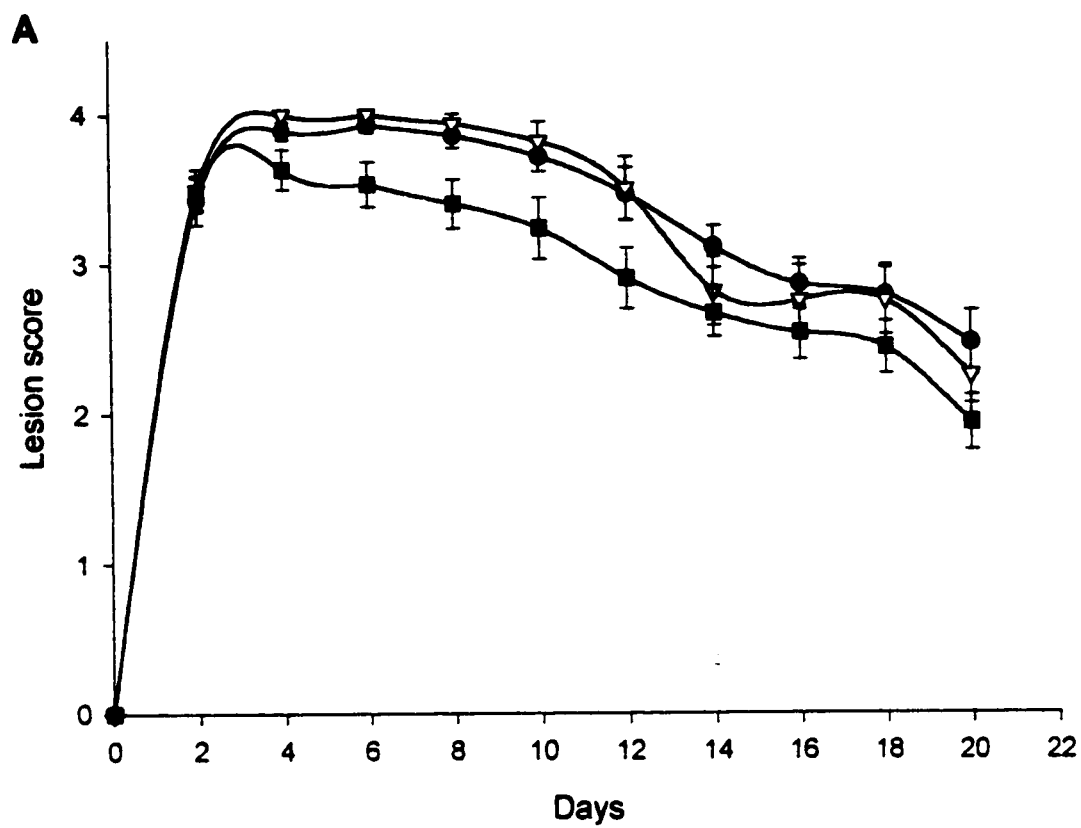


FIGURE 30

The course of an experimental homologous infection with 10^5 CFU of *H. ducreyi* strain 35000 after immunization with rHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rHgbA (n = 15), 350 μ l of PBS (n = 14) or 100 μ g of rFetA (n = 8) in Freund's adjuvant and challenged with 10^5 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesions score ($\pm$ SE) of rabbits vaccinated with rHgbA (red squares) (p=0.706) and rFetA (white inverted triangles) (p=0.974) is compared to sham-immunized rabbits (black circles). **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rHgbA (red bars) (p=0.718) and rFetA (white bars) (p=0.903) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

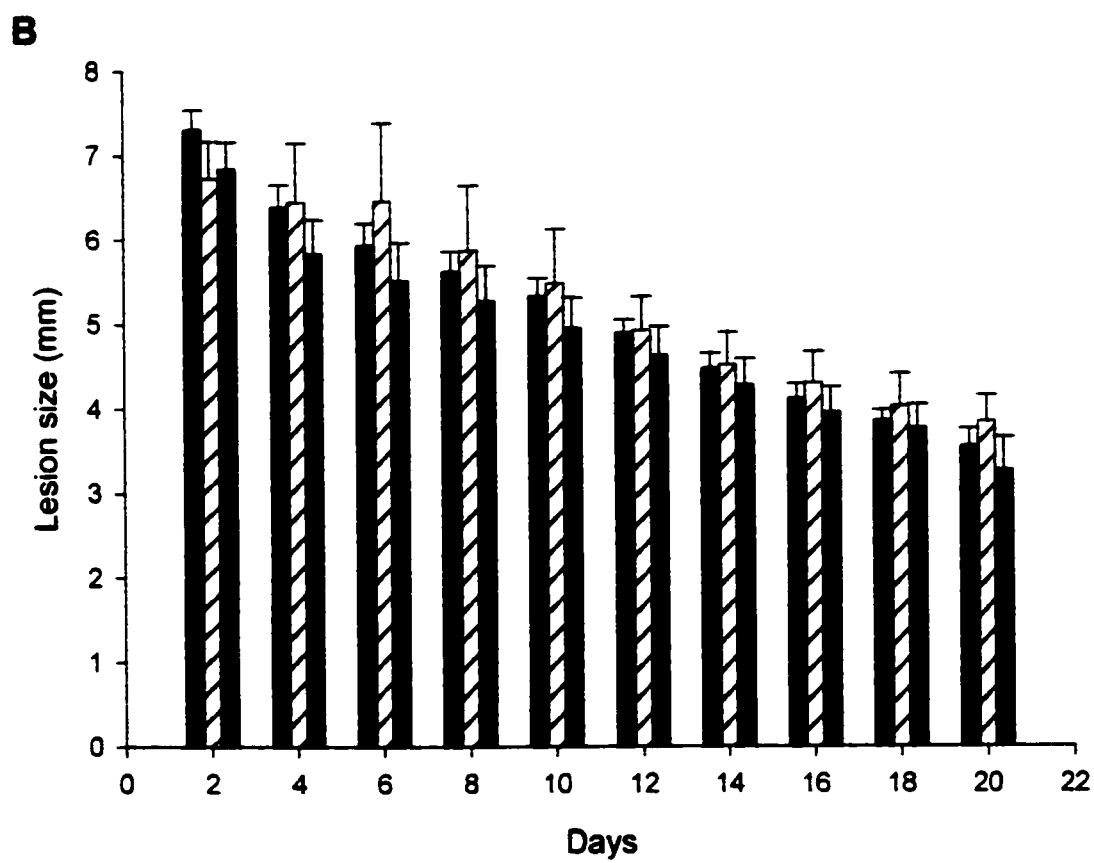
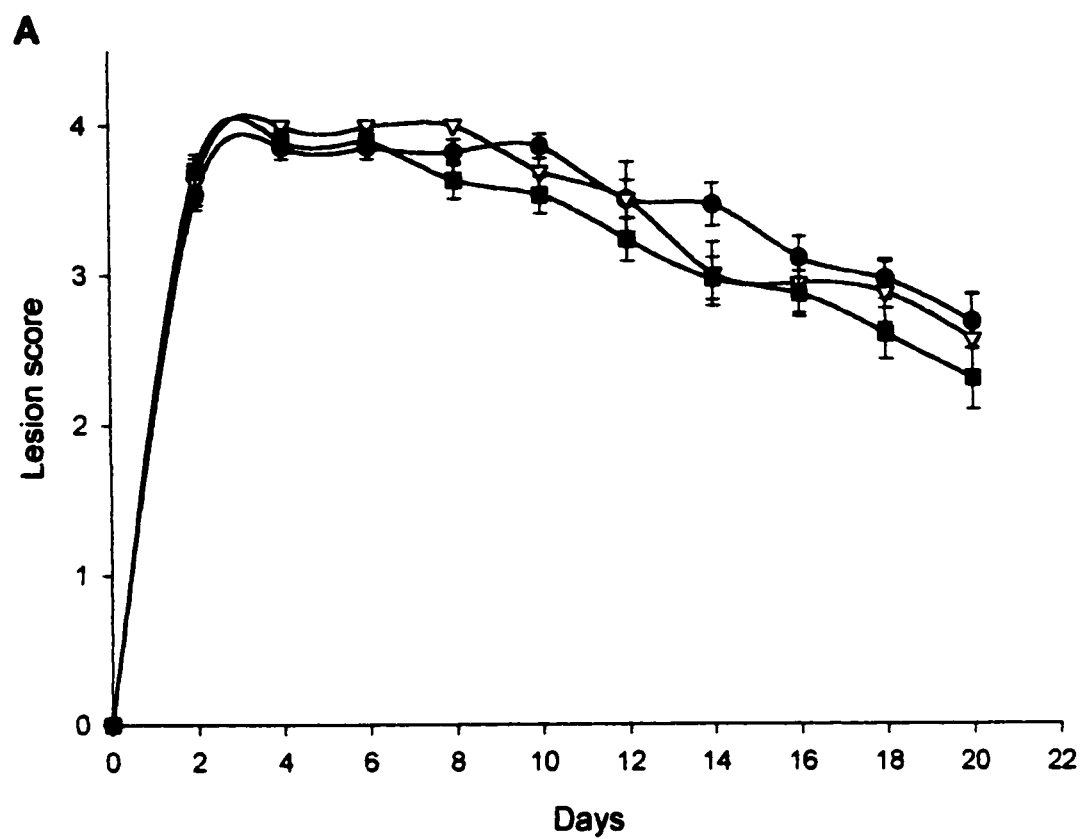


TABLE 9

Virulence of *H. ducreyi* 35000 (homologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs			Mean duration of ulcers (days) ±SD
						N	E	B*	
Sham (n=14)	10 ⁵	5.0±1.9	10.1±4.2	51.4±6.3	4±0	0	1	13	12.0±3.2
	10 ⁴	4.2±2.1	10.7±2.8	47.2±8.8	4±0	0	0	14	11.5±3.3
	10 ³	3.7±2.0	9.1±2.6	30.2±6.9	3.5±0.5	3	5	6	4.7±3.4
rHgbA (n=15)	10 ⁵	6.3±2.5	8.9±2.7	48.3±13	3.9±0.3	1	1	13	9.5±4.4
	10 ⁴	5.6±2.6	9.1±3.5	42.8±13	3.8±0.4	2	3	10	7.6±5.2 ^a
	10 ³	5.2±2.2	7.1±4.1	25.2±17	3.2±0.9	7	1	7	3.9±4.1
rFetA (n=8)	10 ⁵	7.1±4.0	9.0±4.1	52.6±14	4±0	0	0	8	11.8±3.7
	10 ⁴	5.7±2.0	6.8±3.2 ^a	46.0±10	4±0	0	0	8	11.4±3.2
	10 ³	6.0±2.7 ^a	7.8±4.1	29.5±12	3.5±0.7	2	2	4	5.9±4.7

* N = none (0/2); E = either (1/2); B = both (2/2)

^a p≤0.05, Student's t test versus sham

nHgbA. Since rHgbA extraction results in higher yields, the vaccine efficacy of the recombinant form of HgbA was assessed in the TDRM of chancroid.

3.2.2.1 Purity of rHgbA extracts. As described in materials and methods, rHgbA was extracted using a nickel column (figure 27). Although we were unable to find LPS in the rHgbA (data not shown), there were some minor contaminating proteins in the extracts (figure 27D). For this reason, this vaccinogen will be referred to as “rHgbA preparation” rather than purified rHgbA.

3.2.2.2 Vaccine effect of rHgbA immunization on homologous *H. ducreyi* challenge. The lesion score and size of rHgbA-immunized rabbits were not significantly reduced compared to controls (figures 28 to 30) at all inocula tested (10^3 , 10^4 and 10^5 CFU, respectively). In rHgbA-immunized animals challenged with 10^4 CFU of *H. ducreyi* 35000, the mean duration of ulcers (7.6 ± 5.2 versus 11.5 ± 3.3 days, $p=0.025$) and the proportion of lesions that developed into ulcers (77% versus 100%, $p=0.011$) were significantly reduced compared to controls (table 9). Other measures showed consistency in direction of effect without statistical significance (table 9).

3.2.2.3 Immune response to rHgbA immunization in rabbits challenged with a homologous strain. In Western blots of OMP, most of the rabbit sera raised to rHgbA preparation reacted strongly to a band of approximately 100 kDa, the molecular weight of HgbA, and reacted weakly to an unknown protein of approximately 60 kDa (figure 31). The tempo of serologic response on EIA to rHgbA antigen is illustrated in figures 32 and 33. Rabbits vaccinated with rHgbA produced little serologic response to OMP in the form presented in an EIA (figures 32A and 33A). Thus, rabbits immunized with the rHgbA vaccine developed a humoral response that may only recognize the recombinant

FIGURE 31

Immune response of rabbits immunized with the rHgbA vaccine. Screening Western blots of rHgbA antisera to *H. ducreyi* OMP (2 μ g/lane) from strain 35000. The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 100-kDa band of HgbA.

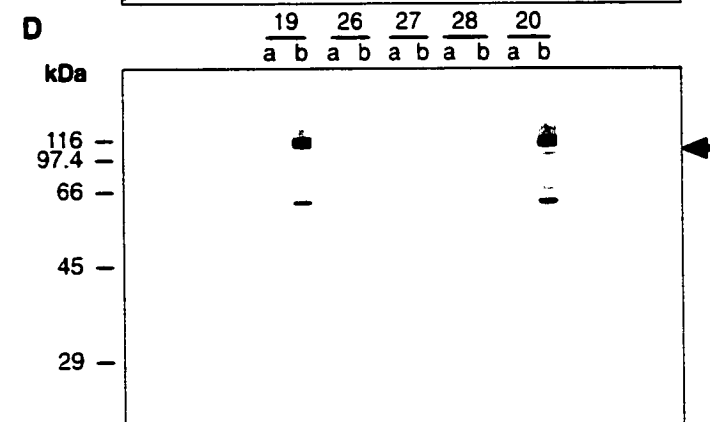
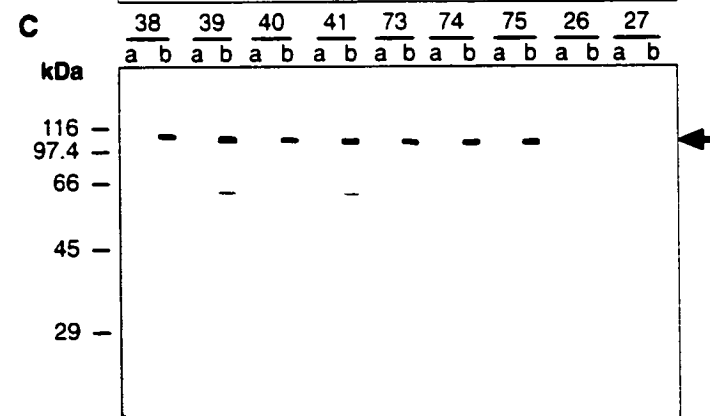
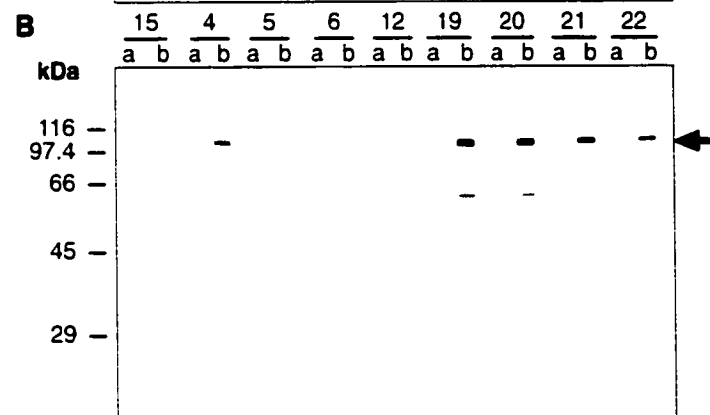
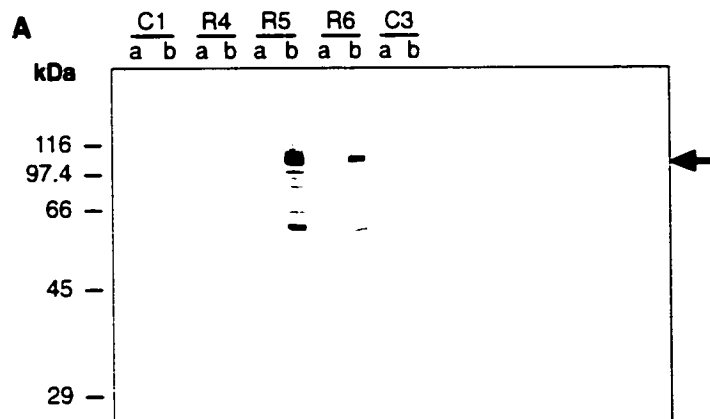


FIGURE 32

Humoral immune response of rHgbA-immunized rabbits in OMP-based (A) and rHgbA-based (B) ELA. The mean $\pm$ SE of optical densities is shown.

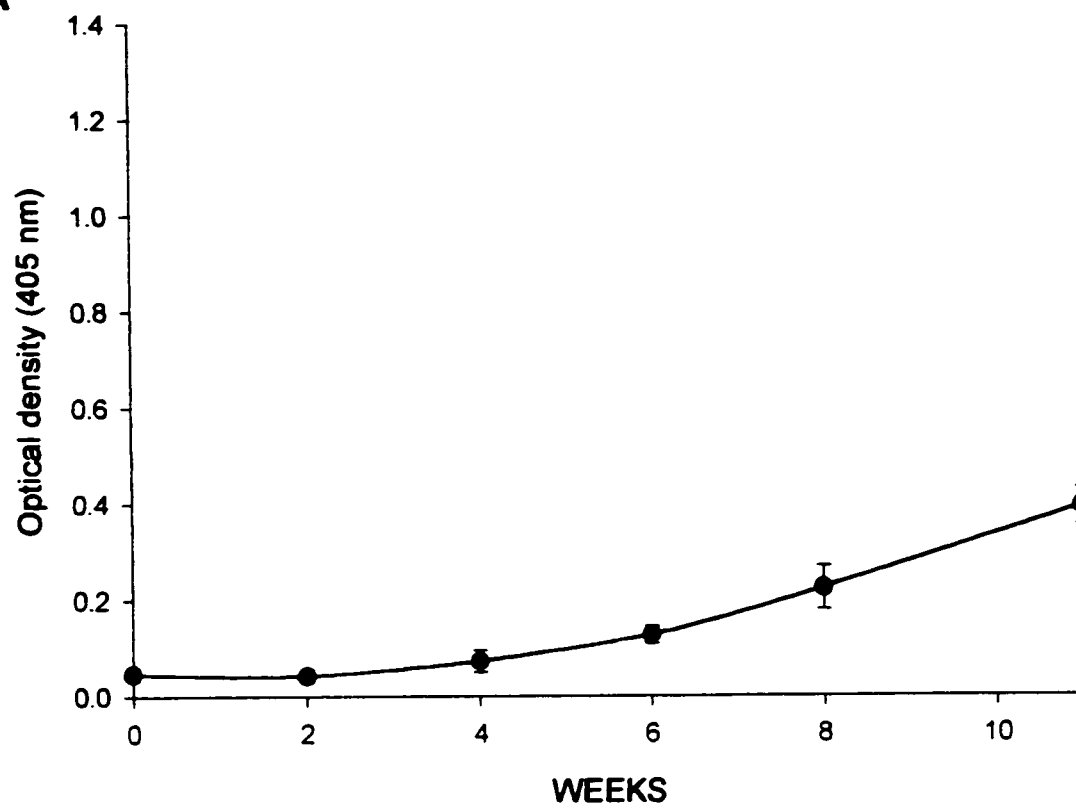
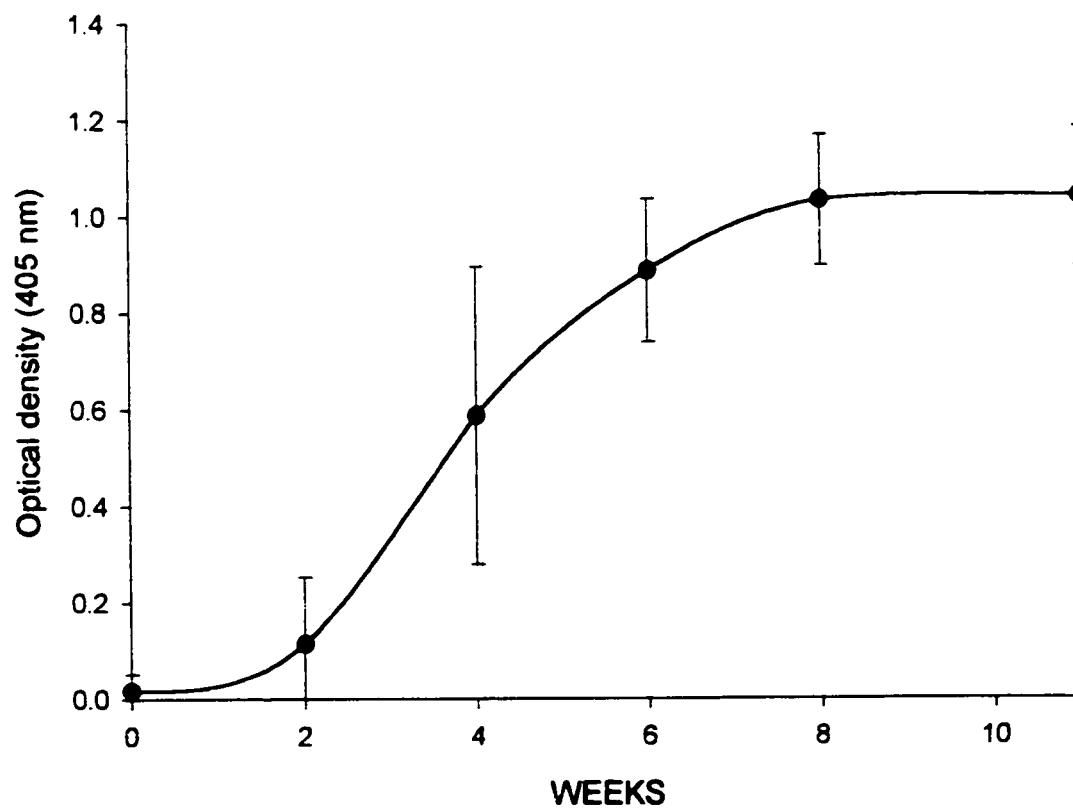
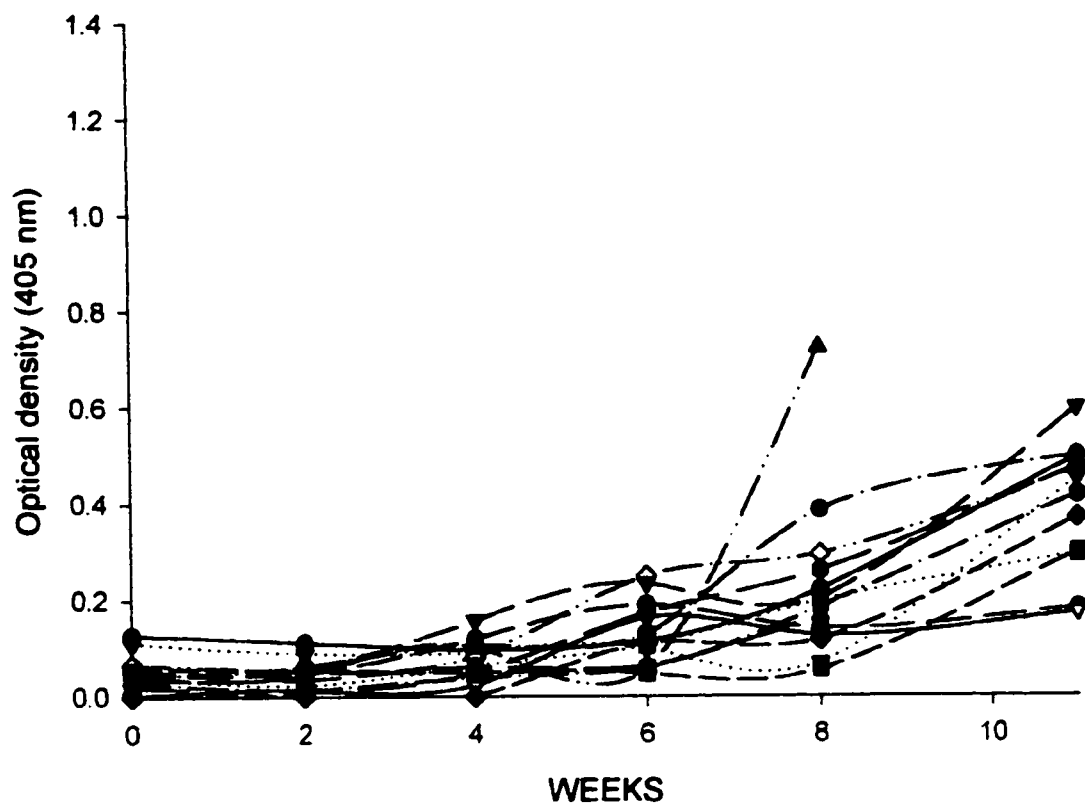
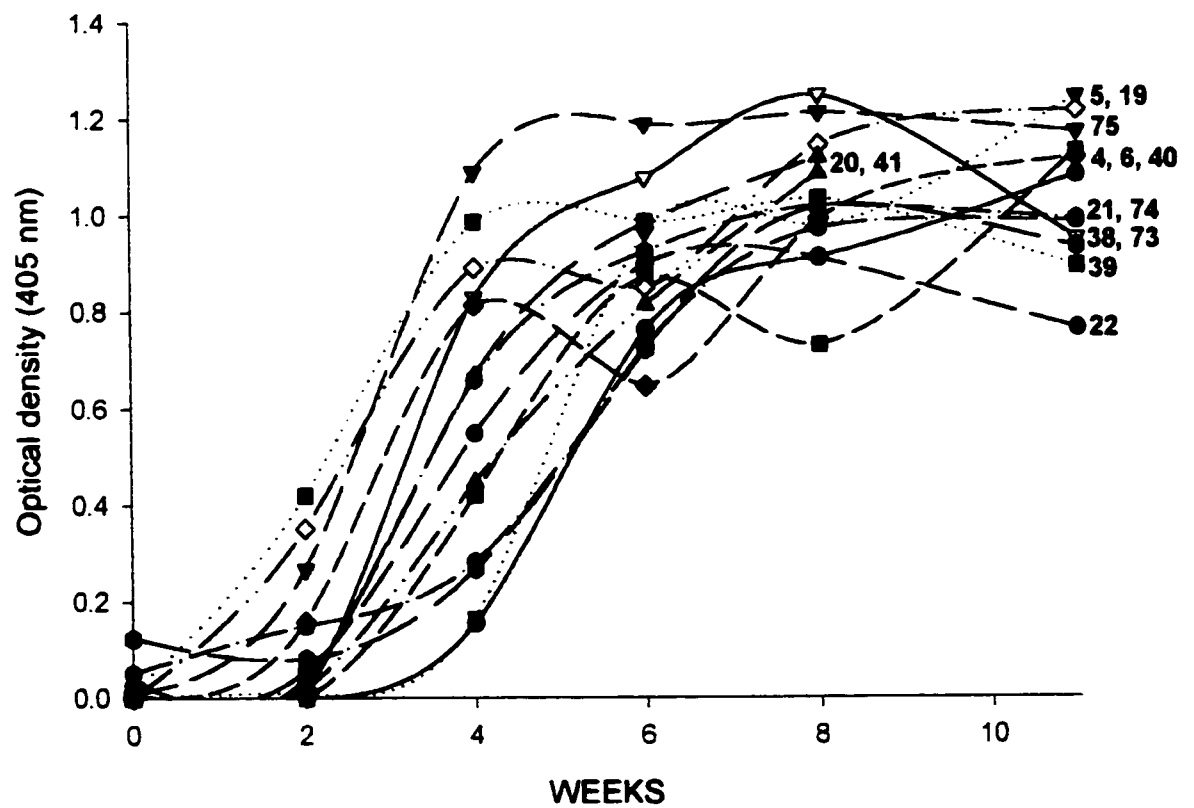
A**B**

FIGURE 33

Humoral immune response of rHgbA-immunized rabbits (per rabbit) in OMP-based (A) and rHgbA-based (B) EIA.

LEGEND: rabbit 4 (black circles), 5 (inverted red triangle), 6 (light green squares), 19 (yellow diamonds), 20 (blue triangles), 21 (pink hexagon), 22 (light blue circle), 38 (grey inverted triangle), 39 (burgundy square), 40 (dark green diamond), 41 (brown triangle), 73 (dark blue circle), 74 (purple circle) and 75 (dark green inverted triangle).

A**B**

protein in EIA or the denatured native protein in Western blots. The humoral immune response of individual rHgbA-immunized rabbits is shown in figure 33.

3.2.2.4 Vaccine effect of rHgbA immunization on heterologous *H. ducreyi* challenge.

rHgbA-immunized rabbits ($n = 6$) had statistically significantly lower lesion scores ($p=0.007$ – figure 34A) and sizes ($p=0.0014$ – figure 34B) compared to sham-immunized rabbits ($n = 3$) when challenged with 10^4 CFU of strain V1157 (figure 34). None of the lesions (0/12) in rHgbA-vaccinated rabbits developed into ulcers, while 50% (3/6, $p=0.025$) of those in the sham-immunized ulcerated (table 10). At an inoculum of 10^5 CFU, the rHgbA vaccine did not affect the course of the experimental *H. ducreyi* infection with strain V1157 (figure 35 and table 10). Thus, partial protection was observed for rHgbA vaccination against a heterologous challenge in this model with 10^4 CFU.

3.2.2.5 Immune response to rHgbA immunization in rabbits challenged with a heterologous strain.

As for rHgbA-immunized rabbits challenged with a heterologous strain, antisera from rabbits challenged with strain V1157 reacted strongly to a 100-kDa band on Western blot, as well as a 60-kDa of unknown origin (figure 36). The tempo of serologic response to rHgbA preparations, measure by EIA, was also similar (figures 37B and 38B) and the rHgbA antisera from rabbits challenged with a heterologous strain reacted poorly with OMP in the EIA format (figures 37A and 38A). Figure 38 represents the humoral immune response of each individual rabbit immunized with rHgbA and challenged with strain V1157.

FIGURE 34

The course of an experimental heterologous infection with 10^4 CFU *H. ducreyi* strain V1157 after immunization with rHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rHgbA (n = 6) and 350 μ l of PBS (n = 3) in Freund's adjuvant and challenged with 10^4 CFU *H. ducreyi* strain V1157 4 weeks later. Lesions were monitored 14 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rHgbA (inverted red triangles) (p=0.007) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rHgbA (red bars) (p=0.014) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

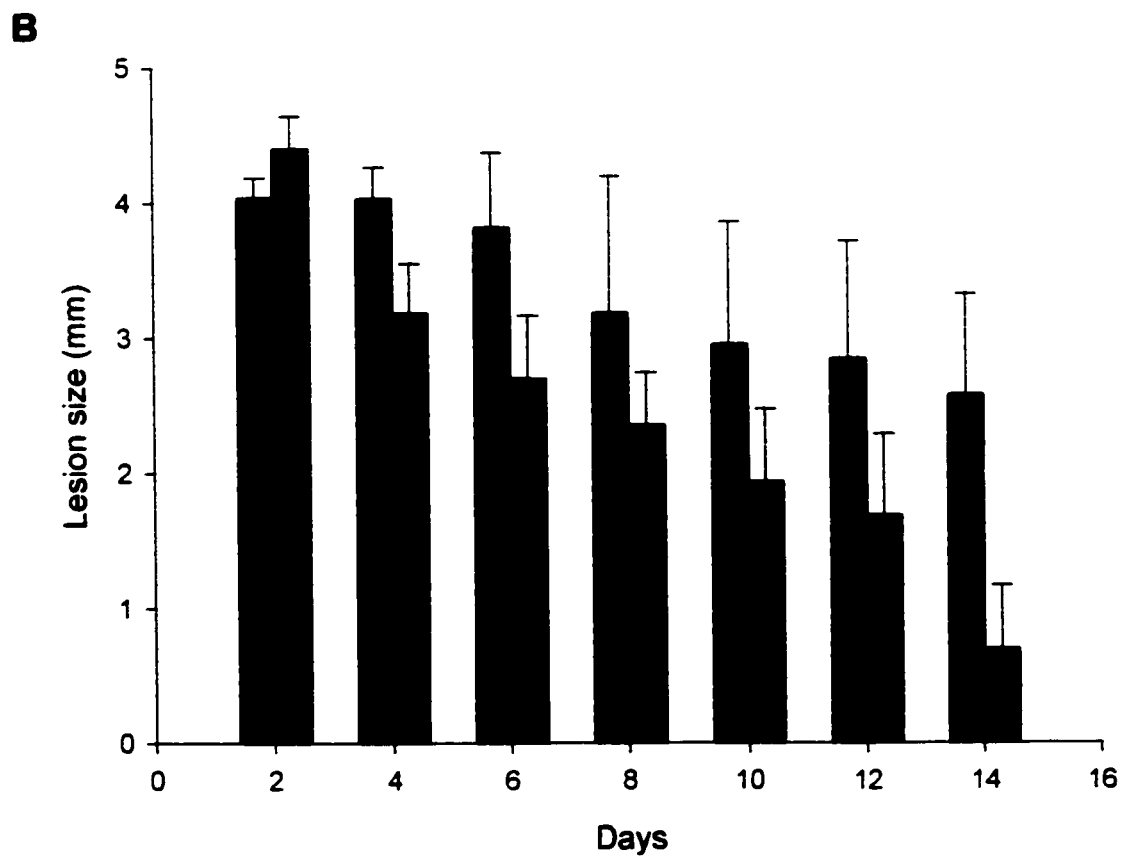
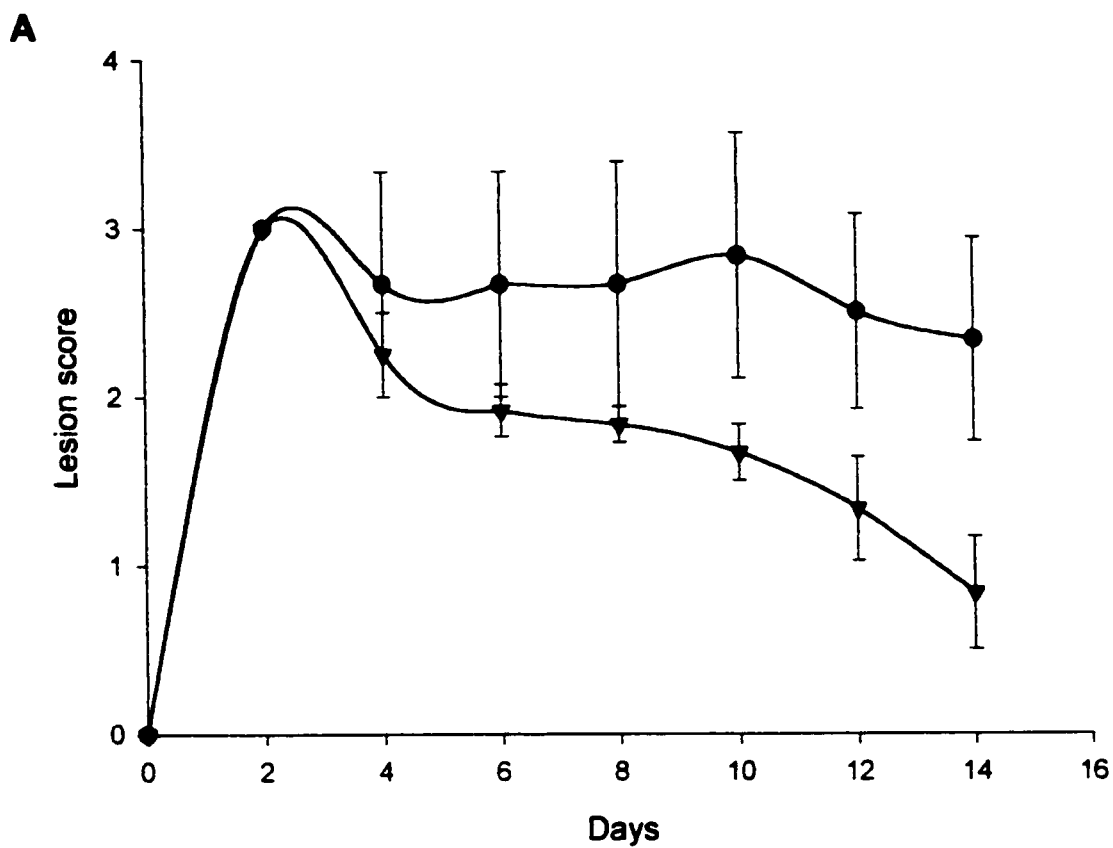


FIGURE 35

The course of an experimental heterologous infection with 10^5 CFU *H. ducreyi* strain V1157 after immunization with rHgbA. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rHgbA (n = 6) and 350 μ l of PBS (n = 3) in Freund's adjuvant and challenged with 10^5 CFU *H. ducreyi* strain V1157 4 weeks later. Lesions were monitored 14 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rHgbA (inverted red triangles) ($p=0.121$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rHgbA (red bars) ($p=0.603$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

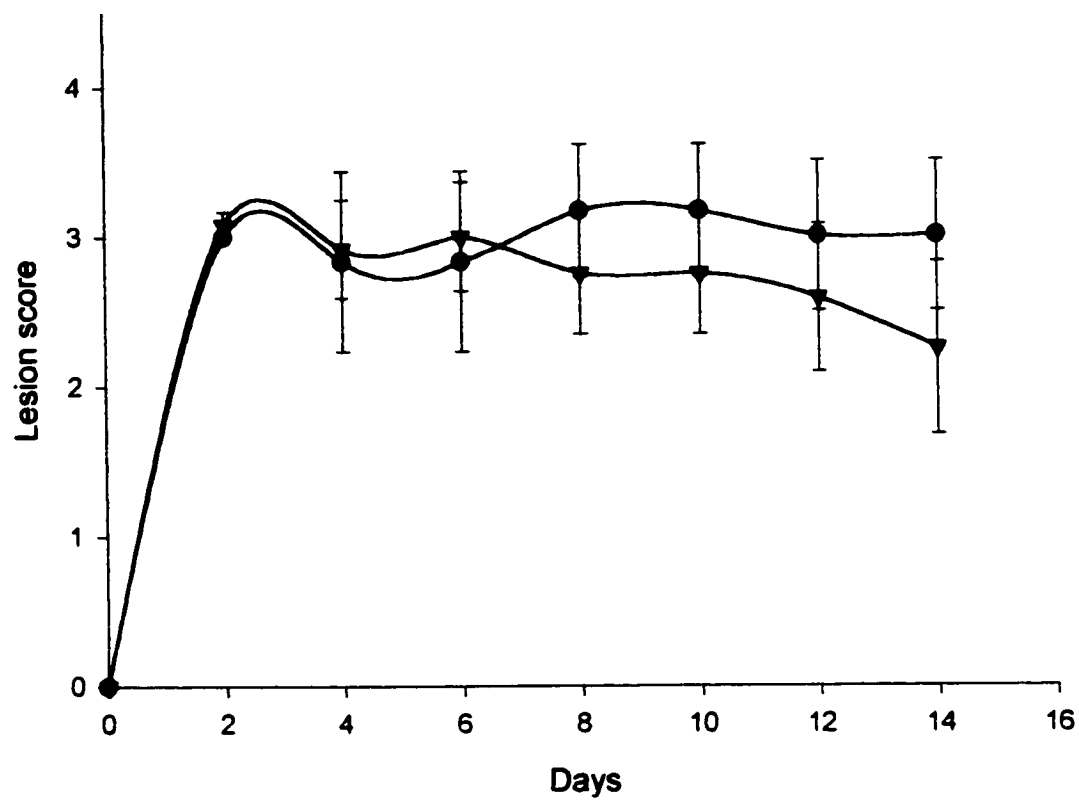
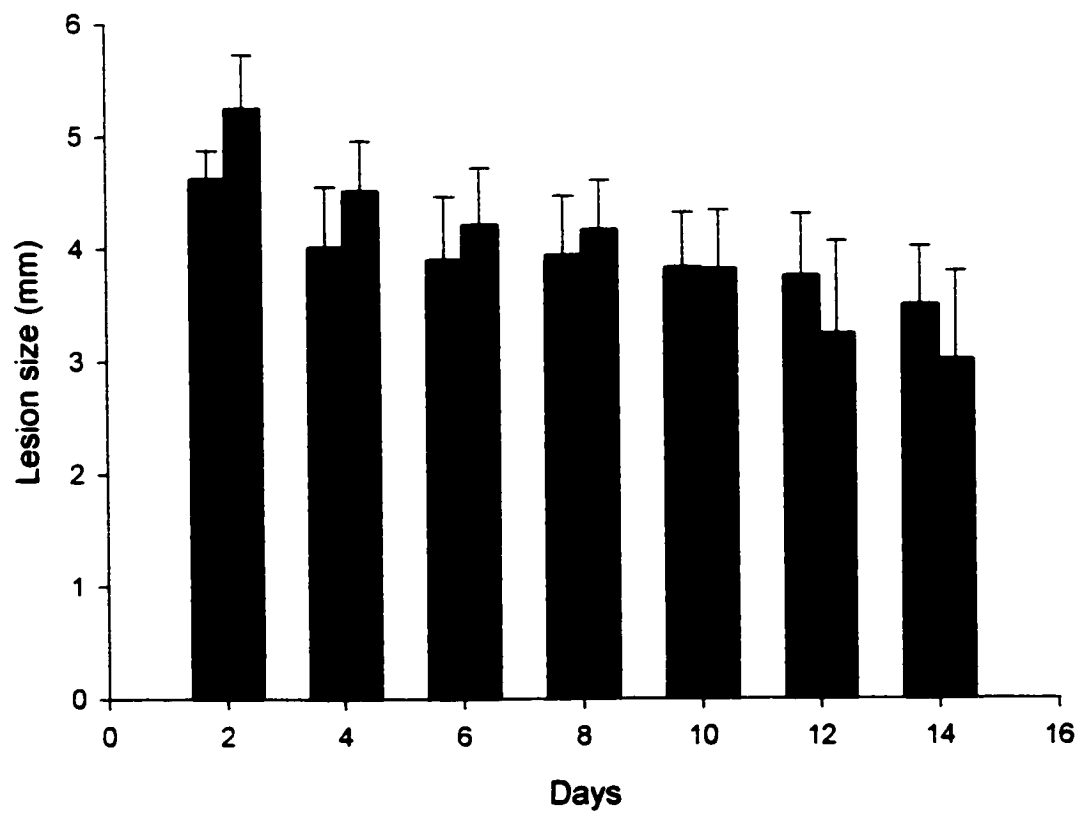
A**B**

TABLE 10

Virulence of *H. ducreyi* V1157 (heterologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean Cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs			Mean duration of ulcers (days) ±SD
						N	E	B*	
Sham (n=3)	10 ⁵	2.0±0.6	6.0±5.3	31.5±12.8	3.3±0.6	2	0	1	5.3±9.2
	10 ⁴	1.2±0.2	6.0±3.5	26.7±12.7	3.7±0.6	1	1	1	6.0±6.6
rHgbA (n=6)	10 ⁵	1.5±0.3	1.7±2.3	34.1±16.0	3.4±0.5	3	1	2	5.5±7.5
	10 ⁴	1.2±0.2	2.0±3.1	18.0 ± 8.0	3±0	6	0	0	0±0

* N = none (0/2); E = either (1/2); B = both (2/2)

FIGURE 36

Immune response of rabbits immunized with the rHgbA vaccine. Screening Western blots of rHgbA antisera to *H. ducreyi* OMP (2µg/lane) from strains 35000 (A and B) and V1157 (C). The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 100-kDa band of HgbA.

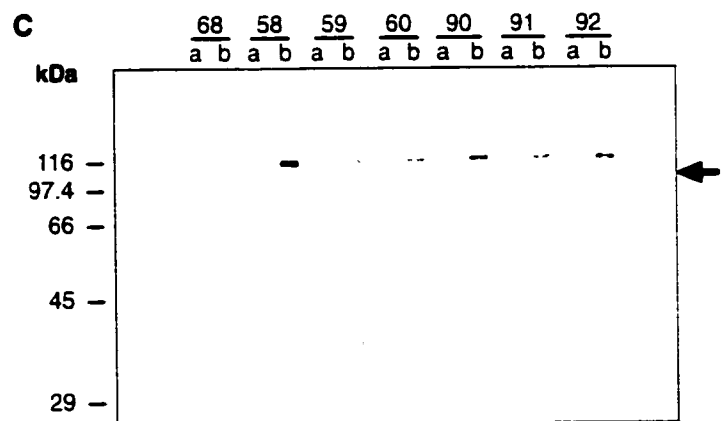
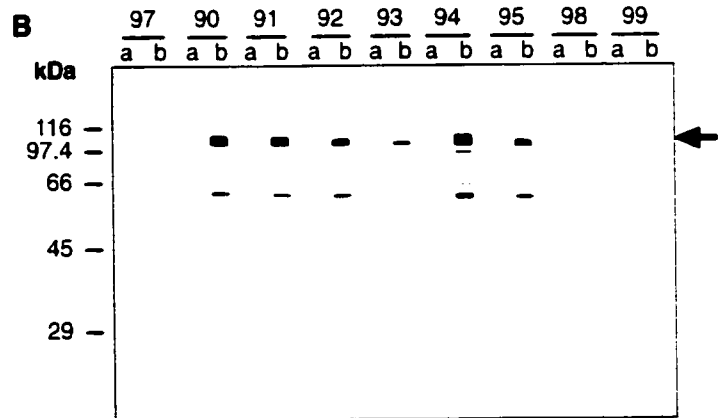
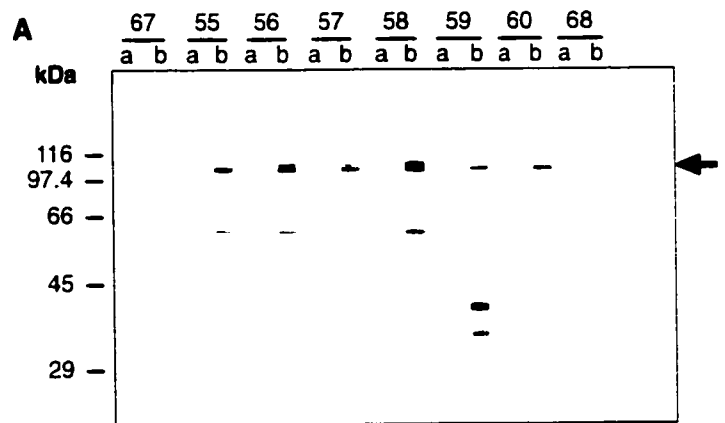


FIGURE 37

Humoral immune response of rHgbA-immunized rabbits in OMP-based (A) and rHgbA-based (B) EIA. The mean $\pm$ SE of optical densities is shown.

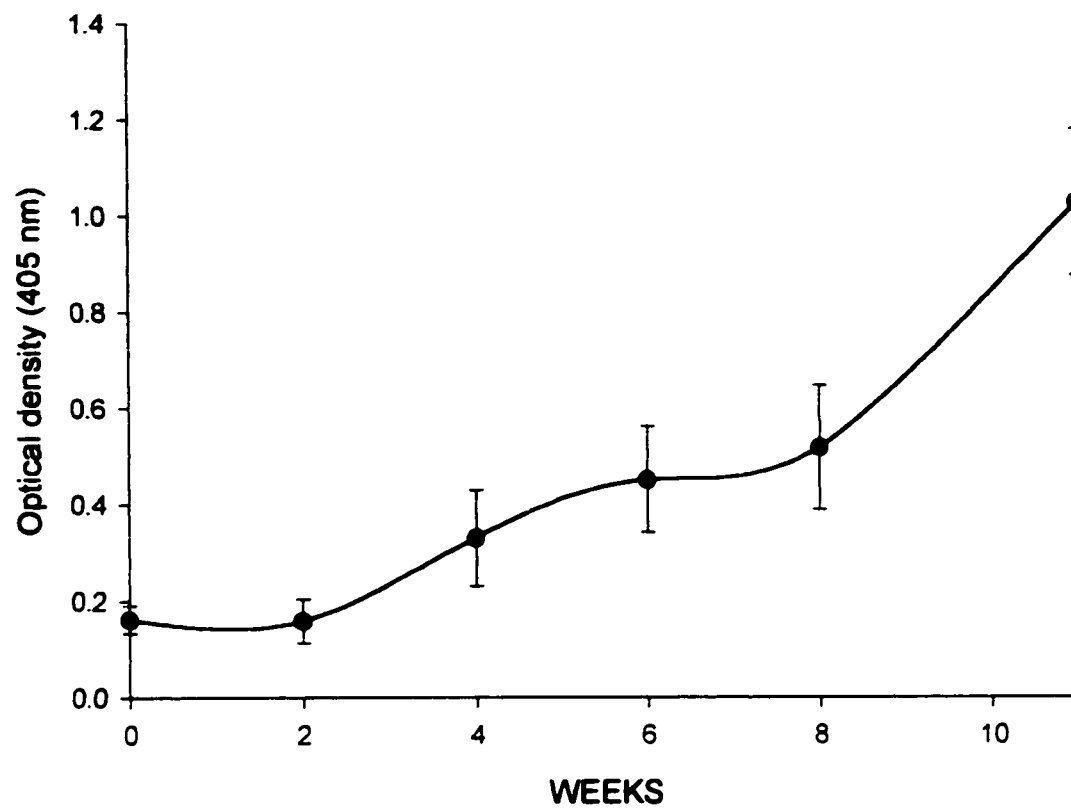
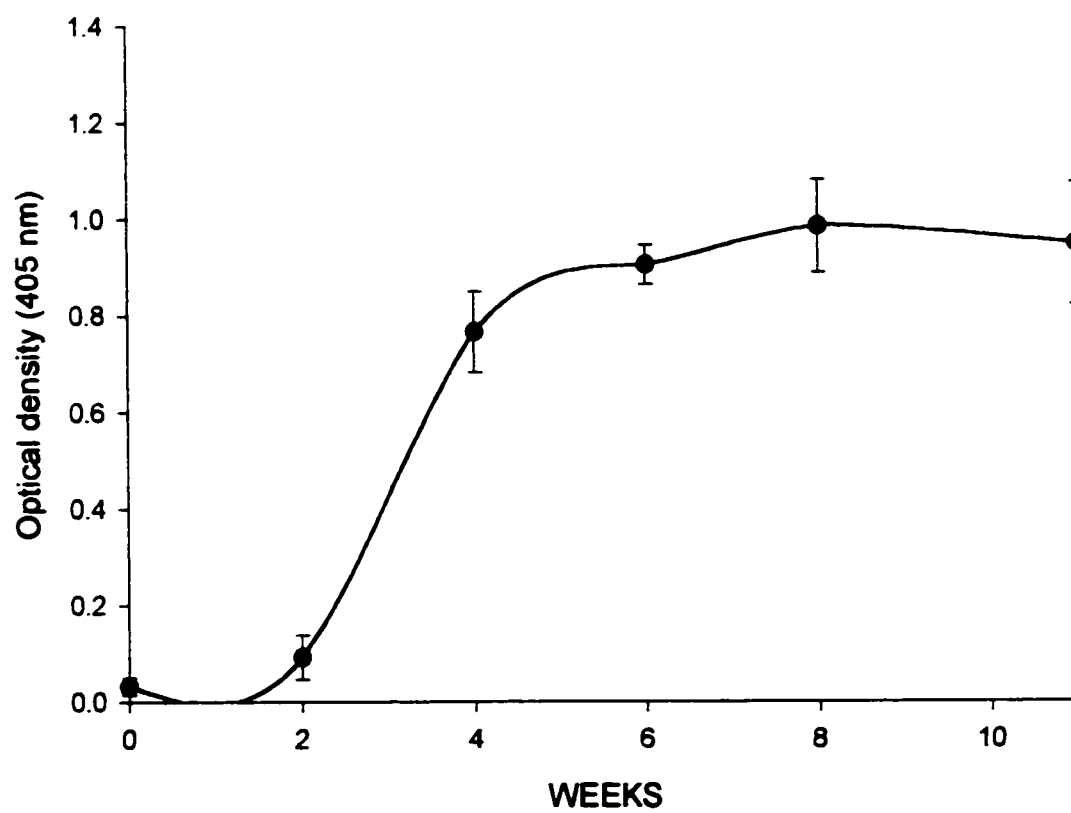
A**B**

FIGURE 38

Humoral immune response of rHgbA-immunized rabbits (per rabbit) in OMP-based (A) and rHgbA-based (B) EIA.

LEGEND: rabbit 58 (black circles), 59 (inverted red triangle), 60 (light green squares), 90 (yellow diamonds), 91 (blue triangles), and 92 (pink hexagons).

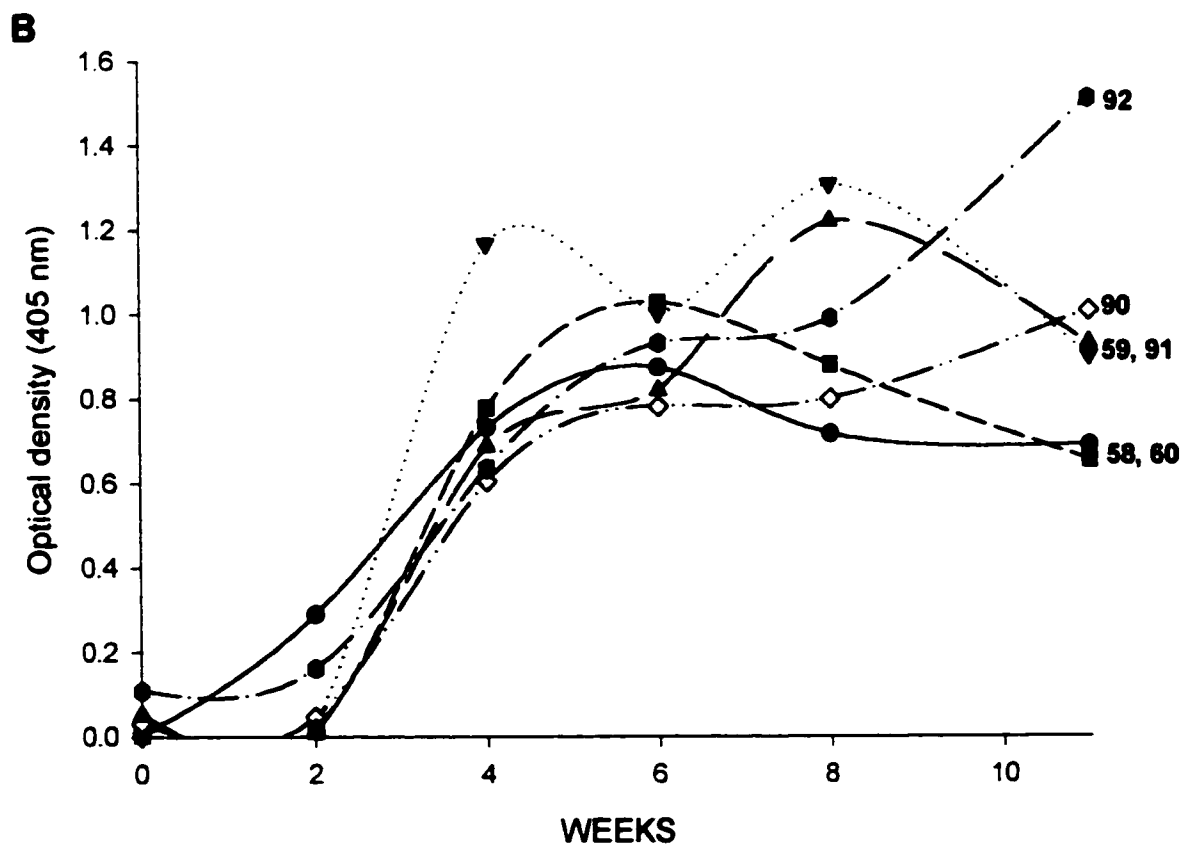
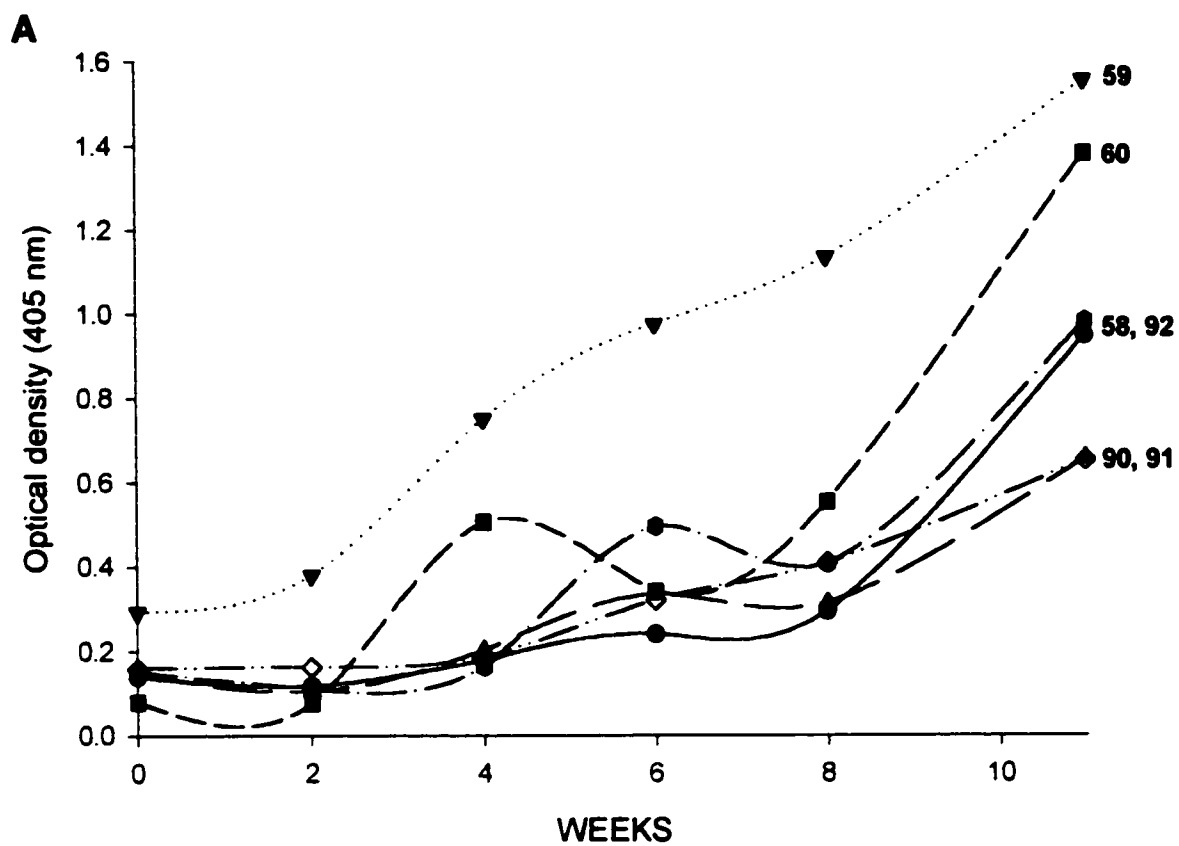


FIGURE 39

SDS-PAGE of the rD15 protein. Two microliters of two different preparations of the rD15 protein (**A** – 1.7 $\mu\text{g}/\mu\text{l}$) and (**B** – 2.7 $\mu\text{g}/\mu\text{l}$) were subjected to SDS-PAGE at 200 volts for 45 minutes and stained with a Coomassie/Bismarck Brown stain. MM = molecular markers; BSA = Bovine Serum Albumin.

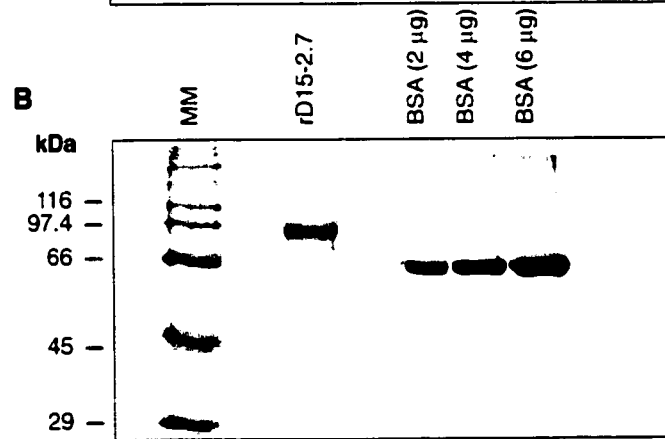
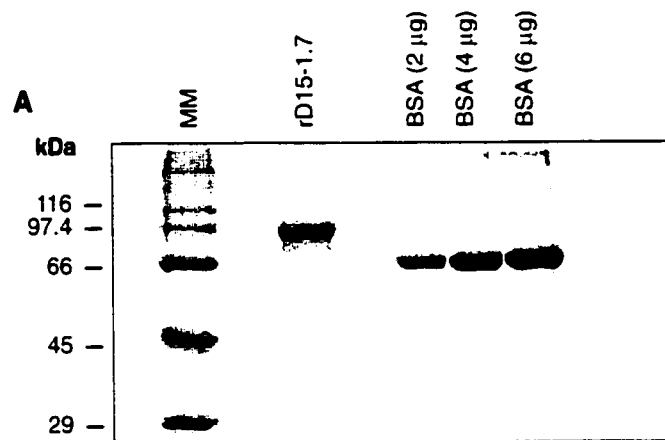


FIGURE 40

The course of an experimental challenge infection with 10^3 CFU of *H. ducreyi* strain 35000 after immunization with rD15. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rD15 (n = 13), 350 μ l of PBS (n = 14) or 100 μ g of rFetA (n = 8) in Freund's adjuvant and challenged with 10^3 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rD15 (red squares) ($p=0.122$) and rFetA (white inverted triangles) ($p=0.974$) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rD15 (red bars) ($p=0.372$) and rFetA (white bars) ($p=0.973$) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

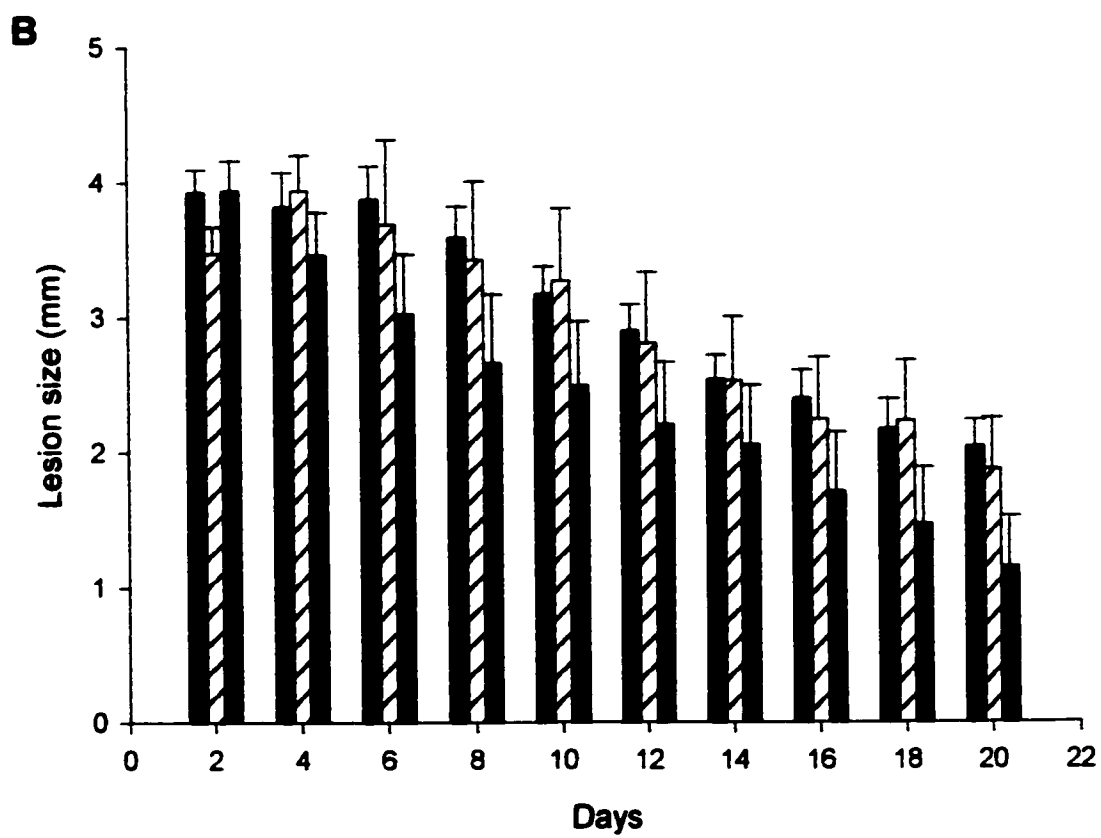
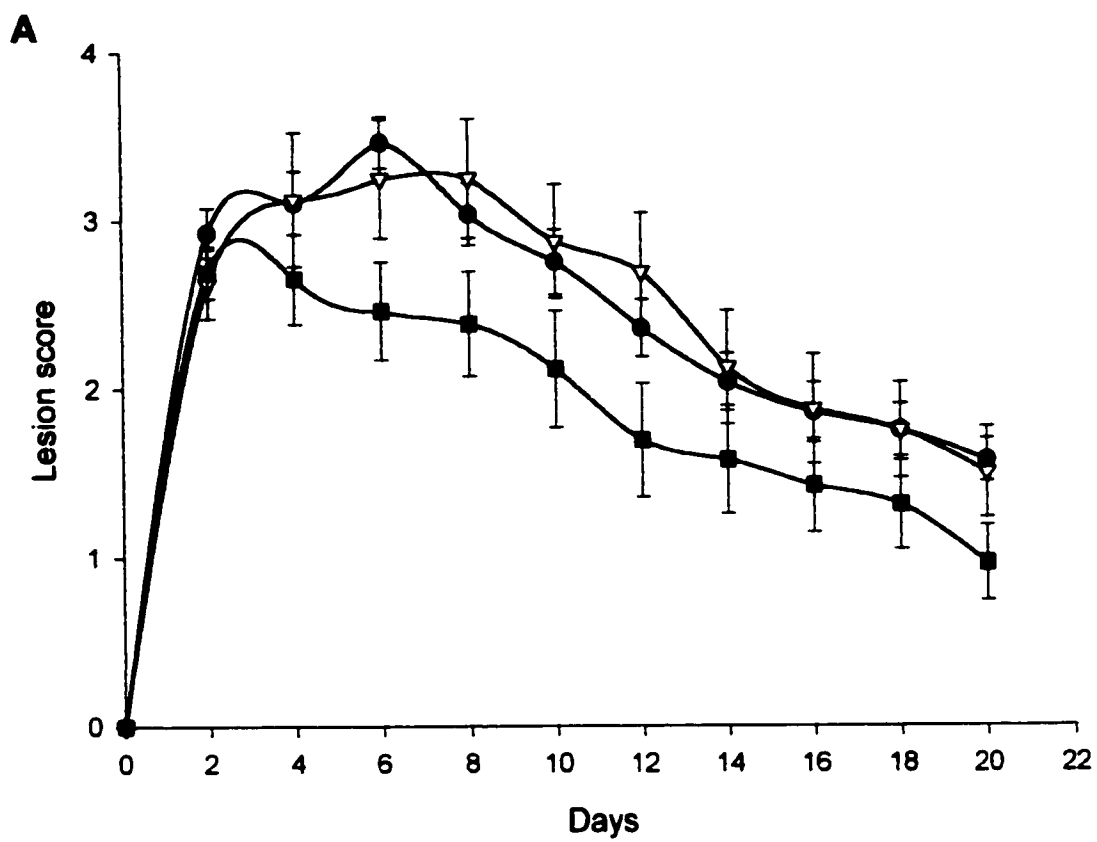


FIGURE 41

The course of an experimental challenge infection with 10^4 CFU of *H. ducreyi* strain 35000 after immunization with rD15. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rD15 (n = 13), 350 μ l of PBS (n = 14) or 100 μ g of rFetA (n = 8) in Freund's adjuvant and challenged with 10^4 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rD15 (red squares) (p=0.381) and rFetA (white inverted triangles) (p=0.844) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rD15 (red bars) (p=0.386) and rFetA (white bars) (p=0.885) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.

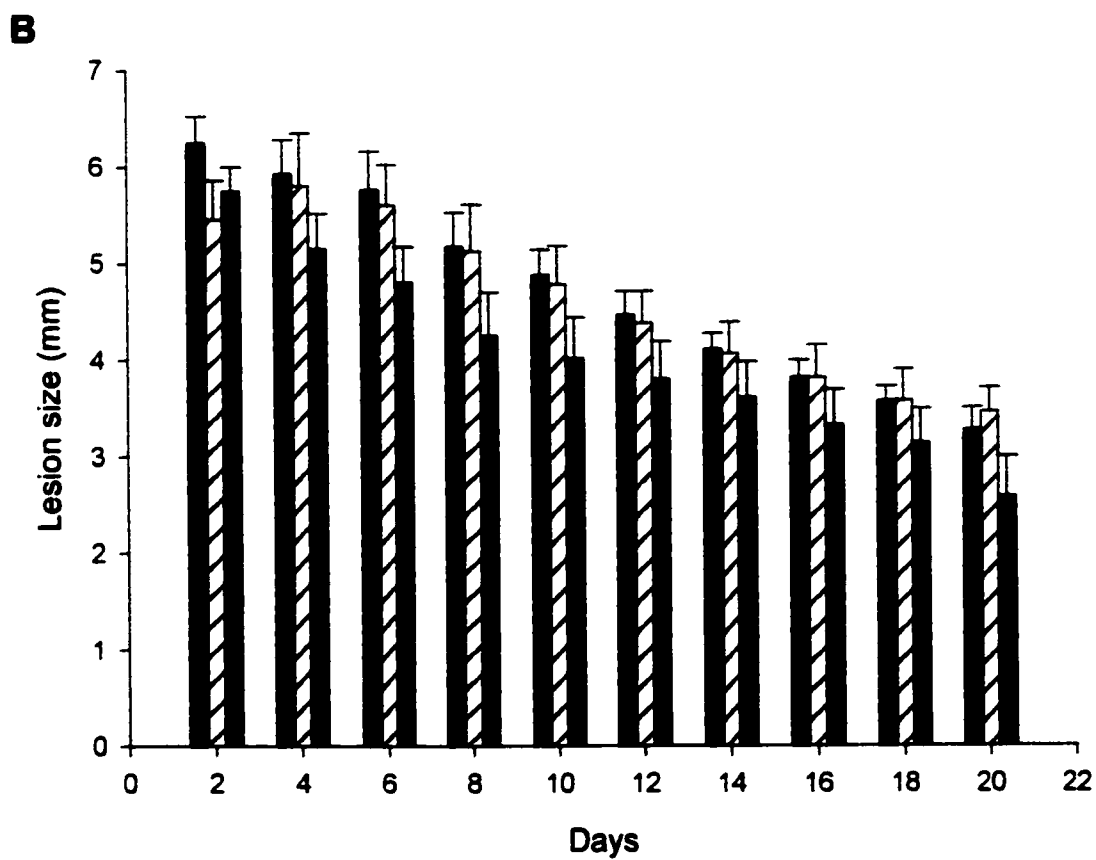
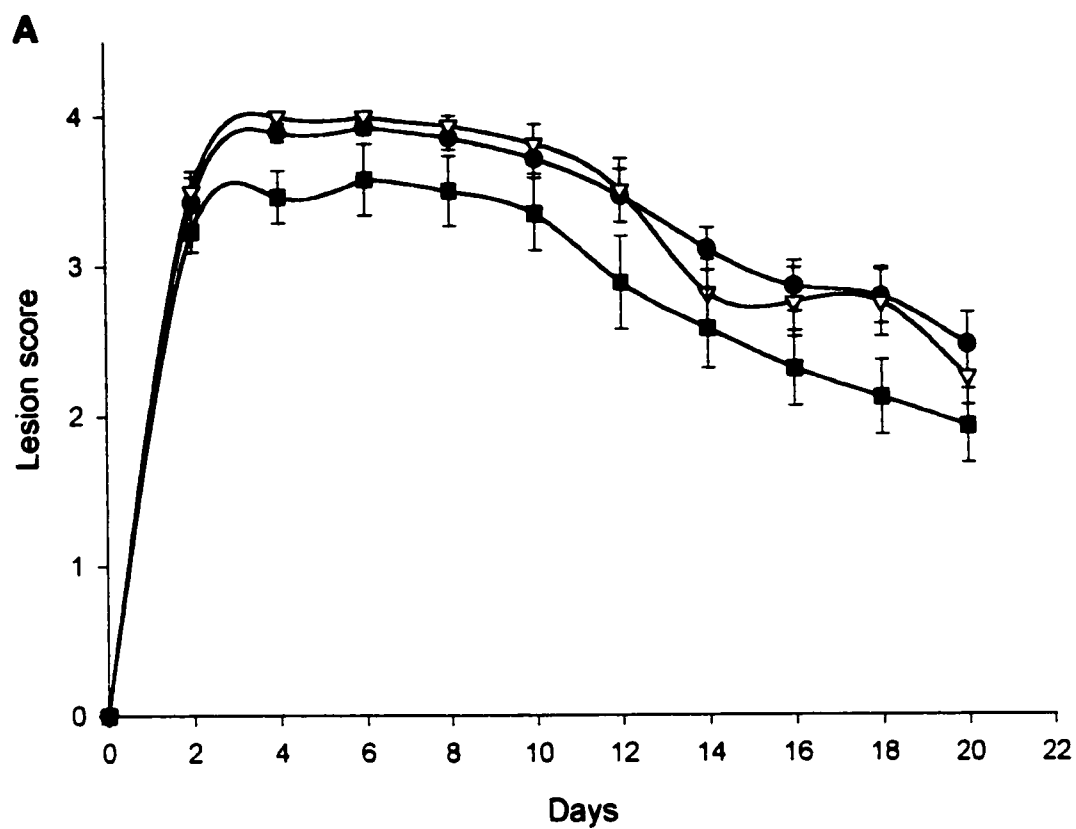
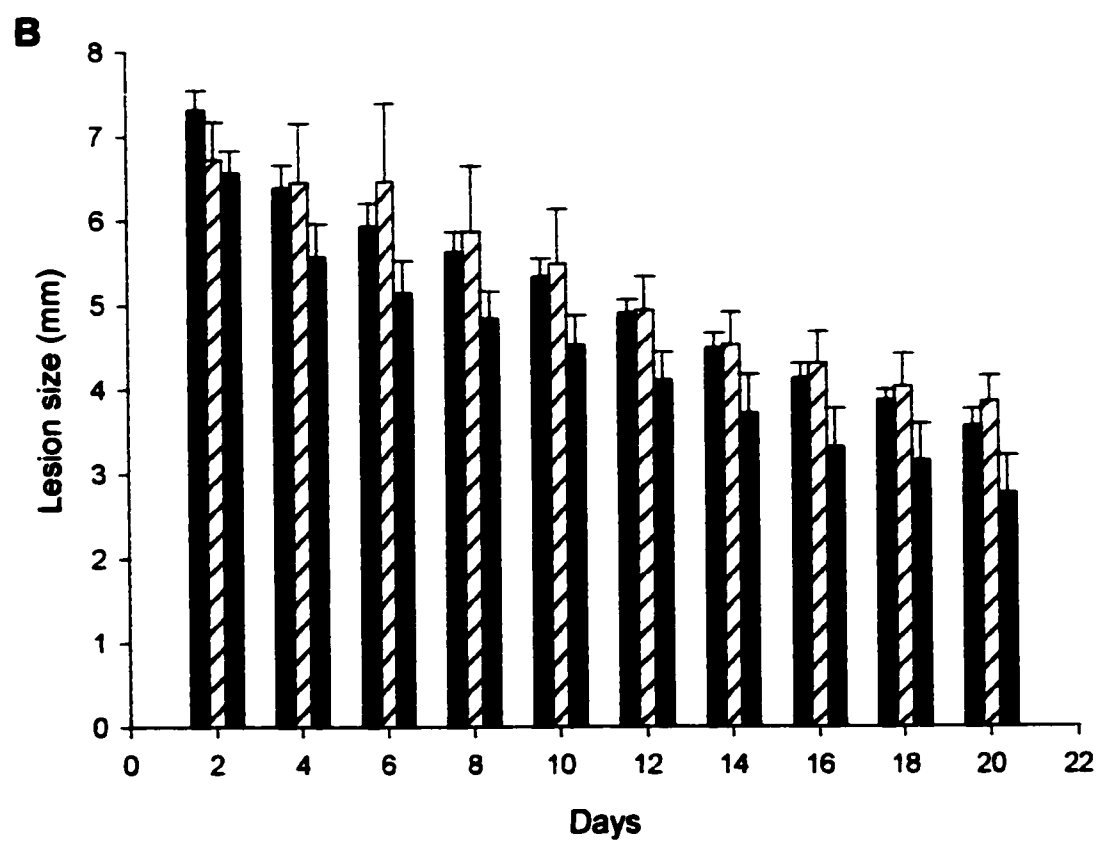
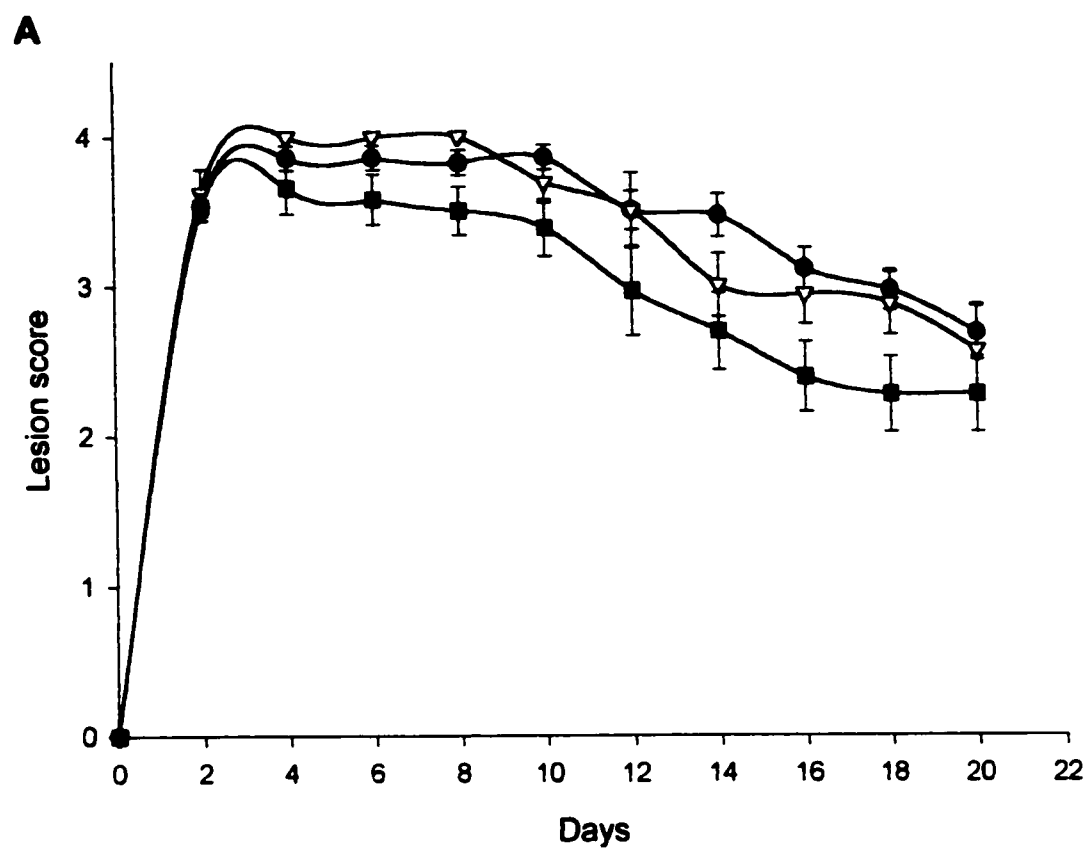


FIGURE 42

The course of an experimental challenge infection with 10^5 CFU of *H. ducreyi* strain 35000 after immunization with rD15. Rabbits were vaccinated and boosted 4 weeks later with 100 μ g of rD15 (n = 13), 350 μ l of PBS (n = 14) or 100 μ g of rFetA (n = 8) in Freund's adjuvant and challenged with 10^5 CFU of *H. ducreyi* strain 35000 4 weeks later. Lesions were monitored 20 days post-challenge. **A** - Lesion score ($\pm$ SE) of rabbits vaccinated with rD15 (red squares) (p=0.202) and rFetA (white inverted triangles) (p=0.965) is compared to sham-immunized rabbits (black circles). Lesions were given a score on a scale of 0 to 4, which is defined as followed: 0 = nil, 1 = redness, 2 = papule, 3 = pustule, and 4 = ulceration. **B** - Lesion size ($\pm$ SE) of rabbits vaccinated with rD15 (red bars) (p=0.252) and rFetA (white bars) (p=0.862) is compared to sham-immunized rabbits (black bars). Parameters were compared using ANOVA.



3.2.3 rD15 as a vaccine candidate in the TDRM of chancroid.

It has been reported that passive transfer of anti-rD15 antibodies protected infant rats from challenge with *H. influenzae* in an animal model of bacteremia (Thomas *et al.* 1990; Loosmore *et al.* 1997). It was of interest to determine whether the *H. ducreyi* D15 homolog would be effective in protecting rabbits against an experimental infection.

3.2.3.1 Purity of rD15 extracts. Christopher Elkins from the University of North Carolina generously provided the histidine-tagged rD15 protein in 2 different protein extracts (table 3 and figure 39). As for rHgbA, there are extraneous bands present in both of the extracts, suggesting that it is not pure (figure 39).

3.2.3.2 Vaccine effect of rD15 immunization on homologous *H. ducreyi* challenge. The lesion score and size of rD15-immunized rabbits were not significantly reduced compared to sham-immunized animals (figures 40 to 42) at all inocula tested (10^3 , 10^4 and 10^5 CFU, respectively). At a challenge inoculum of 10^4 CFU, only the duration of ulcers (8.5 ± 4.0 vs 11.5 ± 3.3 days; $p=0.047$) was significantly reduced in rD15-immunized rabbits compared to sham-immunized rabbits (table 11), and at 10^5 CFU, the number of days culture positive (6.8 ± 2.7 vs. 10.1 ± 4.2 days; $p=0.02$) and the duration of ulcers (7.9 ± 4.5 vs. 12 ± 3.2 days; $p=0.012$) were significantly reduced (table 11). It is important to note that rabbits from experiments 1 and 5 were protected from the experimental chancroid infection, while those from experiments 2 and 3 were not (data not separately shown). Interestingly, rabbits from these latter experiments were immunized with a different batch of rD15 than those from the former (table 3).

3.2.3.3 Immune response to rD15 immunization in rabbits challenged with a homologous strain. To determine if the lack of protection obtained with the rD15

TABLE 11

Virulence of *H. ducreyi* 35000 (homologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs			Mean duration of ulcers (days) ±SD
						N	E	B*	
Sham (n=14)	10 ⁵	5.0±1.9	10.1±4.2	51.4±6.3	4±0	0	1	13	12.0±3.2
	10 ⁴	4.2±2.1	10.7±2.8	47.2±8.8	4±0	0	0	14	11.5±3.3
	10 ³	3.7±2.0	9.1±2.6	30.2±6.9	3.5±0.5	3	5	6	4.7±3.4
rD15 (n=13)	10 ⁵	7.6±2.8 ^a	6.8±2.7 ^a	43.9±13	3.9±0.3	1	3	9	7.9±4.5 ^a
	10 ⁴	6.1±2.5 ^a	8.5±3.2	40.4±13	3.8±0.6	1	2	10	8.5±4.0 ^a
	10 ³	5.8±2.4 ^a	7.4±3.9	24±14.2	3.3±0.7	7	2	4	3.3±4.1
rFetA (n=8)	10 ⁵	7.1±4.0	9.0±4.1	52.6±14	4±0	0	0	8	11.8±3.7
	10 ⁴	5.7±2.0	6.8±3.2 ^a	46.0±10	4±0	0	0	8	11.4±3.2
	10 ³	6.0±2.7 ^a	7.8±4.1	29.5±12	3.5±0.7	2	2	4	5.9±4.7

* N = none (0/2); E = either (1/2); B = both (2/2)

^a p<0.05, Student's t test versus sham

FIGURE 43

Immune response of rabbits immunized with the rD15 vaccine. Screening Western blots of rD15 antisera to *H. ducreyi* OMP (2µg/lane) from strain 35000. The numbers at the top of the blot indicate the rabbit number. Pre- (a) and post-immune (b) sera are indicated. The arrow indicates the 85-kDa band of D15.

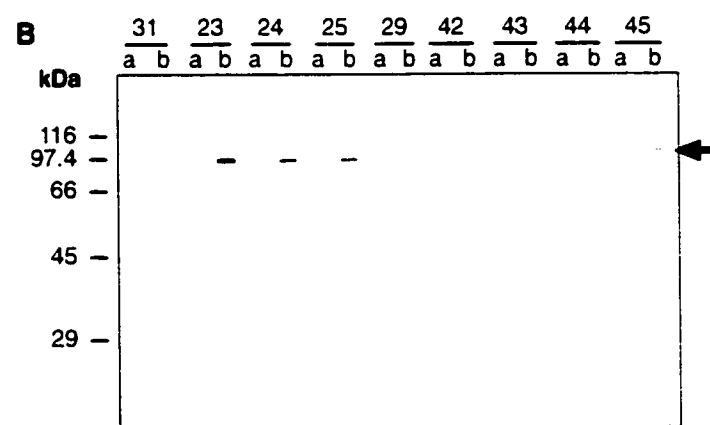
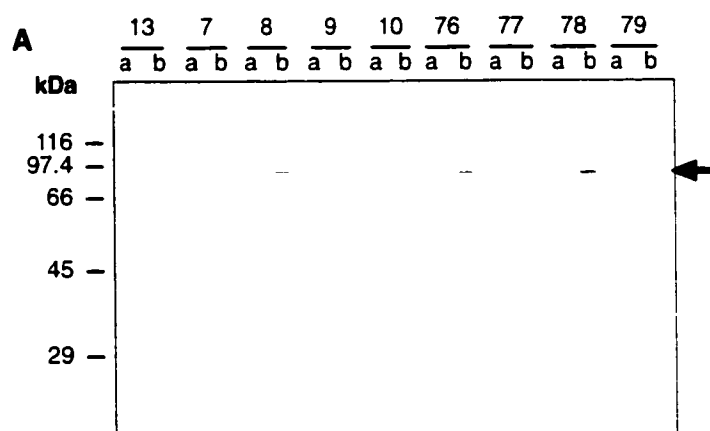


FIGURE 44

Humoral immune response of rD15-immunized rabbits in OMP-based (A) and rD15-based (B) EIA. The mean $\pm$ SE of optical densities is shown.

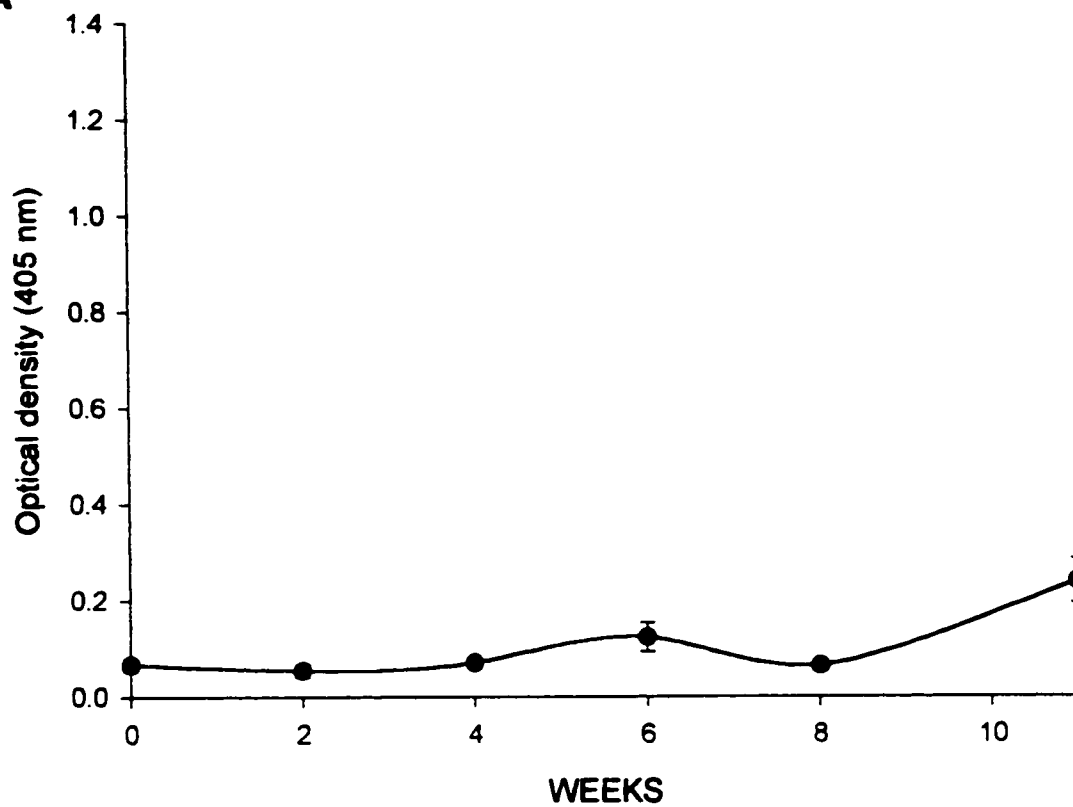
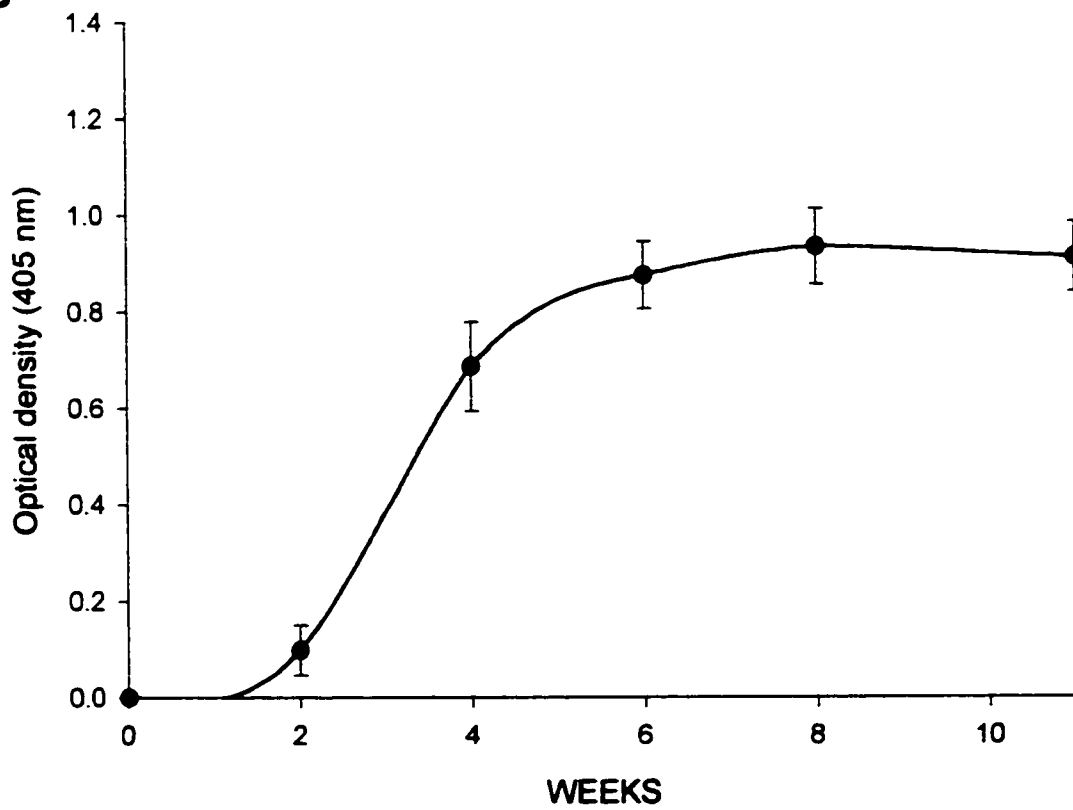
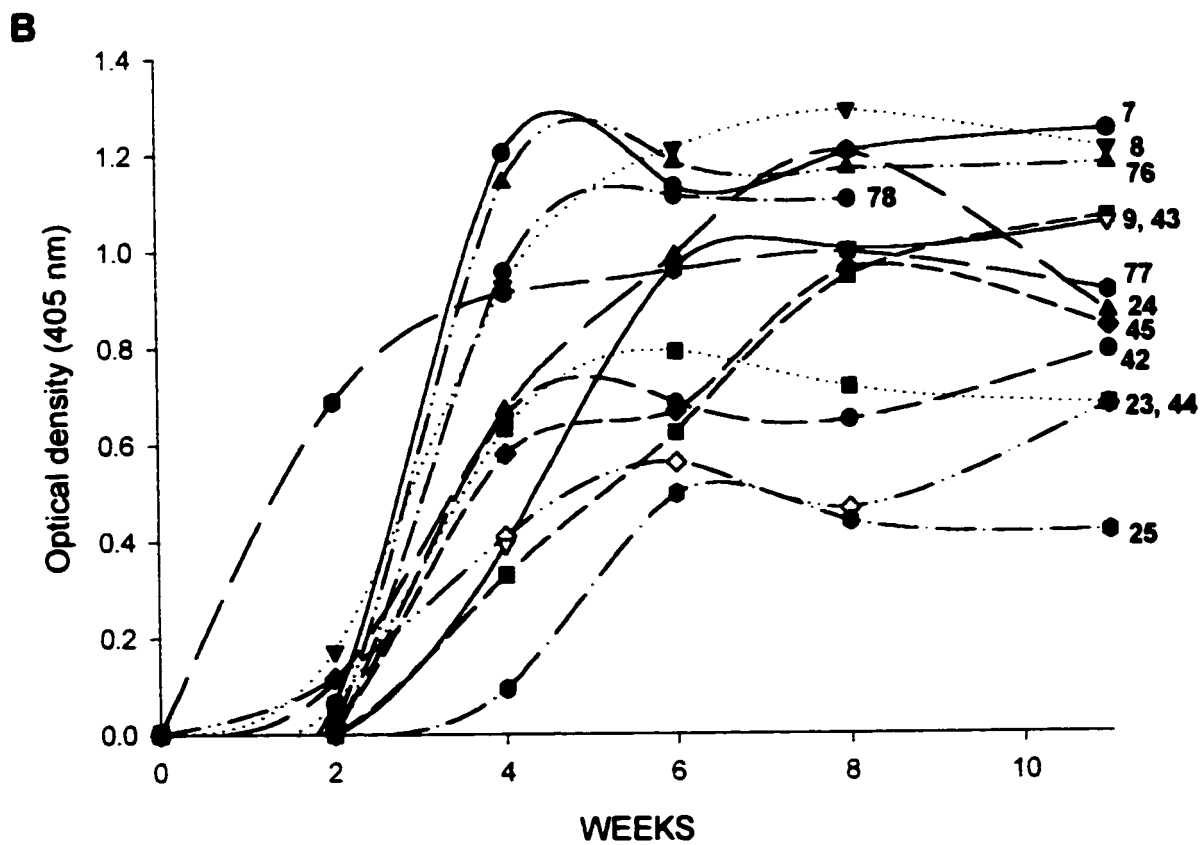
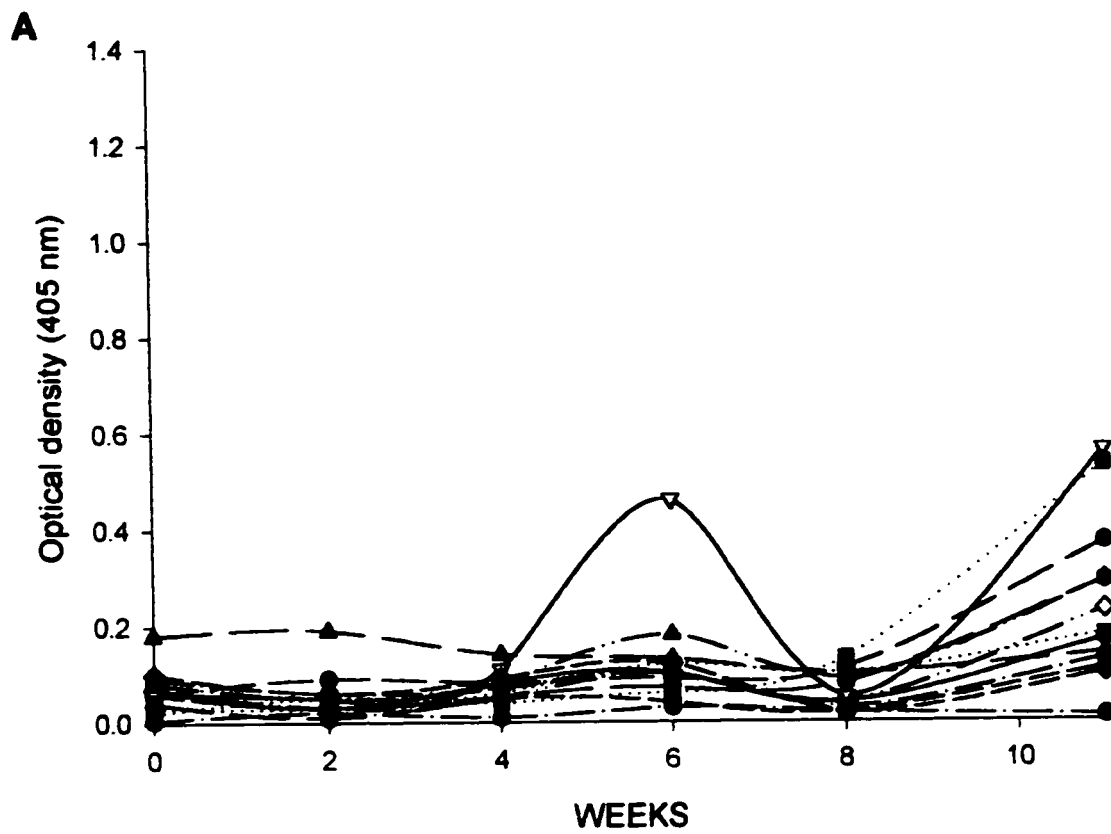
A**B**

FIGURE 45

Humoral immune response of rD15-immunized rabbits (per rabbit) in OMP-based (A) and rD15-based (B) EIA.

LEGEND: rabbit 7 (black circle), 8 (inverted red triangle), 9 (light green square), 23 (yellow diamond), 24 (blue triangle), 25 (pink hexagon), 42 (light blue circle), 43 (grey inverted triangle), 44 (burgundy square), 45 (dark green diamond), 76 (brown triangle), 77 (blue hexagon) and 78 (purple circle).



vaccine reflects a poor immune response by the immunized rabbits, the humoral immune response of these animals was studied. All rD15-vaccinated rabbits reacted strongly to an 85-kDa band on screening Western blots (figure 43). In an EIA format (figures 44 and 45), the rD15 antisera reacted poorly to OMP (figures 44A and 45A), but did so strongly to purified rD15 (figures 44B and 45B). In general, rabbits from experiments 2 and 3 (rabbits 23 to 25 and 42 to 45 – figure 46) had lower OD readings than those from experiments 1 and 5 (rabbits 7 to 9 and 76 to 78 - figure 45B): all rD15-immunized rabbits from experiments 1 and 5 (figure 45B) had optical density (OD) reading above 0.9 at week 8, while most rabbits from experiments 2 and 3 (figure 45B) were below 0.9. These results suggest that a poor humoral immune response may be responsible for the poor protection against the experimental *H. ducreyi* infection. To determine if rabbits with a more marked humoral response (rabbits from experiments 1 and 5) were better protected against an experimental infection than those who had a weaker antibody response (rabbits from experiments 2 and 3), the antibody titer was established for each of the rabbits immunized with the rD15 vaccine. This was calculated by plotting the reactivity of the rD15 antisera, serially diluted from 1:1000 to 1:16000, against purified rD15 (figure 46). The antibody titer was defined as the point before the curve of antibody response flattens (figure 46). The antibody titers of each animal with the number of culture positive days and the duration of ulcer for each inoculum studied are shown in table 12. Interestingly, most of the rabbits from experiments 1 and 5 (except rabbit 9), which were protected from ulceration with the rD15 vaccine against a homologous challenge infection, had antibody titers greater than 1:16000. Conversely, those that were

FIGURE 46

Calculation of the antibody titer of each rD15-immunized rabbit. rD15 antisera was serially diluted from 1:1000 to 1:16000 and assayed against purified rD15 antigens as described in materials and methods.

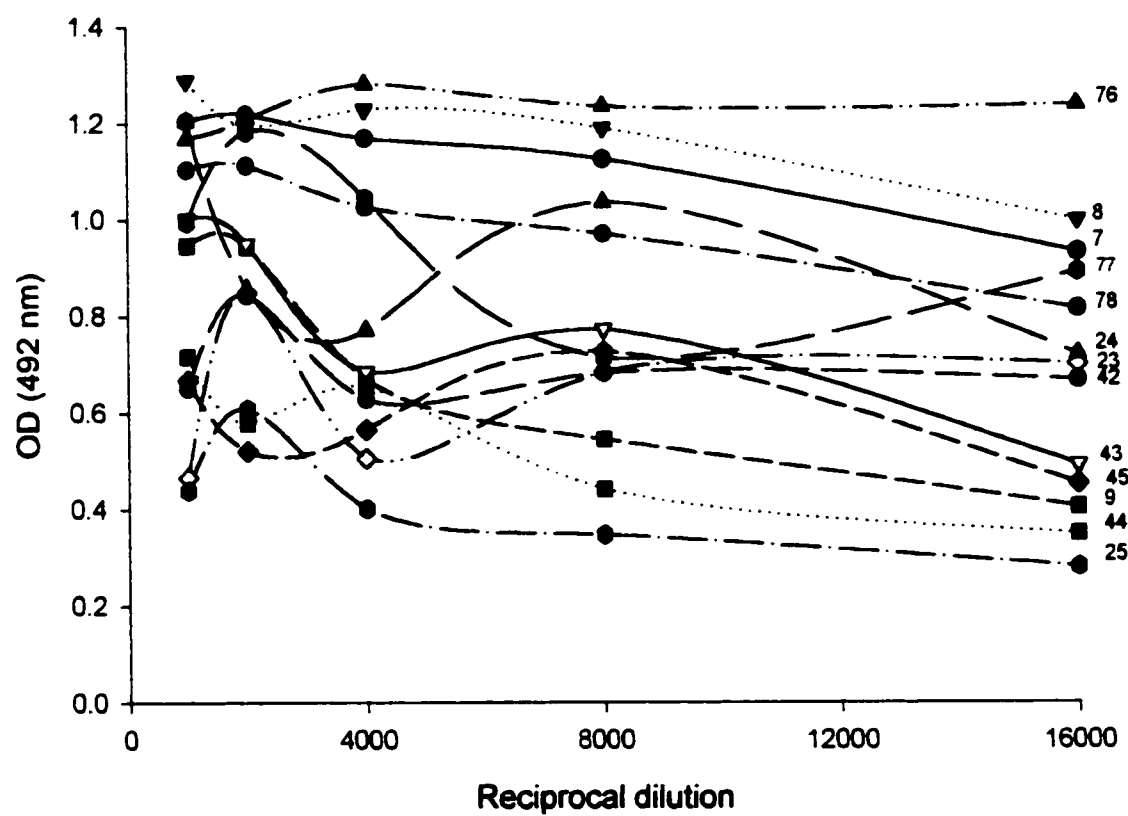


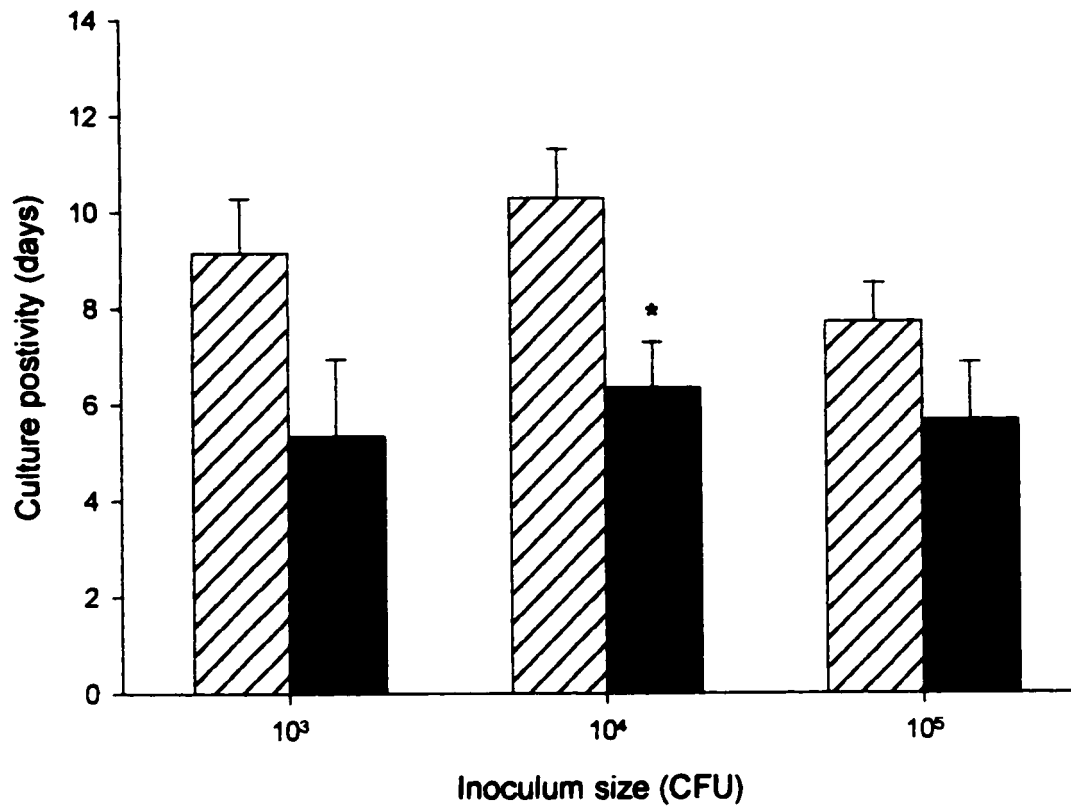
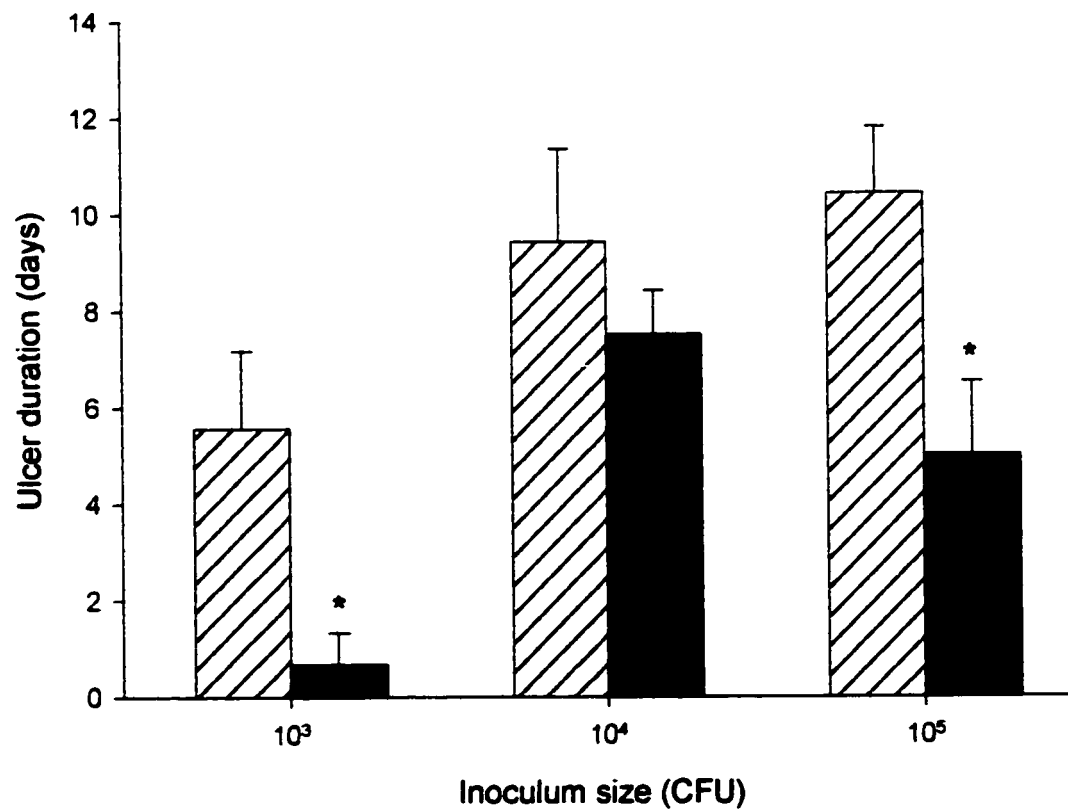
TABLE 12

Antibody titer of each rD15-vaccinated rabbits in relation with culture positivity and ulcer duration. The first column of the table identifies the rabbit and its anti-rD15 antibody titer. The three other columns report on duration of **culture positivity** and (/) **ulcer duration** in days at each inoculum size studied.

Rabbit # - Ab titer (experiment)	10⁵ CFU	10⁴ CFU	10³ CFU
7 – 16000 (1)	6 / 1	8 / 7	2 / 0
8 – 16000 (1)	6 / 7	8 / 7	8 / 4
9 - 8000 (1)	0 / 0	2 / 8	0 / 0
23 – 4000 (2)	8 / 14	14 / 14	12 / 7
24 – 4000 (2)	12 / 4	10 / 0	6 / 0
25 – 4000 (2)	8 / 8	12 / 5	14 / 9
42 – 4000 (3)	6 / 14	8 / 13	8 / 0
43 – 4000 (3)	8 / 9	6 / 10	10 / 9
44 – 4000 (3)	6 / 12	12 / 12	6 / 10
45 – 4000 (3)	6 / 12	10 / 12	8 / 4
76 – 16000 (5)	8 / 5	8 / 4	10 / 0
77 – 16000 (5)	6 / 9	6 / 8	4 / 0
78 – 16000 (5)	8 / 8	6 / 11	8 / 0

FIGURE 47

Correlation between antibody titer and culture positivity (A) or ulcer duration (B) in rabbits immunized with the rD15 vaccine. Red hatched bars = rabbits from experiments 1 and 5 with antibody titers $> 1:4000$; white hatched bars = rabbits from experiments 2 and 3 with antibody titers of $\leq 1:4000$. Stratification of rD15-immunized rabbits in 4 experiments by titer of antibody reaction to rD15 within vaccinees shows consistently shorter duration of culture positivity and ulceration in those with lower titer. * $p < 0.05$ using Student's t test.

A**B**

not protected (rabbits from experiments 2 and 3) had antibody titers smaller or equal ($\leq$) to 1:4000 (table 14).

Controls rabbits in all experiments had developed ulcers as expected, and the apparent differential “protection” was due to ulceration in the rD15 vaccinees in 2 of 4 experiments. To determine if there was a correlation between the lack of protection and a low antibody titer, the mean number of days culture positive and the mean duration of the ulcers were calculated and plotted in a bar graph for groups of rabbits differing in their antibody titers (figure 47). Interestingly, lesions in rabbits with antibody titers greater than ($>$) 1:4000 (red hatched bars of figure 47) were culture positive for a shorter period of time. Furthermore, ulcer duration was also briefer than in rabbits with antibody titers equal or smaller than ($\leq$) 1:4000 (white hatched bars of figure 48). Taken together, these results suggest that rabbits immunized with 100 μg of rD15 protein from a batch at a concentration of 1.7 $\mu\text{g}/\mu\text{l}$ (table 3) had a relatively lower immune response to this protein, which may have left them relatively unprotected against an experimental infection, compared with those immunized with 100 μg rD15 protein from another batch at a concentration of 2.7 $\mu\text{g}/\mu\text{l}$.

CHAPTER 4 – CONCLUSION AND DISCUSSION

Results presented in this thesis explore two features of *H. ducreyi* biology: the heterogeneity of its LOS, and the use of preparations of specific *H. ducreyi* OMP as vaccine candidates in an experimental animal model of chancroid. Study of the electrophoretic heterogeneity of the *H. ducreyi* LOS defined 7 groups, which served as the basis of a typing system to classify strains of this pathogen into easily resolvable groups according to LOS SDS-PAGE band patterns. Examination of the reaction, cross-reaction and its extinction of OMP antisera to purified LOS from all 7 PAGE groups identified 5 categories according to serological profiles. CHO and mass spectrometry analyses revealed structural intrastrain and interstrain diversity in the LOS molecules among the diverse SDS-PAGE groups, which corroborated the electrophoretic and serologic data.

Vaccine feasibility against *H. ducreyi* infection was investigated in the TDRM of chancroid. Immunization with both the native and recombinant forms of HgbA, the hemoglobin receptor of *H. ducreyi*, modified the course of homologous and/or heterologous experimental infections. The recombinant form of D15, an OMP of *H. ducreyi*, also modified the course of a homologous challenge infection.

4.1 *H. ducreyi* LOS as the basis of a typing system.

Much effort has focused on trying to develop a typing system to classify isolates of *H. ducreyi*. None published so far have been suitable (Odumeru *et al.* 1983; Taylor *et al.* 1985; Korting *et al.* 1988; Finn *et al.* 1990; Sarafian *et al.* 1991a; Sarafian *et al.* 1991b; Roggen *et al.* 1992; Roggen *et al.* 1993; Brown and Ison 1993; Pillay *et al.* 1996;) either because the system was not discriminative, or because it necessitated cumbersome

methods and expensive materials. SDS-PAGE analysis of *H. ducreyi* LOS in our laboratory as well as results in some reports (Odumeru *et al.* 1987; Campagnari *et al.* 1991) has suggested that LOS interstrain variation may exist among isolates of *H. ducreyi*. It was of interest to determine if sufficient variation exists in the LOS in a panel of strains to be the basis of a typing system, and if there is a correlation between structural heterogeneity and specific serologic reaction of the LOS.

Contrary to a previous report (Abeck *et al.* 1987), purified LOS were resolved into distinct bands that were simple to read and easy to distinguish since they usually encompass 1 to 6 bands. Neuraminidase treatment of the LOS suggest that some strains may express sialylated LOS, although results are still unclear. There was extensive cross-reactivity of OMP antisera to purified LOS in EIA and Western blots, suggesting that the studied strains shared common LOS epitopes or expressed similar LOS glycoforms at their surface. When the OMP antisera were adsorbed with strains with the most cross-reactive OMP antisera, EIA and Western blots revealed that LOS could be classified in 5 different serologic categories (A to E) according to LOS reaction and/or its extinction. CHO and mass spectrometry analyses revealed that all strains studied expressed more than one LOS glycoform at its surface and that some strains expressed LOS molecules lacking the unique DD-heptose.

4.1.1 Electrophoretic diversity of the *H. ducreyi* LOS. Each band of the SDS-PAGE pattern represents a specific LOS glycoform expressed at the surface of the bacterial cell (Bozue *et al.* 1999). A dark band represents a LOS glycoform that is highly expressed at the cell surface, while a light band corresponds to a less significant glycoform (Melaugh *et al.* 1994). In this manner, it was expected that strains from SDS-PAGE groups 1, 2, 6

and 7 would express a greater diversity of LOS glycoforms at their surface since their band patterns encompass a higher number of bands. However, the LOS from group 1 has 6 bands in its pattern, but strains from this group only express 2 LOS glycoforms at their surface. Conversely, the LOS of groups 3 and 4 has only 2 bands in its pattern, but strains in these groups express 4 and 3 glycoforms at their surface, respectively (table 4). This discrepancy may be explained by the fact that the bands of LOS glycoforms present in lower quantities at the surface of the cell may not be resolved on the SDS-PAGE, some bands may have co-migrated with others or stained at a different intensity. Some minor LOS glycoforms may also have been lost during the purification and deacylation of the extracts, which would not have appeared on the mass spectrometry analysis, or some LOS glycoforms may possess modifications, such as phosphorylation or a different number of fatty acids, that may have affected their band patterns.

The SDS-PAGE banding pattern obtained for strain 35000 (group 2) is consistent with previous reports, which showed that the LOS SDS-PAGE pattern of this strain consists of 6 bands (Campagnari *et al.* 1991; Melaugh *et al.* 1994). For other strains, structural and immunological data about their LOS is available for the first time in this thesis.

4.1.2 Variation of *H. ducreyi* LOS according to culture conditions. LOS glycoform expression, such as the one described above in strain J1159, may vary according to the stress level of the bacteria. It has been reported that the growth phase at which the organisms were harvested may have an impact on the LOS band pattern, or at least epitope expression (Preston *et al.* 1996). Indeed, *H. influenzae* and *N. gonorrhoeae* LOS show compositional and quantitative variation depending on the growth rate of the

culture and the type of growth media. The level of aeration and the pH of cultures have been shown to affect the expression of *N. gonorrhoeae* LOS (Preston *et al.* 1996). In our work, the pattern containing bands of lower molecular weight came from J1159 grown in low-heme conditions or long-term growth on CAP. These results suggest that lower molecular weight LOS may be expressed in situations of stress.

The LOS from *H. influenzae* and *Neisseria* species have been shown to go through phase variation, a process defined as the reversible loss and gain of epitopes corresponding to the synthesis of altered LOS molecules (Preston *et al.* 1996). Three operons, *lic1*, *lic2* and *lic3*, contain genes that are involved in LOS biosynthesis in *H. influenzae* (Preston *et al.* 1996). Some genes in these operons contain multiple tandem repeats of the nucleotide tetramer 5'-CAAT-3', which are involved in *H. influenzae* LOS phase variation (Preston *et al.* 1996). Thus, adding or deleting the number of CAAT repeats produced frame shift mutations and altered expression of the downstream genes. Furthermore, the *lex-2* locus of *H. influenzae* type b strain DL42 also contains the tetrameric repeat 5'-GCAA-3', and there are 6 LOS loci in the strain Rd of *H. influenzae* at which phase variation may operate (Preston *et al.* 1996). Poly-G tracts, which also exist in the *lgt* loci of *N. gonorrhoeae* and *N. meningitidis*, are potential sites of slip-strand mispairing (Preston *et al.* 1996).

It has been reported that the *H. ducreyi lbgA* gene product has similarity to the Lex-1/Lic2A proteins of *H. influenzae* (Stevens *et al.* 1997). However, the nucleotide tetramer CAAT repeats were not present in *lbgA* (Stevens *et al.* 1997). Furthermore, these authors reported the LOS epitope defined by the reactivity to MAb 3E6 did not exhibit phase variation, and others have reported minimal phase variation of the *H.*

ducreyi LOS (Melaugh *et al.* 1994; Schweda *et al.* 1994; Schweda *et al.* 1995; Stevens *et al.* 1997).

4.1.3 Sialylation of the *H. ducreyi* LOS. It has previously been reported that the LOS molecule of *H. ducreyi* is highly sialylated (Schweda *et al.* 1995; Melaugh *et al.* 1996). The 5.1-kDa band, part of the doublet in the SDS-PAGE band pattern of strain 35000, appears to be sensitive to neuraminidase and is likely the sialylated analog of the major glycoform of *H. ducreyi*, represented by a 4.5-kDa band on SDS-PAGE (Melaugh *et al.* 1994). SDS-PAGE groups 1, 2, 5, 6 and 7 have a LOS pattern that contains a 5.1-kDa band, suggesting that strains belonging to these groups may express sialylated LOS. However, mass spectrometry analyses of purified LOS revealed that only strains belonging to SDS-PAGE groups 1, 2, 3 and 6 expressed sialylated LOS. Thus, these experiments reveal unclear results, and further experiments are needed.

It is notable that not all neuraminidase treatment resulted in a clear shift of the 5.1-kDa band to a 4.5 kDa one. Indeed, there was only a change in the intensity of the 5.1-kDa band for some LOS. The neuraminidase activity could have been affected by differences in structures of the LOS. Furthermore, the neuraminidase may not have been left long enough with the purified LOS to break down all the bonds between the sialic acid and the acceptor molecule.

4.1.4 Structural heterogeneity of the *H. ducreyi* LOS. The structural arrangement of the LOS glycoforms expressed at the surface of the strains from each SDS-PAGE group was deduced by combining CHO and mass spectrometry analyses. There was “good resolution” between groups for LOS evaluation by mass spectrometry and CHO analysis.

TABLE 13

Deduced structures of the major LOS glycoform from each SDS-PAGE group based on CHO and mass spectrometry analysis

PAGE Group #	Serologic category	Deduced structure
1, 2, 6	A and E	Neu5Ac-Gal-GlcNAc-Gal-DDHep-Glc-Hep-KDO(P)-lipid A Hep-Hep
3	E	GlcNAc-Gal-DDHep-Glc-Hep-KDO(P)-lipid A Hep-Hep
4	B	GlcNAc-Gal-Glc-Hep-KDO(P)-lipid A Hep-Hep
5	C	DDHep-Glc-Hep-KDO(P)-lipid A Hep-Hep
7	D	Hep-KDO(P)-lipid A Hep-Hep

The major LOS glycoform (100% relative intensity) from strains in SDS-PAGE groups 1 (glycoform B – table 6) and 6 (glycoform H – table 6) are sialylated, while that of strains belonging to SDS-PAGE groups 2 (glycoform E – table 6) and 3 are not. The major LOS glycoform of strains belonging to SDS-PAGE group 3 (glycoform B – table 6) differs from that of groups 1, 2 and 6 since it does not possess the terminal disaccharide Neu5Ac-Gal (glycoforms B and D – table 6 and see structure in table 13). It is identical to that of strain A77 (Sun *et al.* 2000), a galactosyltransferase mutant. This similarity to strain A77 might suggest that strains belonging to this SDS-PAGE group 3 do not express the enzyme involved in the attachment of the second galactose molecule on the LOS glycoform. However, a minor glycoform (C - table 6) of the LOS from SDS-PAGE group 3 has the extra galactose bound to the N-acetyl-glucosamine molecule, presumably to form the terminal disaccharide lactosamine (Gal-GlcNAc). Although this epitope has been found to be the acceptor for the sialic acid (Melaugh *et al.* 1996), this glycoform is not sialylated. Instead, glycoform A seems to be the sialylated glycoform.

Strains belonging to SDS-PAGE group 4 express a LOS molecule that lacks the DD-heptose (glycoform C in table 6 and table 13). This structure has previously been reported for strain 33921 (Melaugh *et al.* 1996), which has lost the ability to add sialic acid. This strain has also lost the ability to bind the MAb 3F11, which forms the epitope formed by the disaccharide Gal-GlcNAc. Interestingly, the minor LOS glycoform A expressed by strains belonging to SDS-PAGE group 4 may possess a terminal hexose (galactose or glucose) residue bound to the N-acetylglucosamine molecule.

The major LOS glycoform from SDS-PAGE group 5 presents itself with 4 heptoses, including the DD-Heptose, and a hexose (glycoforms D and E in table 6 and

TABLE 14**Correlation of SDS-PAGE groups and serologic categories**

	1 (C148)	2 (35000)	3 (1293)	4 (AX557)	5 (V1157)	6 (J1159)	7 (1315)
A	R						
B				R			
C					R		
D							R
E		E_i	E_{ii}			E_{iii}	

R = homologous reaction on Western blot to LOS with specificity, after adsorption of OMP antisera with whole cells from groups 3 and 6

E = extinction of reaction on Western blot to homologous and heterologous LOS, after adsorption of OMP antisera with whole cells from groups 3 and 6 (i), 6 (ii) or 3 (iii).

table 13). On the other hand, the structure of the major LOS glycoform of strains belonging to SDS-PAGE group 7 (glycoforms B and C – table 6) only possesses 3 LD-heptose and is missing the characteristic DD-Hep. These structures have never been reported in any other *H. ducreyi* strain studied, although *H. ducreyi* laboratory generated mutant strains expressing LOS with only one, two or no heptose have been reported (Bauer *et al.* 1998; Bauer *et al.* 1999; Filiatrault *et al.* 2000). Interestingly, the SDS-PAGE band pattern of strain ITM 4747 closely resembles that of SDS-PAGE group 7 (Schweda *et al.* 1994). However, SDS-PAGE group 7 LOS was shown to lack galactose residues, which has not previously been reported.

4.1.5 Correlation of SDS-PAGE groups and serologic categories. Based on primary oligosaccharide structures, previous reports have attributed LOS immunotypes to defined epitopes on the LOS molecule (Verheul *et al.* 1993). Although many unknown factors, such as immunodominance of epitopes and interaction of the LOS with other surface structures, may influence the three-dimensional conformation of the LOS, the authors speculated on the location of the immunotype-specific epitope within the LOS structure. We attempted to find such a correlation by first putting together a table (table 14) with both electrophoretic and serologic types (Western blot-based). There was homologous and heterologous reaction and extinction of antisera to LOS groups by Western blot. Groups 1, 4, 5 and 7 show preserved homologous reaction (Y) after antisera are adsorbed with broadly cross-reactive strains (J1159 and 1293 from SDS-PAGE groups 6 and 3, respectively), and extinction of cross-reaction. Groups 2, 3 and 6 show extinction (E) of homologous and heterologous reaction.

4.1.6 Qualities of a reliable typing system. A typing system can classify strains of a species according to two types of characteristics. A phenotypic typing system examines expressed characteristics, while genotypic typing systems look at the genome of the cell (Maslow and Mulligan 1996). Since typing systems classify strains according to the presence or absence of certain genes or the expression of virulence factors, they identify strains that are more virulent, that cause more important disease or have special genomic or phenotypic characteristics. In epidemiology, stable typing systems can determine if a number of cases in the same area are related or not, if this represents an outbreak from a common source, or if a recurrent infection is caused by the same strain or another one.

Several criteria are used to evaluate the validity of a typing system (Maslow and Mulligan 1996). First, a typing system must give the same unambiguous positive result for one isolate. This refers to its typeability. Second, a typing system must be able to provide this same result at every occasion the same isolate is tested. This is what is called reproducibility. In our hands, SDS-PAGE band patterns and antigenic characteristic obtained for *H. ducreyi* LOS were consistent when bacteria were grown in the same way on the same medium. Discriminatory power of a typing system must also be considered. Indeed, a test used for classification of strains must be able to differentiate between epidemiologically unrelated strains. The fact that one LOS group seemed to cluster in strains from one place and time may support this criterion. Finally, one must also bear in mind the availability, the cost, the technical requirements and the speed of the typing system.

Typing strains using SDS-PAGE band patterns has the disadvantage of having to culture the organism, extract its OMP and perform electrophoresis, which would take

approximately 4 days. However, this technique is cheap, the equipment is readily available, the technique is simple and the results are easy to interpret, contrary to OMP profiles. A typing system based on antigenic properties of the *H. ducreyi* LOS in EIA and Western blots are not simpler or faster to use than the SDS-PAGE pattern technique. However, a serologic typing system could be developed using anti-LOS antibodies specific to the antigenicity of serologic categories. Indeed, strain-specific antibodies might be evaluated in some manner for diagnosis or typing on direct specimens.

4.1.7 Using LOS heterogeneity as the basis of a typing system for *H. ducreyi*. Seven different groups by SDS-PAGE band patterns, and 5 different serologic categories were found among the 91 strains studied. This is not sufficient to discriminate among a large number of strains. However, 93% (85/91) of these clinical isolates came from Nairobi, Kenya in a 2-year period. Interestingly, 3 distinct SDS-PAGE band patterns are present among strains isolated in Kenya between 1986 and 1987, 2 others are found in strains isolated at the same place but 6 years earlier, and the 2 others are from strains isolated in Winnipeg and Seattle, respectively. This suggests that the strains isolated in Kenya may be epidemiologically related. It is notable that except for 35000, only strains found in SDS-PAGE group 2 are from Nairobi. However, some Nairobi strains are found in SDS-PAGE groups other than 2.

4.1.8 Typing systems based on CHO heterogeneity. Classification of *E. coli* strains have long been done according to the type of O-antigen present in the LPS expressed at their cell surface, and this typing method is now combined with pili (K) and flagella (H) typing. However, Gram-negative pathogens that reside on mucosal surfaces, such as members of the genera *Neisseria*, *Haemophilus*, *Bordetella* and *Branhamella*, do not

express the repetitive O-side chain present in enteric LPS and have a more hydrophobic outer membrane surface than enteric bacteria. Nonetheless, it is possible to classify strains based on LOS heterogeneity, although the diversity is not as broad as that observed in enteric bacteria. For example, 6 antigenically distinct serotype antigens were found in *N. gonorrhoeae* (Apicella *et al.* 1981; Preston *et al.* 1996). Twelve immunotypes were distinguished in *N. meningitidis* by combining SDS-PAGE pattern, specific rabbit antisera reactivity and specific MAb (Verheul *et al.* 1992), and 5 serotypically distinct LOS were found in *H. influenzae* (Campagnari *et al.* 1987). However, phase variation has been reported for the LOS molecule of these pathogens, making it an unattractive typing tool. Phase variation has not been evaluated in *H. ducreyi*.

4.1.9 Future research. This study of the structural and antigenic heterogeneity of the *H. ducreyi* LOS should be broadened to include a larger group of strains from more diverse geographical locations. Furthermore, expression of specific LOS epitopes should be studied using MAb for which the reactivity has been well characterized and defined. This data should be correlated with mass spectrometry and sugar analyses performed in this thesis. One goal of future research should be the development, standardization and validation of a simple field-test which might either diagnose or type *H. ducreyi* directly in clinical specimens.

4.2 Vaccine development against chancroid.

In this report, we evaluated prophylactic efficacy against ulcer formation by booster vaccination with *H. ducreyi* OMP in FCA/FIA in an experimental animal model of chancroid. We studied and compared vaccination with native and recombinant

preparations of HgbA, as well as rD15. Preliminary experiments had shown the ability of nHgbA to bind Hg, suggesting that it retained its functional three-dimensional conformation (data not shown), while rHgbA was unable to bind Hg, suggesting an altered three-dimensional structure. Booster immunization of rabbits with a 100µg dose of purified HgbA in both forms and rD15 was shown to alter the course of both homologous and/or heterologous *H. ducreyi* infections in the rabbit model after a challenge inoculum, which in controls produced approximately 50% or 100% ulceration.

4.2.1 HgbA vaccine. Despite significantly higher inoculum titers in the nHgbA vaccine group than in controls, nHgbA provided partial protection against a homologous challenge, although numbers are limited and there was much variation in serial experiments of parallel groups. Both forms of the HgbA vaccine seemed to provide similar protection against a heterologous experimental challenge infection. The heterologous strain V1157 produced ulcers in about 50% of sites at 10^4 CFU, suggesting lower virulence than 35000.

Purity of the immunogen sample may affect vaccine efficacy. Since the lipid A portion of enterobacterial LPS has been shown to possess adjuvant activities (Powell and Newman 1995), LPS or LOS in the protein extract might act as an adjuvant. We show here that only purified nHgbA contains a low-molecular weight contaminant, possibly LOS. Although we have shown that vaccination with LOS alone did not modify infection or disease (Desjardins *et al.* 1995), we cannot exclude the possibility that LOS as adjuvant complexed with HgbA was responsible for the protective effects observed. Likewise, one might expect a carrier effect of protein Ag on immune response to LOS,

which might provide some vaccine effect. Our work showed heterologous protection by nHgbA preparation from strain 35000, and 35000 cross-reacted to V1157 LOS.

The tertiary structure of the proteins in the vaccine preparation might also account for the apparent differences seen in protective activity between the nHgbA and rHgbA vaccines. The crystal structure of two other TonB-dependent receptors, FhuA and FepA, both siderophore receptors of *Escherichia coli*, has been resolved (Buchanan *et al.* 1999; Ferguson *et al.* 1998; Locher *et al.* 1998). The data from crystal structure indicate a complex structure of surface-exposed loops, trans-membrane beta sheets and an internal N-terminal “plug”. Denaturation of nHgbA by boiling in Laemmli sample buffer or rHgbA by solubilizing in 6M-urea destroys the native structure if originally present. Antibodies raised to rHgbA were unable to recognize HgbA in OMP preparations, while those from nHgbA-immunized rabbits bound well to OMP (data not shown). This suggests that the antisera raised to rHgbA react to different epitopes than nHgbA, which may explain differential protective efficacy.

4.2.2 D15 vaccine. Although administration of rD15 in Freund’s adjuvant altered the course of an experimental homologous challenge infection, the results were very variable from run to run, as it was for the rHgbA vaccine. Indeed, rabbits were partially protected from inoculation with strain 35000, even with an inoculum as high as 10^5 CFU, in experiment 1 and 5, but the vaccination did not affect the course of the infection in experiments 2 and 3. Interestingly, rabbits in the latter experiments were vaccinated with a different batch of rD15 immunogen (table 3). Although a SDS-PAGE did not show any physical or structural differences, such as prominent protein degradation or presence of other proteinaceous contaminants between the batches of protein extracts (figure 39),

differences in antigenicity of rD15 preparations may account for the differences seen in protection. I showed that rabbits immunized with 100 µg of the 1.7 µg/µl extract of rD15 (animals from experiments 2 and 3) developed lower antibody titers than animals immunized with the 2.7 µg/µl batch of rD15 (figure 47 and table 14). The presence of lower antibody titers was in turn correlated with development of longer infection and disease. Ulcer duration and culture positivity were higher in animals with antibody titers ≤ 4000 than in animals with antibody titers > 4000 (figure 47). This is the first report of post-immunization antibody titer affecting the course of the experimental chancroid disease.

4.2.3 Which vaccinogen is most promising? Table 15 summarizes the results obtained for the 3 vaccines tested in this thesis. The apparent degree of protection by one or another vaccinogen will be influenced by the inoculum titer and the pathogenicity of the evaluated strain. It is notable that rabbits immunized with both nHgbA and rD15 had inocula significantly higher than in controls. Furthermore, strain V1157 had reduced virulence in this animal model of chancroid, which is why better protection was achieved against this strain.

4.2.4 Qualities of an effective chancroid vaccine. A vaccine against chancroid should greatly reduce the duration of infection and disease, and ideally completely prevent the development of the ulcerative lesion. It has been shown that HIV and, evidently *H. ducreyi*, can be transmitted to uninfected partners via the chancroidal ulcer (HIV may infect CD4⁺ cells of the ulcerative lesion) (Cameron *et al.* 1989; Kreiss *et al.* 1989; Plummer *et al.* 1990; Trees and Morse 1995). Thus, preventing the development of the ulcer may be effective in not only reducing transmission rates of chancroid, but also those

TABLE 15

Virulence of *H. ducreyi* 35000 (homologous strain) by inoculation titer and vaccination status

Immuni- zation group	Titer	Mean inoculum size (CFU) ±SD	Mean no. of days culture positive ±SD	Mean cumulative lesion size (mm) ±SD	Mean peak lesion score ±SD	Ulceration in scored lesion pairs ^c			Mean duration of ulcers (days) ±SD
						N	E	B [*]	
nHgbA (n=12)	10 ⁵	8.1±3.2 ^a	8.2±3.2	45.0±11	4±0	0	2	10	9.2±3.6 ^a
	10 ⁴	6.7±2.3 ^a	7.8±4.9	43.6±12	3.8±0.3	1	2	9	8.2±3.7 ^a
	10 ³	6.8±2.3 ^a	5.0±4.6 ^a	19.2±12 ^a	3.0±0.7 ^a	8	2	2 ^b	2.3±3.8
rHgbA (n=15)	10 ⁵	6.3±2.5	8.9±2.7	48.3±13	3.9±0.3	1	1	13	9.5±4.4
	10 ⁴	5.6±2.6	9.1±3.5	42.8±13	3.8±0.4	2	3	10	7.6±5.2 ^a
	10 ³	5.2±2.2	7.1±4.1	25.2±17	3.2±0.9	7	1	7	3.9±4.1
rD15 (n=13)	10 ⁵	7.6±2.8 ^a	6.8±2.7 ^a	43.9±13	3.9±0.3	1	3	9	7.9±4.5 ^a
	10 ⁴	6.1±2.5 ^a	8.5±3.2	40.4±13	3.8±0.6	1	2	10	8.5±4.0 ^a
	10 ³	5.8±2.4 ^a	7.4±3.9	24±14.2	3.3±0.7	7	2	4	3.3±4.1

* N = none (0/2); E = either (1/2); B = both (2/2)

^a p≤0.05, Student's t test versus sham

^b p=0.023, FET for any ulceration versus sham

^c p=0.05, FET for proportional ulceration across vaccination groups and inoculum titers

of HIV. Elimination of the ulcer in *H. ducreyi* infection would also have the benefit of decreasing the pain associated with the chancroidal ulcer. It would be even better if one could eliminate the formation of the lesions all together, before the pustule and the popule stages, since it has been shown that *H. ducreyi* is shed from these lesions (Al-Tawfiq *et al.* 1998).

The second criterion of an effective chancroid vaccine is elimination of viable *H. ducreyi* organisms at the site of infection, or at least the reduction of the number of days a lesion is culture positive. Indeed, it has been shown in the rabbit and human experimental models of chancroid that replication of virulent bacteria is responsible for the development of ulcers (Purcell *et al.* 1991; Al-Tawfiq *et al.* 1998; Throm *et al.* 2001). Furthermore, replicating organisms in the genitalia of *H. ducreyi*-infected hosts may be involved in the development of the lymphadenopathy characteristic of chancroid.

The activity of vaccine candidates described in this model cannot be called “ideal”, since none completely prevented the development of ulcers or the replication of the organisms after a homologous challenge. However, they did succeed in reducing culture positivity, ulcer duration and the proportion of lesions that ulcerated after an experimental challenge. It is notable that the inocula used to infect rabbits are quite high (10^3 to 10^5 CFU) compared to those used in the human experimental chancroid model (30 CFU) (Al-Tawfiq *et al.* 1998), which may better reflect the inoculum needed to cause natural chancroid. The presence of a very high number of bacteria inoculated in this model or the relative virulence of strains may affect the course of the disease, and the degree of protection conferred by vaccination. This might explain apparent differences in efficacy between homologous and heterologous strains and at different inocula. The

partial protection observed in this model may translate into much greater protection in human infections, likely due to differences in inoculum.

4.2.5 Improving the HgbA/D15 subunit vaccine. To improve a bacterial subunit vaccine, one must consider the type of adjuvant with which it was administered. Freund's complete adjuvant, a strong promoter of cell-mediated response was chosen in this study since it had previously been reported that the cellular immune response was important in bacterial clearing and reduction in ulcer formation in the experimental rabbit model of chancroid (Desjardins *et al.* 1995; Desjardins *et al.* 1996). However, granuloma and abscess formation is a common side effect of the administration of this adjuvant, which prevents its use in humans. Conversely, alum might not provide the necessary immune response to clear an *H. ducreyi* infection since it has been shown to preferentially stimulate a humoral response (Powell and Newman 1995), unless it would promote opsonophagocytosis or bactericidal antibodies.

4.2.6 Strengths and weaknesses of the animal model of chancroid. One feature of the TDRM of chancroid is the genetic variability of the animals. Since the rabbit model of chancroid uses partially out-bred New Zealand White rabbits, this increases the biological variability in their immune response to a particular antigen, or infection, mimicking the anticipated response in an outbred human population. We observed differences in immunogenicity and vaccine effect between experiments of groups of 12-18 rabbits, which may be related to such variability. The TDRM of chancroid is complex, and also operator-dependent. Blinding of an observer performing serial measurements is also an important part of comparative outcomes in controlled experiments. This may explain

why these results seem less pronounced than those obtained for the vaccine containing pilus preparation (Desjardins *et al.* 1995).

The experimental rabbit model lets the disease run its course to the end, allowing for full development of the chancroidal ulcer and the natural regression of the lesion over a 3-week period. The experimental human model of chancroid uses inocula (~30 CFU) that are probably closer to the inoculum size physiologically encountered in natural infection (Al-Tawfiq *et al.* 1998), although no report has thus far been published on that subject. Protective effects of vaccines can be overcome at high inocula, so the partial protection demonstrated here may translate into great protection in a different, more natural setting. Indeed, 10^5 *H. ducreyi* organisms, the highest inoculum used in the above-described experiments, can cause an important immune reaction when inoculated intraepithelially in the skin of the animal, independent of infection itself. Such an inflammatory response has been shown to play a role in ulcer development (San Mateo *et al.* 1999). Thus, at high inocula, if much of the ulceration is more directly due to the immune response, then one might not expect a vaccine to maintain efficacy. No experimental vaccine has totally protected animals in the TDRM (Hansen *et al.* 1994; Desjardins *et al.* 1995; Desjardins *et al.* 1996;).

4.2.7 Other *H. ducreyi* vaccines. Several other vaccines have been tested against an experimental *H. ducreyi* infection (Hansen *et al.* 1994; Desjardins *et al.* 1995; Dutro *et al.* 1999). The first experimental vaccine for chancroid contained acellular preparations from two strains of *H. ducreyi*, 35000 and Cha-1 (Hansen *et al.* 1994). Interestingly, although both strain provided protection against a homologous challenge infection, only preparations from the Cha-1 strain protected animals against a heterologous challenge.

Pilus preparation and LOS were subsequently studied in the TDRM of chancroid (Desjardins *et al.* 1995). While LOS immunization did not affect the virulence of *H. ducreyi* strain 35000, the vaccine containing the pilus preparation protected against both homologous and heterologous challenges (Desjardins *et al.* 1995). Another group tested the *H. ducreyi* hemolysin as a possible vaccinogen against experimental chancroid (Dutro *et al.* 1999). Although immunization with hemolysin did not affect the development of the lesions, it reduced the recovery of WT *H. ducreyi* strain 35000 from lesions, but not that of the hemolysin-negative mutants (Dutro *et al.* 1999).

4.2.8 Future work. This may include studying the efficacy of HgbA and rD15 vaccines in the experimental human model of chancroid. Adjuvancy appropriate to the presumably desired cell-mediated immunity in human vaccination is a developmental research need.

4.3 General conclusions.

In summary, there are serologic and structural LOS features that corroborate a simple SDS-PAGE grouping system for *H. ducreyi*. We have identified 2 new LOS molecular structures in clinical specimens. A large panel of diverse *H. ducreyi* isolates should be evaluated for LOS PAGE group and serologic category. This may permit serogroups to be identified for epidemiologic analysis in localized or sustained chancroid outbreaks.

Although a trend in vaccine effect was observed after the immunization with nHgbA, it is not clear whether it is this antigen or other antigens in the preparations that afforded partial protection against a *H. ducreyi* challenge.

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