

FEASIBILITY OF VACCINATION FOR CHANCROID:
SERO-IMMUNOLOGY AND VIRULENCE ASSAY IN AN EXPERIMENTAL
MODEL OF INFECTION

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of Graduate Studies
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ABSTRACT

Because of the well established epidemiologic and biologic interactions between human immunodeficiency virus (HIV) transmission and genital ulcer disease, measures to control chancroid could have a significant impact on the epidemic of HIV. Strategies to control chancroid might include vaccination as well as detection, treatment and prophylaxis through education.

My first goal was to standardize a serologic enzyme linked immunosorbent assay (ELISA) for measurement of human immunoglobulin G and M antibodies to *Haemophilus ducreyi*.

I developed an IgG and IgM antibody ELISA using pooled sera from clinically and microbiologically proven cases of chancroid as positive controls, and pooled sera from normal individuals without a history of sexually transmitted diseases as negative controls. Cross reactivity was minimized by adsorption of serum samples with a sorbent prepared from three other *Haemophilus* species. Standardization of ELISA for measurement of antibody to crude soluble bacterial antigen of *H. ducreyi* 35000 was performed with a panel of serum samples from clinically and microbiologically proven cases of chancroid and from controls. Test interpretation was standardized for optimal sensitivity and specificity using receiver operator characteristic curve (ROC) analysis on chancroid positive standards. Performance of the ELISA was enhanced in the period of early convalescence from acute primary chancroid and was not diminished in the presence of HIV coinfection, reflecting the tempo of the natural serologic response to an acute infection.

I also developed an inhibition ELISA to determine antigenicity of potential vaccine candidates in human *H. ducreyi* infection, using lipooligosaccharide (LOS) as the test antigen. In a panel of 10 sera from cases of primary natural *H. ducreyi* infection reactive to *H. ducreyi* 35000 soluble antigen, only 4 samples were identified with reaction to *H. ducreyi* 35000 LOS. Inhibition ELISA confirmed that pre-adsorption with LOS substantially diminished reactivity of these sera to LOS but not to soluble antigen, suggesting that LOS might be antigenically heterogeneous between strains and have more potential for a serogrouping system than a vaccinogen.

Using the ELISA to detect immunogenicity by measuring the serologic response to vaccine candidates in rabbits, we further tested the feasibility of three bacterial antigen preparations to induce protective immunity against infection and disease. LOS, carbohydrate and a pilus preparation were purified from *H. ducreyi* 35000 and used in a booster immunization procedure. The serologic response to each immunogen was monitored by ELISA. Using a temperature-dependent rabbit model of *H. ducreyi* infection, virulence was assayed by intra-epithelial challenge and measurement of disease for homologous strain 35000 or the virulent clinical isolate RO-34. LOS and the pilus preparation both induced humoral responses to the corresponding antigen, but the carbohydrate preparation did not. The kinetics of the LOS antibody response is suggestive of a type I T-independent response, whereas the pilus preparation induced an anamnestic response. A 100 μ l inoculum of 10⁵ CFU/mL of *H. ducreyi* 35000 or RO-34 suspended in Mueller-Hinton broth, consistently produced ulcerative lesions in naive rabbit controls. Immunization with LOS or carbohydrate did not modify virulence of infection with *H. ducreyi* 35000. Immunization

with the strain 35000 pilus preparation significantly reduced the severity of disease, and the duration of infection and disease compared with controls, with both homologous and heterologous infection. Serial histology of lesions from rabbits vaccinated with the pilus preparation compared with that of lesions from controls revealed accelerated lymphoid cell recruitment and more prominent plasma cell infiltrate.

To study the mechanisms of inducible immunity with the pilus preparation in this animal model, I conducted passive immunization experiments, and characterized the inflammatory infiltrate of chancroidal lesions. Polyclonal IgG was purified from hyperimmune serum raised against *H. ducreyi* 35000 whole bacterial cell lysate, the pilus preparation and from unvaccinated controls. Naive rabbits were passively immunized with 24 or 48 mg of purified polyclonal IgG intravenously and 24 hours after infusion, challenged with the homologous strain 35000. With controls, I compared virulence upon infectious challenge for each IgG preparation. Despite sustained antibody levels throughout the experimental period following passive transfer, no significant difference in disease resulting from infection with the homologous strain was observed.

I then comparatively evaluated the serial immunohistology of lesions produced by infectious challenge with the homologous strain in sham-immunized or rabbits immunized with the pilus preparation. Flow cytometric analysis of rabbit peripheral blood leucocytes with CD5 and CD4 rabbit lymphocyte markers prior to infectious challenge revealed two T lymphocyte populations: CD5+CD4+ and CD5+CD4-. Immunohistochemical stains for CD5 and CD4 markers were performed on sectioned lesion biopsies on days 4, 10, 15, and 21 after infection. Pilus preparation vaccinee lesions showed significant quantitative

acceleration and increase in T lymphocyte infiltration, and similar early increased recruitment of the CD4+ T lymphocyte subset preceding early lesion sterilization and early healing without ulceration. These phenotypic changes in lymphocyte population are seen to precede sterilization and healing after ulceration in control rabbits.

Intra-dermal challenge of pilus vaccinated rabbits with 100µg of the pilus preparation produced large indurated lesions at 48 hours with prominent lymphoid and plasma cell infiltration, edema and erythrocyte extravasation .

I conclude that both the LOS and pilus preparations are immunogenic and that the latter induces homologous and heterologous strain protection in this model of infection and disease. This inducible immunity is associated with cell mediated immune responses. With the development of vaccine adjuvants suitable for human use with similar efficacy to Freund's adjuvant, the *H. ducreyi* pilus may be a candidate or component of future human vaccines.

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This thesis is dedicated to
my wife Anik and son Sacha,
the two most important people in my life

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	v
DEDICATION	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvii
CHAPTER 1: INTRODUCTION	1
1. Chancroid	1
Historical perspective	1
Taxonomy	2
Clinical presentation	2
Diagnosis	4
Treatment	5
Epidemiology	6
2. <i>H. ducreyi</i> physiology	7
Biochemistry	7
Iron requirements	8
Cell wall composition	9
3. Virulence determinants of <i>H. ducreyi</i>	10
Adherence and Invasion	11

Exotoxins	13
Lipooligosaccharide	14
Pili	16
4. Interaction of <i>H. ducreyi</i> with the human immunodeficiency virus	17
5. Control strategies for chancroid	19
6. Immunity to <i>H. ducreyi</i>	20
Immune responses to natural infections	20
Vaccine development strategies	21
7. Animal models of chancroid	21
8. Statement of objectives and hypothesis	25
Specific aims:	25
CHAPTER 2: MATERIALS AND METHODS	27
1. Bacterial strains and culture conditions:	27
2. Antigen Preparation	28
Soluble bacterial antigen extract	28
Lipooligosaccharide antigen preparation	28
Pilus preparation	29
3. Standardization of an enzyme immunoassay for antibody to <i>Haemophilus</i> <i>ducreyi</i>	29
Sorbent preparation	29
Enzyme immunoassay to soluble antigen	30
Inhibition ELISA and rheumatoid factor	31

Patient population and sera	31
Standardization of ELISA	32
Comparative evaluation	33
4. Inhibition ELISA for human antibody to <i>H. ducreyi</i> Lipooligosaccharide ..	33
LOS ELISA	33
Inhibition ELISA	34
Serum population	34
5. Feasibility of experimental vaccination for chancroid	35
Animals and housing conditions	35
Immunization with LOS, carbohydrate and the pilus preparations	35
Virulence of <i>H. ducreyi</i> in immunized rabbits	36
Histopathology	37
ELISA	38
Western blotting	38
6. Evaluation of humoral immunity to pilus preparation immunization	39
Generation of whole cell or pilus preparation antisera	39
Fractionation of polyclonal IgG from hyperimmune and naive sera	40
Passive immunization with purified polyclonal IgG	40
Virulence of <i>H. ducreyi</i> in passively immunized rabbits	41
ELISA	41
7. Characterization of cellular infiltrate in chancroidal lesions	41
Quantitation of cellular infiltrate in chancroidal lesions	41

Isolation of rabbit peripheral blood leucocytes	42
Flow cytometric analysis of rabbit PBL	43
Intra-dermal testing in pilus preparation immunized rabbits	44
8. Descriptive and comparative statistical analysis	44
CHAPTER 3: RESULTS	46
1. <i>H. ducreyi</i> ELISA standardization	46
Reactivity of control sera	46
Standardization of the IgG and IgM ELISAs	46
Assay performance for detection of primary infections	52
HIV serology	52
Past history of genital ulcer disease	52
LOS reactivity	56
Inhibition of LOS and soluble antigen reactivity	56
2. Inducible immunity to <i>H. ducreyi</i> infection	60
Antigen extracts	60
Serologic response to immunization with LOS, carbohydrate and the pilus preparation	60
Infection with <i>H. ducreyi</i> 35000 in controls	66
Virulence of <i>H. ducreyi</i> in LOS and Carbohydrate immunized rabbits ...	70
Virulence of <i>H. ducreyi</i> in pilus immunized rabbits	70
Heterologous strain protection	70
Histopathology of chancroidal lesions in pilus immunized rabbit	78

3. Passive immunization with protective anti-sera	81
Serum antibody levels following passive immunization	81
Virulence of <i>H. ducreyi</i> in passively immunized rabbits	83
4. Characterization of cellular infiltrates in lesions of pilus vaccinated rabbits ..	89
Peripheral blood lymphocyte immunofluorescence	89
Immunophenotyping of lymphocytic infiltrate of chancroidal lesions ..	89
Intra-epithelial challenge of pilus vaccinated rabbits immunized with the pilus preparation	96
CHAPTER 4: DISCUSSION	98
1. Standardization of an <i>H. ducreyi</i> ELISA	98
2. Antigenicity of <i>H. ducreyi</i> LOS	102
3. Rabbit immunology	103
4. Rabbit model of chancroid	105
5. Inducible immunity in the temperature dependent rabbit model of chancroid	107
Immunization with carbohydrate and LOS antigen	107
Immunization with the pilus preparation	109
Passive Immunization	110
Characterization of cellular infiltrates in chancroidal lesions	111
Intradermal skin challenge of pilus vaccinated rabbits	112
6. Pilus vaccination in other models	114
7. Conclusions	116

REFERENCES 118

APPENDICES 132

LIST OF TABLES

1. IgG EIA performance with respect to time from onset of genital ulceration	50
2. IgM EIA performance with respect to time from onset of genital ulceration	51
3. IgG EIA performance by HIV serology	54
4. IgM EIA performance by HIV serology	55
5. IgG EIA performance in populations with a past history of genital ulcer disease	57
6. Homologous strain protection at 10^5 CFU/ml in rabbits immunized with the pilus preparation or LOS	69
7. Heterologous strain protection at 10^5 CFU/ml in rabbits immunized with the pilus preparation from strain 35000 and challenged with the RO-34 isolate	77
8. Virulence of <i>H. ducreyi</i> 35000 in rabbits passively immunized with 24 mg of polyclonal IgG from whole cell or pilus preparation specific antisera	87
9. Virulence of <i>H. ducreyi</i> 35000 in rabbits passively immunized with 48 mg of polyclonal IgG from whole cell or pilus specific antisera	88

LIST OF FIGURES

1. Serologic reactivity of normal sera with and without adsorption with sorbent	47
2. Inhibition of reactivity of the IgG and IgM EIAs	48
3. Receiver operator characteristic curve for the IgG and IgM EIAs	49
4. Specificity and sensitivity of the IgG and IgM EIA overall and at weekly intervals from time of onset of genital ulcers to phlebotomy	53
5. Mean reactivity of ten <i>H. ducreyi</i> antibody positive sera 4 weeks from onset of chancroid	58
6. Inhibition of reaction to LOS and soluble bacterial antigen	59
7. SDS-PAGE of the three antigen preparations for rabbit immunization	61
8. OMP profile of <i>H. ducreyi</i> 35000 and the Kenyan clinical isolate RO-34	62
9. IgM and IgG serologic response in rabbits immunized with LOS and the pilus preparation	63
10. Western blot analysis of seven rabbits immunized with the pilus preparation	64
11. Western blot analysis of IgG serologic response to the carbohydrate preparation	65
12. Virulence of <i>H. ducreyi</i> 35000 at 10^5 CFU/ml in naive control rabbits and sham- immunized rabbits	67
13. Appearance of disease in sham-immunized and naive control rabbits	68
14. Appearance of disease in LOS immunized and naive control rabbits	71
15. Virulence of <i>H. ducreyi</i> 35000 at 10^5 CFU/ml in LOS and naive control rabbits	72
16. Appearance of disease in carbohydrate immunized and naive control rabbits	73

17. Homologous strain disease protection in pilus preparation immunized	74
18. Homologous strain virulence at 10^5 CFU/ml in pilus immunized and naive control rabbits	75
19. Heterologous strain disease protection in pilus preparation immunized and naive control rabbits	76
20. Heterologous strain virulence at 10^5 CFU/ml in pilus immunized and naive control rabbits	79
21. Hematoxylin and eosin stained biopsy sample of chancroidal lesions at 10^5 CFU/ml from pilus immunized and naive control rabbits	80
22. Serum titers of rabbits passively immunized with whole cell or pilus preparation polyclonal IgG	82
23. Virulence of <i>H. ducreyi</i> 35000 at 10^5 CFU/ml in rabbits passively immunized with whole cell polyclonal IgG	84
24. Virulence of <i>H. ducreyi</i> 35000 at 10^5 CFU/ml in rabbits passively immunized with the pilus preparation polyclonal IgG	85
25. Appearance of disease 7 days after challenge with <i>H. ducreyi</i> 35000 in rabbits passively immunized with naive, whole cell or pilus preparation polyclonal IgG	86
26. Phenotypic characterization of peripheral T lymphocytes in pilus preparation immunized rabbits	90
27. Immunophenotyping of T lymphocyte infiltrate in biopsies of sham-immunized rabbits	92

28. Immunophenotyping of T lymphocyte infiltrate in biopsies of pilus preparation immunized rabbits	93
29. Comparative serial T lymphocyte infiltrate phenotype quantification in chancroidal lesions from infection, sterilization, and healing	94
30. Intradermal skin challenge of pilus preparation immunized rabbits	97
31. Silver stain and Coomassie blue stain of LOS and the uncharacterized carbohydrate extracts.	Appendix 1
32. Immunophenotyping of spleen T lymphocyte for CD5 and CD4 expression and negative control.	Appendix 2
33. Protein G purification of normal anti sera.	Appendix 3
34. Protein G purification of whole cell sera.	Appendix 4
35. Protein G purification of pilus preparation anti-sera.	Appendix 5
36. List of publications and published abstracts.	Appendix 6

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAP	Chocolate agar plate
CMP-NANA	Cytosine 5'-monophospho-N acetyl neuraminic acid
CFU	Colony forming units
DMK	Dimethyl menaquinone
DNA	Deoxy ribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FBS	Fetal bovine serum
GUD	Genital ulcer disease
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MIN	Minute

MLR	Mixed lymphocyte reaction
NBCS	New born calf serum
PBL	Peripheral blood lymphocytes
NZW	New Zealand white
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonucleocyte
ROC	Receiver operator characteristic curve
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UPEC	Uropathogenic <i>Escherichia coli</i>

CHAPTER 1: INTRODUCTION

1. Chancroid

Historical perspective

There are five classical venereal diseases, and except for gonorrhoea, the others which include syphilis, herpes genitalis, lymphogranuloma venereum and chancroid, are sexually transmitted genital ulcer diseases (GUD). Chancroid was first distinguished from syphilis in the early 1800s on the basis that chancroid could be auto-inoculated to adjacent anatomical sites, a feature not associated with syphilis (Kampmeir 1982). In 1889, Auguste Ducrey described the microscopic appearance of small streptobacilli from the pus of chancroidal lesions, which he could identify after passage by serial auto-inoculation (Kampmeir 1982; Davies 1905). For want of supportive artificial growth conditions and media, recovery of the organism in pure culture was not possible until 1897. Istamanov and Akspianz, using media consisting of macerated human skin isolated the “ bacillus of Ducrey” (Albritton 1989; Davies 1905). It was not until the early 1900's that Bazencour successfully reproduced chancroidal lesions after serial passage of *Haemophilus ducreyi* from ulcer exudate on blood agar medium (Albritton 1989) that *H. ducreyi* was established as the etiologic agent of chancroid. Because of inconsistent microbiologic isolation of *H. ducreyi* in clinical settings, and the availability of effective antibiotic therapy, interest in the disease declined (Ronald 1989; Clarridge *et al.* 1990). It was not until 1975, following an outbreak of chancroid in Winnipeg, Canada, that interest in chancroid was renewed and more reliable culture media and culture conditions became available for consistent recovery of the organism (Hammond

et al. 1979a; Hammond *et al.* 1978b).

Taxonomy

Classification of *H. ducreyi* within the genus *Haemophilus* of the family *Pasteurellaceae* is based on its haem requirement, the presence of nitrate reductase activity and a guanosine-cytosine content similar to the type species *Haemophilus influenzae* (Casin *et al.* 1985; Kilian 1976). Although *H. ducreyi* shows extensive serologic cross reactivity with other *Haemophilus* species (Ronald 1989), its classification within the *Haemophilus* genus has been contested. Arguments for new classification are based on features unique to *H. ducreyi* (Carlone *et al.* 1988; Casin *et al.* 1985; Kilian 1975). Major taxonomical differences include a lack of DNA relatedness (Casin *et al.* 1985), a larger requirement for haemin (Albritton *et al.* 1986) and the presence of both dimethyl menaquinone (DMK) and menaquinone as electron transport molecules rather than DMK alone or in combination with ubiquinone (Carlone *et al.* 1988). However, since no new and acceptable taxonomical classification has been proposed, *H. ducreyi* still remains in the *Haemophilus* genus.

Clinical presentation

The portal of entry for *H. ducreyi* is thought to be small abrasions on the epithelial surfaces of the genitalia which may occur during sexual intercourse (Jessamine and Ronald 1990; Morse 1989). With natural inoculation, mechanisms of tissue adherence and potential target cells of *H. ducreyi* which lead to infection and disease are still poorly characterized. Inflammatory lesions become visible within 7 to 14 days and appear as small papules with

an erythematous background, becoming pustular and rupturing to become a frank ulcer (Abeck and Johnson 1992). Chancroidal ulcers appear as painful irregular lesions with undermined borders and a friable purulent base (Jessamine and Ronald 1990; Clarridge *et al.* 1990). Clinical presentation of chancroid is often variable and differential diagnosis may include other (GUD) such as syphilis and herpes (Morse 1989; Clarridge *et al.* 1990). Up to half of the patients can go on to develop inguinal lymphadenitis, forming a bubo which may suppurate through the overlying skin (Morse 1989).

Several different clinical variants of chancroidal lesions have been described (Morse 1989 and King and Nicol 1975). Follicular lesions originate around hair follicles. Dwarf chancroid ulcerative lesions are similar in size and appearance to herpetic lesions. Transient chancroid are small lesions which heal rapidly but can progress after resolution to inguinal lymphadenitis within 3 to 4 weeks. Papular chancroid refers to typical painful ulcerative lesions of chancroid whereas giant chancroid are lesions originating from ruptured inguinal buboes which spread to other sites by auto-inoculation. Finally, phagedenic chancroid are the most severe ulcerative lesions and may be accompanied by complications. These lesions originate from several small typical ulcers which coalesce into large ulcerative lesions and may be accompanied by extensive destruction of the external genitalia. Complications include phimosis and urethral fistulas. In men, the most commonly affected sites include the mucosal surfaces of the prepuce, the distal prepuce, the coronal sulcus and less commonly, the glans, the shaft of the penis or the anus (Ronald, 1989). Urethritis due to *H. ducreyi* is also now recognized (Kunimoto 1988). In women, common sites include the labia, clitoris, fourchette, vestibule, and the medial aspects of the thighs (Morse 1989). In women,

chancroid is less symptomatic, and under-diagnosed owing to the slower recognition of disease and lesser visibility of lesions (Ronald 1989). Multiple lesions in women are common, and untreated cases may resolve 4 to 6 months from onset of disease compared to 3 to 8 weeks in men (Ronald 1989).

Histological features of chancroidal lesions are characterized by a three-zone architecture. The first zone consists of a narrow band of necrosis with degenerative polymorphonuclear (PMN) leucocytes, fibrin, and red blood cells. The second zone is a layer of edematous tissue, neovascular endothelial cells and reactive fibroblasts. One feature characteristic of chancroid is the prominent angiogenic response characterized by numerous small blood vessels and degenerative vessels with infiltrating neutrophils. The third zone underlying the first and second zones consists of infiltrating lymphocytic cells (Heyman 1945; Freinkel 1987).

Diagnosis

Diagnosis of chancroid has relied on clinical presentation of disease and microbiologic culture in settings with high prevalence. Classical chancroid is clinically differentiated from syphilis by virtue of pain and tenderness of the ulcer, and from herpes genitalis by the depth, purulence and undermined ulcer borders. Direct microscopic examination of ulcer exudate with Gram stain, reveals typical morphologic appearance of *H. ducreyi* consisting of parallel chains of streptobacilli often described as a “school of fish” or “train tracks” (Albritton 1989). Atypical presentation of chancroid requires exclusion of syphilis by dark field examination and appropriate serology. Unfortunately, non-expert or

inadequate microscopy makes the diagnosis of chancroid unreliable (Jessamine and Ronald 1990). Following the 1975 chancroid outbreak in Winnipeg, an appropriate medium for primary isolation of *H. ducreyi* was developed, consisting of gonococcal agar supplemented with haemoglobin and fetal bovine serum (FBS). Sensitivity of microbiologic culture has been reported to be anywhere from 60 to 80% depending on the geographic location (D'Costa *et al.* 1986; Kunimoto *et al.* 1986; Fast *et al.* 1984).

With a reported accuracy of 57% for clinical diagnosis of chancroid (O'Farrell *et al.* 1994) and inconsistent sensitivity of diagnosis by culture, alternative methods have been developed for diagnostic or epidemiologic applications. Such measures include the use of monoclonal antibodies in immunologic detection (Hansen and Loftus 1984; Karim *et al.* 1989; Schalla *et al.* 1986; Sloothman *et al.* 1985), enzyme profile (Shawar *et al.* 1990; Hannah and Greenwood 1982) or molecular approaches such as ribotyping (Sarafian *et al.* 1991), the use of DNA probes (Parsons *et al.* 1991) or PCR assays (Chui *et al.* 1993). Since these techniques are relatively expensive and rely on the availability of ulcer specimens, their practicality remains limited.

Treatment

Over the last few years, recommendations for the treatment of chancroid have been modified to accommodate increasing microbial resistance (Fast *et al.* 1984) and single dose treatment failure associated with infection with the human immunodeficiency virus (HIV) (Cameron 1995a; Cameron *et al.* 1988; Tyndal *et al.* 1993). In many endemic areas, tetracyclines, sulfonamides and trimethoprim, once recommended therapeutic approaches

for chancroid are no longer reliably effective (Plourde *et al.* 1992a; Schmid 1989 and McNicol *et al.* 1984). Recent guidelines by the U.S. Center for Disease Control and Prevention (CDC) for chancroid therapy (CDC 1993) include single doses of azithromycin or ceftriaxone or a 7 day regimen with erythromycin. Alternative regimens include amoxicillin or ciprofloxacin (CDC 1993). Recently, treatment failures with ceftriaxone have been reported in association with HIV seropositivity (Tyndal *et al.* 1993) underscoring the need for new, and more effective therapeutic approaches for populations at risk for chancroid and HIV.

Epidemiology

Chancroid is now believed to be the most common GUD with a world wide incidence which may surpass that of syphilis (Plourde *et al.* 1992b; Plummer *et al.* 1985). The disease is relatively uncommon in most industrialized nations with sustained outbreaks associated with illicit drug use (such as crack and cocaine) and prostitution (Martin and DiCarlo 1994; Plourde *et al.* 1992b; DesJarlais *et al.* 1991). Although chancroid is endemic in large urban centres of the United States such as New York or South Florida (Ronald 1989), control of outbreaks in Winnipeg and Orange County, California, was achieved with appropriate public health intervention such as screening and treatment (Blackmore *et al.* 1984; Hammond *et al.* 1978). In the United States, chancroid has been on the decline since 1987 when over 4500 cases were reported (Martin and DiCarlo 1994; Morse 1989). Interestingly, there has been a noticeable migration of outbreaks from coastal cities towards Midwestern rural settings (Martin and DiCarlo 1994). This trend may partially account for the decline in chancroid

incidence since under-reporting of cases may be associated with a general lack of clinical expertise in these settings (Martin and DiCarlo 1994).

Chancroid is endemic in many developing countries of Southeast Asia, South America and Sub-Saharan Africa. In some geographically distinct areas, chancroid accounts for more than 50% of all reported cases of GUD (Plummer *et al.* 1990). Historically, low socioeconomic status and poverty in third world nations has promoted the practice of prostitution. The lack of basic public health support and effective health care leads female prostitutes and their clientele to become a high prevalence reservoir for sexually transmitted diseases (Ronald 1989; Kreiss *et al.* 1989; Kreiss *et al.* 1986). The prevalence of chancroid in Kenyan prostitutes with GUD is nearly 80% (Plourde *et al.* 1992b; Plummer *et al.* 1985). In Kenyan men, infection with *H. ducreyi* accounts for 60% of all GUD compared to 11% and 4% for syphilis and genital herpes, respectively (Nsanze *et al.* 1989). Lack of circumcision has also been found to increase susceptibility to infection. Uncircumcised men were 3 times as likely to have a recent history or active chancroid infection compared to circumcised men (Cameron *et al.* 1989; Simonsen *et al.* 1988). The increased susceptibility to chancroid in uncircumcised males may be related to the moist mucosal preputial epithelium and the underlying glans providing ideal conditions for facilitated transmission and survival of *H. ducreyi* (Hand 1989).

2. *H. ducreyi* physiology

Biochemistry

H. ducreyi is essentially asaccharolytic with no glycosidase activity, but is capable

of reducing nitrite and does have a wide range of phosphatase activity (VanDyck and Piot 1987). Some weak reactions have been detected for glucose and maltose metabolism but vary depending on test method (Sottneck *et al.* 1980). A wide spectrum of aminopeptidase activity was also noted in a large number of geographically distinct isolates (VanDyck and Piot 1987). Although most strains were found to have similar aminopeptidase profiles, several enzymatic forms were unique to some isolates (VanDyck and Piot 1987).

Iron requirements

Iron is essential for the growth of *H. ducreyi*, and as the case with other microorganisms, it plays a central role in numerous metabolic processes including oxygen transport and as a co-factor in the electron transport chain (Salyers and Whitt 1994). An effective means for the host to prevent or control the growth of pathogens in the body is to limit the availability of iron. In the host body, free iron is in low concentration and primarily sequestered by iron binding proteins such as lactoferrin, transferrin, ferritin and haemin. Microorganisms scavenge iron by releasing into the environment high affinity iron chelators such as siderophores. Uptake of the iron chelator complex is mediated by specific outer membrane receptors (Salyers and Whitt 1994). Other strategies include the ability to utilize alternative iron sources such as the host iron-binding proteins or other haem-containing proteins. *H. ducreyi* can utilize haemin and several haem-binding proteins such as haemoglobin, catalase and cytochrome C_{III}, but not lactoferrin, transferrin or inorganic iron (Lee 1991). Although siderophore production has not been demonstrated in *H. ducreyi*, several inducible outer membrane proteins have been detected when *H. ducreyi* is grown

under haem limiting conditions (Elkins 1995; Lee 1991; Abeck *et al.* 1990). One of these, a 100 kDa outer membrane haem binding protein has been purified and characterized. The specificity of the haem binding protein was found not to be limited to human haemoglobin, which contrasts with the *H. influenzae* haem binding protein. Several sources of haem such as cat, dog, horse, cow and rabbit haemoglobin can support the growth of *H. ducreyi* (Elkins 1995).

Cell wall composition

Ultrastructural examination of the cell wall of *H. ducreyi* revealed morphological features common with other Gram negative bacteria (Kilian and Theilade 1975). One report described the presence of a discontinuous extracellular capsule visible by ruthenium red staining on electron microscopy (Albritton 1989). However, the presence of an extracellular polysaccharide on *H. ducreyi* has never been confirmed. The composition of the outer membrane also appears typical of other Gram negative bacteria. Analysis of outer membrane proteins by electrophoresis, revealed five or six major protein constituents and several minor species (Odumeru 1983). Comparison of the protein profile in the 24 to 50 kDa range permitted the presumptive classification of clinical isolates into 7 subtypes. Other investigators using similar approaches (Taylor *et al.* 1985) or immunoblot analysis (Roggen *et al.* 1992; Saunders and Folds 1986) have since confirmed the heterogeneity of the outer membrane proteins of *H. ducreyi*. Outer membrane protein profiles were not modified by different culture conditions or by serial passage (Abeck *et al.* 1987a; Odumeru *et al.* 1983). Several antigenically conserved proteins with molecular masses of 18, 29, 40, and 62 kDa

have been identified (Spinola *et al.* 1992; Finn *et al.* 1990; Schalla *et al.* 1986; Hansen and Loftus 1984). One of these, the 40 kDa protein, has been shown to be present in all tested strains (Roggen *et al.* 1992; Abeck and Johnson 1987b; Taylor *et al.* 1985; Odumeru *et al.* 1983). Purification and characterization of this major outer membrane protein revealed similarities in amino acid composition and serologic cross reactivity to the outer membrane OmpA porin of *Escherichia coli* (Spinola *et al.* 1993). Other members of this porin family include protein 5 of *H. influenzae*, protein F of *Pseudomonas aeruginosa*, and pIII protein of *Neisseria gonorrhoeae* (Spinola *et al.* 1993). Members of the OmpA protein family may represent a unique class of porins since they do not appear to form typical ion permeable channels (Yoshihara and Nakae 1989 and Nakaido *et al.* 1991). The role of this protein in the pathogenicity of chancroid is not known.

3. Virulence determinants of *H. ducreyi*

Pathogenesis can be defined as the mechanism by which a microorganism causes infection or disease in a particular host, and virulence as the degree of pathogenicity measured by disease severity and invasiveness (Salyers and Whitt 1994). Survival and multiplication are the primary concerns of invading pathogens, and disease an outcome of the interaction between the parasite and its host. Thus, all factors that contribute to infection and disease by ensuring survival, such as haem-binding proteins, could be considered virulence determinants (Mekalanos 1991).

There is a lack of consensus and experimental tools that allow the consistent distinction between virulence and avirulence of *H. ducreyi* strains. Although all described

strains were originally clinical isolates, virulent and avirulent strains have been described in relation to polymyxin resistance (Odumeru *et al.* 1984), sensitivity to serum bactericidal activity (Odumeru *et al.* 1987) or based on the production of typical chancroidal lesions in the classical intra-dermal rabbit test (Hammond *et al.* 1978). However, type strain CIP 542, which is classified as avirulent according to these criteria, still retained its virulence following accidental laboratory exposure (Lagergård 1995); this reflects the need for improved and standardized approaches to define virulence of *H. ducreyi*.

How *H. ducreyi* causes disease, and what are the principal virulence factors are still unknown. Presumptive identification of potential virulence determinants is largely based on functional comparisons with virulence determinants of other Gram negative mucosal pathogens such as *H. influenzae* and *N. gonorrhoea*. Several virulence traits and virulence factors have been described: Adherence and invasion, cytotoxins, haemolysins, lipooligosaccharide (LOS) and pili.

Adherence and Invasion

To cause disease, a pathogen must enter the host, adhere to target tissues, evade host defence mechanisms, replicate and cause disease. *H. ducreyi* has been shown to adhere to cultured cells such as primary human foreskin fibroblasts (HFF) (Alfa *et al.* 1995; Hollyer *et al.* 1994; Alfa *et al.* 1993; Lammel *et al.* 1993), human keratinocytes (Brentjens *et al.* 1994; Totten *et al.* 1994a), cell lines such as HeLa or HEC-1-B (Lammel *et al.* 1993; Lagergård *et al.* 1993; Purvén and Lagergård 1992) and extracellular matrix components such as fibrinogen, fibronectin, collagen, gelatin and laminin (Abeck and Johnson 1992).

Many of these studies have identified *in vitro* phenotypic features such as induction of cytopathic effects (CPE), microcolony formation and cytoadherence (Alfa *et al.* 1995; Hollyer *et al.* 1994; Alfa *et al.* 1993; Lammel *et al.* 1993; Lagergård *et al.* 1993; Purvén and Lagergård 1992). Alfa and co-workers (1995) correlated expression of these phenotypic traits with virulence in a temperature dependent rabbit model of infection. Avirulence in this system correlated well with loss of one or more of these three phenotypes, defined by the absence of disease. The significance of these observations relating to natural human infection is not known.

Adherence to target cells can be mediated by different types of bacterial adhesins including pili and other surface exposed outer membrane components such as LOS. Alfa and co-workers (1993a) did observe a fibrillar matrix in the gap separating adherent *H. ducreyi* cells and cultured primary HFF cells. The nature of this matrix however has not been defined.

Several groups examining adherence of *H. ducreyi* to primary and transformed cell described the localization of adherent strains either on the surface of cultured cells, within interstitial spaces or become internalized (Totten *et al.* 1994a; Lammel *et al.* 1993; Lagergård *et al.* 1992). However what appear to be cytoplasmic vacuoles containing microorganisms may be invaginations or folds in the cell membrane to which *H. ducreyi* has adhered. This issue was addressed by Totten and co-workers (1994a) by performing the gentamicin protection assay originally described by Devenish and Schiemann (1981). This technique relies on the fact that internalized microorganisms are protected from the actions of gentamicin because of poor intra-cellular penetration of this antibiotic. Surviving bacteria

released from the cells by disruption can then be enumerated. Invasiveness was tested on cultured human foreskin epithelial cells, which are equivalent to keratinocytes. Positive controls included a highly invasive *Salmonella* strain and an adherent but non-invasive *E. coli* as negative control. The results indicated that although invasion of cell cultures by *H. ducreyi* was significantly higher than that of *E. coli*, it was significantly lower than that of *Salmonella*. Microfilament inhibitors such as cytochalasin B and D reduced invasiveness as measured in this assay, suggesting that *H. ducreyi* may in fact be an intra-cellular pathogen (Totten *et al.* 1994a). Results obtained by Alfa and co-workers (1993a) and Hollyer and co-workers (1994), using similar approaches, did not correlate with internalization of *H. ducreyi* into primary HFF, contrasting the results of Totten and co-workers in an epithelial cell line (1994a). The lack of agreement between studies may reflect differences in experimental methods, including cell lines examined, interpretation of results, and definition of what is considered to be significant invasion.

Exotoxins

Cytopathic effects (CPE) on cultured cell lines resulting from the adherence of *H. ducreyi* have been demonstrated (Alfa *et al.* 1995; Hollyer *et al.* 1994; Lammel *et al.* 1993; Lagergård *et al.* 1993; Purvén and Lagergård 1992). The CPE was subsequently characterized by Hollyer and co-workers (1994) and was shown to be contact-dependent without identifiable diffusible toxins (Hollyer *et al.* 1994). Furthermore neither bacterial sub-fractions nor spent media from *in vitro* cell culture grown *H. ducreyi* could induce CPE. These results appear to contrast with those of Purvén *et al.* (1992) and Lagergård *et al.*

(1993), who identified several strains that may express soluble exotoxins. The relevance of this cytotoxic activity is difficult to evaluate since CPE was only observed on transformed cell lines and not on primary HFF. The bacterial fraction responsible for the cytotoxicity was not well characterized and consisted of osmotic or sonic cellular preparations and spent culture media. Heat and pronase treatment could only partially abolish cytotoxicity suggesting that LOS or other heat stable factors may be partially responsible for the observed effects.

The haemolytic activity of *H. ducreyi* can be readily detected using horse red blood cells (Palmer *et al.* 1994). Low level haemolysis was also detected with sheep, rabbit and human erythrocytes. Recently, the gene encoding a putative *H. ducreyi* haemolysin was cloned and sequenced, and shown to share extensive homology with *Proteus mirabilis* and *Serratia marcescens* haemolysins (Palmer *et al.* 1995). The relevance of this putative haemolysin or cytotoxin in disease is not known.

Lipooligosaccharide

Lipopolysaccharide (LPS) is a major constituent of the outer membranes of Gram negative bacteria and it plays an important role in maintaining cellular integrity (Qureshi and Takayama 1990). The structure of LPS can be divided into three regions. Lipid A (also known as the endotoxin since it harbours the toxicity of LPS) anchors the LPS into the outer leaflet of the outer membrane. The core polysaccharide is linked to the lipid moiety via a unique 3-deoxy-D-manno-2 octulosonic acid (KDO), which in turn is linked to the O antigen side chain consisting of repeating units of oligosaccharide units of variable length (Qureshi

and Takayama 1990 and Jimenez-Lucho et al. 1990).

Electrophoretic analysis of LPS revealed that *H. ducreyi* like to other Gram negative mucosal pathogens, expresses a truncated oligosaccharide that is now being referred to as lipooligosaccharide (LOS) (Abeck *et al.* 1987b; Odumeru *et al.* 1987). Characterization of the molecular structure revealed a branched terminal oligosaccharide of approximately 8 sugar residues (Melaugh *et al.* 1992). The terminal trisaccharide unit of *H. ducreyi* LOS is conserved among several Gram negative non-enteric pathogens such as *H. influenzae* and *N. gonorrhoeae* and binds to the monoclonal antibody 3F11, specific for the same epitope present on gonococcal LOS (Campagnari *et al.* 1990). This 3F11 epitope has also been shown to be homologous to the paragloboside precursors of the human I blood group antigens (Mandrell *et al.* 1992). By mimicking host antigens, this antigenic component of LOS may serve to protect *H. ducreyi* from host defences.

Analysis of *H. ducreyi* LOS revealed electrophoretic heterogeneity among different strains and these differences might be related to virulence (Abeck *et al.* 1987c; Odumeru *et al.* 1987). Susceptibility to bactericidal activity of normal or convalescent serum was associated with the type of LOS (Odumeru *et al.* 1985). Serum sensitivity was also shown to be associated with avirulence in the classical intra-dermal rabbit model (Odumeru *et al.* 1987). Furthermore, the potential role of LOS in disease was demonstrated when intradermal injection of purified LOS induced inflammatory lesions similar to those caused by heat-killed *H. ducreyi* cells or LOS from an unrelated species (Campagnari *et al.* 1991). However, limitations of this animal model make interpretation of the results difficult. Similar challenges in the temperature-dependent rabbit model of infection failed to produce

lesions (Purcell et al 1992).

Much of our understanding of *H. ducreyi* LOS as a virulence factor in chancroid comes from comparisons with the LOS of other Gram negative mucosal pathogens. Similar to *N. gonorrhoeae*, *H. ducreyi* LOS epitopes can be sialylated *in vivo* (Mandrell *et al.* 1992). However unlike *N. gonorrhoea*, cytosine 5'-monophospho-N acetyl neuraminic acid (CMP-NANA) synthase activity has been detected suggesting that *H. ducreyi* is capable of synthesizing the necessary precursors for *in vitro* sialylation of LOS (Tullius *et al.* 1995). Although the role of sialylation of *H. ducreyi* LOS is unknown, sialylated gonococcal LOS has been shown to be an important virulence factor (Mandrell and Apicella 1993). Since sialic acid is an ubiquitous component of mammalian tissues, sialylation may provide pathogens with the means of evading host immunity by mimicking host antigens. Sialylation has also been shown to decrease the binding of opsonizing antibody, decrease binding to neutrophils and inhibit the activation of the classical and alternative complement pathways. LOS has also been shown to form divalent cation bridges with outer membrane proteins as well as mediate binding to eukaryotic cells such as human keratinocytes (Campagnari *et al.* 1995).

Pili

Pilus-like structures have been identified by negative staining and electron microscopy in 10 of 12 *H. ducreyi* clinical isolates (Spinola *et al.* 1990). The structures appeared as short, fine and disorganized bundles with a cross sectional diameter of 3nm. Purification and characterization of the 24 Kd pilin protein from all 12 clinical isolates

revealed an amino acid composition similar to that of the pili of other Gram negative non-enteric pathogens such as *H. influenzae*. The significance of pili in the pathogenicity of chancroid is not known. However, because of the small surface area of pili, electrostatic repulsive forces would not affect the binding to receptors on surfaces of target cells. This interaction may provide long range, reversible binding until more stable interactions can be established. Although no serological studies were done to examine cross reactivity, electrophoretic mobility and amino acid composition of the pilin monomer appeared to be stable among all isolates tested and after more than 80 in vitro passages with three strains (Spinola *et al.* 1990).

4. Interaction of *H. ducreyi* with the human immunodeficiency virus

Chancroid has become a public health concern in the last 10 to 15 years because of its biological co-factor or promoter status for sexually transmitted human immunodeficiency virus (HIV) (Behet *et al.* 1995; Augenbraun *et al.* 1994; Cameron *et al.* 1989; Greenblatt *et al.* 1988; Holmberg *et al.* 1988; Simonsen *et al.* 1988). Up to 49% of HIV-1 seropositive females attending a sexually transmitted disease clinic were infected with *H. ducreyi* or had a recent history of chancroid compared to 17% for a similar seronegative group. The HIV acquisition rate for uncircumcised men after a single sexual contact with an HIV infected partner with chancroid was 43% (Cameron *et al.* 1989). Some association between HIV and other sexually transmitted disease can be attributed to a shared risk of exposure and modes of transmission (Cameron and Padian 1991).

It is believed that the biological interaction between GUD and sexual transmission

of HIV consists of a complex bi-directional association. Type I interactions refer to increased susceptibility to or infectiousness of HIV in the presence of GUD. This facilitated transmission of HIV in the presence of GUD is thought to arise from the breakdown of the protective barrier of the epithelial surfaces, with ensuing inflammatory responses associated with the recruitment of lymphocytes and monocytic cells, and shedding of HIV from ulcerative lesions (Plummer *et al.* 1990; Kreiss *et al.* 1989; Greenblatt *et al.* 1988; Kreiss *et al.* 1986). Epidemiologic surveys are also suggestive of an interaction between HIV and augmented virulence of GUD pathogens. Evidence for this type II interaction comes from reports showing increased GUD prevalence associated with HIV seropositivity (Simonsen *et al.* 1988), and reduced effectiveness of antibiotic therapy for GUD among HIV patients despite antimicrobial drug sensitivity (MacDonald *et al.* 1989). Increased likelihood of treatment failure with single dose of oral antibiotic therapy for chancroid was associated with HIV seropositivity. MacDonald and co-workers found a 27% failure rate for fleroxacin in HIV positive patients compared to 5% among HIV negative individuals. Success rates for therapy with trimethoprim-sulfamethoxazole or quinolones was 94% for HIV seronegative and 60% for seropositive patients, with treatment failures a predictor for HIV seropositivity (Cameron *et al.* 1988). Moreover, failure rates with ceftriaxone were 60% among HIV patients compared to 27% for a similar seronegative group (Tyndall *et al.* 1993). Thus, because of the biologic positive feedback interaction of GUD and HIV, chancroid control has become doubly important to HIV control strategies. Design of such control programs to limit HIV transmission must also consider the control of GUD (Cameron and Padian 1991).

5. Control strategies for chancroid

There are several features of chancroid that suggest it is a controllable disease: 1) core groups have been identified 2) there are no reservoirs other than humans, and 3) there are no carrier states associated with chancroid. Public health control measures directed towards chancroid may include contact tracing and empirical antibiotic therapy for *H. ducreyi*, and prophylaxis by education and promotion of the use of condoms. Antibiotic therapy may be effective in controlling sporadic localized outbreaks, but may not be effective in areas of established, sustained dynamic outbreaks. One major concern with this approach is the demonstrated potential for selection of antibiotic resistance. Furthermore, antibiotic treatment does not prevent re-infection, may not be as effective in HIV seropositive individuals and is largely dependent on patient compliance. Promotion of the use of condoms might provide protection against GUD and HIV in HIV seronegative patients (Cameron *et al.* 1991). However, because of the enhanced virulence of GUD pathogens in HIV seropositive individuals and lower than 100% compliance by the clientele of prostitutes, condoms have not been particularly effective as a control tactic for GUD (Plourde *et al.* 1992b; Cameron *et al.* 1991).

Effective vaccination, if feasible, would offer an attractive control strategy through durable, practical, and cost effective chancroid prevention. Several features of chancroid suggest that vaccination may be feasible. The disease is localized and does not disseminate beyond regional lymph nodes. The disease does resolve naturally, without intervention, with a serologic response, and HIV seropositive patients have a more severe clinical course with longer disease duration and higher rates of single dose antibiotics treatment failures. It

would appear therefore that there might be a potential role for the immune system in clearance of the disease.

6. Immunity to *H. ducreyi*

Immune responses to natural infections

The lack of information concerning the nature and relevance of the immune response to *H. ducreyi* is an obstacle for vaccine development. Re-infection and auto-inoculation are features common to chancroid.

Humoral immune responses to *H. ducreyi* antigens have been detected in acute and convalescent sera following natural infection with *H. ducreyi* (Roggen *et al.* 1994; Alfa *et al.* 1992; Museyi *et al.* 1988). Since detection of serologic reactivity relies on the use of crude bacterial preparations, specificity of the antibody response has been questioned because of cross reactivity of *H. ducreyi* with other *Haemophilus* species (Lagergård 1995). Because chancroid produces a localized infection, the magnitude of the immune response may be such that a single natural infection is insufficient to induce protective immunity (Lagergård 1995). Furthermore, since there are no standardized epidemiological tools available to detect antigenic strain heterogeneity or lack thereof, recurrent chancroid episodes may be associated with heterogenous populations of *H. ducreyi*. Thus, protective acquired immunity following natural infection may only be induced after multiple infections or following exposure to diverse populations of *H. ducreyi* to ensure cross strain protection.

Studies have demonstrated a potential role for cell mediated immune responses (CMI) in clearance of the organism. Intradermal injection of sterile ulcer exudate in

convalescent chancroid patients produced papular lesions visible within 48 hours and the reaction remained positive for their lifetimes (Heyman *et al.* 1945). Histological examination of lesions from human experimental infections revealed prominent perivascular infiltration of T lymphocytes and macrophages characteristic of CMI responses (Spinola *et al.* 1994).

Vaccine development strategies

In the course of natural infection, exposure to irrelevant decoy antigens or suppressor epitopes may interfere with the induction of appropriate protective immune responses (Lowell 1990). There are several approaches to vaccine development that could circumvent these problems and induce effective protective immunity. One of these approaches is to design a subunit vaccine, such that potential suppressor epitopes or decoy antigens are excluded from the final vaccine preparation (Lowell 1990). One design strategy is to characterize protective antigens from pathogens causing the animal equivalent of a human disease. The fact that *H. ducreyi* does not naturally infect animals however does not necessarily preclude the use of this approach in chancroid vaccine development. Animals can be used as effective tools by carefully designing and developing a viable model of disease that could be used to identify bacterial fractions associated with inducible immunity.

7. Animal models of chancroid

There are 4 important constraints that must be considered for the successful development and use of an animal model in vaccine development (Smith 1989): 1) The

growth of the pathogen must be quantifiable, such as with the use of colony counts expressed as colony forming units (CFU). 2) Disease must be produced with relatively small inocula and be similar in appearance, duration and course to natural human disease. 3) Disease must be measurable and 4) there must be a measurable immune response.

Animals such as mice, rabbits and monkeys have been evaluated as potential pathogenesis or virulence models of *H. ducreyi* (Totten *et al.* 1994b; Trees *et al.* 1991; Tuffrey *et al.* 1988; Freiner *et al.* 1945). The classical test for *H. ducreyi* virulence is the rabbit intradermal test (Freiner *et al.* 1945). In this model, intradermal injection of *H. ducreyi* induces necrotic ulcerative lesions. Both IgG and IgM humoral responses are also detectable in this model (Saunders and Folds 1986). Similar to other bacterial pathogens, antibodies of these classes are directed towards antigens with molecular masses of 79, 62, 55, 49 and 26 Kd (Morse 1989). Campagnari and co-workers (1991) demonstrated that intradermal inoculation of purified LOS from *H. ducreyi* or other Gram negative pathogens induced similar lesions to those induced by challenge with viable bacteria. Intradermal inoculation of *H. ducreyi* into the flanks of mice produced similar lesions to those observed in the rabbit model (Tuffrey *et al.* 1988). However, neither one of these animal systems represents an appropriate model of infection, since large inocula sizes ($>10^7$ CFU) are required to produce ulcerative lesions. The models may also lack specificity since challenge of rabbits with heat killed bacteria or purified LOS could induce lesions (Campagnari *et al.* 1991; Tuffrey *et al.* 1990).

A primate model of *H. ducreyi* infection was recently developed by Totten and co-workers (1994b). In this system, adult pig-tail macaques were intradermally injected with

10^7 to 10^8 CFU of *H. ducreyi* on the exposed surfaces of the foreskin of the penis in males or the opening of the vaginal surfaces in females. Lesions developed only in males and not in female Macaques, and were clinically and histologically similar to those described in natural human infection. Inocula consisting of heat killed *H. ducreyi* cells failed to induce lesions. However, production of disease required high inoculum sizes and the financial restrictions imposed by the use of primate models would limit interpretation of small number of observations.

To circumvent problems associated with the classical rabbit model, Purcell and co-workers (1992) developed a temperature dependent rabbit model of *H. ducreyi* infection. In this model, rabbits are housed at an ambient temperature of 15°C. Intradermal challenge of rabbits with *H. ducreyi* consistently produced ulcerative lesions at an inoculum size of 10^5 CFU/mL, clinically and histologically similar to natural human chancroid. Inoculation with 10^4 CFU/mL inconsistently produced ulcerative lesions. The specificity and validity of the model was shown by demonstrating the absence of lesions in rabbits challenged with heat-killed organisms or in rabbits pre-treated with antibiotics prior to infectious challenge with viable *H. ducreyi* (Purcell *et al.* 1992). Recently, induction of protective immunity was demonstrated in this rabbit model of disease (Hansen *et al.* 1994). Immunization with a cell envelope preparation successfully protected rabbits against subsequent challenge with both the homologous and a heterologous strain. A method for use of this model of infection as a quantitative virulence assay has been developed (Meloche *et al.* 1992). With this model, alterations in *H. ducreyi* virulence following immunization with vaccine candidates can be measured. Several quantitative parameters such as lesion size, score, number of days lesions

remain culture positive, duration of ulcerative lesions, peak lesion size and number of ulcerous lesions were defined to assess modifications in *H. ducreyi* virulence. When considered with chancroidal histology of these lesions, the temperature dependent rabbit model of *H. ducreyi* infection is a viable and appropriate system to examine the feasibility of vaccination for human chancroid.

The final requirement needed to develop a successful model for experimental evaluation of vaccination is a measurable and detectable immune response. Several immunological techniques can be applied towards the detection of immunologic reactivity to vaccine candidates. Enzyme-linked immunosorbent assays (ELISA) can be utilized to detect IgG and IgM class antibody serologic responses to immunizing antigens as a marker for specific immune responses, as well as to define kinetics of the antibody responses in the animal model. Similarly, an ELISA might permit seroepidemiologic detection and measurement of the prevalence of chancroid in targetable reservoirs or core groups and evaluation of medical public health STD control programs. Such serologic tests might also be applied in measuring the individual or population response to a human vaccine.

A desirable serologic test must not only have technical simplicity and be of low cost, but it must also have high or at least well defined performance characteristics. Likewise, identification of *H. ducreyi* specific antigens for which serum antibody is uniformly present in geographically diverse natural infections may permit, in addition to developing a more specific assay, the evaluation of the general presence of potential vaccinogens in diverse natural infecting strains. Identification of such antigens present in bacterial fractions may be pursued through studies of specific antibody reaction and inhibition with the vaccinogen.

8. Statement of objectives and hypothesis

Because of the demonstrated association between chancroid and HIV, the increased incidence of microbial resistance and single dose treatment failures associated with HIV seropositivity, chancroid control strategies have become important. The primary aim of this work was to examine the feasibility of vaccination against chancroid using the temperature-dependent rabbit model of infection. Since a temperature-dependent rabbit model of chancroid has already been established, a sensitive assay for the characterization of serologic reactivity to immunizing antigens and for future evaluation of control strategies needed to be developed and standardized. Inducible immunity of purified *H. ducreyi* fractions was assessed in this animal model, with the subsequent characterization of the immune response.

I hypothesized that induction of durable cross strain protective immunity to *H. ducreyi* infection by immunization with purified, exposed and conserved antigens using the temperature dependent rabbit model of chancroid, would demonstrate the feasibility of vaccination for human chancroid.

Specific aims:

- 1) Develop and improve upon an IgG and IgM ELISA for *H. ducreyi*, and optimally standardize the performance of assay using receiver operator characteristic curves.
- 2) Evaluate the performance of the ELISA in natural human infections with respect to time from onset of disease and HIV co-infection.
- 3) Develop an inhibition ELISA to define the antigenicity of *H. ducreyi* LOS.
- 4) Demonstrate immunization potential and cross strain protection of purified bacterial

components.

- 5) Evaluate the humoral and cell mediated components of detectable protective acquired immunity, by measuring vaccine effects of passive transfer of immunity with "protective" anti-sera and to perform immunocytochemistry of biopsies from lesions of naive and vaccinated rabbits to characterize the cellular infiltrate associated with disease convalescence and protection.

CHAPTER 2: MATERIALS AND METHODS

1. Bacterial strains and culture conditions:

H. ducreyi 35000 was originally isolated from a 1975 Winnipeg outbreak and was subsequently characterized (Hammond *et al.* 1978a). This strain has been shown to be virulent in the temperature dependent rabbit model (Purcell *et al.* 1991; Meloche *et al.* 1992). *H. ducreyi* strain RO-34 is a Kenyan clinical isolate. Growth of *H. ducreyi* was carried out on chocolate agar plates (CAP) supplemented with 1% Iso Vitale X (BBL Microbiology Systems, Cockeysville, Md.) and 5% fetal bovine serum (FBS) or in broth (α Minimal essential medium and Mueller-Hinton agar with 5% FBS [1:1 ratio {vol/vol}]). Plates were incubated for 48 hours at 33°C in 5% CO₂ and broth cultures were incubated for 16 hours at 33°C in an environmental shaker. Inocula for subsequent infection in the temperature dependent rabbit model of infection were prepared by harvesting *H. ducreyi* from late mid-log phase of growth in broth by centrifugation at 3000 x g for 20 minutes (min). Pellets were washed once in phosphate-buffered saline (PBS, 0.01 M NaHPO₄, 0.1M NaCl [pH 7.2]). Sarcosyl insoluble outer membrane proteins were isolated for comparison of *H. ducreyi* 35000 and strain RO-34 as previously described (Odumeru *et al.* 1983). Distinct protein profiles were compared by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% acrylamide gel and Coomassie blue stain.

2. Antigen Preparation

Soluble bacterial antigen extract

Soluble crude bacterial antigen for detection of *H. ducreyi* antibody by enzyme immunoassay (ELISA) was prepared from 20 CAP grown *H. ducreyi* 35000. Bacterial lawns were harvested and suspended in 10mL of sterile PBS, and the suspension washed four times and collected by centrifugation at 1500 x g for 15 min at 4°C. The pellet was suspended in 10mL PBS with 1% sodium dodecyl sulfate (SDS) and sonicated three times on ice using 30 second pulses with 15 second pauses (maximum microtip limit; 70% duty cycle). The suspension was rocked for 2 hours at room temperature (RT), and the supernatant was collected after centrifugation at 50000 x g for 90 min. The protein concentration of the supernatant was determined for future adjustments with the Bio Rad protein assay kit (Bio Rad Laboratories, Richmond, Calif.), and the supernatant was stored in aliquots at -70°C.

Lipooligosaccharide antigen preparation

Purified LOS from *H. ducreyi* 35000 for development of the inhibition ELISA and for immunization in vaccine feasibility studies, was extracted from 160 CAP (150 mm in diameter) cultures with the hot phenol extraction method of Westphal and Jann (1965). Final purification was done with three 3-hour ultra-centrifugation cycles at 105000 x g, yielding LOS in the pellet and an uncharacterized carbohydrate fraction in the supernatant. Both the purified LOS and carbohydrate preparations were lyophilized and stored at 4°C. Purity of the LOS extract was evaluated by SDS-PAGE with 12% polyacrylamide gel and silver staining (Bio Rad Laboratories, Richmond, Calif.). The uncharacterized carbohydrate extract

was subsequently evaluated as a potential vaccine candidate in the rabbit model.

Pilus preparation

The pilus preparation for vaccine feasibility studies was extracted by harvesting *H. ducreyi* 35000 from 160 CAP (150mm) cultures as previously described by Spinola *et al.* (1990). Briefly, bacteria were harvested from the agar and suspended in 0.1M Tris-HCl, pH 8.0. Pili were sheared from the surface with an Omnimixer 2000 (Diamed Laboratories) and centrifuged at 8000 x g. Pili in the supernatant were purified with 3 cycles of precipitation and re-solubilization at differential pH. Pili were precipitated by dialysis with PBS (pH 5.0), centrifuged at 8000 x g and re-solubilized by dialysis with 0.05M Tris-HCl (pH 10.5). Insoluble contaminants were removed by centrifugation at 23000 x g for 40 min. The protein concentration was determined by the Bio Rad protein assay. Aliquots were stored at -70°C. Purity of the extract was assessed by SDS-PAGE with 12% polyacrylamide gels silver staining.

3. Standardization of an enzyme immunoassay for antibody to *Haemophilus ducreyi*

Sorbent preparation

Respiratory isolates of *Haemophilus parainfluenzae*, *Haemophilus parahaemolyticus* and *Haemophilus influenzae* obtained from the Ottawa General hospital Microbiology laboratory were grown on CAP supplemented with 1% Iso Vitale X at 37°C in 5% CO₂ for 24 hours. Antigen was prepared in the same manner as described above for *H. ducreyi* crude soluble antigen. The protein was adjusted to 250 µg/mL in PBS with 1% newborn calf

serum (NBCS) and 0.1% Tween 80. Equal volumes of each sorbent were mixed, and the mixture stored at -70°C. Test serum samples were diluted to 1:50 by adding the sorbent, shaken for 1 hour at RT and incubated at 4°C.

Enzyme immunoassay to soluble antigen

Ninety six well round bottom plates (Nunc Polysorb U96) were coated with 20 µg/mL of *H. ducreyi* antigen suspended in 0.1M carbonate buffer (pH 9.6), incubated for 2 hours at 37°C, and washed three times with washing buffer (PBS, 0.1% Tween 80). Plates were wrapped in foil and stored at 4°C.

Adsorbed test serum (100µl per well) was applied at final dilutions of 1:200 and 1:400 in PBS (1% NBCS, 0.1% Tween 80), incubated for 30 min at 37°C, and washed five times with washing buffer. A total of 200µl of a 1:2000 dilution of peroxidase conjugated goat anti-human immunoglobulin G (IgG) (Tago, Burlingham, CA.) diluted in PBS (5% NBCS, 0.1% Tween 80) was added to each well. A 1:1400 dilution was used with peroxidase conjugated goat anti-human IgM. The plates were incubated for 30 min at 37°C and washed five times with washing buffer. Bound conjugated antibody was detected by adding 100µl of 0.36mM 2,2'-azino-bis (3-ethyl benthizoline-6-sulfonate) (Boehringer Mannheim) and 0.03% H₂O₂ dissolved in citrate buffer (0.1M citric acid and 0.02M sodium phosphate [pH 4.25]). Plates were kept at RT for 25 min and the A₄₀₅ nm was determined spectrophotometrically. Each plate included one positive and one negative control sample prepared by serially diluting pooled sera from eight highly reactive cases and five non reactive donors respectively.

Inhibition ELISA and rheumatoid factor

Control sorbent adsorbed ELISA positive sera were selected from a panel of clinically and microbiologically proven cases of chancroid and pooled. Pooled positive sera at a 1:1000 dilution were adsorbed to seven serial twofold dilutions (160 to 2.5 µg/mL) of *H. ducreyi* antigen, or *E. coli* 055.B5 purified LPS (Sigma Chemicals Ltd. St-Louis, Mo.), and the solutions were incubated for 2 hours at 37°C. A total of 100µl of adsorbed sera was then tested as described above for extinction of reactivity.

Fifteen *H. ducreyi* IgM positive serum samples from patients with no history of other STD were tested for the presence of rheumatoid factor by using the Ortho rheumatoid antibody test (Ortho Diagnostic Systems, Beersel, Belgium).

Patient population and sera

Characterized sera were banked and available from male and female populations of the Nairobi City Commission Dermatovenerology Clinic. Male urethritis was the most common STD diagnosis and 95% of the GUD cases were clinically if not microbiologically diagnosed chancroid (Cameron *et al.* 1989). Patients with non STD dermatologic conditions were also represented. Standardization of the ELISA was performed by using a panel of 432 serum specimens characterized according to: 1) Clinical or microbiologic diagnosis of chancroid, 2) non STD diagnosis, 3) HIV antibody serology as described previously (Cameron *et al.* 1989), 4) past history of genital ulceration reported by the patient and 5) for chancroid, the time from the appearance of genital ulcers to the time of phlebotomy.

Selected positive control sera were used for the standardization of the assay. These

sera were from patients with primary *H. ducreyi* infection and included those with clinically acute chancroid, an ulcer specimen culture positive for *H. ducreyi*, the absence of previous history of GUD and HIV seronegativity. Negative control sera were obtained from non STD clinic patients without STD, with other infectious or dermatologic diseases and no history of GUD.

Standardization of ELISA

Standardization was carried out with the positive sera representing a primary *H. ducreyi* infection, and negative sera as described above, both at a 1:400 dilution. Both IgG and IgM ELISA were standardized by using receiver operator characteristic (ROC) curves (Metz 1978). Threshold values for each plate were generated using the following arithmetic formula:

$$\frac{CN_p + (2 \times SD_{(\text{mean } CN, OD)})}{X}$$

where CN_p is the optical density of the plate negative control, $SD_{(\text{mean } CN, OD)}$ is the standard deviation of the mean optical density for over 30 plate negative controls and X represents arbitrary serial multiples of 0.025 to 1.0 for IgG and 0.1 to 2.4 for IgM. Test results were evaluated by determining the ratio of the optical density of a test serum sample at a dilution of 1:400, to the threshold for the plate by ROC curve analysis. Positive test results were defined as those that had a ratio of 1.0 or higher. Sensitivity and specificity were calculated for each arbitrary threshold. ROC curves were constructed by plotting sensitivity versus the false positive rate. The accuracy and the positive and negative predictive values of the assay

were calculated for the threshold that performed optimally (Nettleman 1988; Hanley and McNeil 1982; Metz 1978).

Comparative evaluation

The performance of the ELISA was compared by using the larger group of positive serum specimens from patients with chancroid with respect to time between the onset of acute chancroid and phlebotomy, presence of a history of genital ulcer disease and HIV antibody serology.

4. Inhibition ELISA for human antibody to *H. ducreyi* Lipooligosaccharide

LOS ELISA

Polysorb U96 round bottom plates were coated with 20 µg/mL of either the soluble bacterial or LOS extracts (100µl/well) suspended in carbonate buffer (0.1M, pH9.6). The remainder of the ELISA protocol was performed exactly as described above. Alternatively, solubilized bacterial antigen and LOS ELISA were performed as previously described by Maclean *et al.* (1991), with slight modifications. Extracts diluted to 10 µg/µL in PBS containing 1 mg/mL of chenodeoxycholate was heated for 15 min at 97°C, and applied, 100µl per well, to Polysorb U96 plates and incubated for 2 hours at 37°C. Plates were washed 3 times with wash buffer and wells were blocked with 2% bovine serum albumin (BSA) for 1 hour at 37°C. All plates were stored at 4°C.

All sera were adsorbed with the sorbent and applied in serial twofold dilutions from 1:200 to 1:25600 in PBS (1% NBCS, 0.1% Tween 80). A 1:2000 dilution of peroxidase

conjugated goat anti-human IgG (Tago, Burlingame, Calif.) diluted in PBS (5% NBCS, 0.1% Tween 80) was added to each well (100µl/well). Plates were incubated at 37°C for 30 min and washed 5 times with washing buffer. Bound conjugated antibody was detected as described above. All ELISA assays included one control positive sample and one control negative sample from 8 highly reactive cases and 5 normal donors, respectively.

Inhibition ELISA

LOS reactivity in samples identified as reactive to LOS from *H. ducreyi* 35000, was evaluated using an inhibition ELISA. Four variants of the same inhibition assay were used, such that serum samples were adsorbed with either LOS or soluble bacterial extracts, and tested for residual LOS and soluble antigen reactivity. The serum dilution (in PBS (1% NBCS, 0.1% Tween 80)) that yielded 75% of the ELISA value of the serum dilution with the highest reaction, measured as described above. Sera were adsorbed with four serial twofold increments from 10 to 80µg of either purified LOS or soluble antigen, and incubated for 2 hours at 37°C. Residual reactivity to LOS or soluble bacterial antigen was determined as described for the LOS ELISA.

Serum population

Serum samples from the Nairobi City Commission Dermatovenerology Clinic were selected on the basis of clinical and microbiologic diagnosis of chancroid, time from onset of genital ulceration, absence of past history of genital ulcer disease and negative HIV serology. For evaluation of anti-LOS antibody, sera from 10 cases of clinically and

microbiologically proven primary chancroid collected 4 weeks from ulceration and with high soluble antigen IgG ELISA reactivity were selected. Negative control samples were obtained from 5 healthy volunteers without any history of STD.

5. Feasibility of experimental vaccination for chancroid

Animals and housing conditions

A total of 118 2.5 Kg New Zealand white (NZW) male rabbits were purchased from Charles River Canada and housed in an 11.7m² room. Ambient room temperature was kept at 14±1°C with a Thermo Air Plus air conditioning unit. Rabbits for all experiments were age matched from acquisition and housed in identical conditions for the duration of the experiments.

Immunization with LOS, carbohydrate and the pilus preparations

Six and fourteen NZW rabbits were immunized with 100µg of LOS or 100µg of the pilus preparation in PBS (pH 7.2), respectively, suspended in Freund's complete adjuvant (FCA). One half of the 500µl dose was administered intramuscularly, and the other half subcutaneously in the back of the neck. Four weeks after primary immunization, rabbits were given boosters of identical doses of each antigen suspended in Freund's incomplete adjuvant (FIA) by the same route of administration. Control rabbits included ten naive unimmunized rabbits and five rabbits sham immunized with PBS in FCA and FIA. Seven pilus preparation immunized rabbits and six LOS-immunized rabbits were ear-bled weekly for measurement of IgG and IgM serological response to each antigen as determined by

ELISA and Western blot for IgG (see below).

Three NZW rabbits were immunized intramuscularly and subcutaneously with either 1, 10, or 100 μ g of the carbohydrate extract suspended in FCA, and boosted four weeks later with an identical dose in FIA. All rabbits were ear bled and sera were assayed for IgG and IgM serologic responses.

Virulence of *H. ducreyi* in immunized rabbits

Homologous strain virulence was assayed by challenge with five triplicate 100 μ l doses of 10^7 to 10^3 CFU/mL of viable late mid-log phase *H. ducreyi* 35000 from broth cultures, four weeks after booster with the pilus preparation or three weeks after booster with the LOS extract. Colony counts for each inoculum in each rabbit were directly determined in duplicate, by plating 100 μ l of the inoculum from the injecting syringe to agar plates. Inocula were delivered by intra-epithelial injection in triplicate in a grid across the shaven backs of the animals. As 10^4 CFU has been found to be the minimum inoculum density consistently producing uicers (Meloche *et al.* 1992), comparisons of lesions produced at this inoculum were performed by measuring the transverse lesion diameter which were also scored for severity on a scale of 0 to 4 (0: nil, 1: erythema, 2: induration >2mm, 3: suppuration and 4: ulceration). The third lesion at each inoculum size was cultured to determine the presence or absence of *H. ducreyi* by lateral injection of 0.1 mL sterile PBS, and aspiration back into the syringe. Aspirates were plated onto chocolate agar and incubated at 33°C for 48 hours. Identification of *H. ducreyi* was based on the characteristic colonial morphology, push test and microscopic examination with Gram's stain for

characteristic appearance (D'Costa *et al.* 1986). Lesions were cultured until negative for four consecutive days. Lesions that were used for culture were not scored or measured for virulence due to the potential influence of serial injection of 0.1 mL PBS and manipulations in culturing upon lesion size and characteristics. Naive and sham-immunized adjuvant control rabbits were challenged with in an identical fashion.

Eight rabbits immunized with the pilus preparation from *H. ducreyi* 35000 and 5 naive age-matched controls, were challenged with the heterologous strain RO-34. Virulence was assayed as described above..

Histopathology

On days 4, 10, 15 and 21 after inoculation, ten rabbits; two naive rabbits, 3 sham-immunized rabbits and 5 rabbits immunized with the pilus preparation were given 0.1 mL/Kg of Innovar (Fentanyl Droperidol; Jansen Pharmaceutical, Mississauga, Ontario) with 0.05 mg/Kg of atropine sulfate (MTC pharmaceutical, Cambridge, Ontario) intramuscularly. Biopsies of lesions at 10^5 CFU/mL (full skin thickness including fat and muscle) were taken from the ulcer or lesion edge with 6 mm disposable biopsy punches. No suturing or antibiotic ointment were necessary. Biopsy specimens from the two naive rabbits and two pilus-immunized rabbits were fixed in 10% formalin, embedded in paraffin, thin sectioned and stained with haematoxylin and eosin for microscopic identification of cellular infiltrates by nuclear morphology. Biopsies from the remaining three sham-immunized rabbits and three pilus-immunized rabbits were sectioned in half down the centre of the lesion. One half of the section was fixed in formalin, embedded in paraffin and stained with haematoxylin

and eosin. The second half was frozen in OCT freezing compound (Miles Laboratories Inc (Ames division) Indiana) and cut in 7 μ m thick sections for microscopic identification of cellular infiltrates with haematoxylin and eosin staining and quantitation of lymphocytic infiltrate by immuno-phenotyping (see below).

ELISA

Serologic response to immunization with LOS was measured by ELISA as described above. Serologic response to immunization with the pilus preparation was measured using the standardized ELISA as described above, and using the pilus preparation as the ELISA detection antigen. Serologic reactivity to the carbohydrate preparation was measured with both the LOS or standardized ELISA protocol as described above, with the carbohydrate antigen substituted as the detection antigen in each assay. Since pre-bleed sera of all immunized rabbits had no significant background reactivity to *H. ducreyi* 35000 antigen, pre-adsorption of sera with the sorbent was not necessary.

Western blotting

Pilus preparation, soluble bacterial antigen and the carbohydrate extract were separated by SDS-PAGE (approximately 10 μ g of antigen preparation per well), and electro blotted onto nylon membranes (Boehringer Mannheim) at 100 volts for 1 hour at 4°C. Transfer buffer consisted of 25 mM Tris (Bio Rad Laboratories), 195 mM Glycine (Bio Rad Laboratories) and 20% v/v methanol. Nylon membranes were blocked with 2% (w/v) skim milk powder in PBS for 1 hour at 37°C then washed for five minutes five times with PBS and

0.1% Tween 20. Individual lanes were cut in separate strips.

Sera from the seven pilus-immunized rabbits, taken 49 days after primary immunization and prior to infection, were diluted 1:200 in 2% skim milk powder in PBS and incubated with pilus preparation blotted nylon strips for 2 hours at 37°C. Serum from one rabbit immunized with 100µg of the carbohydrate extract, at 1:50 and 1:100 dilutions in 2% skim milk/PBS was incubated with carbohydrate blotted nylon strips for 2 hours at 37°C. Finally, acute chancroid serum from one patient shown to be highly reactive to *H. ducreyi* soluble antigen by ELISA, was incubated at a 1:100 dilution in 2% skim milk in PBS for 2 hours at 37°C with nylon membranes blotted either with the carbohydrate antigen or the *H. ducreyi* soluble antigen. After washing, strips were incubated for 1 hour with horse radish peroxidase-conjugated goat anti-human IgG (1:1000 dilution) (Tago Burlingham, CA) or horse radish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) (Tago, Burlingham, CA) diluted in 2% skim milk/PBS. Strips were washed with PBS and colour was developed with 0.8 mM 3 amino-9 ethyl carbazole and 0.1% H₂O₂. The reaction was stopped with 3 washes of 5 minutes each in double distilled water.

6. Evaluation of humoral immunity to pilus preparation immunization

Generation of whole cell or pilus preparation antisera

New Zealand white rabbits were immunized with 100µg of the pilus preparation or 10⁸ CFU of heat killed whole cell suspension of *H. ducreyi* 35000 suspended in FCA. One half of the dose was administered intramuscularly the rest was administered subcutaneously in the back of the neck. Rabbits were boosted four weeks after primary immunization with

an identical dose and route.

Fractionation of polyclonal IgG from hyperimmune and naive sera

A total of 10 whole cell immunized, 10 pilus preparation immunized rabbits and 13 naive uninfected normal rabbits were exsanguinated by cardiac puncture after anaesthesia with 0.1 mL Innovar (Fentanyl-Droperidol, Jensen Pharmaceutical, Mississauga, Ont, Canada). Sera from rabbits of each group were pooled and polyclonal IgG purified by protein G affinity chromatography with a Hi Trap affinity column [5 mL bed volume (Pharmacia biotech Inc, Piscataway, NJ)] as per manufacturer's instruction. Eluted fractions were collected in 1 mL aliquots and assayed for IgG spectrophotometrically at 280 m μ . Positive fractions were pooled, dialysed against PBS and filter sterilized with a 0.22 μ m filter. Immunoglobulin concentration in pooled fractions was determined by relating the extinction at 280 m μ to the specific extinction coefficient of 13.5 O.D./mg/mL for rabbit IgG (1% solution and 1 cm light path) with a Varian DMS 200 UV spectrophotometer as previously described (Little and Donahue 1976; Crumpton and Wilkinson 1963). Reactivity of the purified polyclonal IgG fraction to whole cell or pilus preparation antigens was verified by enzyme immunoassay (ELISA) to the corresponding antigen (see below).

Passive immunization with purified polyclonal IgG

Passive immunization was done on three groups of eight rabbits, infused with the polyclonal IgG from rabbits immunized with either the pilus preparation, whole cells or with IgG from normal rabbits. Within each group, four rabbits were passively immunized by slow

intravenous infusion (30 min IV drip) with 24 mg, and 4 with 48 mg of the IgG fractions. One rabbit in each group was phlebotomized at 0, 8, 24 72 hours after infusion, then on a weekly basis for 4 weeks for IgG end point titers, and the elimination half lives of transferred antibody was measured by ELISA (see below).

Virulence of *H. ducreyi* in passively immunized rabbits

Three rabbits passively immunized with the IgG from rabbits immunized with whole cells or pilus preparation or naive, and three naive control rabbits (total of 12) were challenged 24 hours after infusion with five triplicate 100 μ l doses from 10^7 to 10^3 CFU/mL of viable late mid log *H. ducreyi* 35000 from broth cultures, as described above.

ELISA

Immunoassay for detection of transferred whole cell polyclonal IgG was performed as described above without pre-adsorption using the soluble bacterial antigen extract or the pilus preparation as the ELISA detection antigen.

7. Characterization of cellular infiltrate in chancroidal lesions

Quantitation of cellular infiltrate in chancroidal lesions

Frozen sections were mounted on glass slides pre-treated with 2% 3-amino propyl-ethoxy silane (ICN biochemicals, Aurora, Ohio) in acetone and dried overnight at room temperature. Sections were fixed in acetone for five minutes and washed five minutes three times in PBS containing 2% BSA. Sections were incubated with a 1:100 dilution of either

mouse anti-rabbit T cell CD4 monoclonal antibody (IgG_{2a}) (Bio Source International, Camarillo, Calif, Lot#11157-05S) or mouse anti-rabbit T cell CD5 monoclonal antibody (IgG₁) (Bio Source International, Camarillo, Calif, Lot#11157-06S) for 30 minutes at room temperature (Kotani et al. 1992). After washing for 5 minutes three times, sections were incubated for 30 minutes at room temperature with a 1:100 dilution of peroxidase conjugated goat anti-mouse IgG (Tago, Burlingham, Calif). Slides were washed and colour was developed with a substrate containing 3-amino-9-ethyl carbazole for 30 minutes in the dark. Reactions were stopped by washing slides in double distilled water. Sections were counter stained with a 20 second dip in haematoxylin. Optimal dilutions for both the primary and secondary conjugated antibody were first defined by titration on normal rabbit spleen sections. Normal mouse serum served as a negative control.

For each section from the chancroid biopsies the CD4 and CD5 positive cells were counted in 30 to 40 fields (at a 40x magnification). Each section was counted three times. An average total cell count per field was determined in each section for each of three biopsies, these were then averaged for vaccine and control animals.

Isolation of rabbit peripheral blood leucocytes

A total of 10 mL of whole blood was obtained from the ear vein of three pilus-immunized or three sham-immunized rabbits, with 2.7% EDTA as the anticoagulant. Peripheral blood leucocytes (PBL) were isolated as previously described with slight modifications (Wilkinson *et al.* 1992). The blood was diluted with an equal volume of RPMI-1640, pipetted onto an equal volume of Ficoll-Hypaque (Pharmacia Biotech Inc,

Piscataway, NJ) and centrifuged at 400 x g for 30 minutes. PBLs were carefully removed from the interface, resuspended in wash buffer (PBS-0.1% NaN₃, 1% BSA) and centrifuged for 10 minutes at 200 x g. The pellets were washed, centrifuged for 5 minutes at 200 x g and resuspended in wash buffer. Cells were then counted with a Coulter cell counter (Coulter Electronics Inc. Hialeah, Florida) or with 1% methylene blue in a haemocytometer.

Flow cytometric analysis of rabbit PBL

A total of 10⁵ cells from isolated rabbit PBLs were incubated simultaneously for 10 minutes at room temperature with a 1:10 dilution of mouse anti-rabbit T cell CD4 (IgG_{2a} isotype) and mouse anti-rabbit T cell CD5 (IgG₁ isotype) diluted in PBS with 0.1% NaN₃ and 1% BSA. Cells were washed with wash buffer (PBS, 1%NaN₃, 1% BSA), centrifuged at 200 x g for 5 minutes, then incubated simultaneously for 10 minutes at room temperature with a 1:10 dilution of FITC-conjugated goat anti-mouse IgG_{2a}, for CD4 labelling (Cedarlane Labs, Hornsby Ontario, Canada) and with R-Phycoerythrin (PE)-conjugated goat anti-mouse IgG₁, for CD5 labelling (Cedarlane Labs, Hornsby Ontario, Canada). After washing with the wash buffer, stained cells were analysed with an argon laser Epics XL-MCL Coulter Flow cytometer (Coulter Electronics, Hialiah, Florida) using Epics XL flow cytometry analysis software version 1.5. Appropriate isotypic controls for each monoclonal antibody were included, and FITC was compensated at 0.4% of PE and PE was compensated to 28.3% of FITC. Optimal primary and secondary antibody dilutions were first determined by titration using normal rabbit PBL. Lymphocyte populations were identified by their forward scatter and granular characteristics, and separated from other cells for analysis by appropriate gating

(Wilkinson *et al.* 1992).

Intra-dermal testing in pilus preparation-immunized rabbits

A total of three pilus preparation-immunized rabbits and three naive control rabbits were challenged with 0, 1, 10 and 100 μ g of the purified pilus preparation suspended in PBS by intra-epithelial injection on the rabbit's back. After 48 hours, the lesions were scored as in the virulence assay and measured for size. Biopsies of indurated lesions at the site of challenge with 100 μ g of the pilus preparation were taken from one pilus preparation-immunized and one naive control rabbit, sectioned and stained with haematoxylin and eosin as described above for microscopic evaluation.

8. Descriptive and comparative statistical analysis

Comparative analysis of sensitivity and specificity of ELISA with respect to time from onset of acute chancroid to phlebotomy or HIV serology was performed with the chi-square test. Statistical analysis of mean reactivity (as optical density) of *H. ducreyi* antibody positive sera to soluble bacterial or LOS preparation was performed with Student's t test and one way analysis of variance (ANOVA) for comparison of inhibition curves.

Lesions produced by inocula of 10⁵ CFU/mL of *H. ducreyi* in the rabbit model of infection were compared by averaging the scores of the two non manipulated lesions (not used for culture) in each rabbit to generate a single lesion size measurement and score for each rabbit. Statistical analysis of mean lesion sizes and scores serially measured during 21 days of observations following inoculation of 10⁵ CFU/mL were performed with the one-way

repeated measures ANOVA and the Wilcoxon signed rank test, respectively. Comparative evaluation of inoculum sizes, duration of culture positivity, lesion sizes, and peak lesion score was performed with the Student's t test. Analysis of CD4 positive T cell fraction was done with the chi-square test or the Mantel-Haenszel chi-square test. All computational statistics were evaluated with Sigma Plot[®] Sigma Stat[®] statistical software version 2.0 (Jandell Scientific, San Rafael, CA).

CHAPTER 3: RESULTS

1. *H. ducreyi* ELISA standardization

Reactivity of control sera

The mean ratio of positive control sera to negative plate control sera for the IgG ELISA at a 1:400 serum dilution was 9.0 ± 2 ; for the IgM ELISA, it was 18.1 ± 3.3 . Adsorption of sera with the sorbent was necessary to minimize cross reactivity between common epitopes of *H. ducreyi* and other *Haemophilus* species (Figure 1). For both the IgG and IgM ELISA, up to 80% of the positive reactivity was inhibited by pre-adsorption with the highest concentration of soluble bacterial antigen (Figures 2A and B). No inhibition was produced by pre-adsorption with *E. coli* 055:B5 LPS. None of the IgM positive samples were positive for rheumatoid factor.

Standardization of the IgG and IgM ELISAs

Each ELISA was standardized with ROC curves for optimal performance (Figure 3, Tables 1 and 2). For the IgG ELISA, optimal performance (defined by the highest sensitivity and lowest false positive rate) was identified for an arbitrary threshold of 10 standard deviations (0.18 OD units) of the mean value for the negative plate controls (0.092 OD units) above the individual negative control value of that plate. Improved performance was observed by using positive control sera taken at 4 weeks from time of disease onset. The sensitivity was 100% at 4 weeks, whereas sensitivity of the ELISA on all sera was 94%, and the specificity was 84% (Figure 3A and Table 1). For the IgM assay, optimal performance

Figure 1

Scrologic reactivity of normal sera from 3 volunteers with no history of STD or past history of GUD before (open circles) and after adsorption (open squares) with the sorbent. Data represent mean OD (\pm SD).

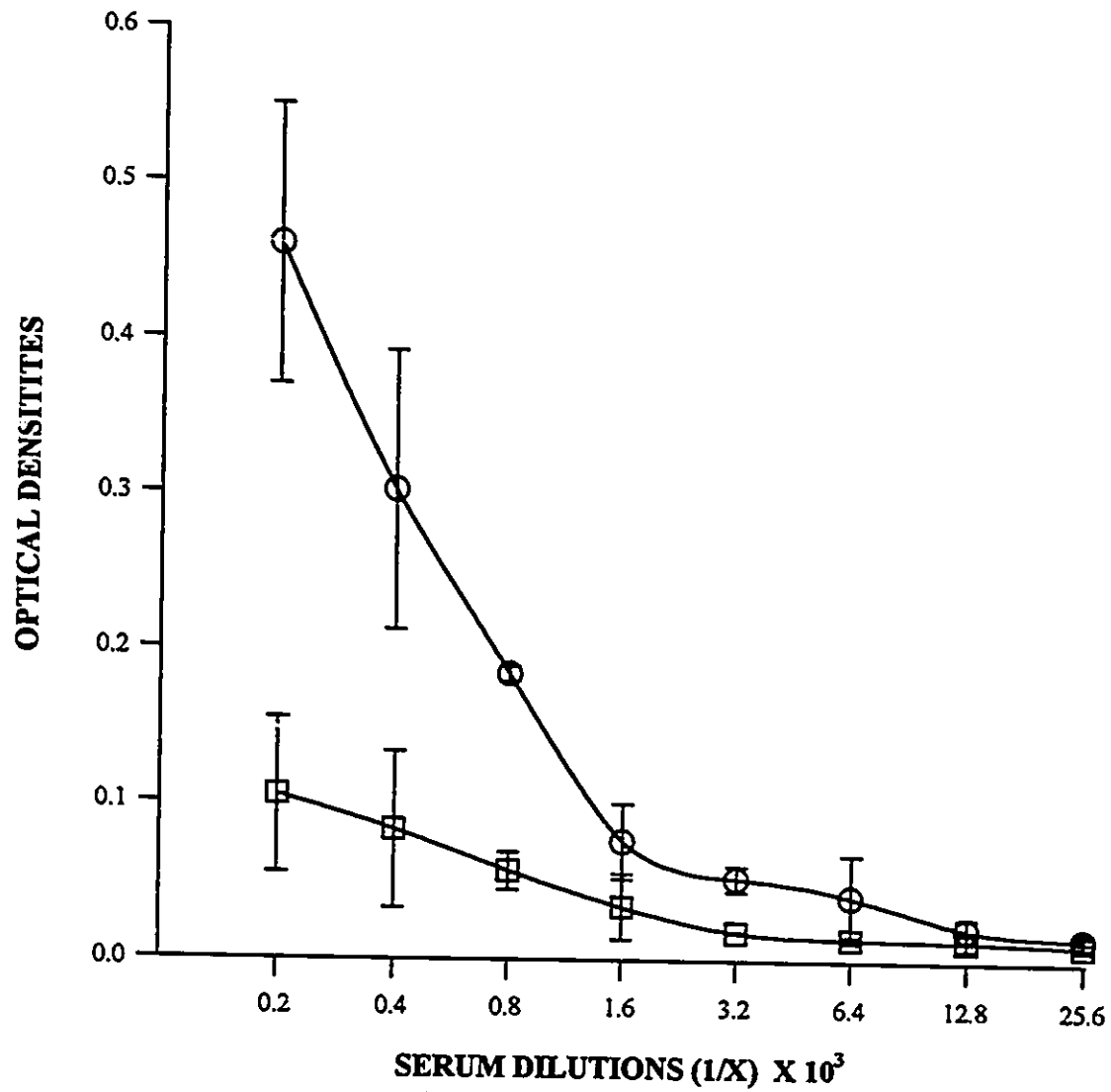


Figure 2

Inhibition of reactivity by soluble *H. ducreyi* antigen in the IgG (A) and IgM (B) ELISAs (\pm SD). Positive (circles) and negative (squares) control samples were adsorbed with serial twofold dilutions of soluble *H. ducreyi* antigen extract (open symbols) or *E. coli* 055:B5 lipopolysaccharide (closed symbols) and tested for inhibition of reactivity by soluble antigen. Substantial inhibition only by soluble antigen confirmed the specific antigen-antibody reaction in the ELISA.

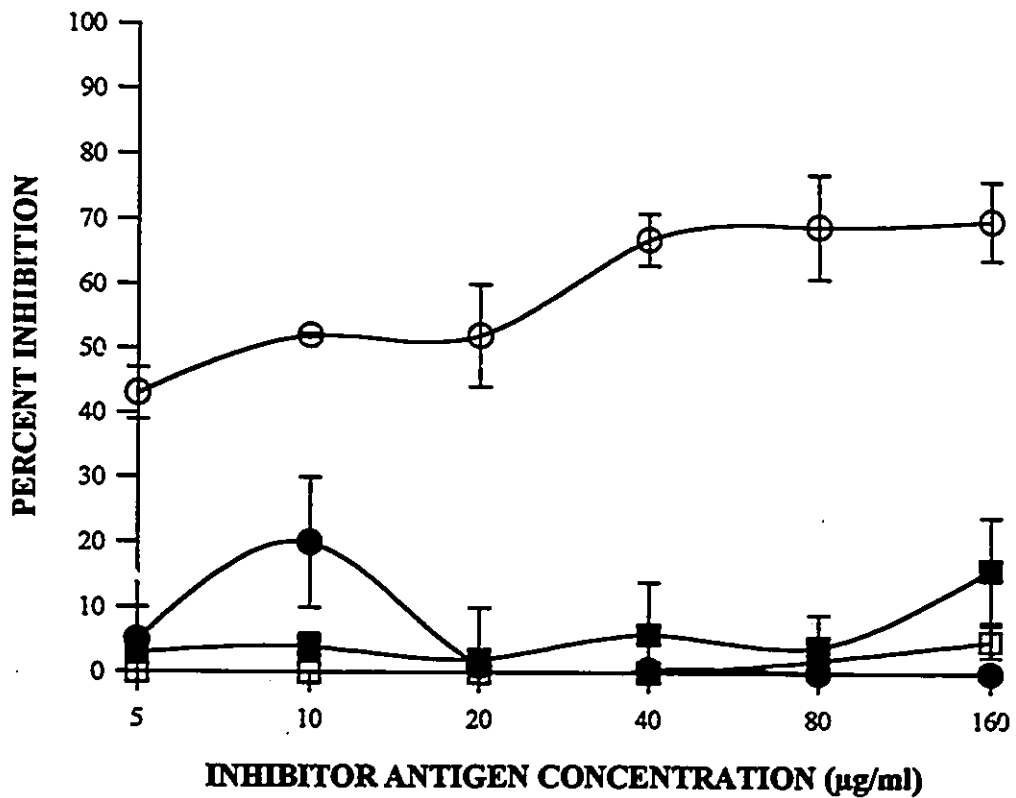
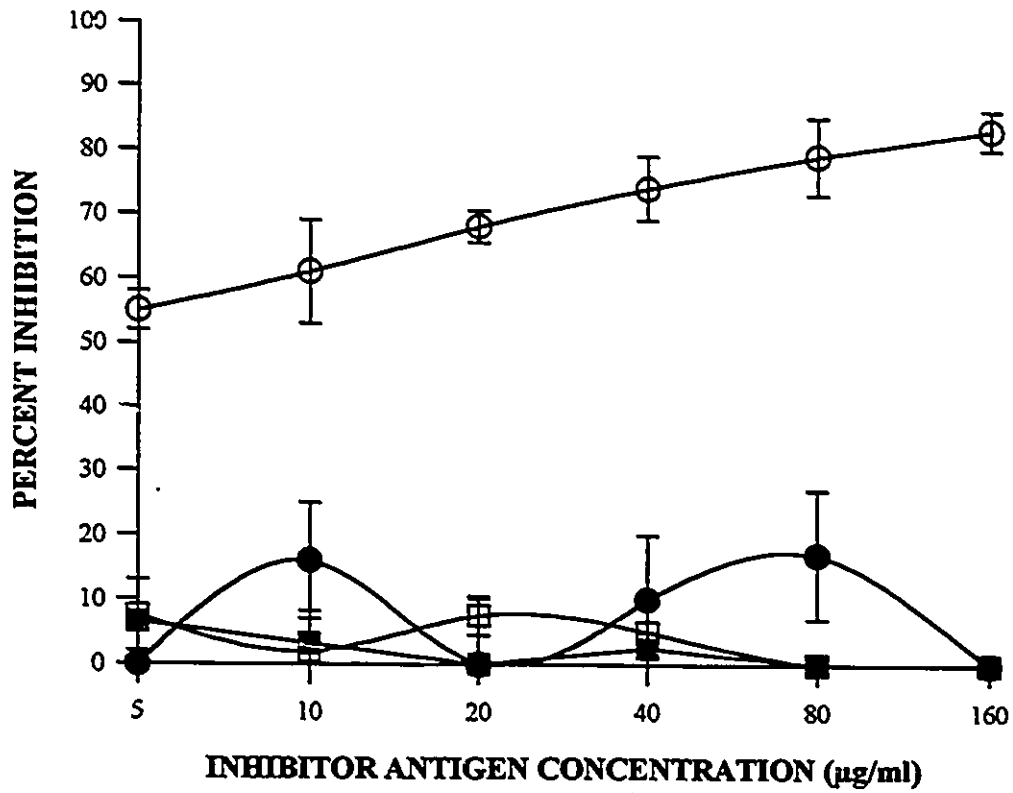


Figure 3

Receiver operator characteristic curve for the IgG (A) and IgM (B) ELISAs. Curves for sera at 4 weeks from the time of disease onset (open circles) and all sera (closed circles) for the IgG ELISA and at 3 weeks from time of disease onset (open circles) and all sera (closed circles) for the IgM ELISA were compared. Optimally performing thresholds, indicated by the arrows, were 10 standard deviations above the value for the negative plate control for the IgG ELISA and 2 standard deviations above the value for the negative plate control for the IgM ELISA.

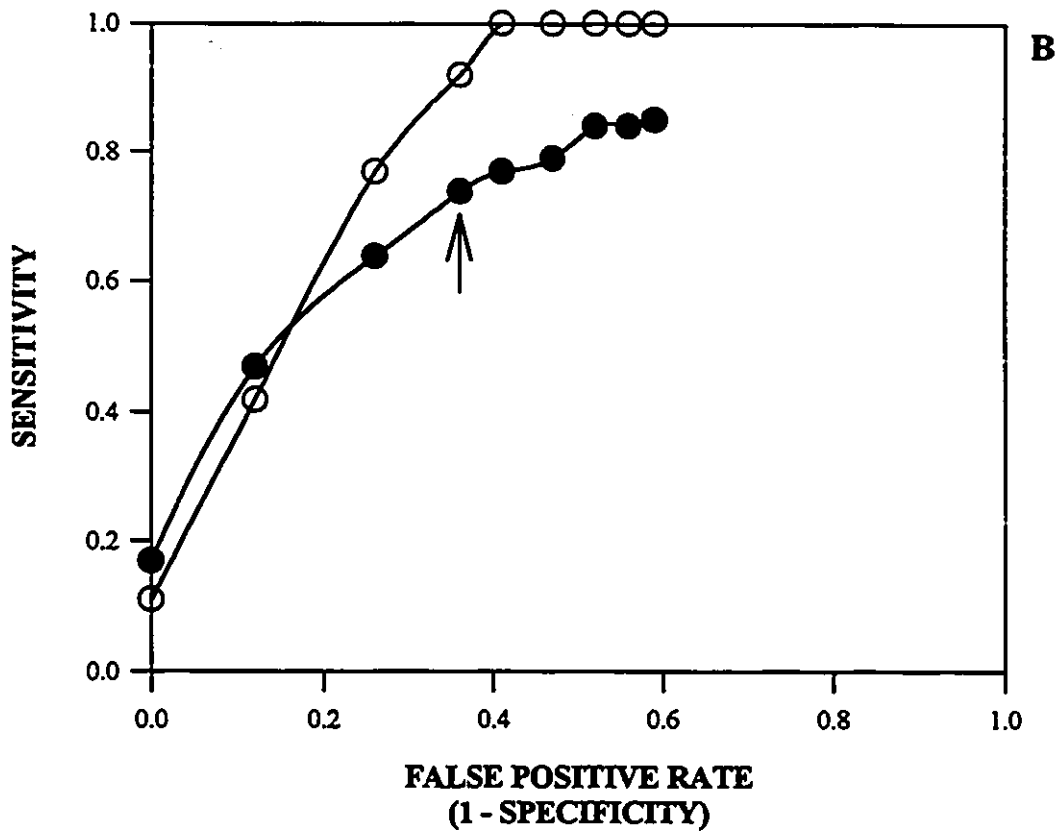
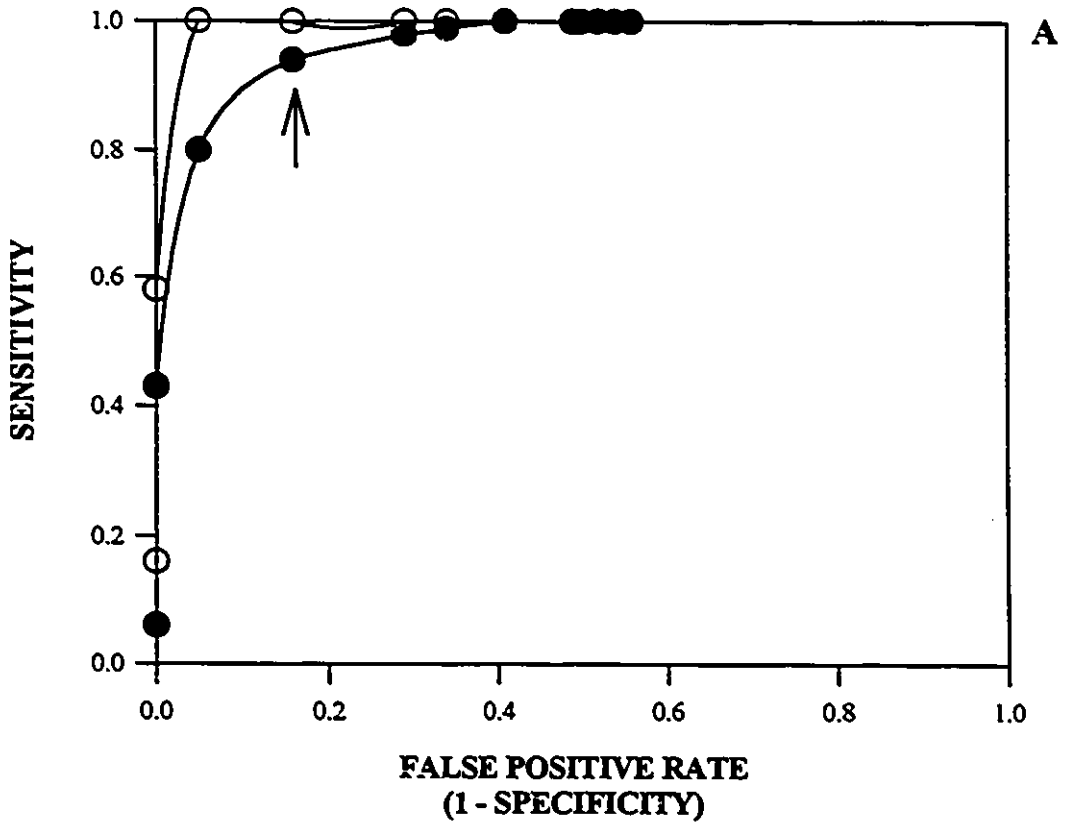


Table 1
 IgG ELISA performance with respect to time from onset
 of genital ulceration^a

ELISA results	No. (%) of serum samples with the following microbiologic and clinical diagnosis		
	STD-naive sera (n=128)	Chancroid positive sera (n=134)	Sera positive \geq 4 weeks (n=43)
Positive	21	126 (94) ^b	43 (100) ^c
Negative	107 (84) ^d	8	0

^a Accuracy and positive and negative predictive values for assay performance were 89, 86, and 93%, respectively, for IgG.

^b Sensitivity of EIA for all *H. ducreyi* positive chancroid sera.

^c Sensitivity of EIA at 4 weeks from ulceration (p was not significant compared with sera described in footnote b).

^d IgG EIA specificity.

Table 2
 IgM ELISA performance with respect to time from onset
 of genital ulceration*

ELISA results	No. (%) of serum samples with the following microbiologic and clinical diagnosis		
	STD-naive sera (n=128)	Chancroid positive sera (n=171)	Sera positive 3 weeks from ulceration (n=26)
Positive	57	126 (74) ^b	24 (92) ^c
Negative	81 (64) ^d	45	2

* Accuracy and positive and negative predictive values for assay performance were 69, 73, and 64%, respectively, for IgM.

^b Sensitivity of EIA for all *H. ducreyi* positive chancroid sera.

^c Sensitivity of EIA at 3 weeks from ulceration ($p < 0.01$ compared with sera described in footnote b).

^d IgM EIA specificity.

was similarly identified for a threshold of 2 standard deviations (0.034 OD units) of the mean value for the negative plate controls (0.061 OD units), above the individual negative control value for that plate. The sensitivity was 92% for case sera at 3 weeks from the time of disease onset, whereas the overall sensitivity was 74% and specificity was 64% (Figure 2B and Table 2).

Assay performance for detection of primary infections

Only the IgM and not the IgG ELISA demonstrated time related performance. Peak sensitivity occurring at 3 weeks from time of disease onset for the IgM ELISA, and at or after 4 weeks for the IgG ELISA (Figure 4A and B). The reduced sensitivity of the IgG ELISA before 4 weeks from time of onset of genital lesions (Figure 4A) was not statistically significant. For the IgM ELISA, there was a gradient in sensitivity, which peaked at 3 weeks from the time of disease onset, followed by a significant decline ($p = 0.002$) (Figure 4B).

HIV serology

Comparison of overall assay performance between HIV positive and HIV negative sera revealed significantly but moderately improved specificity for the IgG ELISA (Table 3) and a significantly but moderately improved sensitivity for the IgM ELISA in HIV seropositive case and control sera (Table 4).

Past history of genital ulcer disease

By using the threshold as standardized for acute, primary episode of chancroid, the

Figure 4

Specificity (dashed boxes) and sensitivity (open boxes) of the IgG (A) and IgM (B) ELISAs overall and at weekly intervals from the time of onset of genital ulcers to phlebotomy. Fractions refer to the number of case serum samples that were found to be positive at weekly intervals. The sensitivity of detection of IgG was greater at 4 weeks from the time of onset of genital lesions, but it was not significantly different from that at other times of detection. The sensitivity of IgM peaked at 3 weeks and was significantly different from that of other times of detection ($p < 0.001$; chi-square test for trend).

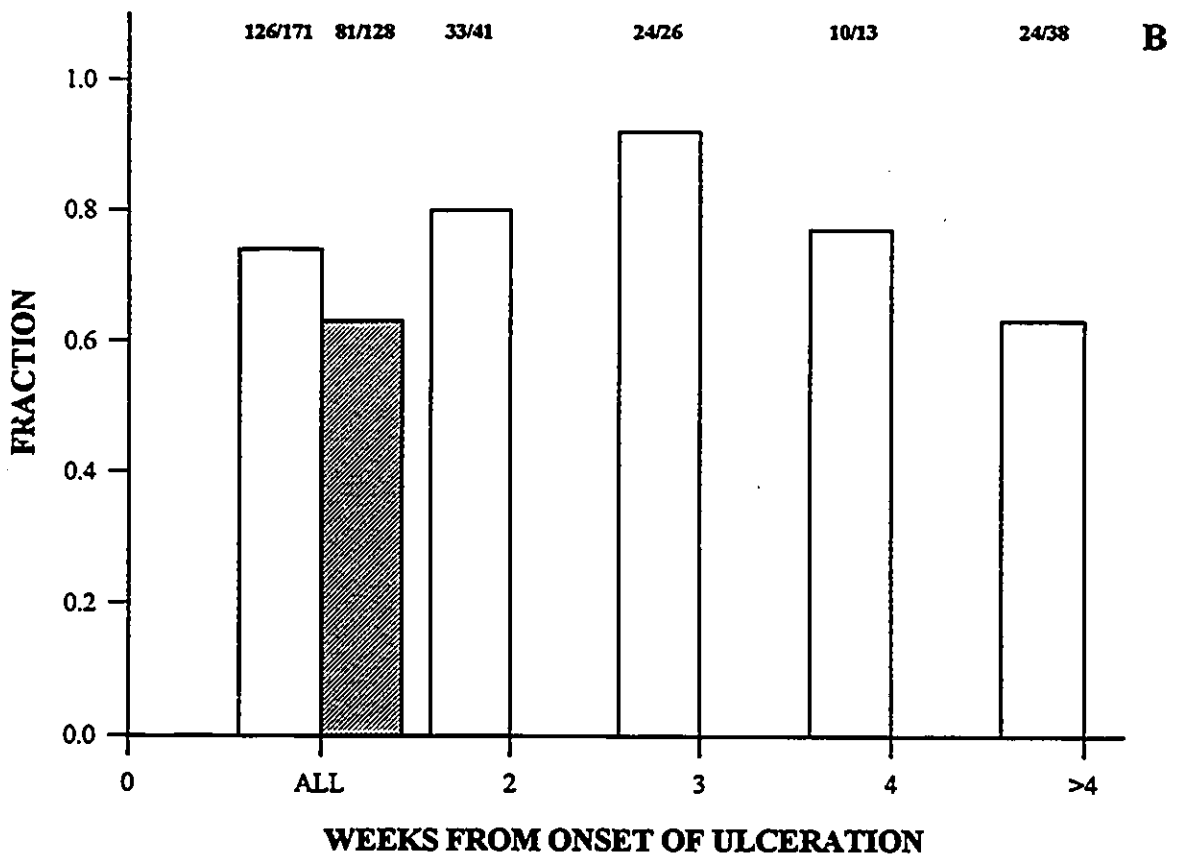
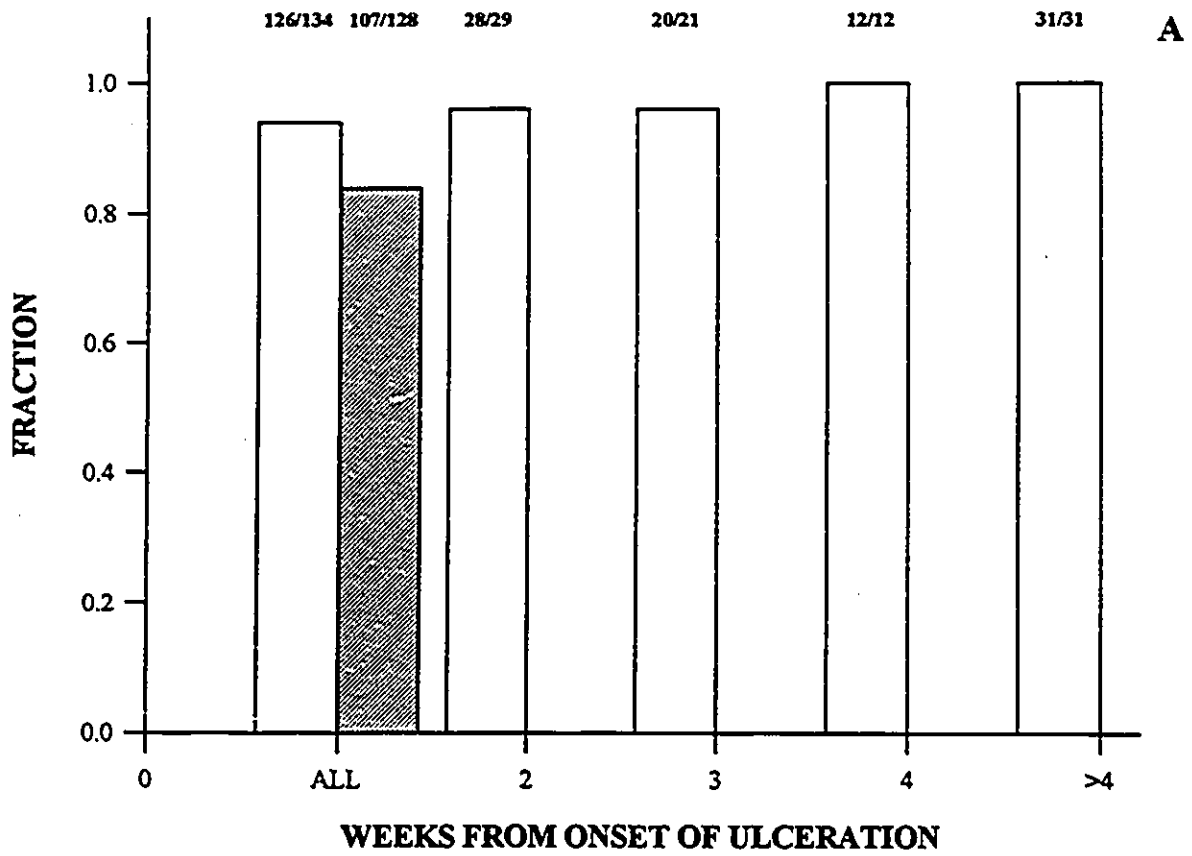


Table 3
IgG ELISA performance by HIV serology^a

ELISA results	No. (%) of serum samples			
	HIV positive		HIV negative	
	STD-naive sera (n=80)	Chancroid positive sera (n=54)	STD-naive sera (n=128)	Chancroid positive sera (n=134)
Positive	0	52 (98) ^b	21	126 (94) ^b
Negative	80 (100) ^c	2	107 (84) ^c	8

^a Accuracy and positive and negative predictive values for assay performance were 99, 100, and 99%, respectively, for IgG.

^b Assay sensitivity was not significantly affected by HIV seropositivity ($p=0.4$).

^c Assay specificity was significantly increased in the presence of HIV seropositivity ($p < 0.001$).

Table 4
IgM ELISA performance by HIV serology^a

ELISA results	No. (%) of serum samples			
	HIV positive		HIV negative	
	STD-naive sera (n=80)	Chancroid positive sera (n=53)	STD-naive sera (n=128)	Chancroid positive sera (n=171)
Positive	25	46 (87) ^b	47	126 (74) ^b
Negative	55 (69) ^c	7	81 (64) ^c	45

^a Accuracy and positive and negative predictive values for assay performance were 76, 65, and 89%, respectively, for IgM.

^b Assay sensitivity was significantly increased in the presence of HIV seropositivity ($p < 0.01$).

^c Assay specificity was not significantly affected by HIV seropositivity.

ELISA detected IgG seropositivity with 90% sensitivity and 64% specificity in a panel that includes 72 case serum samples from individuals who reported a past GUD and 261 control serum samples from individuals without a current or past GUD attending an STD clinic (Table 5) ($p < 0.001$).

LOS reactivity

IgG ELISA reactivity to soluble bacterial antigen was detected in all 10 samples tested using either carbonate or chenodeoxycholate buffers for antigen binding to plates (Figure 5A and B). Anti-LOS IgG reactivity was only detectable if LOS was bound to plates in chenodeoxycholate (Figure 5B), but not in carbonate buffer (Figure 5A). Of ten samples reactive to soluble bacterial antigen, only four samples had positive IgG reactivity to LOS from *H. ducreyi* 35000 (Figure 5B).

Inhibition of LOS and soluble antigen reactivity

Substantial inhibition of LOS reactivity as mean change in OD after adsorption with either LOS or soluble antigen did not significantly differ between these two adsorption antigens (Figure 6A) ($p = 0.119$). Adsorption with LOS or soluble antigen consistently reduced LOS reactivity, with a mean inhibition of $86 \pm 11\%$ and $89 \pm 5\%$ respectively for $40\mu\text{g}$ of adsorbing antigen. Conversely, inhibition of reactivity to soluble antigen with LOS adsorption was significantly less than with adsorption with soluble antigen ($p = 0.0004$) (Figure 6B). Percent inhibition of reactivity was reduced $47 \pm 10\%$ versus $87 \pm 8.5\%$ for LOS and soluble antigen, respectively.

Table 5
**IgG ELISA performance in populations with a past history of genital
ulcer disease^a**

ELISA results	No. (%) of serum samples			
	Presence of a history of genital ulcer disease		Absence of a history of genital ulcer disease	
	Chancroid negative sera (n=261)	Chancroid positive sera (n=72)	Non-STD sera (n=128)	Chancroid positive sera (n=134)
Positive	94	65 (90) ^b	21	126 (94) ^b
Negative	167 (64) ^c	7	107 (84) ^c	8

^a Accuracy and positive and negative predictive values for assay performance were 99, 100, and 99%, respectively, for IgG.

^b Assay sensitivity was not significantly affected by the presence of a history of genital ulcer disease ($p=0.475$).

^c Assay specificity was significantly decreased in the presence of a past history of genital ulcer disease ($p < 0.001$).

Figure 5

Mean reactivity of 10 *H. ducreyi* antibody positive sera 4 weeks from onset of chancroid. (A) Carbonate buffer fixed antigen: Reactivity to soluble bacterial extract (open circles) or to LOS (Open squares). (B) Chenodeoxycholate buffer fixed antigen: All ten samples were found to have reactivity to soluble antigen (open circles). Of these only four were reactive to LOS antigen (Closed squares) while the remaining six were LOS non reactive (open squares). Data represent mean OD (\pm SD).

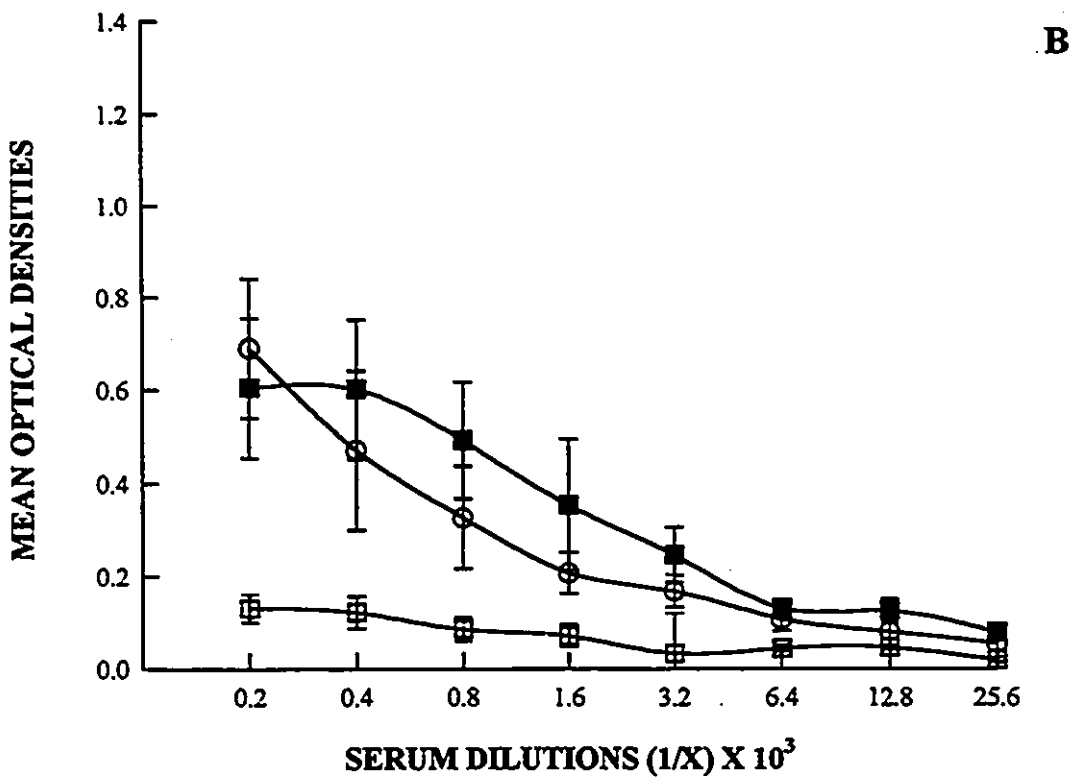
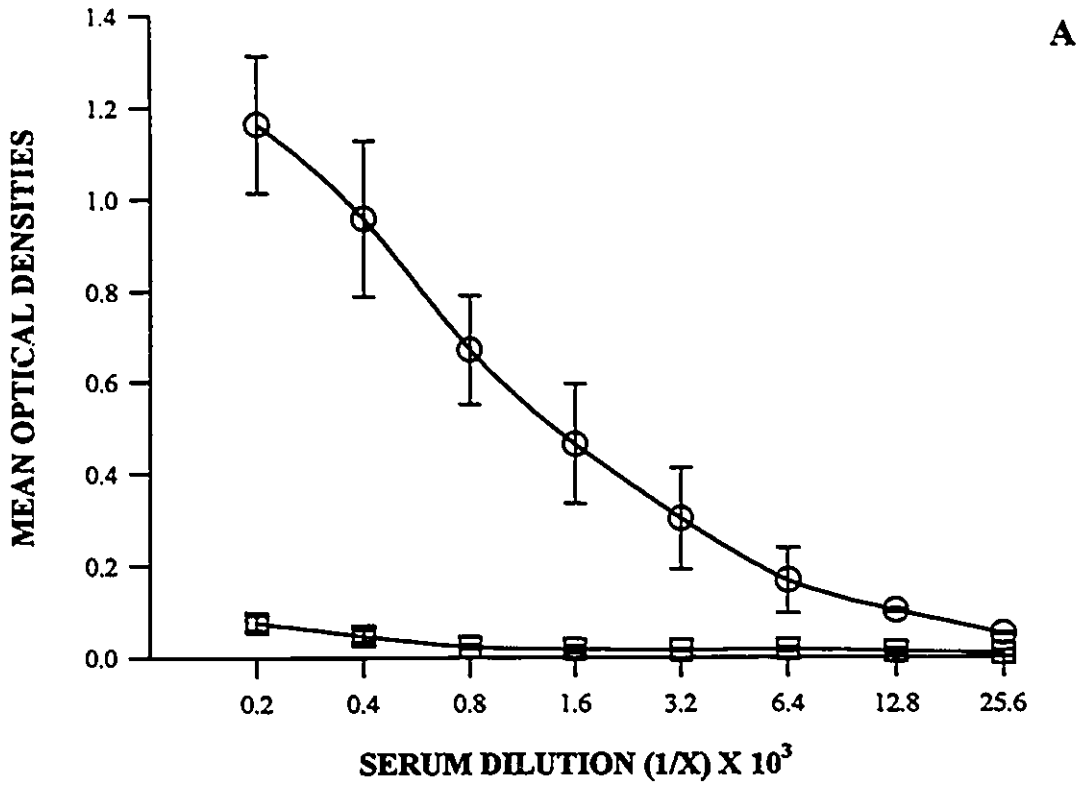
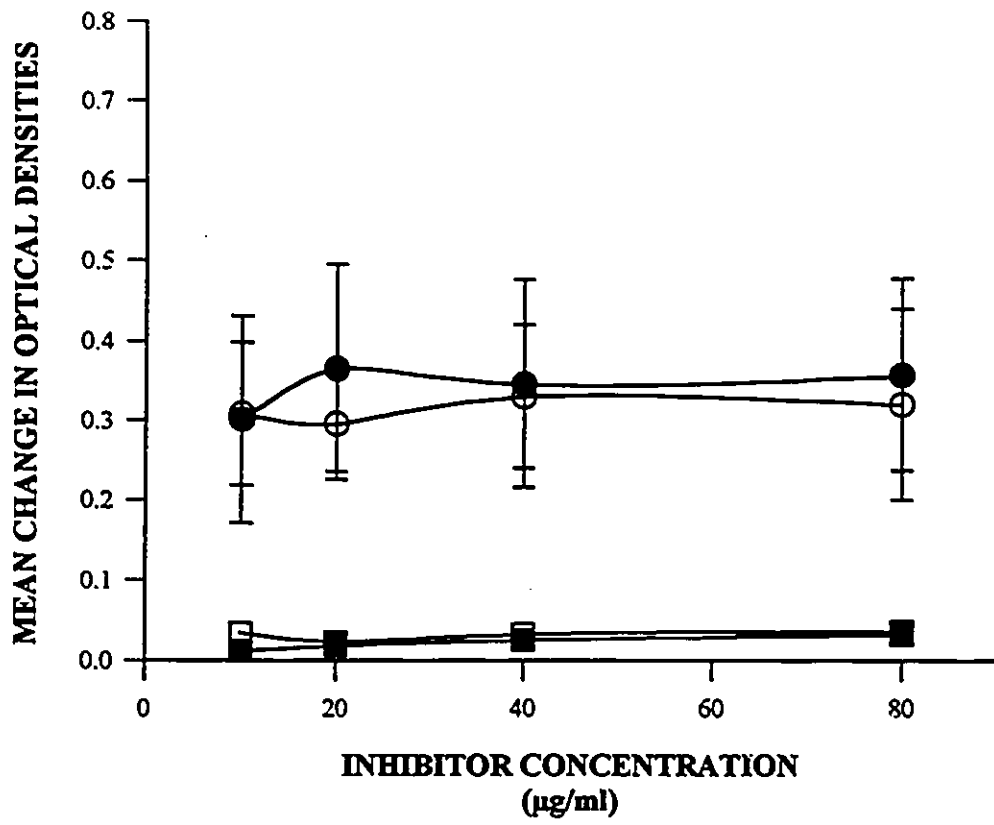


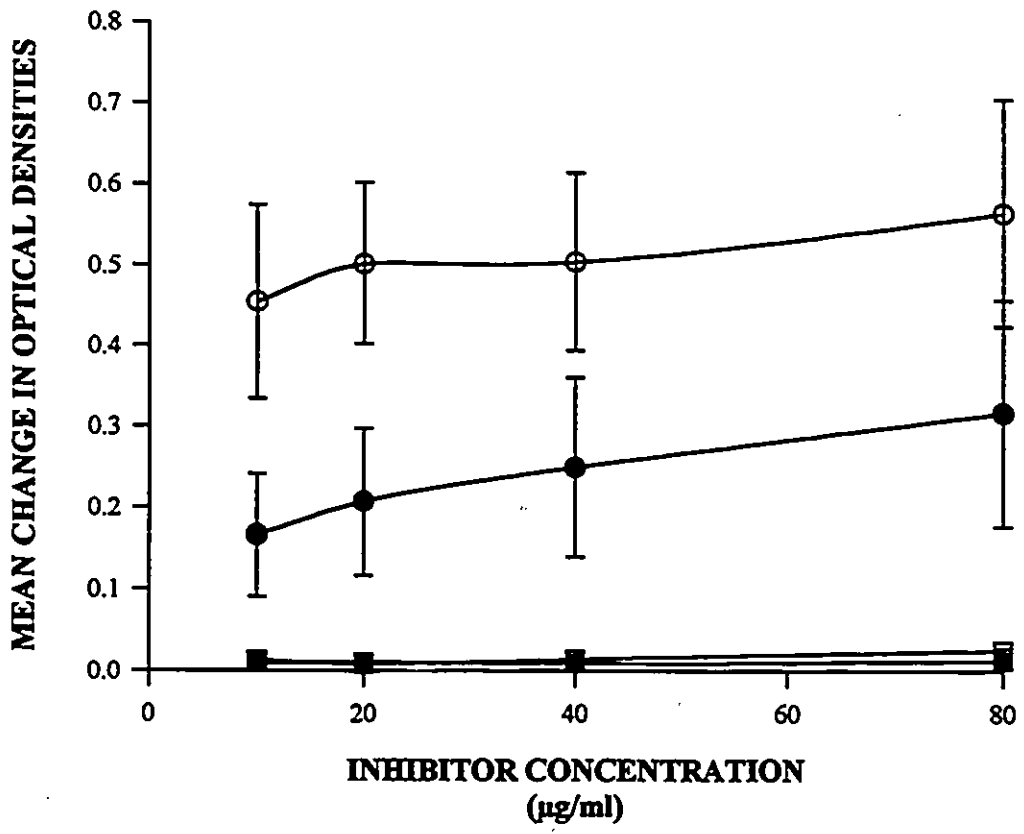
Figure 6

Inhibition of reaction to LOS and soluble bacterial antigen for four LOS reactive samples. (A) Mean change in optical densities (\pm SD) of LOS reactivity by adsorption with soluble bacterial antigen (open circles) was not significantly different from adsorption with LOS antigen (closed circles) ($p = 0.119$). (B) Mean change in optical densities (\pm SD) of soluble bacterial antigen by adsorption with the soluble antigen (open circles) was significantly different than by LOS adsorption (closed circles) ($p < 0.001$). Adsorption of negative control samples with LOS (closed squares) or soluble antigen (open squares) did not affect reaction to either LOS or the soluble antigen.

A



B



2. Inducible immunity to *H. ducreyi* infection

Antigen extracts

The purity of the LOS and the pilus preparation was assessed by SDS-PAGE and silver staining, and both were found to be relatively pure. Similar to the results of Spinola and co-workers (1991), minor protein bands, in addition to the 24 kDa pilus antigen were seen (Figure 7). Outer membrane protein profiles of both *H. ducreyi* 35000 and the clinical isolate RO-34 were found to be distinct (Figure 8).

Serologic response to immunization with LOS, carbohydrate and the pilus preparation

Immunization of rabbits with purified LOS produced a strong IgM response peaking at 7 days following primary immunization, with no detectable response or booster effect for IgG (Figure 9A). No LOS specific IgG or IgM was detectable by ELISA in naive (Figure 9A) or naïve rabbits.

Immunization of rabbits with the pilus preparation induced a strong IgG response detectable within 14 days and peaking 35 days after primary challenge and one week after booster immunization (Figure 9B). Only a weak IgM response was observed after immunization with the pilus preparation (Figure 9B). Again, naive control rabbits were negative. The presence of IgG serologic reaction in all seven rabbits tested is illustrated by Western blot (Figure 10). Dominant and consistent reaction to the 24 kDa protein was present in all seven, with weaker and inconsistent reaction to bands at 25 to 26 kDa and higher molecular masses.

Immunization of rabbits with the carbohydrate extract failed to induce any detectable

Figure 7

SDS-PAGE of the three antigen preparations used for immunization of rabbits. MW, molecular mass markers (in kilodalton). Lane A, uncharacterized carbohydrate extract; Lane B, LOS extract; Lane C, pilus preparation. Extracts were run on a 12.5% acrylamide gel and bands were visualized with silver stain.

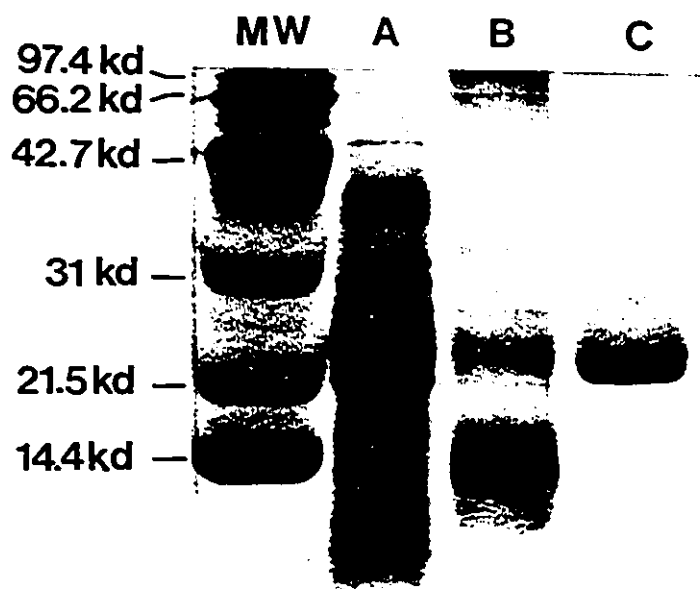


Figure 8

Outer membrane protein profiles of the *H. ducreyi* Kenyan clinical isolate RO-34 (A) and strain 35000(B). MW, molecular mass standards. Extracts were run on a 12.5% acrylamide gel and stained with silver stain.

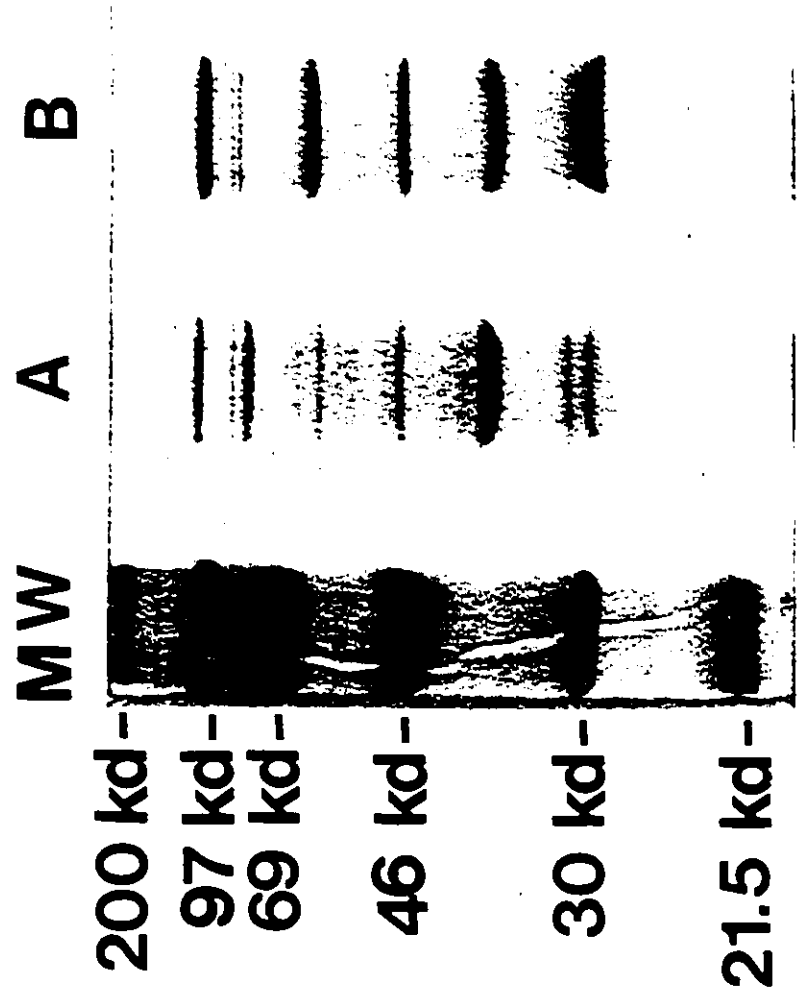


Figure 9

IgG and IgM serologic response in rabbits immunized with LOS (A) and the pilus preparation (B) from *H. ducreyi* strain 35000. Rabbits were immunized with 100 μ g of each antigen and given boosters 4 weeks later as indicated by the asterisks. IgG (closed inverted triangles) and IgM (closed triangles) serologic responses to LOS (A) or the pilus preparation (B) and IgG (closed circles) and IgM (closed squares) of naive control rabbits were measured by ELISA. Data represent the mean OD (\pm SD) of 6 LOS, 7 pilus immunized rabbits and 7 naive controls.

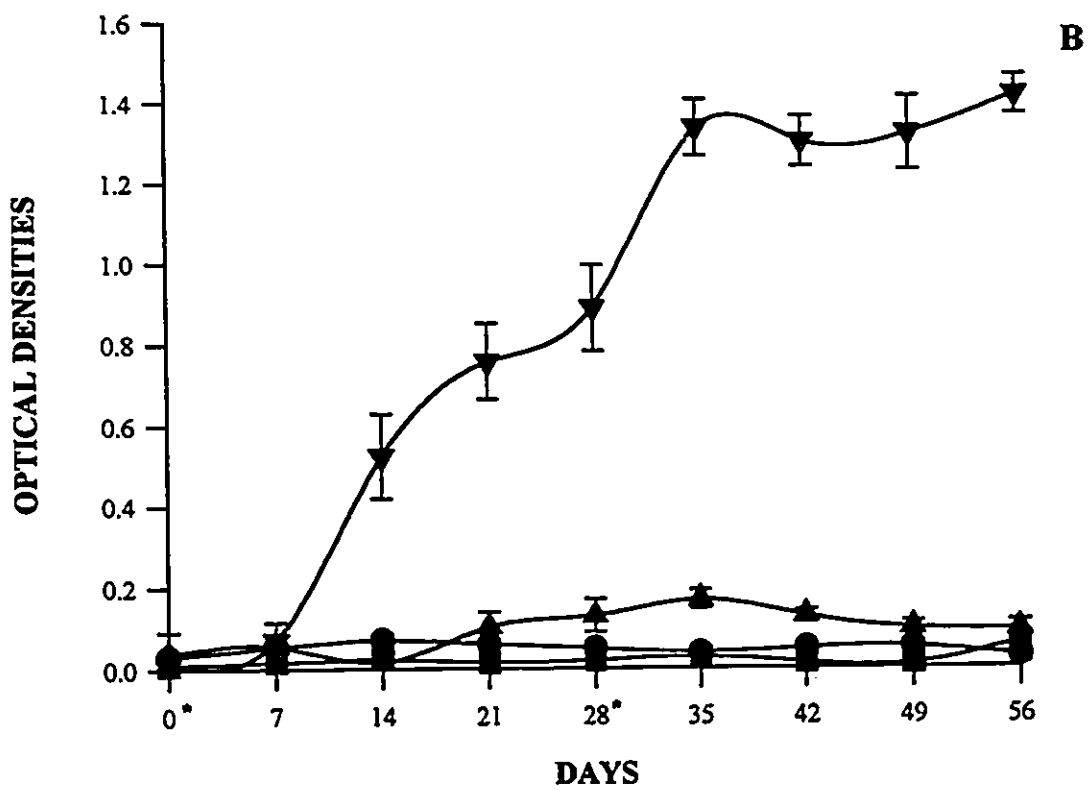
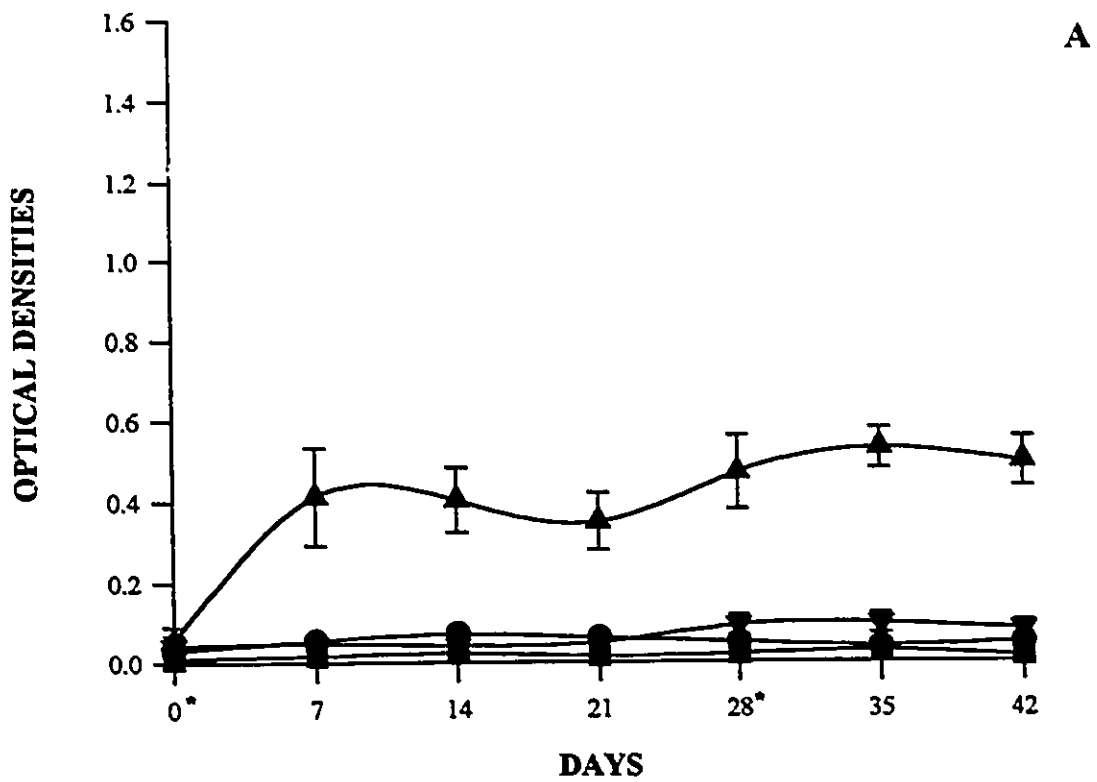


Figure 10

Western blot analysis of sera taken 49 days after primary immunization, or 21 days after booster immunization of seven rabbits with the pilus preparation and prior to infectious challenge (lanes A through G). MW, molecular mass standards.

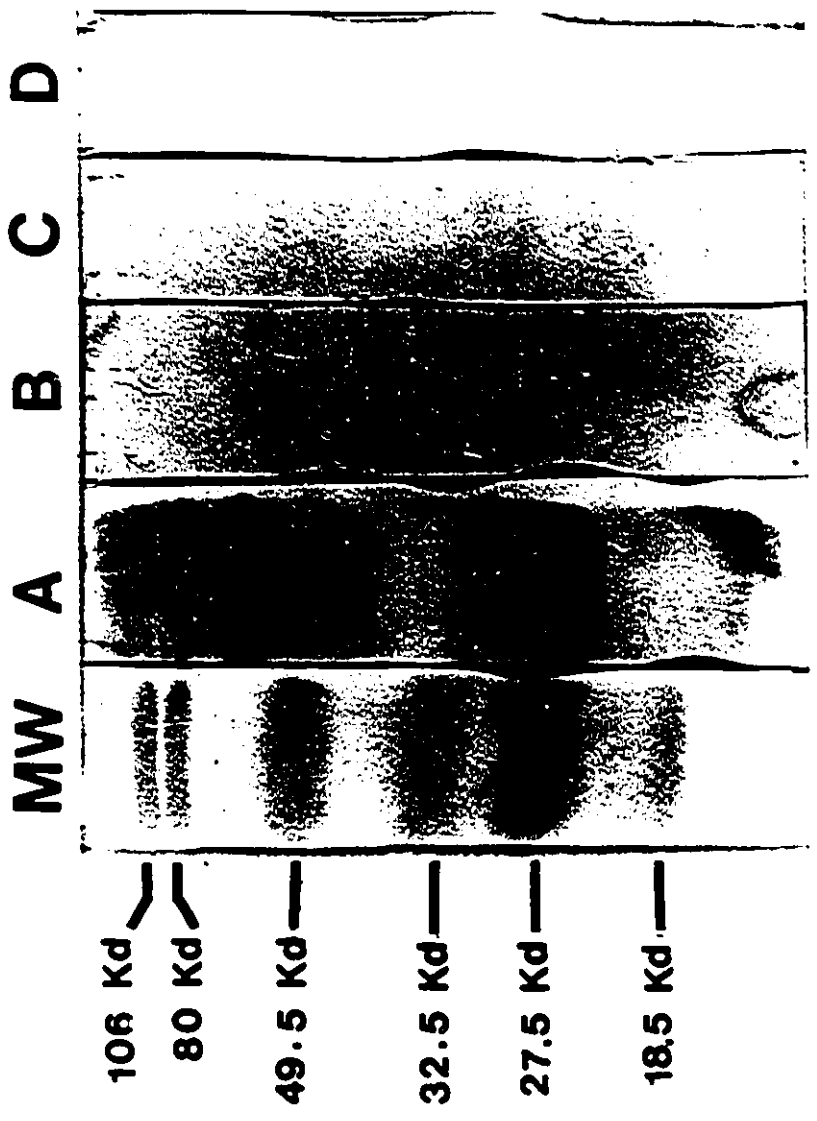
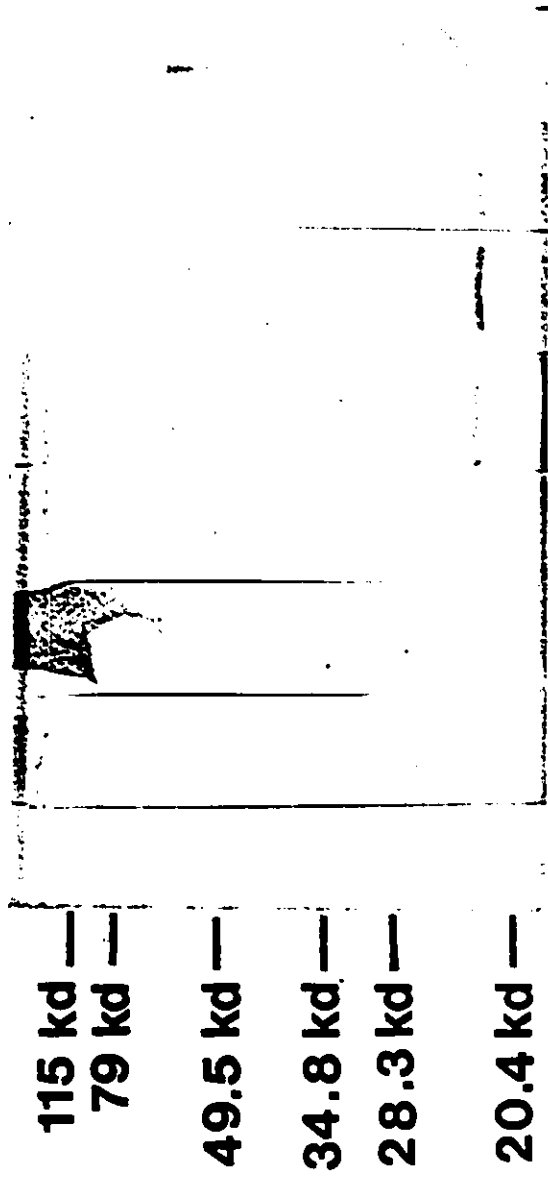


Figure 11

Western blot of analysis of rabbit IgG serologic reaction to *H. ducreyi* 35000 carbohydrate antigen. MW, molecular masses (in kilodaltons): Lane A, *H. ducreyi* 35000 soluble bacterial antigen blotted with acute chancroid human serum; Lane B, carbohydrate extract blotted with 1:100 dilution of acute chancroid human serum; Lane C and D, carbohydrate extract blotted with a 1:50 and 1:100 dilution of serum from the rabbit immunized with 100 μ g of the carbohydrate antigen.

MW A B C D E F G



IgG responses by Western blotting (Figure 11 lanes C and D). An acute chancroid human serum from a culture-proven case of chancroid had strong IgG response to several components of soluble bacterial antigens (Figure 11 lane A), but as seen in immunized rabbits, there was no detectable IgG response to the carbohydrate antigens (Lane B). No serologic reaction was detected by ELISA with carbonate or chenodeoxycholate-fixed carbohydrate antigen in any of the three immunized rabbits (data not shown).

Infection with *H. ducreyi* 35000 in controls

In the rabbits, the onset of disease following challenge with *H. ducreyi* 35000 was similar to that described by Purcell and co-workers (1991). Rabbits developed erythematous nodular indurated inflammatory lesions within 24 hours of infectious challenge with 10^4 CFU of *H. ducreyi* strain 35000 (100 μ l inoculum), and these become suppurative (score of 3) within 2 days. Lesions became ulcerative 3.8 ± 0.9 days after challenge with a mean duration until re-epithelialization of 11.4 ± 1.2 days (Table 6). All naive control rabbits developed ulcerative lesions with a scabbing, thick purulent, bleeding base (Figure 13 B, 14 B, 16 B, 17 B). Lesions produced at 10^6 and 10^5 CFU were also ulcerative and generally larger and more severe. Inocula at or below 10^3 CFU failed to consistently induce ulcerative lesions. *H. ducreyi* was recovered from lesions between 24 hours and 16.9 ± 1.7 days after challenge with 10^4 CFU (Table 6). There was no significant difference in disease between naive control rabbits and adjuvant control rabbits after challenge with *H. ducreyi* strain 35000 (Figure 12 and 13).

Figure 12

Virulence of *H. ducreyi* 35000 in rabbits challenged with 100 μ l of 10⁵ CFU/mL. Lesion sizes (bar graph) and lesion score (plot) of 9 naive control rabbits (open bars and open circles) (\pm SE) and 5 sham-immunized controls (shaded bars and open squares), with last culture positive day indicated for controls (closed circles) and sham vaccinees (closed squares) are shown (\pm SE). No significant reduction in lesion size or score was observed over the 21 days of observation ($P > 0.5$).

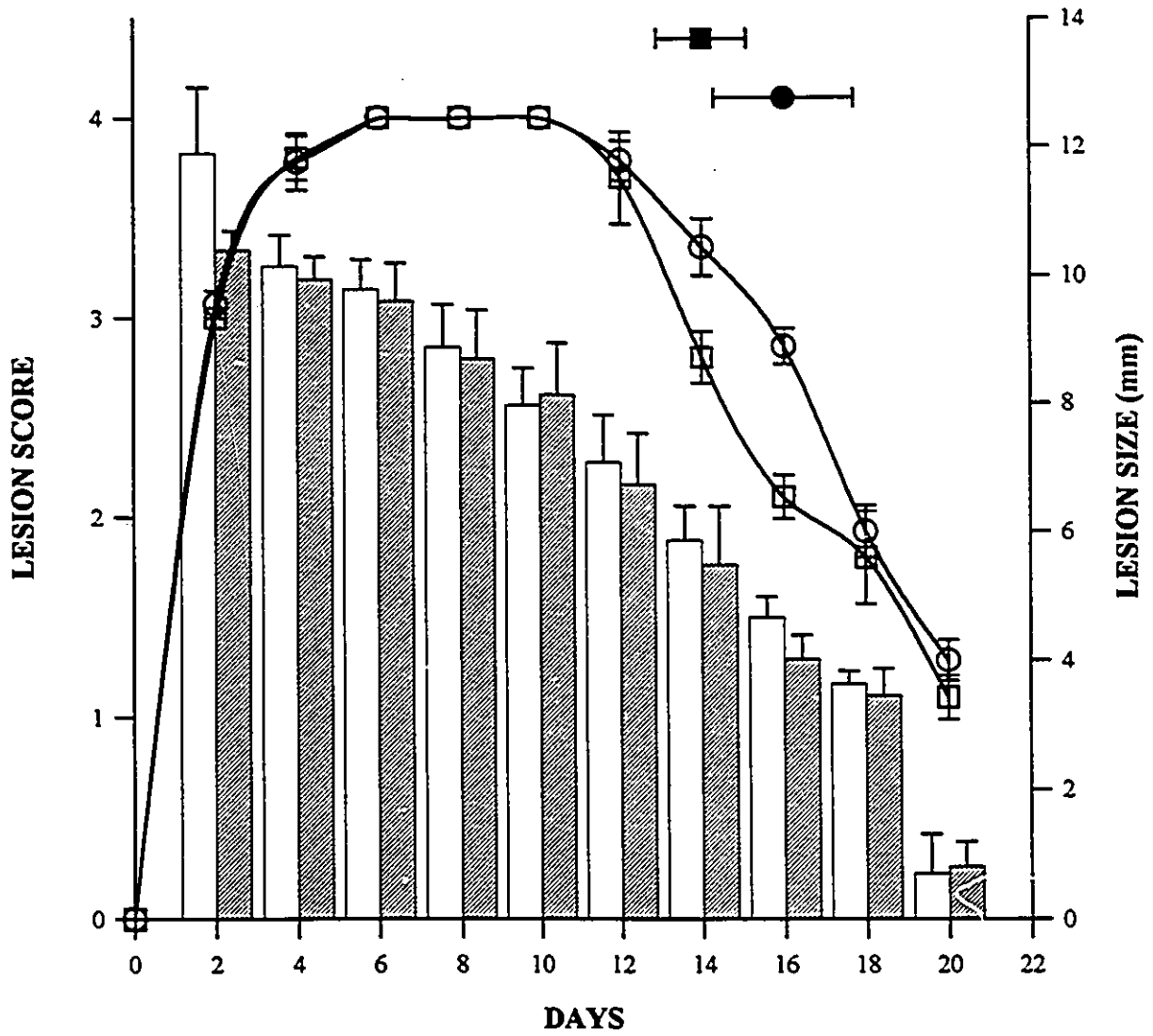


Figure 13

Appearance of disease in sham-immunized (A) or naive control rabbits (B), seven days after infectious challenge with inocula (100 μ l) from 10^7 to 10^3 CFU/mL of *H. ducreyi* 35000. No significant difference in disease is shown.



B

10^7

10^6

10^6

10^4

10^3



A

Table 6

Homologous strain protection at 10^7 CFU/ml in rabbits immunized with the pilus preparation or LOS

	Controls (n=9)	LOS vaccinated (n=6)	Pilus preparation vaccinated (n=14)
Inoculum size (CFU X10 ⁵ /mL) ^a	1.12 ± 0.424	2.45 ± 0.75 (p = 0.26) ^b	2.5 ± 1.4 (p = 0.37) ^c
Number of days culture positive ^a	16.9 ± 1.7	16 ± 0.632 (p = 0.72) ^b	6.2 ± 1.7 (p < 0.001) ^c
Lesion size (mm)			
Cumulative (21 days)	148.7 ± 25.7	135.83 ± 29.7 (p = 0.52) ^b	48.6 ± 10.9 (p < 0.001) ^c
Average ^a	7.1 ± 1.2	6.46 ± 1.7 (p = 0.06) ^b	2.32 ± 0.5 (p < 0.001) ^c
Lesion score			
Peak score ^a	4 ± 0	4 ± 0	3.1 ± 0.14 (p < 0.01) ^c
No. of ulcerative lesions/total	9/9	6/6	3/14
Ulcer duration (days) ^a	11.4 ± 1.2	9.0 ± 1.4 (p=0.9) ^b	1.4 ± 2.7 (p < 0.001) ^{c,d}

^a Data represent means ± standard deviations.

^b Comparative evaluation of LOS-vaccinated rabbits versus naive controls (Students t test).

^c Comparative evaluation of rabbits vaccinated with the pilus preparation versus naive controls (Student's t test).

^d Data represent the duration of five ulcers in three rabbits.

Virulence of *H. ducreyi* in LOS and carbohydrate immunized rabbits

Although rabbits did respond serologically to LOS, no significant protection against disease was observed (Figures 14 and 15; Table 6). In six LOS vaccinated rabbits challenged with the homologous strain, disease progressed and resolved in a manner similar to that in nine control rabbits (Figure 15). No significant difference was observed in peak lesion size ($p = 0.097$) or score ($p = 0.244$) over 21 days, or at any point of observation. Similar to LOS immunization, immunization of rabbits with the carbohydrate extract failed to produce any significant difference in disease in any of the three immunized rabbits (Figure 16).

Virulence of *H. ducreyi* in pilus immunized rabbits.

Within 24 hours of challenge with *H. ducreyi* strain 35000, rabbits vaccinated with the pilus preparation developed indurated lesions similar in size and severity to those of controls. However, only 3 of 14 vaccinated rabbits developed ulcerative lesions which lasted an average of 1.4 ± 2.7 versus 11.4 ± 1.2 days in controls ($p < 0.001$) (Table 6) at 10^5 CFU/mL. The duration and severity of disease was significantly reduced in all 14 pilus-immunized rabbits (Figure 17 and 18; Table 6). Lesion size and score throughout the period of observation were significantly reduced in pilus preparation-vaccinated rabbits compared to naive controls ($p < 0.001$) (Figure 18).

Heterologous strain protection

Five naive control rabbits infected with 10^5 CFU/mL of the RO-34 clinical isolate developed suppurative lesions within 24 hours, becoming ulcerative within 3.8 ± 0.4 days

Figure 14

Appearance of disease at day seven in LOS immunized (A) and naive control (B) rabbits challenged with titered inocula from 10^7 to 10^3 CFU/mL of the homologous strain 35000.



B
→ 10^7 ←

→ 10^6 ←

→ 10^5 ←

→ 10^4 ←

→ 10^3 ←



A

Figure 15

Virulence of *H. ducreyi* 35000 at 10^5 CFU/mL in rabbits immunized with LOS. Lesion sizes (bar graph) and lesion score (plot) for 6 rabbits vaccinated with the LOS extract (shaded bars and open squares) and 9 naive control rabbits (open bars and open circles) (\pm SE), with last culture positive day indicated for vaccinees (closed square) and controls (closed circle) in reference to number of days on the x axis, are shown (\pm SE). No significant reduction in lesion sizes and scores was observed over the 21 days of observation ($p > 0.05$).

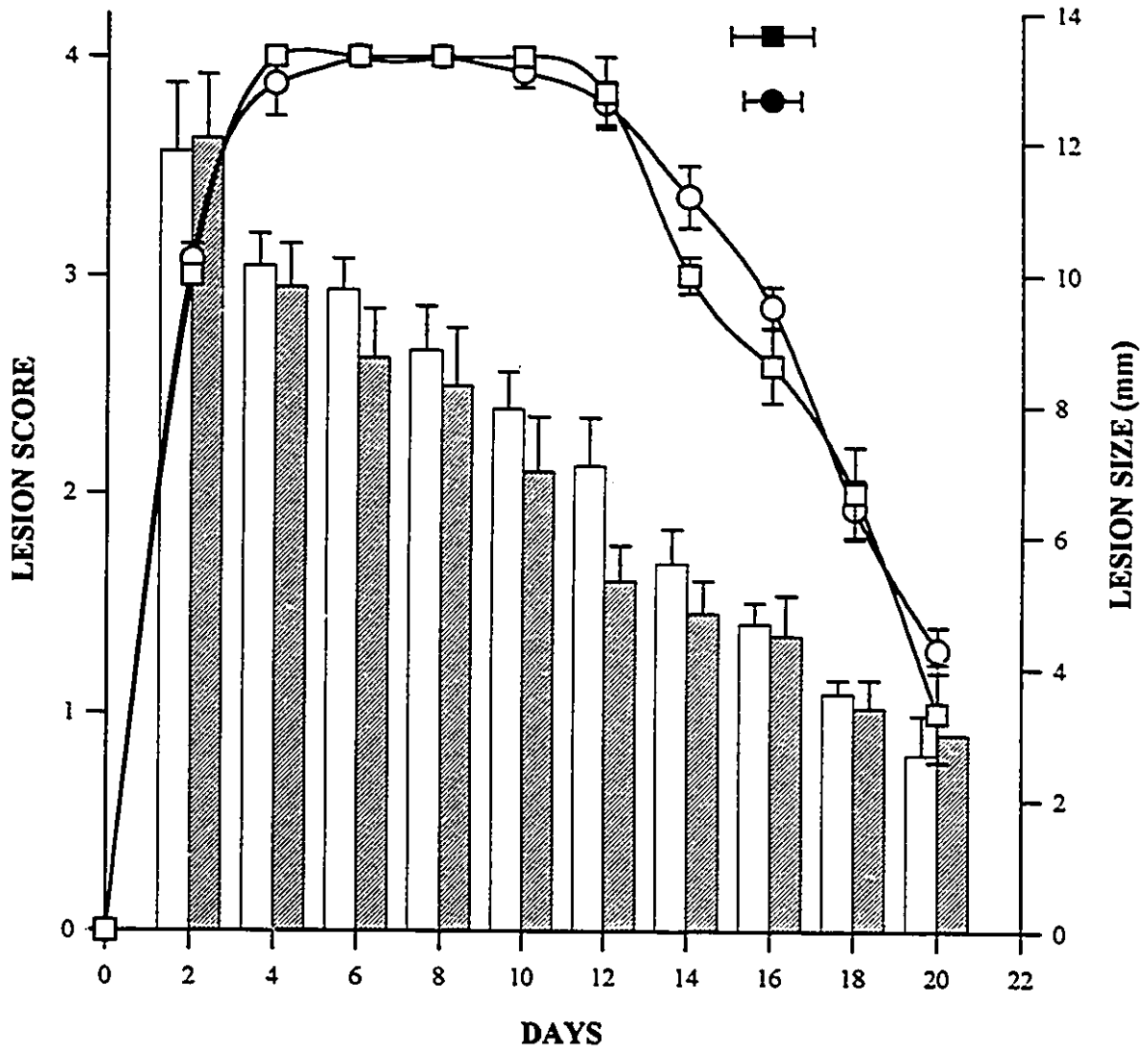


Figure 16

Appearance of disease in carbohydrate (A) and naive control (B) rabbits 7 days post infectious challenge with titered inocula (100 μ l) from 10^7 to 10^3 CFU/mL of the homologous strain 35000.



B

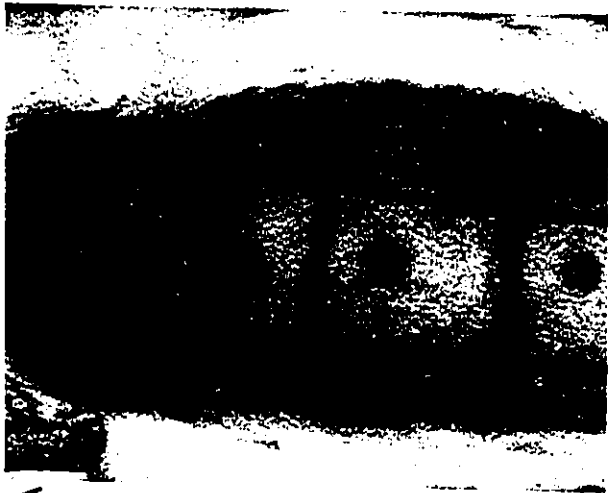
10^7

10^6

10^5

10^4

10^3



A

Figure 17

Homologous strain disease protection in rabbits vaccinated with the pilus preparation. The appearance of disease at 7 days in immunized (A) and control (B) rabbits challenged with titered inocula (100 μ l) from 10^7 to 10^3 CFU/mL of the homologous strain is shown.



B

10^7

10^6

10^5

10^4

10^3



A

Figure 18

Homologous strain (35000) protection following challenge with 10^5 CFU/mL (100 μ l) produced by immunization with the pilus preparation. Lesions (bar graph) and lesion score (plot) for 14 rabbits vaccinated with the pilus preparation (shaded bars and open squares) and 9 naive control rabbits (open bars and open circles) (\pm SE), with last culture positive day indicated for vaccinees (closed squares) and controls (closed circles) are shown (\pm SE).

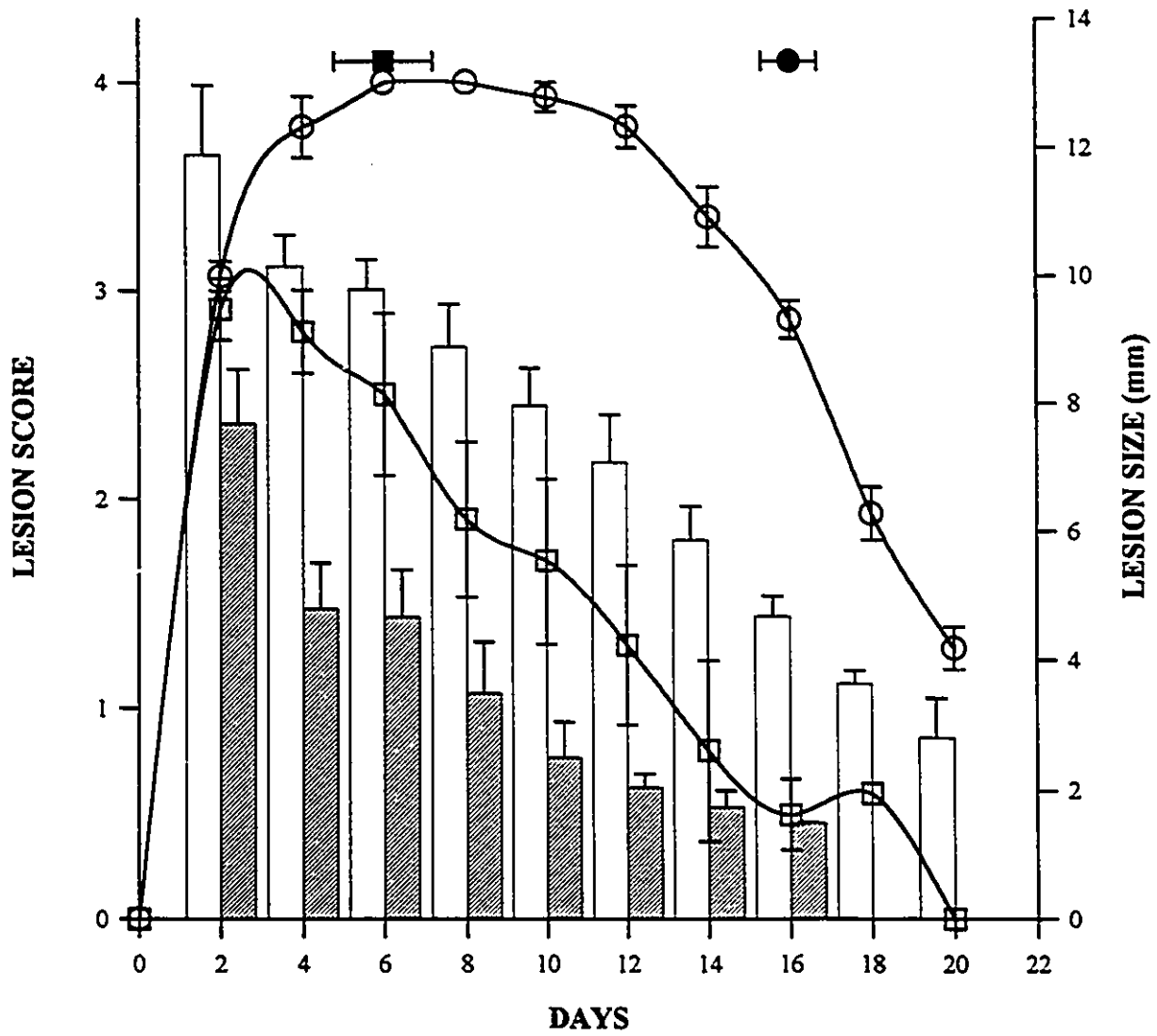
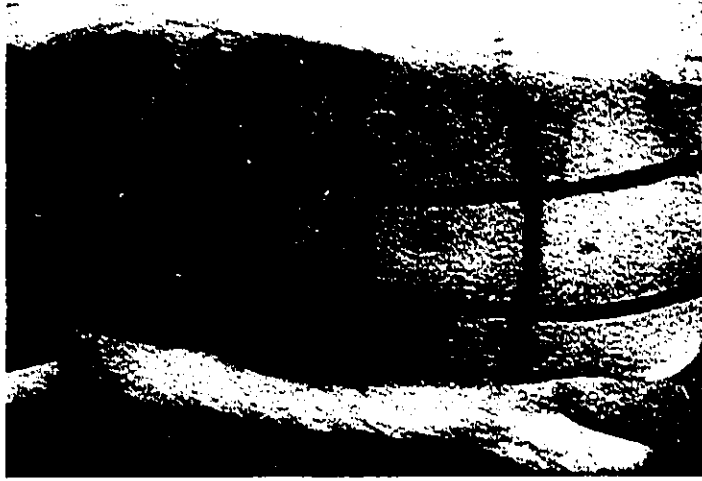


Figure 19

Heterologous strain disease protection in rabbits immunized with the pilus preparation. The appearance of disease in rabbits immunized with the pilus preparation of *H. ducreyi* 35000 (A) and a naive control rabbit (B), all of which were challenged with titered inocula from 10^7 to 10^3 CFU/mL of the heterologous clinical isolate RO-34.



B

$\leftrightarrow 10^7 \leftrightarrow$

$\leftrightarrow 10^8 \leftrightarrow$

$\leftrightarrow 10^5 \leftrightarrow$

$\leftrightarrow 10^6 \leftrightarrow$

$\leftrightarrow 10^3 \leftrightarrow$



A

Table 7

Heterologous strain protection at 10^5 CFU/ml in rabbits immunized with the pilus preparation from strain 35000 and challenged with the RO-34 isolate

	Controls (n=5)	Pilus preparation vaccinated (n=8)
Inoculum size (CFU $\times 10^3$ /mL) ^a	1.78 \pm 0.56	1.62 \pm 0.47 (p = 0.35) ^c
Number of days culture positive ^a	12.6 \pm 1.5	1.88 \pm 2.6 (p < 0.005) ^c
Lesion size (mm)		
Cumulative (21 days)	144 \pm 41	83.5 \pm 32 (p < 0.01) ^c
Average ^a	6.85 \pm 1.5	3.98 \pm 1.5 (p < 0.001) ^c
Lesion score		
Peak score ^a	4 \pm 0	3.38 \pm 0.35 (p < 0.001) ^c
No. of ulcerative lesions/total ^b	5/5	5/8
Ulcer duration (days) ^a	10.2 \pm 1.9	3 \pm 2.7 (p < 0.001) ^{c,d}

^a Data represent means \pm standard deviations.

^b An ulcerous lesion had a score of 4.

^c Comparison of mean values by the Student's t test.

^d Data represent the duration of six ulcers in five rabbits.

with a mean duration of 10.2 ± 1.9 days (Figure 19; Table 7). At 10^5 CFU/mL of the heterologous strain RO-34 all eight rabbits immunized with the pilus preparation from strain 35000 developed nodular indurated lesions within 24 hours of challenge. Five of eight rabbits developed ulcerative lesions within 7.0 ± 0.7 days, with an average duration of 3.0 ± 2.7 days (Table 7). Lesions at 10^5 CFU/mL were culture positive for 1.9 ± 2.6 days compared to 12.6 ± 1.5 days for naive controls ($p < 0.005$) (Table 7). Disease severity and duration were significantly reduced by immunization with the pilus preparation (Figure 19). Lesion size and score were also significantly reduced in immunized rabbits challenged with the heterologous strain RO-34 ($p < 0.001$) (Figure 20).

Histopathology of chancroidal lesions in pilus immunized rabbits

In pilus vaccinated, naive and sham-immunized control rabbits, ulcerative lesions at 4 days from infection with strain 35000 were similar and typical of chancroidal lesions, which are characterized by three architectural zones (Heyman *et al.* 1945). The first zone consists of an area of necrosis with cellular debris, degenerative polymorphonucleocytes (PMN) and fibrin, corresponding to the ulcer surface. The second zone consists of PMN and histiocytic infiltrate characteristic of an acute necrotizing inflammation. The third, regenerative or edematous zone with PMN, reactive fibroblasts, edema and neo-vascular endothelial cells (Figures 21 A-1, 21 B-1, 27 A-1 and 28 A-1) underlies zone 1 and 2. Cellular infiltrate of day 4 lesions of pilus vaccinated rabbits consisted of a mixture of both acute and chronic inflammatory cells with both PMN and abundant lymphoid cells. At 10 days after infection, lesions of control rabbits were still acutely inflamed with abundant PMN

Figure 20

Heterologous strain protection at 10^5 CFU/mL in rabbits immunized with the pilus preparation from strain 35000 and challenged with the clinical isolate RO-34. Lesion sizes (bar graph) and lesion scores (plot) for eight rabbits vaccinated with the pilus preparation (shaded bars and open squares) and five control rabbits (open bars and open squares) (\pm SE). The last culture positive day is indicated for controls (closed circles) and vaccinees (closed squares) (\pm SE).

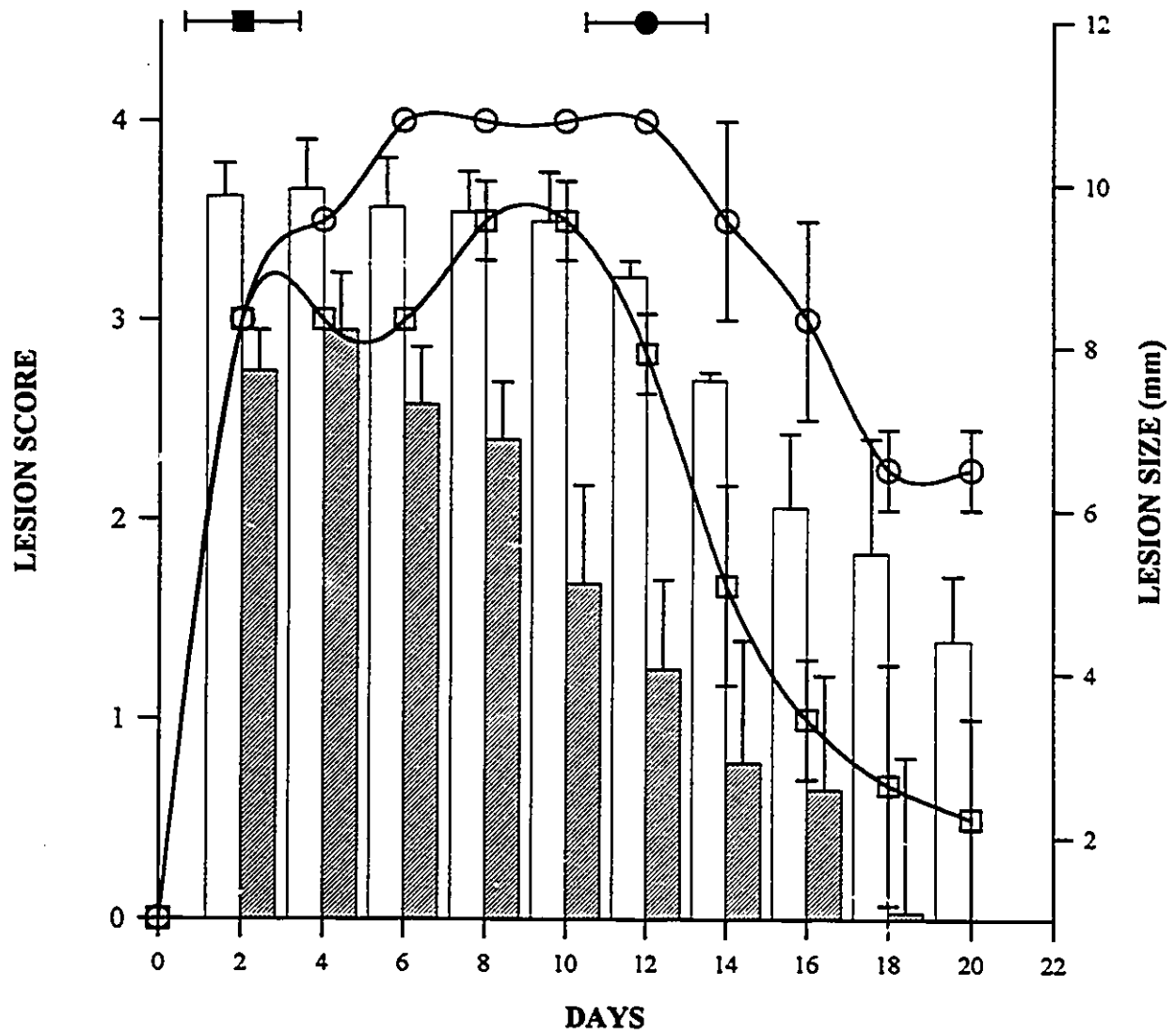


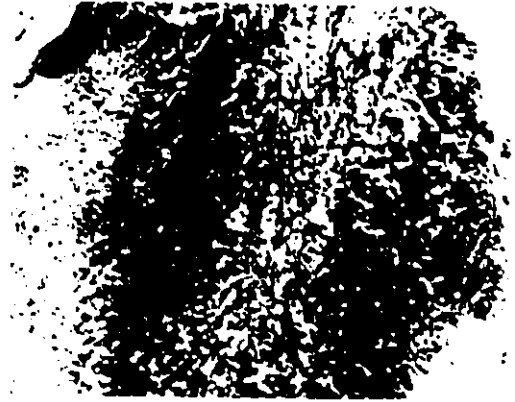
Figure 21

Hematoxylin and eosin stained biopsy samples of chancroidal lesions following challenge (100 μ L) with the homologous strain 35000 in 10⁵ CFU/mL of naive control rabbits (A) and rabbits immunized with the pilus preparation (B). Biopsies were performed on days 4 (A-1, B-1), 10 (A-2, B-2), 15 (A-3, B-3), and 21 (A-4, B-4) following infection with the homologous strain 35000. The day 4 biopsy samples show typical organization of chancroidal lesions into three well-defined zones, whereas samples from day 10, day 15, and day 21 biopsies are high power views of the infiltrative zones. Accelerated recruitment of lymphoid and plasmacytoid infiltrate is illustrated, corresponding to more rapid healing of the lesions in vaccinees.

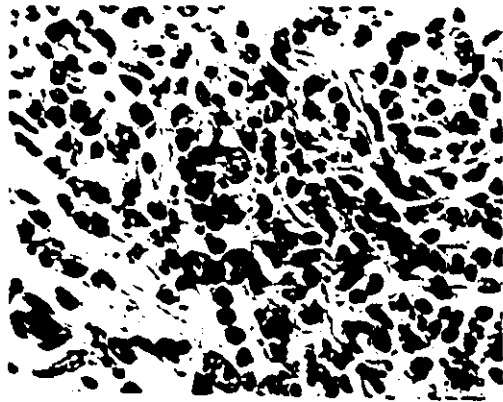
A-1



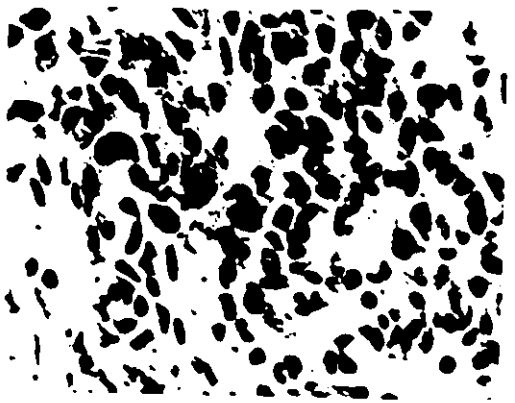
B-1



A-2



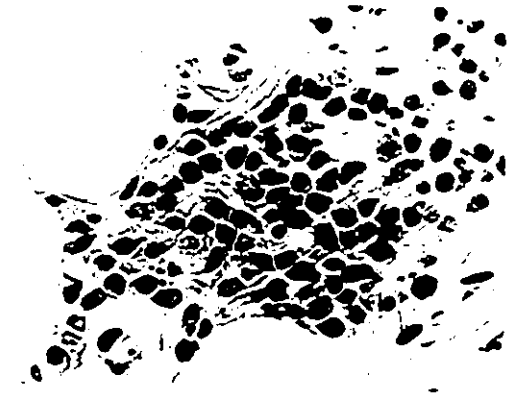
B-2



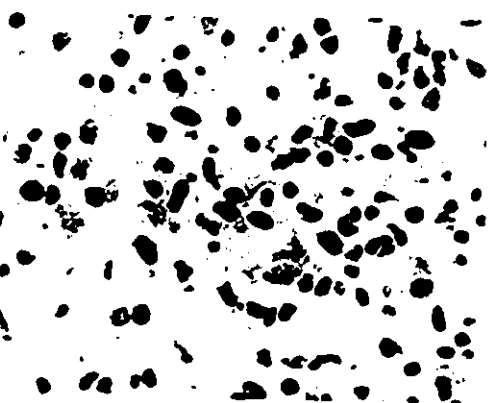
A-3



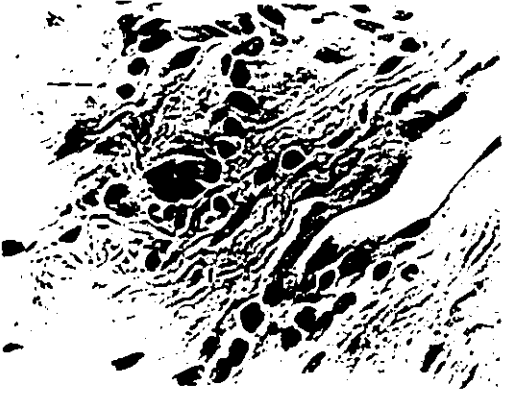
B-3



A-4



B-4



and few lymphoid cells (Figures 21A-2 and 27B-2). By day 10, cellular infiltrate from lesions of vaccinees were consistent with chronic inflammation with a predominantly lymphocytic and histiocytic infiltration and numerous plasma cells (Figures 21 B-2 and 28 B-1). Similar to day 10, day 15 lesions of naive control and sham-immunized rabbits were ulcerative with necrotic debris and acute ulcerative inflammation. Cellular infiltrates consisted of a mixture of PMNs, lymphoid and scattered plasma cells and few histiocytes (Figures 21A- 3 and 27 C-1). Cellular infiltrates of day 15 lesions of pilus vaccinated rabbits were consistent with chronic inflammation and contained abundant lymphoid and plasmacytoid cells and disseminated clusters of histiocytes (Figures 21 B-3 and 28 C-1). On day 21 after infection lesions of both vaccinees and controls were similarly characterized by heavy infiltrates of foamy histiocytes, lymphoid cells and lipid vacuoles, typical of a granulomatous reaction. The presence of multi-nucleated giant cells, abundant plasma cells and the presence of Russ bodies were distinguishing features of day 21 lesions of pilus preparation vaccinated (Figures 21B-4 and 28 D-1) rabbits compared to controls (Figures 21 A-4 and 27 D-1).

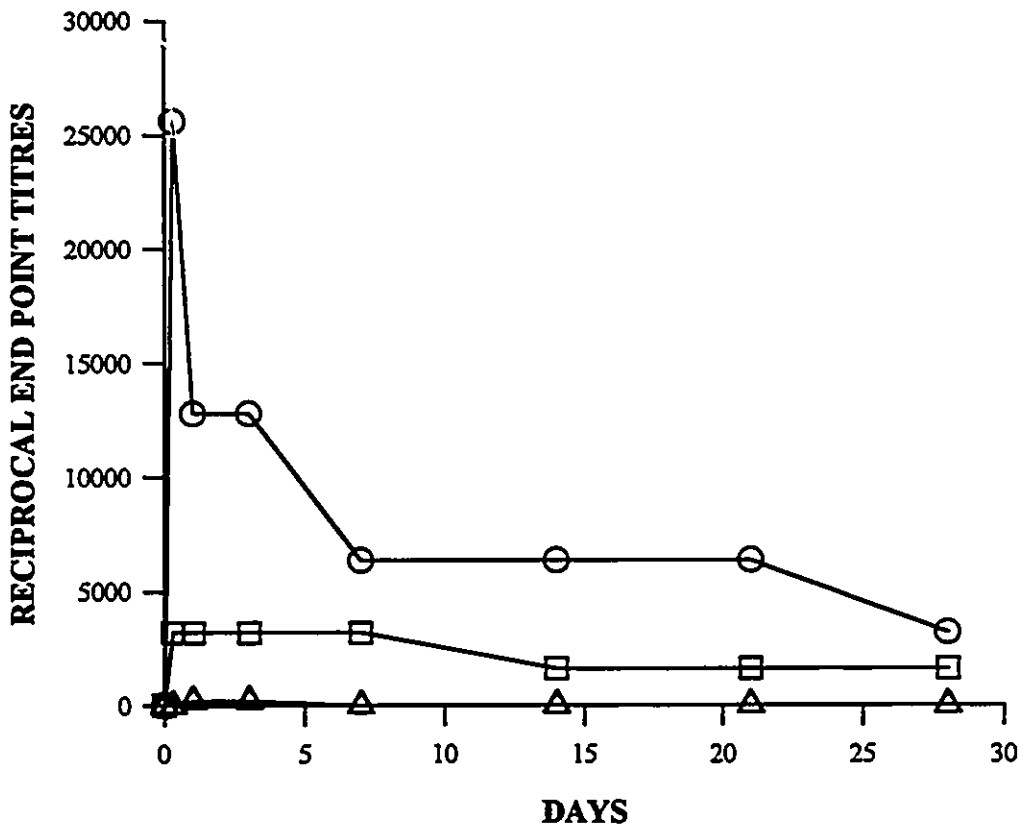
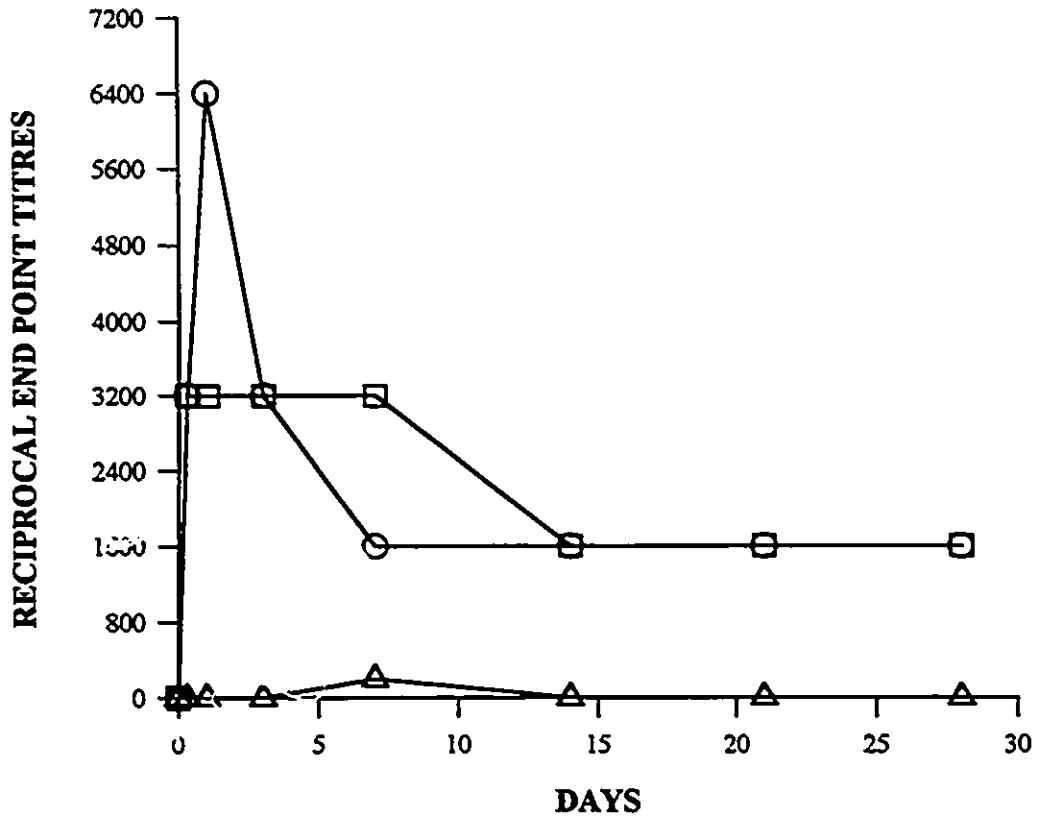
3. Passive immunization with protective anti-sera

Serum antibody levels following passive immunization

Slow intravenous infusion of rabbits with either 24 or 48 mg of affinity purified IgG from anti-sera of whole cell immunized rabbits produced ELISA end point titers to the soluble bacterial antigen of 1:3200 1 week, and 1:1600 3 weeks after infusion. Elimination half life was calculated to be 22 days (Figure 22). No ELISA reactivity to the *H. ducreyi*

Figure 22

End point dilutions of rabbits passively immunized with IgG from rabbits immunized with the pilus preparation, whole cells or naive controls. One rabbit from each group of 4 was infused with 24 mg (A) or 48 mg (B) of IgG from rabbits immunized with whole cells (open circles), pilus preparation (open squares) or naive controls (open triangles). Serum antibody end points were defined by ELISA with the soluble bacterial antigen for rabbits infused with the whole cell IgG, or by ELISA with the pilus antigen for rabbits infused with the pilus preparation IgG. Serum antibody levels in test rabbits were sustained through the experimental period, with a calculated half life of 11 days for passive immunization with 24 or 48 mg of whole cell IgG, and 24 and 18 days for 24 and 48 mg of the pilus preparation IgG respectively.



soluble antigen were detectable in rabbits passively immunized with either 24 mg or 48 mg of polyclonal IgG from sera of un-immunized rabbits (Figure 22). Passive immunization with 24 mg of the pilus preparation specific polyclonal IgG produced peak end point titers on ELISA to the pilus antigen of 1:6400 at 48 hours, 1:1600 1 week and 1:1600 at 3 weeks after infusion (Figure 22). With 48 mg infusion of the pilus IgG end point titer of 1:25600 at 24 hours and 1:6400 1 and at 3 weeks after infusion were detected on ELISA (Figure 22). Serum elimination half life was calculated at 24 days for passive immunization with 24 mg of the pilus preparation specific IgG, and 18 days for passive immunization with 48 mg of the pilus preparation specific IgG.

Virulence of *H. ducreyi* in passively immunized rabbits

Development of disease in un-immunized rabbits was similar to that described above. One day after challenge with *H. ducreyi* 35000 all four unimmunized controls developed erythematous nodular lesions which became ulcerative 3.33 ± 2 days after infection, with a mean ulcer duration of 9 ± 1 days, and remained culture positive for 11 ± 2 days, and resolving by 19 ± 1 days to a score of <2 (under 2mm lesion size) (Table 8). Disease in rabbits passively immunized with 24 mg of the polyclonal IgG from the sera of naive uninfected rabbits progressed in a manner similar to that in negative controls (Table 8). No significant differences in peak lesion size or score were detected in rabbits passively immunized with the whole cell (Figures 23 A and 25; Table 8) or the pilus-specific IgG fractions (Figures 24 A and 25; Table 8) compared to rabbits passively immunized with IgG from naive rabbits or with unimmunized controls. Similarly, disease in rabbits

Figure 23

Virulence of *H. ducreyi* 35000 at 10^5 CFU/mL in rabbits passively immunized with IgG from rabbits immunized with whole cells. Lesion sizes (bar graph) and lesion scores (plot) of 3 rabbits infused with 24 mg (A) or 3 rabbits infused with 48 mg (B) of IgG from rabbits immunized with whole cells (shaded bars and open squares) and 3 control rabbits infused with 24 mg and 3 rabbits infused with 48 mg polyclonal IgG from naive rabbits (open bars and open circles) (\pm SE). Last culture positive day is shown for passively immunized rabbits (closed squares) and control rabbits (closed circles) (\pm SE).

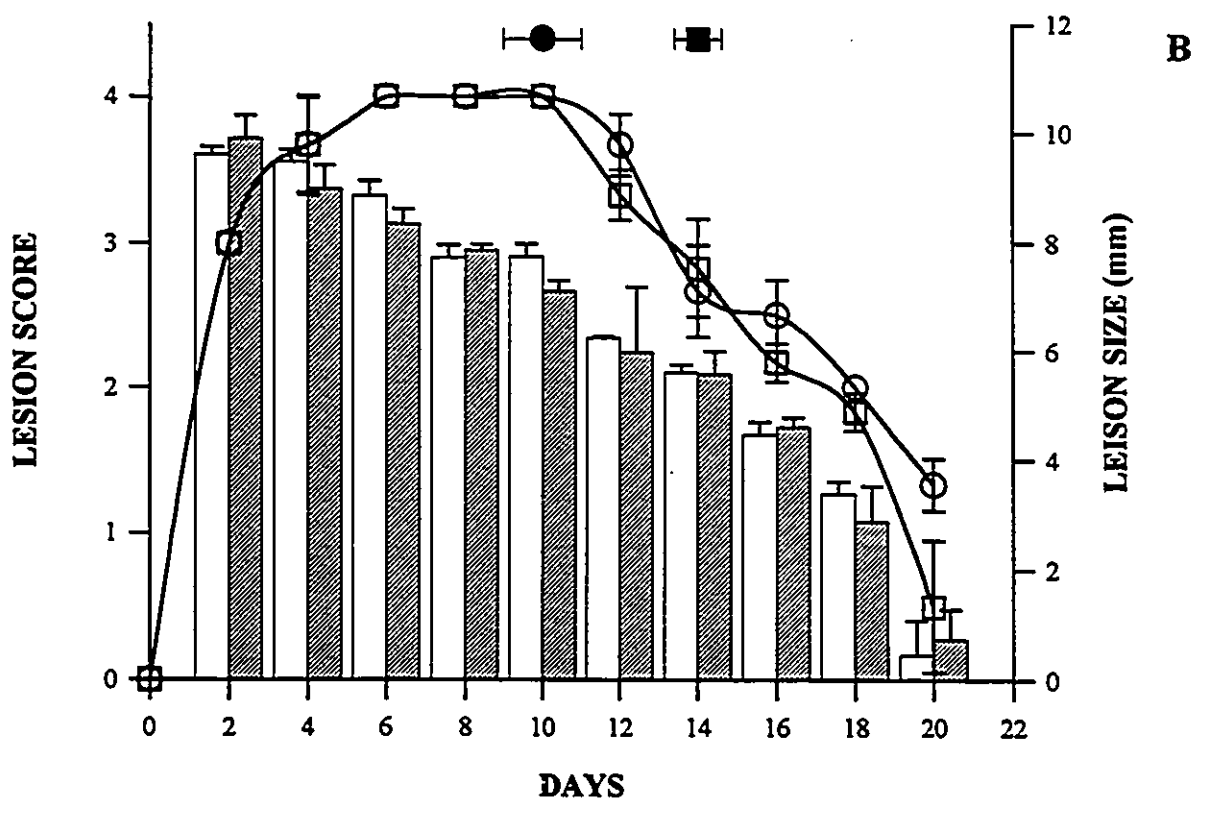
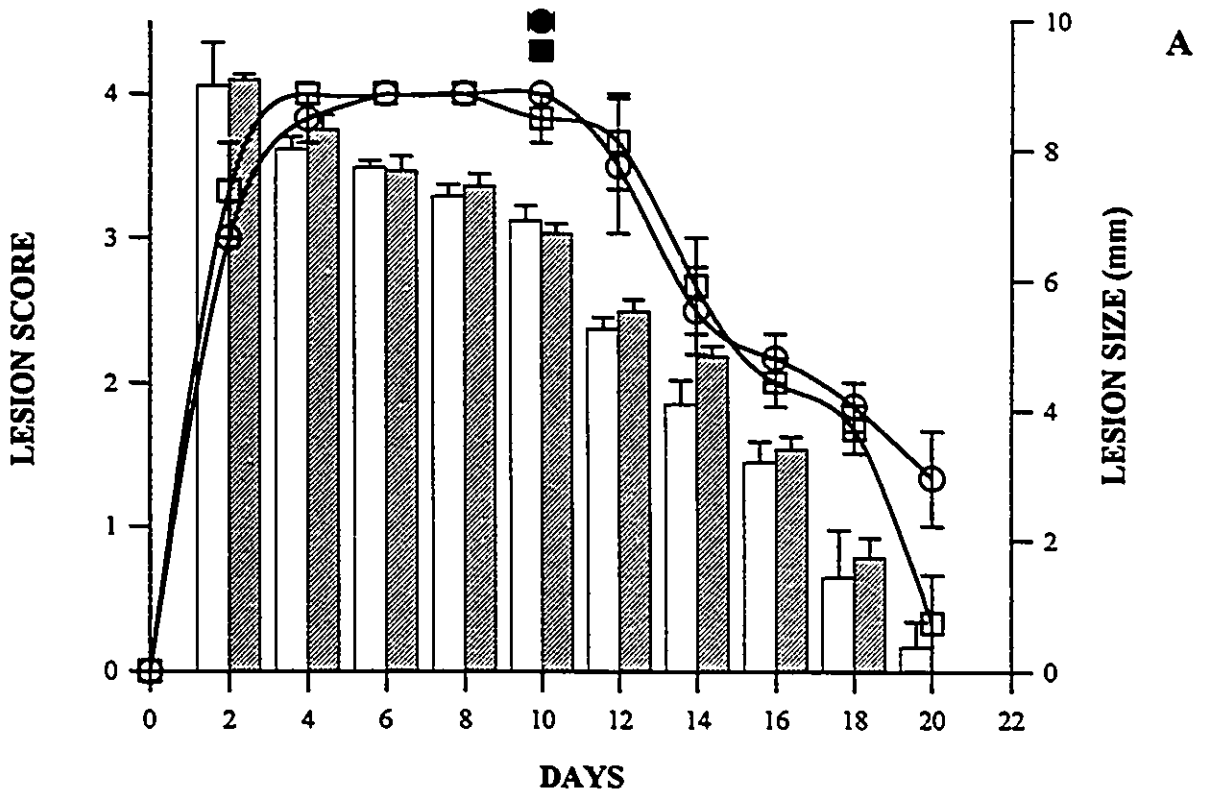


Figure 24

Figure legend as for figure 23 except that rabbits were passively immunized with IgG from rabbits immunized with the pilus preparation.

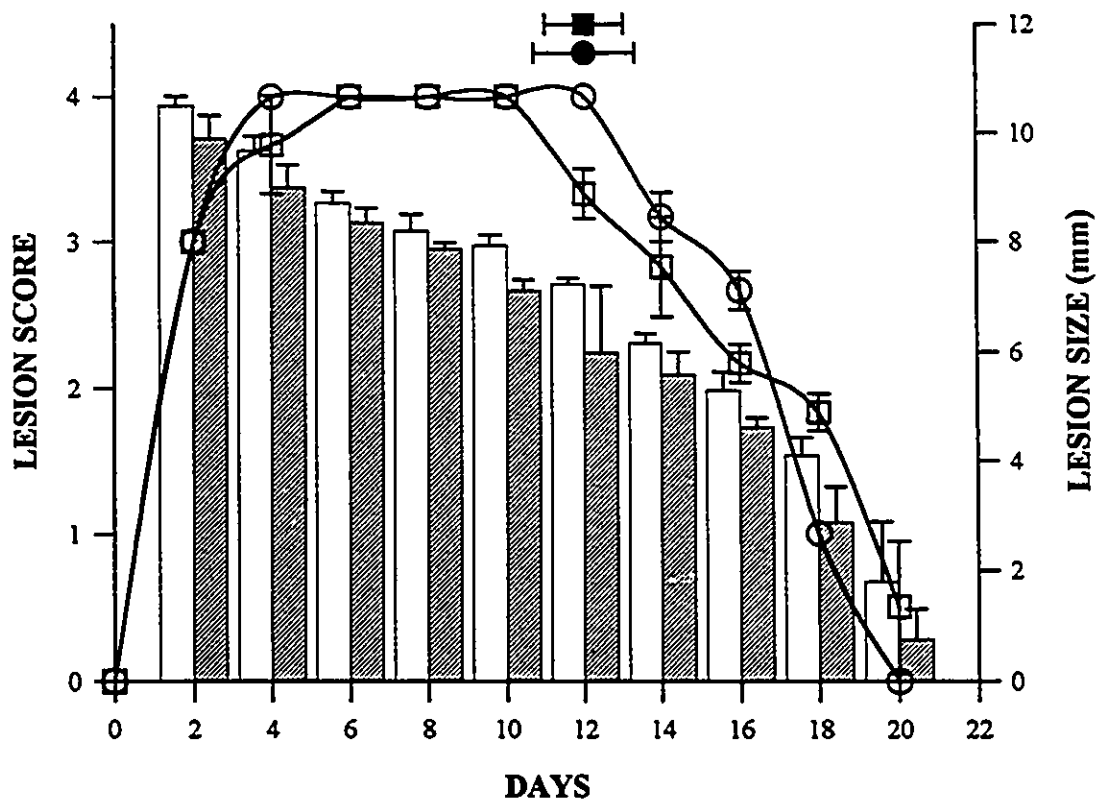
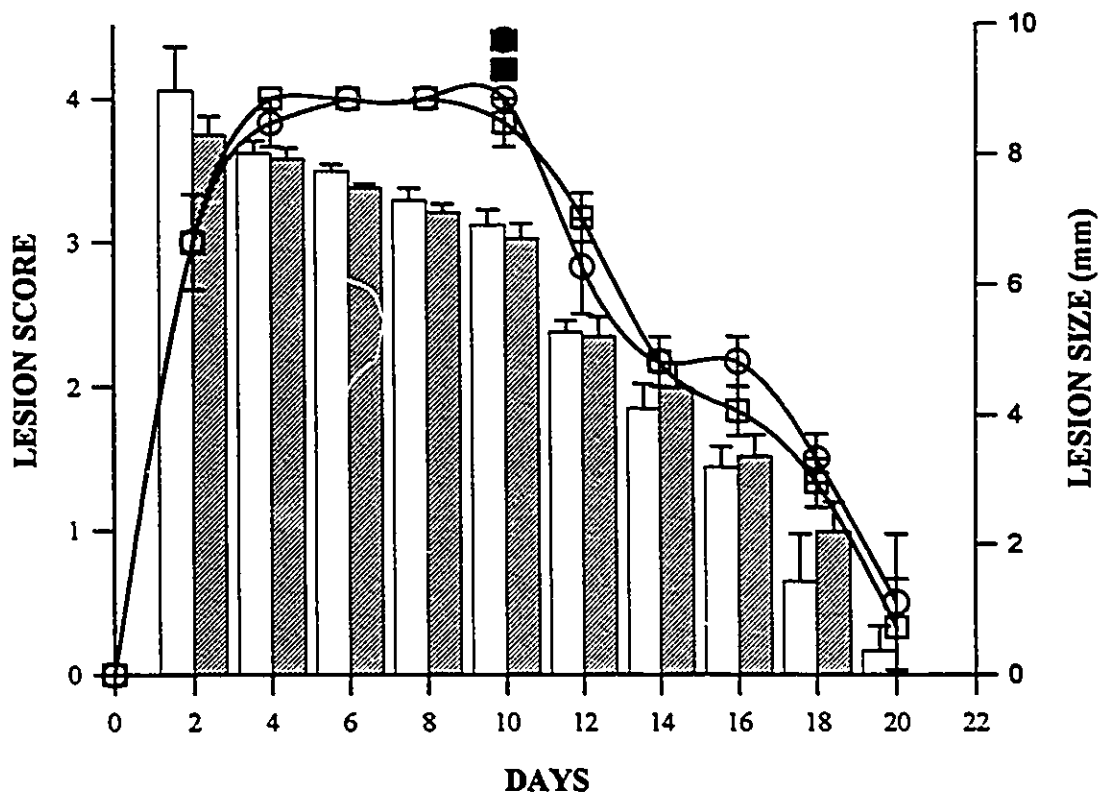
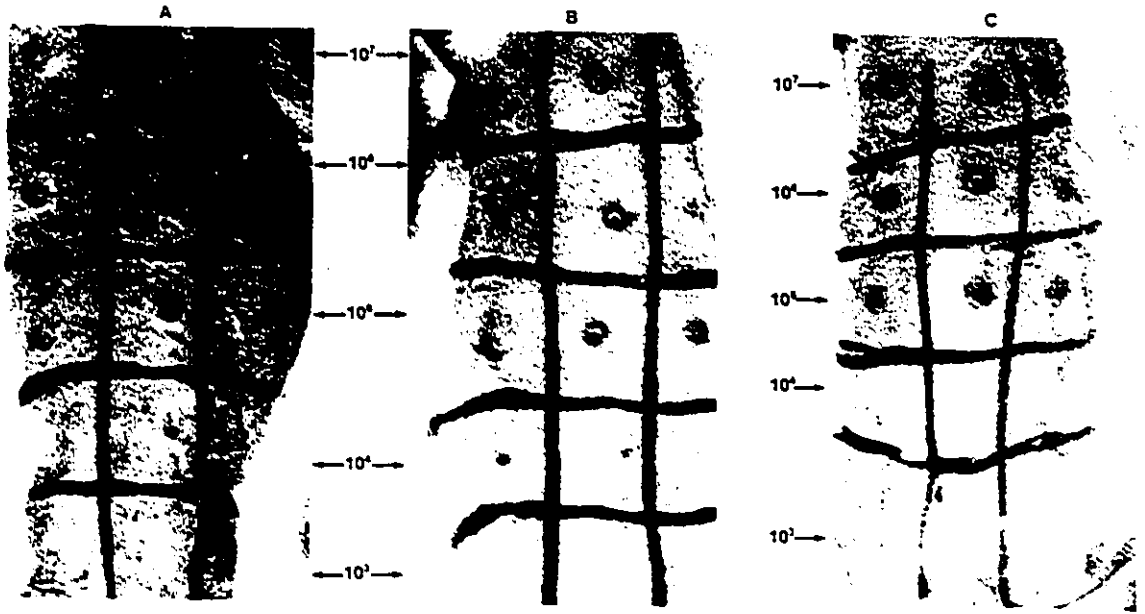


Figure 25

Appearance of disease 7 days after challenge with titered inocula from 10^7 to 10^3 CFU/mL of *H. ducreyi* 35000 in rabbits passively immunized with IgG from rabbits immunized with whole cells or the pilus preparation. Rabbits were intravenously infused with either 24 mg (1) or 48 mg (2) of IgG from normal un-immunized (A) or rabbits with whole cell IgG (B) or with pilus preparation IgG (C).

1



2

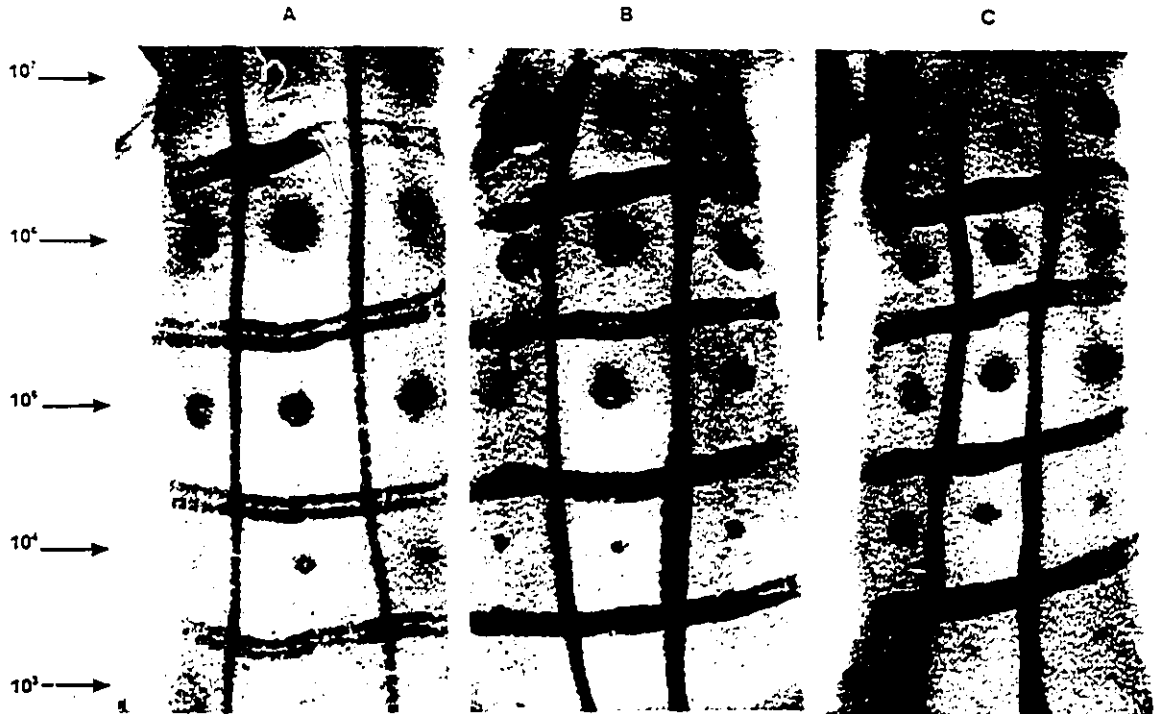


Table 8

Virulence of *H. ducreyi* 35000 at 10^7 CFU/ml in rabbits passively immunized with 24 mg of IgG from rabbits immunized with whole cells or pilus preparation.

	Source of IgG for passive immunization		
	Naive controls (n=3)	Normal rabbits (n=3) ^a Whole cell (n=3) ^b	Rabbits immunized with pilus preparation (n=3) ^c
Inoculum size (CFU x10 ⁷ /mL) ^a	2.8 ± 0.67	2.5 ± 0.36 (p = 0.52)	2.3 ± 0.55 (p = 0.34)
Number of days culture positive ^a	11 ± 2	10.7 ± 0.6 (p = 0.77)	10.7 ± 0.58 (p = 0.89)
Lesion size (mm)			
Cumulative (21 days)	119 ± 8.74	110 ± 11.8 (p = 0.23)	110 ± 8.35 (p = 0.85)
Average ^a	5.68 ± 0.48	5.37 ± 0.32 (p = 0.24)	5.2 ± 0.4 (p = 0.85)
Lesion score			
Peak score ^a	4 ± 0	4 ± 0	4 ± 0
Lesion duration (>2mm)	19 ± 1	17.3 ± 1.5 (p = 0.19)	17.7 ± 0.58 (p = 0.23)
Ulcer duration (days) ^a	9 ± 1	7.33 ± 0.6 (p = 0.23)	7.67 ± 0.58 (p = 0.52)

^a Data represent means ± standard deviations.

^b Comparative evaluation of rabbits passively immunized with normal IgG versus naive controls (Student's t Test).

^c Comparative evaluation of rabbits passively immunized with whole cell or pilus polyclonal IgG versus rabbits infused with normal IgG (Student's t Test).

Table 9

Virulence of *H. dicreya* 35000 at 10^5 CFU/ml in rabbits passively immunized with 48 mg of IgG from rabbits immunized with whole cells or pilus preparation.

	Source of IgG for passive immunization		
	Naive controls (n=3)	Normal rabbits(n=3) ^a	Rabbits immunized with Whole cells (n=3) ^b Rabbits immunized with pilus (n=3) ^c
Inoculum size (CFU x10 ⁵ /mL) ^d	2.8 ± 0.67	1.9 ± 0.36 (p = 0.42)	2.2 ± 0.51 (p = 0.2) 2.5 ± 0.31 (p = 0.28)
Number of days culture positive ^e	11 ± 2	11 ± 1.73 (p = 0.98)	13.7 ± 1.15 (p = 0.31) 12 ± 2.1 (p = 0.44)
Lesion size (mm)			
Cumulative (21 days)	119 ± 8.74	130 ± 12.8 (p = 0.14)	134 ± 10.5 (p = 0.42) 145 ± 11 (p = 0.068)
Average ^f	5.68 ± 0.48	6.17 ± 0.61 (p = 0.16)	6.46 ± 0.5 (p = 0.48) 6.9 ± 0.5 (p = 0.65)
Lesion score			
Peak score ^g	4 ± 0	4 ± 0	4 ± 0
Lesion duration (>2mm)	19 ± 1	19.3 ± 1.2 (p = 0.12)	19 ± 1 (p = 0.7) 19.5 ± 1.2 (p = 0.98)
Ulcer duration (days) ^h	9 ± 1	7.67 ± 0.58 (p = 0.23)	8.33 ± 1.15 (p = 0.42) 8.7 ± 0.6 (p = 0.1)

^d Data represent means ± standard deviations.

^e Comparative evaluation of rabbits passively immunized with normal IgG versus naive controls (Student's t Test).

^f Comparative evaluation of rabbits passively immunized with whole cell or pilus polyclonal IgG versus rabbits infused with normal IgG (Student's t Test).

passively immunized with 48 mg of IgG from immunized rabbits, was not different from control rabbits that did not receive any IgG (Figures 23 B, 24 B and 25; Table 9).

4. Characterization of cellular infiltrates in lesions of pilus vaccinated rabbits

Peripheral blood lymphocyte immunofluorescence

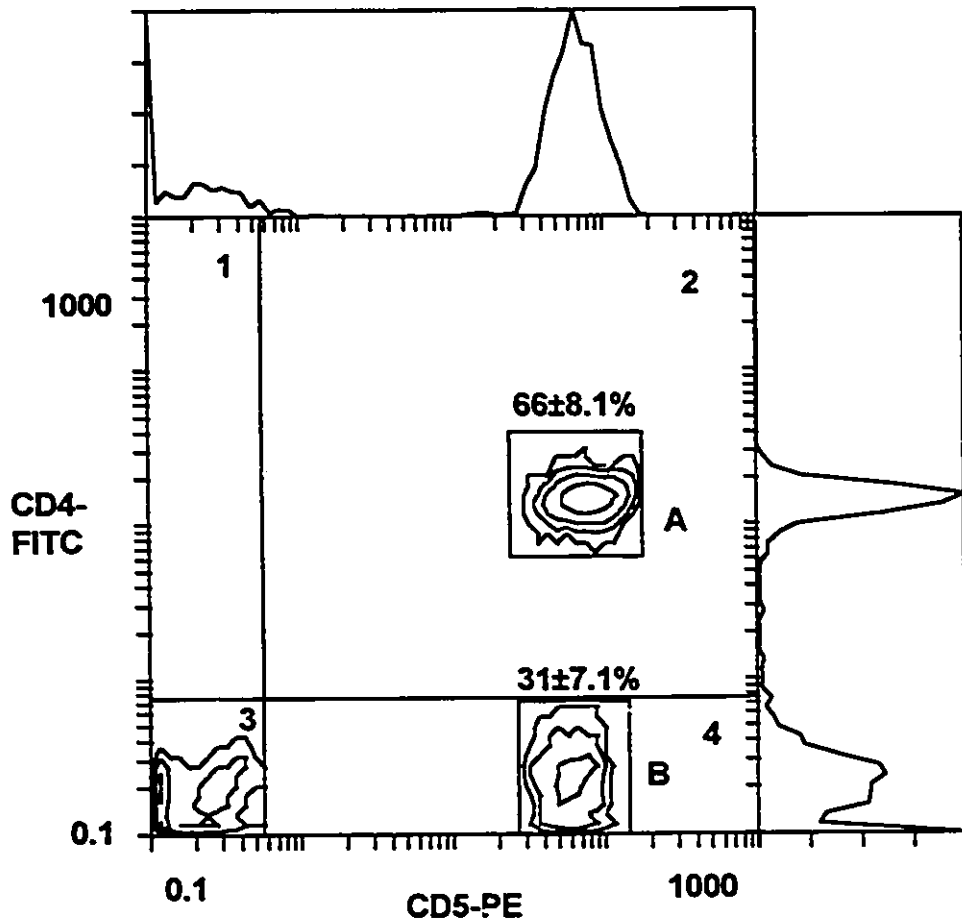
To confirm the existence of two T lymphocyte populations, PBLs were isolated and probed for the expression of the CD5 and CD4 T cell markers. T cell populations were identified in rabbit PBL by their forward light scatter and granularity as previously described (Wilkinson *et al.* 1992) and their reaction to the CD5 pan T cell marker. Other cells consisting of macrophages and monocytes recognized by their high forward angle scatter were gated out (Wilkinson *et al.* 1992). Two peripheral T lymphocyte populations were identified. One subset was positive for both the CD5 and CD4 cell surface markers, and the other was positive only for CD5 (Figure 26). Doubly positive cells accounted for $66 \pm 8.4\%$ of the total peripheral CD5 positive population, whereas the CD4 negative subset accounted for $31 \pm 7.1\%$ of the peripheral CD5 positive T lymphocytes (Figure 26).

Immunophenotyping of lymphocytic infiltrate of chancroidal lesions

Lymphocytic infiltrates in chancroidal lesions of sham-immunized and pilus-vaccinated rabbits were characterized by immunohistocytology for expression of the CD5 and CD4 T cell markers. Biopsies of lesions were flash frozen, sectioned and stained with the anti-CD5 and CD4 monoclonal antibodies. Both CD4 and CD5 monoclonal antibodies identified cell populations within T-dependent areas of the spleen, such as in germinal

Figure 26

Phenotypic characterization of peripheral T lymphocytes from three pilus and three sham-immunized rabbits. Rabbit peripheral blood leucocytes were stained for rabbit CD5 and CD4 surface markers and analysed by flow cytometry. Two distinct peripheral T lymphocyte subsets can be identified, with the CD5⁺CD4⁺ T lymphocytes (A) the most abundant phenotype and the CD5⁺CD4⁻ phenotype (B) the minor subset.

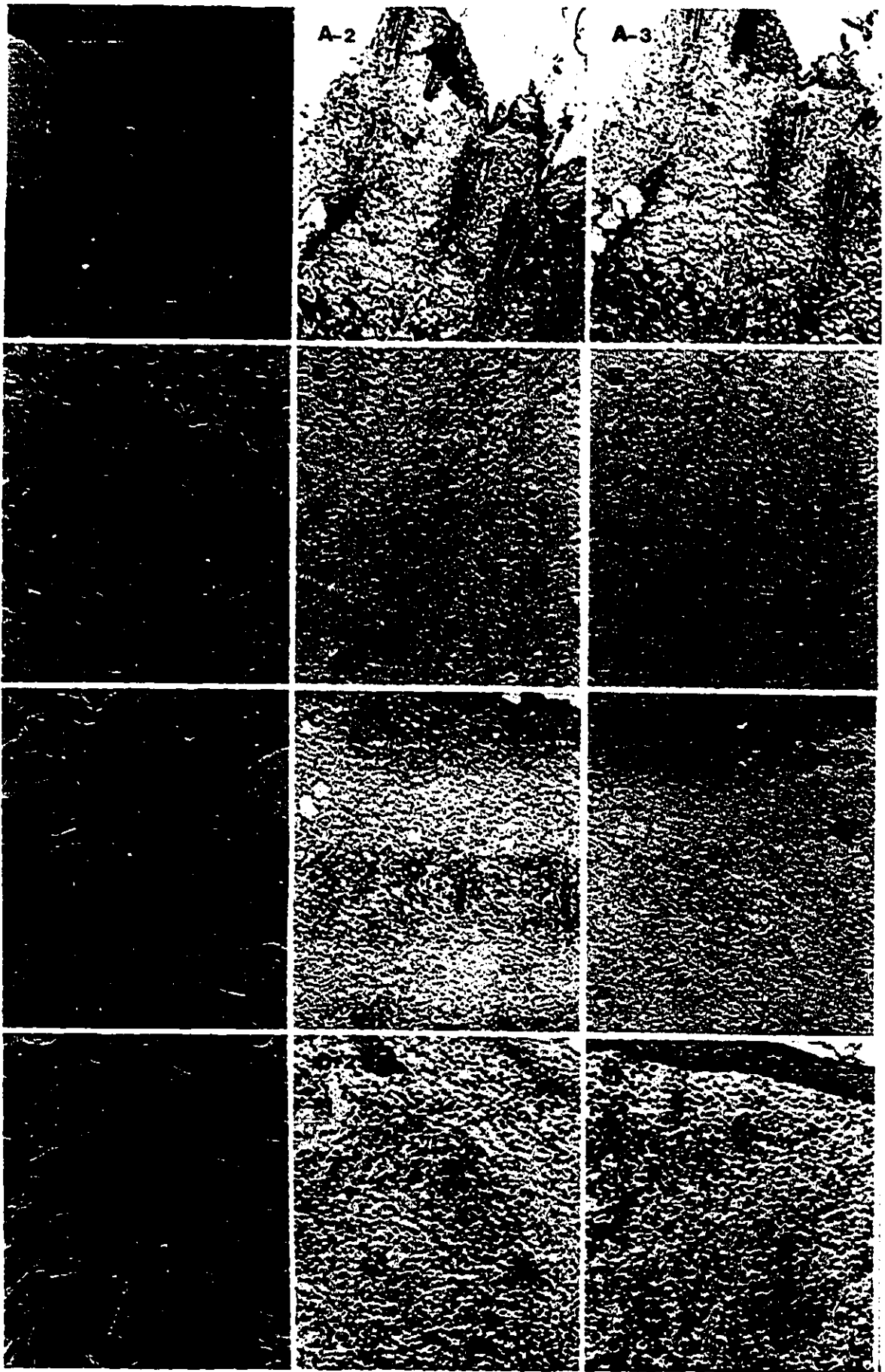


centres and perivascular areas (Appendix). Few CD5 and CD4 positive lymphocytes could be seen in day 4 lesions of sham-immunized rabbits (Figure 27 A-2 and A-3). The cellular infiltrate of control lesions was consistent with acute necrotizing inflammation (Figure 27 A-1), with infiltrating CD5 and CD4 positive lymphocytes confined to the superficial dermis of chancroidal lesions (Figure 27 A-2 and A-3). Cellular infiltrate of day 4 lesions in pilus vaccinated rabbits was consistent with a conversion of acute inflammatory responses to chronic inflammation. A more prominent CD5 positive lymphocyte population with more abundant CD4 positive lymphocytes was evident in sections 4 days after infectious challenge in pilus vaccinated rabbits when compared to sections from sham-immunized rabbits (Figure 28 A-2, A-3). In day 10 and 15 lesions of sham-immunized rabbits, infiltrating CD5 positive (Figure 27 B-2 and C-2) and CD4 positive (Figure 27 B-3 and C-3) lymphocytes were confined to a narrow band within the centre of the vascularization zone of chancroidal lesions. In vaccinees, infiltrating CD5 positive (Figure 28 B-2 and C-2) and CD4 positive lymphocytes (Figure 28 B-3 and C-3) were predominantly perivascular. At 21 days after challenge, infiltrating CD5 positive T cells in both vaccinees and controls were primarily of the CD4 phenotype, consistent with the granulomatous reaction seen in haematoxylin and eosin stained sections (Figure 27 and 28).

Earlier in disease, lesions of pilus vaccinated rabbits consistently demonstrated a more abundant CD5 positive T lymphocyte infiltrate compared to controls (Figure 29 A). At 4 days after challenge, an average of 359 ± 178 CD5 positive T lymphocytes per section were counted in lesions of pilus vaccinated rabbits compared to 49 ± 12.7 for sham-

Figure 27

Immuno-phenotyping of T lymphocyte infiltrate in biopsies of chancroidal lesions of sham-immunized control rabbits at 4 (A), 10 (B), 15 (C) and 21 (D) days post infectious challenged with an inocula (100 μ L) of 10^5 CFU/mL. Lymphocytes in serial biopsies of control lesions were stained with haematoxylin and eosin (A-1, B-1, C-1, D-1) (60x magnification) and for expression of the CD5 (A-2, B-2, C-2, D-2) (40x magnification) and CD4 (A-3, B-3, C-3, D-3) (40x magnification) T cell markers, appearing as red stained cells.



A-2

A-3

Figure 28

Immuno-phenotyping of T lymphocyte infiltrate in biopsies of chancroidal lesions at of pilus preparation immunized rabbits at 4 (A), 10 (B), 15 (C) and 21 (D) days post infectious challenged with an inoculum (100 μ L) of 10⁵ CFU/mL . Lymphocytes in serial biopsies of control lesions were stained with haematoxylin and eosin (A-1, B-1, C-1, D-1) (60x magnification) and for expression of the CD5 (A-2, B-2, C-2, D-2) (40x magnification) and CD4 (A-3, B-3, C-3, D-3) (40x magnification) T cell markers appearing as red stained cells.

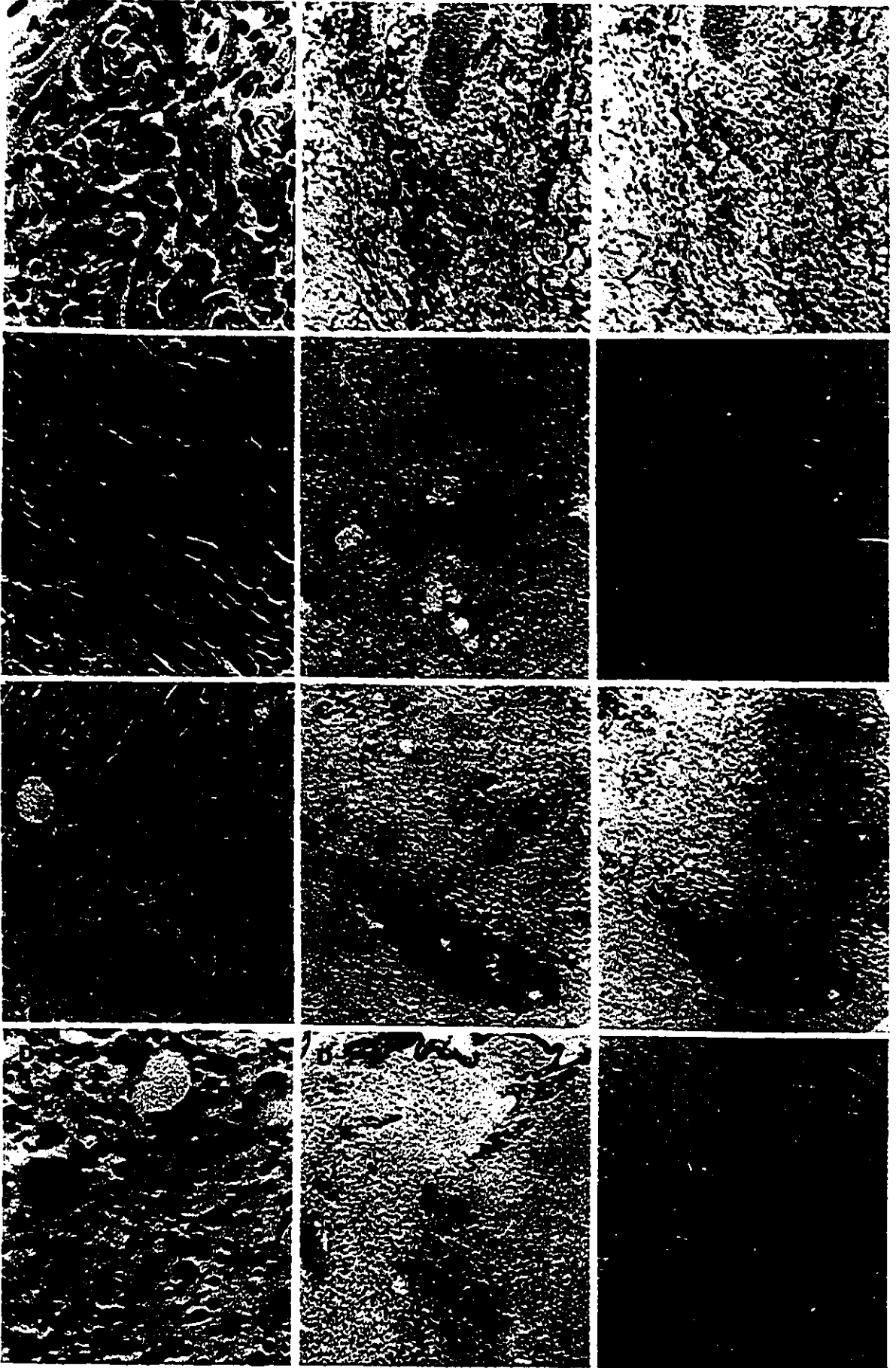
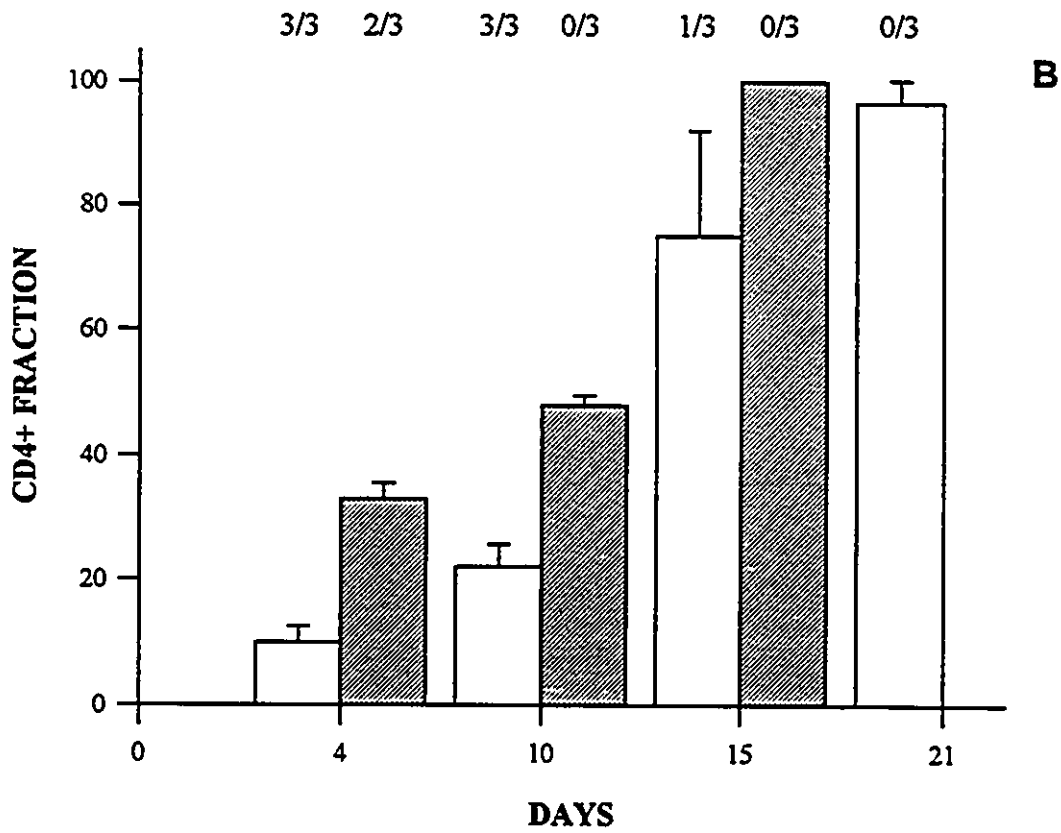
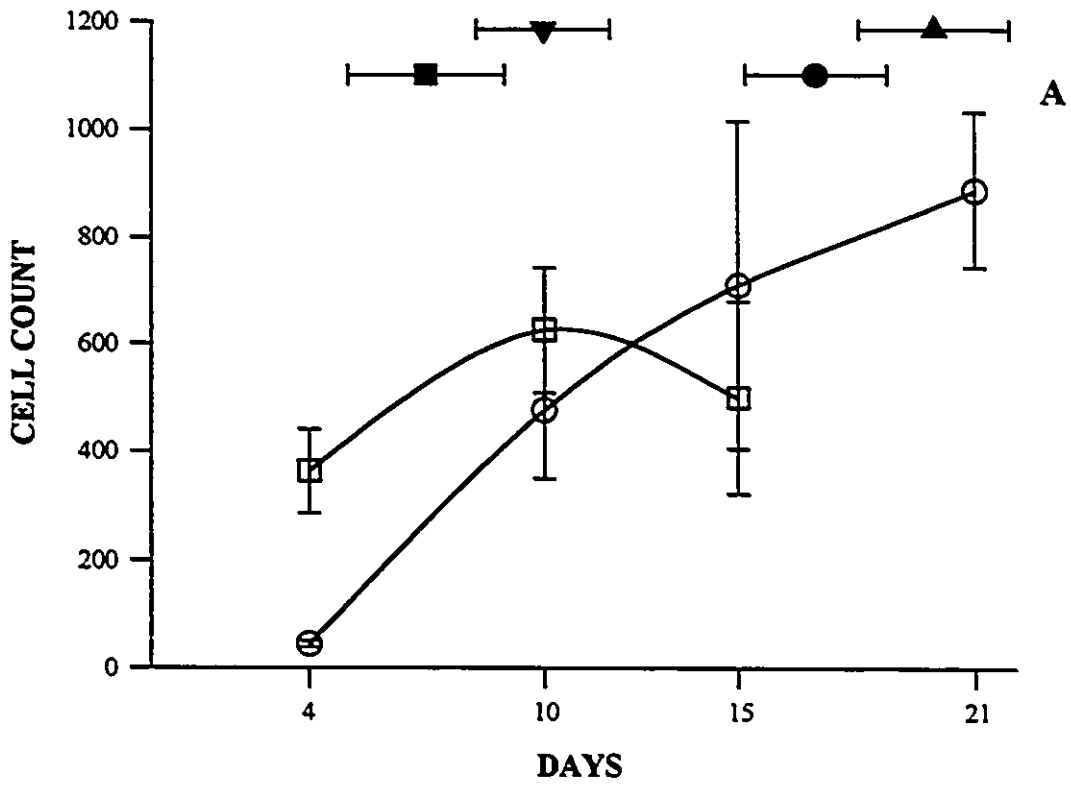


Figure 29

Quantification of T lymphocyte subsets infiltrating chancroidal lesions. Total CD5 positive lymphocyte counts (A), expressed as mean \pm SD cell count per section (35 to 40 fields at 40x magnification) of lesions in three pilus vaccinated rabbits (open squares) and three sham-immunized rabbits (open circles) in serial biopsies over the course of infection, disease and resolution. Lesions in vaccinated rabbits had completely resolved in all rabbits by day 15. The number (mean \pm SD) of days following infection that lesions remained culture positive in controls (closed circles) and vaccinees (closed squares), or with a score ≥ 2 (2mm induration) in controls (closed triangles) and vaccinees (closed inverted triangles). Proportion of CD5 positive T lymphocyte with the CD4 phenotype (B) in lesions of pilus vaccinated rabbits (shaded bars) and sham-immunized rabbits (open bars). Fractions above bars refer to the number of rabbits culture positive for *H. ducreyi* on the indicated day after inoculation, in control rabbits above the open bars, and in vaccinees above the shaded bars.



immunized controls ($p=0.04$) (Figure 29 A). By day 10, total CD5 positive T cell counts in sections of sham-immunized lesions in controls were similar to the vaccinees (Figure 29 A). Four days after infectious challenge, $33 \pm 5.5\%$ of the infiltrating lymphocytes were of the CD4 phenotype in vaccinees with two of three cultured lesions (total of 3 rabbits) remaining culture positive for *H. ducreyi*. In sections of lesions from sham-immunized rabbits, $9.7 \pm 2\%$ of the infiltrating lymphocytes were CD4 positive ($p = 0.002$). By day 10, CD4 positive T cells accounted for $21 \pm 7.2\%$ of infiltrating lymphocytes in sham immunized rabbits compared to $50 \pm 2.8\%$ for sections of pilus vaccinated rabbits ($p = 0.0018$). At this time, all 3 cultured lesions of vaccinated rabbits were sterile, and all 3 lesions of control rabbits still remained culture positive. At 15 days after infectious challenge, CD4 positive cells accounted for 100% of the total lymphocyte population in sterile lesions of vaccinees, compared to $75 \pm 20\%$ for infiltrates of lesions of control rabbits with one lesion of three still culture positive (Figure 29 B). By day 21, all lesions of controls were sterile and 100% of infiltrating lymphocytes were of the CD4 phenotype. Lesions of vaccinees had resolved.

A more rapid and pronounced CD5 and CD4 positive T lymphocyte infiltrate was consistently evident in sections of pilus vaccinated rabbits reaching 100% by day 15 (Figure 29 B). Conversion of lesion histology from a necrotizing acute inflammatory response to chronic inflammation characterized by a CD4 positive T lymphocyte infiltrate was not evident until 15 days after challenge in sham-immunized rabbits (Figure 27 C-3 and 29 B) compared to 4 to 10 days in lesions of vaccinees (Figure 28 B-3, C-3 and 29 B). Sterilization and onset of healing of chancroidal lesions in vaccinated rabbits and in controls corresponded with the appearance of a prominent CD4 positive lymphocyte infiltration observed within

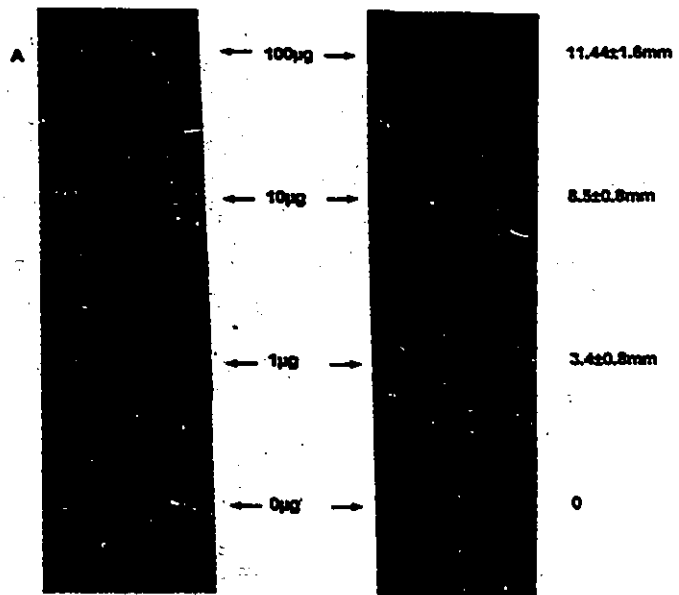
4 days following infection in vaccinees (Figures 28 A-3 and 29 B) and required at least 15 days for controls (Figures 27 C-3 and 29 B).

Intra-epithelial challenge of pilus vaccinated rabbits immunized with the pilus preparation

To characterize the type of cellular immune response associated with pilus vaccination, naive control and immunized rabbits were challenged with titered doses of the pilus antigen. Rabbits were examined 48 hours later for the presence of nodular lesions at the site of inoculation, and biopsies were stained with hematoxylin and eosin to define the cellular infiltrate. Pilus vaccinated rabbits developed large indurated nodular lesions 48 hours after challenge with 1, 10 and 100 μ g of the pilus preparation (Figure 30 A-2). No lesions were visible in naive unimmunized controls (Figure 30 A-1). Extravasation of red blood cells, extensive edema and tissue destruction were evident in lesions of pilus immunized rabbits challenged with 100 μ g of the pilus preparation. A prominent lymphocytic infiltrate was evident with scattered plasma cells (Figure 30 C). Biopsies of skin from naive rabbits challenged with the pilus preparation were normal without any evidence of alterations in the epidermal, superficial dermal or deep dermal layers (Figure 30 B).

Figure 30

Intradermal skin challenge of rabbits immunized with the pilus preparation. Three naive control rabbits (A-1) and three pilus immunized rabbits (A-2) were intra-dermally challenged with serial 10 fold dilutions (from 0 to 100 μ g) of the pilus preparation. 48 hours after inoculation, lesions were measured (\pm SD). Biopsies were taken from the area challenged with 100 μ g of the pilus preparation on the control rabbit (B) and the pilus immunized rabbit (C) and stained with haematoxylin and eosin.



CHAPTER 4: DISCUSSION

1. Standardization of an *H. ducreyi* ELISA

To successfully develop and establish an experimental model of vaccination, there must be an ability to measure the timing, degree, and specificity of the immune response. Detection of the serologic response to immunizing antigens can be a useful marker of specific immune activation. ELISAs offer fast and reproducible approaches to defining IgG or IgM serologic reactions to test antigens in addition to measuring temporal events or the kinetics of the serologic responses. Furthermore, such an assay of known performance characteristics would be valuable in identifying groups at risk for chancroid, and evaluating public health control efforts as part of larger intervention against STDs and the transmission of HIV.

The assay must be simple, inexpensive, and sensitive with a predictable overall performance. The simplest measure of the quality of a test is its accuracy defined as the fraction of correct cases the assay can identify, or predictive values (Metz 1978). However, the usefulness of accuracy as an index of performance is limited and can be misleading since disease prevalence is an integral component of accuracy (Metz 1978). Sensitivity, which reflects the rate of detection of positive samples, and specificity, the rate of detection of negative samples are best suited as indices of test performance. ROC curve analysis can provide useful insight into the relationship between sensitivity and specificity over a range of cut off or threshold values, allowing the receiver of the test to select an appropriate

decision threshold (Metz 1978). The performance of an *H. ducreyi* ELISA was evaluated by using ROC curves, which allowed the selection of a threshold for obtaining optimal assay performance for future epidemiologic applications. In the past, estimation or comparison of chancroid prevalence in a population has been limited by the absence of a well characterized, practical sensitive screening assay.

An *H. ducreyi* ELISA was developed using a modification of the ELISA previously described by Museyi and co-workers (1988). Important modifications include the pre-adsorption of test sera and the use of a different antigen preparation. Because *H. ducreyi* shares extensive serologic cross reactivity with other members of the *Haemophilus* genus, pre-adsorption of serum samples with an antigen mixture of three cross reactive *Haemophilus* species is necessary to ensure specificity. The antigen preparation described by Museyi and co-workers (1988) consisted of the insoluble fraction of *H. ducreyi* whereas I used the soluble fraction. With the *H. ducreyi* ELISA described by Museyi and co-workers (1988), a seropositive rate of 89% was reported in a similar population of chancroid cases, with a specificity of 95% with sera from an unrelated source, compared to 94% and 84% respectively for my ELISA. These differences in performance between the assay of Museyi *et al.* and my ELISA is due to different antigen preparations and my preadsorption of test sera.

The performance of the ELISA as determined by the standardization technique depends on biological and systematic limitations. Biological limitations include the quality of information used to characterize the serum specimens. Sera defined to be positive or negative were chosen by using several concurrent clinical and microbiologic parameters that

indicate if an acute phase primary *H. ducreyi* infection or if *H. ducreyi* is absent, respectively. There is potential recall bias of past GUD by study subjects. This bias was minimized for standardization by using negative sera from clinic patients without any current or past GUD.

Systematic limitations on the accuracy of standardization include the specificity of the antigen-antibody reaction and non-antigen binding of immunoglobulins, particularly for sera from HIV infected persons with non specifically elevated immunoglobulin levels. The fact that the assay measured antibody specific to *H. ducreyi* antigen is supported by the extinction of reactivity by the soluble bacterial test antigen and the lack of extinction of reactivity by an unrelated bacterial antigen. It has been suspected that HIV infection and associated immune-related disease alter the serologic reaction to vaccination (Collier *et al.* 1988, Carne *et al.* 1987, Huang *et al.* 1988 and Ragni *et al.* 1988) and natural infections (Janoff *et al.* 1988). The serodiagnosis of syphilis in HIV positive populations has been carefully evaluated (Janier *et al.* 1991). In this study, I observed enhanced specificity of IgG and sensitivity of IgM ELISAs for sera of HIV positive patients. There may be several explanations for this. A past GUD that was known to be associated with HIV seropositivity in this population may have been under reported. Altered bacterial virulence in HIV positive individuals (Cameron and Padian 1990) may modify the serologic reaction, resulting in increased ELISA sensitivity of the IgM ELISA in individuals with *H. ducreyi* and HIV coinfection. Since the assay specificity for both the IgG and IgM ELISAs was preserved, HIV-related non-specific hyperglobulinemia is a less likely explanation.

Recently, Roggen and co-workers (1994) developed a similar ELISA for the

detection of IgG and IgM serologic responses to *H. ducreyi* that included adsorption of serum samples with three antigens cross reactive *Haemophilus* species. The reported sensitivity of the ELISA in a similar Kenyan population was 90% and 40% with a specificity of 93% and 99% for IgG and IgM antibody, respectively (Roggen *et al.* 1994). Improved performance was observed for the IgG ELISA with both an increase in time from disease onset and HIV seropositivity. Important differences between the two ELISAs included the antigen preparation, which in the test described by Roggen *et al.*, consisted of a cocktail of a whole cell fraction from 9 geographically disparate *H. ducreyi* isolates and a different approach to ELISA standardization. Although there were overall differences in assay performance, the results of Roggen *et al.*, do corroborate the findings described in this thesis.

The performance of the ELISA in settings other than the one described here needs further evaluation. Application of the ELISA in settings of high or a low prevalence of chancroid will alter the performance of this test with respect to positive and negative predictive values. The best application of this assay would be to identify and measure the prevalence of chancroid in individuals who may be or who are likely to be STD reservoirs, such as urban prostitutes and clientele or at STD treatment clinics. Such seroepidemiologic application was evaluated for identification and measurement of *H. ducreyi* antibody in the setting of the Ministry of Health AIDS control program and mandatory national chancroid case reportage in Costa Rica (Cameron *et al.* 1995b). A significant decrease in *H. ducreyi* antibody seropositivity corroborated the decrease in national reportage of chancroid. Interestingly, seroprevalence in prostitutes was found to increase in the same period, and may justify the establishment of targeted STD control programs for HIV intervention (Cameron

et al. 1995b), even though increasing prevalence of antibody in a relatively stable population may reflect stable incidence of infection, due to lasting seropositivity.

2. Antigenicity of *H. ducreyi* LOS

In the antibody ELISA described in this thesis, the selection of one strain of *H. ducreyi* for antigen was based upon the reported broad cross reactivity of antiserum to *H. ducreyi* 35000 by immunofluorescence (Sloothmans *et al.* 1985) and enhanced ELISA reaction to LOS from this strain in a Kenyan population (Alfa *et al.* 1993b). Although specificity using appropriate controls for detection of antibody to crude soluble bacterial antigens could be improved by adsorption of non specific antibody to antigen of other bacteria, systematic performance characteristics of the LOS ELISA are inadequate for a specific serodiagnostic or seroepidemiologic test.

LOS from *H. ducreyi* may be relevant to virulence (Odumeru *et al.* 1987) and pathogenesis in the classical intradermal rabbit test (Campaganri *et al.* 1991). Purification of *H. ducreyi* 35000 LOS permitted us to identify that chenodeoxycholate fixation of antigen allowed the detection of IgG antibody by ELISA. However, only 4 in a panel of 10 HIV negative sera positive for antibody to soluble bacterial antigen had detectable anti-LOS antibody. Extinction of reactivity in these 4 sera by adsorption with soluble bacterial antigen and with LOS test antigen confirmed the specificity of the antigen antibody reaction detected in this LOS-specific antibody ELISA. The heterogeneity of LOS IgG in this panel of *H. ducreyi* positive sera is corroborated by independent findings. Serodiagnostic evaluation of an ELISA using pooled LOS antigen from two *H. ducreyi* strains had a sensitivity of

approximately 40% (Maclean *et al.* 1991). This suggests that LOS antigen of *H. ducreyi* may have group or strain specificity, or perhaps low immunogenicity in natural infections.

Incomplete extinction of serologic reaction in ELISA to soluble antigen suggests that antigens, other than LOS, present in a soluble bacterial extract, may contribute to serologic reactivity of the ELISA. These other antigenic determinants might account for the enhanced performance of a similar LOS ELISA developed by Alfa and co-workers (1993b). The Inzana protocol for LOS purification (Inzana 1983) yields extracts that, although apparently devoid of proteinaceous bands, as determined by Coomassie blue staining following SDS-PAGE, contain numerous carbohydrate species observed following silver staining. The purified products described by Alfa *et al.*, are analogous to the LOS extract prepared according to the method of Westphal and Jann (1965) prior to ultra-centrifugation (Figure 7 lane A and B).

Because of its limited antigenicity, LOS from *H. ducreyi* 35000 is not a suitable antigen for use in a serodiagnostic test. However, LOS may be a suitable antigenic basis for a sero-grouping system for *H. ducreyi*. Although immunogenic, the use of LOS from a single isolate as a potential broadly reactive vaccine candidate might be limited owing to its restricted antigenicity, as shown by the less than 50% inhibition of the serologic response to soluble bacterial extract, and by only a 40% seroprevalence.

3. Rabbit immunology

Rabbits have served as experimental models of infection and disease in several biomedical research areas such as the study of rheumatoid arthritis (Wilkinson 1988),

familial hypercholesterolemia. (Buja *et al.* 1983), syphilis (Sell *et al.* 1985 and Lukehart *et al.* 1980), and leprosy (Mshana *et al.* 1983) among others. Knowledge of the rabbit humoral immune system is quite extensive, especially concerning the nature and function of rabbit immunoglobulins, both at the functional and molecular levels. There is a wealth of information available regarding rabbit allotypes, immunoglobulin gene structure and the organization of the rabbit class I and II of the major histocompatibility gene cluster (MHC) (Sittisumbut and Knight 1986, Bernstein *et al.* 1985, Gallarda *et al.* 1985, Le Guern *et al.* 1985 and Marche *et al.* 1985). However the paucity of information regarding the rabbit cell mediated immune response (CMI) may limit efforts to design and evaluate vaccine candidates.

Recently, several reagents capable of identifying rabbit leucocyte surface markers have been identified. Numerous pan T cell markers have been described (For review see Wilkinson 1988), but only a limited number of markers are specific to T lymphocytes (Wilkinson *et al.* 1992, De Smet *et al.* 1983, McNicholas *et al.* 1981a, McNicholas *et al.* 1981b, and Watkins *et al.* 1981). Using some of these reagents, several groups have described the existence of two peripheral T cell populations (Kotani *et al.* 1993, Wilkinson *et al.* 1992 and Watkins *et al.* 1984). Kotani and co-workers (1993) described two T cell specific monoclonal antibodies, one which identified T dependent areas of the spleen and mesenteric lymph nodes by immuno-phenotyping, and precipitated a 67 kDa protein similar to the CD5 marker of T lymphocytes from other animal species. Furthermore, all of these CD5 positive cells were surface immunoglobulin negative and proliferated in response to T cell mitogens. The second monoclonal antibody identified a T cell subset from the larger

CD5 positive T lymphocyte population, and precipitated a protein antigen similar to the CD4 T cell marker. It phenotypically identified HTLV-1 transformed cell lines and, similar to anti-class II MHC antibody, suppressed the allo-mixed lymphocyte reaction (MLR). Two other monoclonal antibodies with similar specificities were describe by McNicholas and co-workers (1981) and Watkins and co-workers (1984). A helper function for secondary *in vitro* antibody responses in the larger lymphocyte population, analogous to the CD5 positive cells, was described (McNicholas *et al.* 1981). However, which subset harboured the helper activity was not defined. Limited cytotoxicity assays were performed on separated T cell subsets (Watkins *et al.* 1984). Although both the CD4 positive and CD4 negative populations demonstrated cytotoxic activity against xenogenic target cells, the CD4 negative subset consistently expressed higher activity. Since each preparation was found to be cross contaminated with cells of the opposite phenotype, primary cytotoxic effector cells or functionality could not be properly identified.

The results of these limited qualitative studies suggest that rabbit peripheral T cells can be classified into two subsets based on phenotypic expression of CD4 and that some CD4 positive T lymphocytes may harbour helper like activity analogous to human helper T cells.

4. Rabbit model of chancroid

Animal models of infection and disease have been used to assess the feasibility of specific inducible immunity in humans. Their use in identifying vaccine feasibility and specific vaccine candidates, and in characterising mechanisms of inducible immunity are

limited by the adequacy of an experimental animal infection to precisely mimic natural human infection, pathogenesis and immunity. In the pursuit of human vaccine, the ideal animal model will parallel human disease in terms of route of infection, size of inoculum, nature and course of disease (Smith 1989). Few models would survive such scrutiny, yet useful knowledge of the phenomenon or feasibility of vaccination has been derived from imperfect animal models (Anderson 1994, Casadevall and Scharff 1994, and Sell *et al.* 1985).

Original rabbit models of infection were not adequate models of chancroid. The disease produced by intradermal inoculation of *H. ducreyi* did not correlate with natural human disease in inoculum size ($>10^7$ CFU), route of administration and histological appearance. The models also lacked specificity such that heat killed organisms or purified products of *H. ducreyi* or other Gram negative pathogens could produce lesions similar to those induced by viable organisms (Campagnari *et al.* 1991). Purcell and co-workers (1992) addressed these deficiencies by developing a reproducible temperature-dependent rabbit model of infection. By housing the rabbits at an ambient temperature of 15°C ulcerative lesions were observed only in with viable inocula as low as 10^3 CFU, and closely resembled natural disease in clinical appearance and histopathology. However the model was developed as a model of infection rather than a quantitative virulence assay and was not amenable to statistical comparisons of virulence. The model was subsequently developed into a quantitative virulence assay in which homologous inducible immunity was demonstrated with a whole cell preparation (Meloche *et al.* 1992). The temperature-dependent rabbit model of *H. ducreyi* infection has several desirable traits as a model of

human chancroid. We and Purcell *et al.* (1992) have demonstrated that intra-epithelial inocula of the same order of magnitude (Spinola 1994), produced similar measurable disease effects in the temperature-dependent rabbit model and in experimental human infections. The temperature-dependency of the rabbit model may parallel the predilection for natural human disease to occur on the relatively cool extremities, and to confine itself to the skin, with usually sterile regional lymphadenitis in the minority of cases. The clinical pathology of the rabbit disease produced by experimental infection is similar in nature and course to human chancroid, with sequential acute suppurative inflammation, necrotic ulceration, lymphocytic infiltration, sterilization and re-epithelialization in convalescence over a period of weeks.

Using this model, I assessed the ability of three purified bacterial fractions to induce measurable serologic responses and protective immunity in rabbits. Some characterization of the immune response can be made by defining the class and kinetics of the serologic antibody response, measuring vaccine effects by passive immunization with "protective" anti-sera and characterizing and quantitating the cellular infiltrates by histopathology and immunophenotyping.

5. Inducible immunity in the temperature dependent rabbit model of chancroid

Immunization with carbohydrate and LOS antigen

Immunization of rabbits with 1, 10 or 100 μ g of an uncharacterized carbohydrate extract failed to induce detectable IgG serologic responses as detected either by Western blotting or ELISA. Immunoblotting of the soluble bacterial antigen with the serum of one

human patient highly reactive to *H. ducreyi* antigen on ELISA, identified several antigenic determinants but, similar to rabbit immune sera, no reactivity to the carbohydrate antigen was detectable. Lack of detectable serologic reactivity could result from the inability of the carbohydrate antigen to effectively transfer to nylon membranes, even in the absence of detectable bands on SDS-PAGE by silver stain after transfer. Alternatively, similar to other bacterial species, the carbohydrate antigen may be of low immunogenicity in this purified form. None of the rabbits immunized with the carbohydrate antigen were protected against challenge with the homologous strain. It is therefore unlikely that the carbohydrate antigen in this form would be a desirable vaccine candidate.

Immune responses to type I, thymus-independent antigens such as LPS are dominated by IgM antibody with little or no class switching, minimal affinity maturation, the lack of a booster effect and no memory (Möller *et al.* 1991 and Mosier and Subbarao 1982). It is not surprising therefore that the kinetics of the serologic response to purified LOS immunization in rabbits resembles that of thymus-independent type I antigens (Figure 9A). However, I and others have been able to detect LOS specific IgG in sera of patients with chancroid (Alfa *et al.* 1993). Immune response to LOS presented with bacterial protein antigens in the course of natural infection could lead to antibody class switching and affinity maturation. The role of IgG anti-LOS antibodies in the clearance of infection is unknown, and since vaccination did not provide protection against homologous strain infection, the suitability of LOS by itself as a candidate vaccinogen is likely limited. In addition, serologic recognition of LOS may not be consistent in natural infection since only 40% of sera from chancroid patients reacted in LOS ELISA, perhaps reflecting antigenic heterogeneity of *H.*

ducreyi LOS in nature.

Immunization with the pilus preparation

In rabbits vaccinated with the pilus preparation, early sterilizing protective immunity was apparent for homologous and one heterologous unrelated clinical isolate. The pilus preparation would exist in distinct forms, as monomers under denaturing conditions for immunoassay and as partially reassembled pili in solution at physiologic pH for immunization. Immunization led to protection, with a significant reduction in lesion size, severity and number of culture positive days and accelerated healing. At the minimum ulcer-producing inoculum, the duration of infection was reduced as measured by the number of days lesions remained culture positive. Since *H. ducreyi* virulence was not altered in rabbits immunized with either PBS, carbohydrate or LOS, in the presence of FCA or FIA, it is unlikely that adjuvant mediated immune activation (Cooper 1994) is responsible for the observed protection of rabbits immunized with the pilus preparation.

Comparative histology of these chancroidal lesions corroborated the differences in clinical outcome (Figure 21). The more rapid resolution and re-epithelialization of lesions in vaccinated rabbits corresponded with a switch from an acute inflammatory response and neutrophilic infiltrate to a chronic lymphocytic infiltrate, which also preceded later convalescence from disease produced in naive control rabbits. Thus, vaccination with the pilus preparation accelerated recruitment of lymphoid cells to the site of infection, with a more pronounced infiltration of plasma cells. The histology during healing of chancroidal lesions in naive rabbits and vaccinees appeared to be consistent with CMI responses.

Passive Immunization

The specific role of humoral mechanisms cannot be defined by histology, despite the more prominent plasma cell infiltrate in lesions of vaccinees and the presence of IgG antibody to the 24 kDa protein in sera of vaccinees before infection. The convalescent histology of control rabbits and vaccinees is supportive of a role for CMI responses in clearance of disease. Antibody in serum and the increased number of plasma cells in infiltrates in controls and vaccinees following healing may suggest a role of humoral immunity in protection against infection. To elucidate the potential role of antibody, groups of rabbits were passively immunized with IgG purified from sera of rabbits that had been immunized with either the pilus preparation or whole cells. Passive immunization could be useful for STD control programs in HIV seropositive individuals because of the high failure rates of hepatitis B, influenza and pneumococcal vaccination in HIV infected individuals (Collier *et al.* 1988, Carne *et al.* 1987, Huang *et al.* 1987 and Ragni *et al.* 1987).

Passive immunization with IgG from rabbits immunized with either the pilus preparation or whole cells, produced sustained antibody levels throughout the infection period (3 weeks). Identical antibody levels in rabbits passively immunized with 24 or 48 mg of IgG from rabbits immunized with whole cells may reflect failure to adequately detect serum antibody to the whole cell antigen using the ELISA established for soluble bacterial antigen. Studies in humans have described three phases in the distribution of passively immunized immunoglobulins, based on serum levels (Compendium of pharmaceutical and specialities 1995). The first phase, referred to as the α -phase, corresponds to early delivery of immunoglobulins into the circulatory compartment. The second or β -phase, occurs within

a few days, and corresponds to the rapid re-distribution of the infused immunoglobulin from the circulatory compartment into extravascular spaces. In the final or γ -phase, passively transferred immunoglobulins are slowly leached from the tissues back into the circulatory system followed by clearance over a period of weeks. Similar distribution and elimination kinetics were observed in rabbits infused with the IgG prepared from rabbits immunized with the pilus preparation or whole cells prior to infection.

Despite sustained antibody levels, no protection against infection was observed in rabbits passively immunized with the pilus or whole cell specific IgG. It is unlikely therefore that passive immunization under these conditions provides protection against infectious challenge with the homologous strain, and may be unlikely that IgG antibody alone can play a protective role in this model. The persistence of *H. ducreyi* in a mouse chamber model, despite serologic response supports (Trees *et al.* 1990) the observation that passive transfer of antibody provides no protection in this rabbit model of infection.

Characterization of cellular infiltrates in chancroidal lesions

Immunization with the pilus preparation was shown to accelerate recruitment of T lymphocytes into lesions of vaccinees and preceded accelerated healing of lesions. In vaccinated rabbits, despite the presence of neutrophils in day 4 lesions, CD5 and CD4 positive lymphoid cells were a major component of the cellular infiltrate. By day 10, complete conversion to chronic inflammation was evident with more than 50% of infiltrating T lymphocytes of the CD4 phenotype. Conversion to a CD4 positive infiltrate preceded sequential sterilization and resolution of lesions in vaccinated rabbits. Although the relative

CD5 lymphocyte count in day 10 lesions in control rabbits was similar to vaccinees. phenotypic characterization was consistent with the recruitment of a predominantly CD4 negative rather than a CD4 positive lymphocyte subset, until 15 days after challenge. Again, the later conversion to a predominant CD4 positive lymphocyte infiltrate preceded sterilization and resolution in controls. By day 15 in vaccinees and day 21 in controls, 100% of the infiltrating T lymphocytes were CD4 positive.

In control rabbits, infection with *H. ducreyi* was associated with the early recruitment of CD4 negative T cells, and clearance of disease correlated with conversion to a CD4 positive infiltrate. In pilus vaccinated rabbits, resolution and lesion sterilization occurred earlier and also correlated an earlier prominent CD4 positive infiltrate. Thus, immunization of rabbits with the pilus preparation accelerated the recruitment of the CD4 positive lymphocytic cells, corroborating the clinical outcome. The temporal events associated with lymphocyte subset recruitment following infection and prior to sterilization and re-epithelialization is supportive of a role for the CD4 positive subset and cellular immunity in clearance of disease.

Intradermal skin challenge of pilus vaccinated rabbits

To characterize immunity resulting from immunization with pili, vaccinees and naive controls were challenged 4 weeks after booster immunization with the pilus preparation, in a similar fashion to the Mantoux skin test for tuberculosis with purified protein derivative or tuberculin.. After 48 hours visible lesions were measured and biopsies were taken from the area challenged with the highest dose of organism. Although LOS was not visualized

in SDS-PAGE, concentrations below the detection limits of the silver stain could still be responsible for the inflammatory lesions observed. However, since no reaction to the pilus preparation was visualized in controls, it is unlikely that LOS is responsible for the nodular lesions observed in pilus vaccinated rabbits. LOS may be responsible for the vascular endothelial damage with extravasation of red blood cells, in lesions of vaccinees, in a Schwartzman-like reaction (Abbas *et al.* 1992). However, this type of reaction, mediated by the local release of TNF, is largely non specific and does not account for the extensive lymphocytic and plasmacytoid infiltrate observed in lesions of vaccinees. It is more likely that the recruitment of these lymphoid cells represents specific activation of memory responses to the pilus preparation in a reaction similar to delayed type hypersensitivity (DTH). No lymphocytic infiltrate was observed in the intact skin of controls. Compatible features and the tempo of the cellular infiltrate associated with healing of chancroidal lesions in pilus-vaccinated and sham-immunized control rabbits also support this conclusion. Perivascular recruitment of CD4 positive T cells (by day 4 vaccinees and by day 21 controls), a prominent histiocytic infiltrate and abundant activated fibroblasts preceded healing, and are features associated with activation of DTH responses.

This response also parallels the type of cellular infiltrate described for lesions in an experimental human model of chancroid (Spinola *et al.* 1994). Although phenotypic characterization of T cells into CD4 positive or negative subsets was not performed, presence of perivascular lymphocytes, activated endothelial cells, recruitment of monocytic cells and expression of HLA-DR on keratinocytes and T cells is consistent with activation of DTH responses. Elevated levels of soluble IL-2 receptor were detected in the urine and sera of

patients with chancroid (Abeck *et al.* 1990). Furthermore, sera from patients with acute chancroid were found to suppress production of IL-2 by mitogen stimulated lymphocytes. Moreover, suppression was more pronounced when sera from patients with persistent inguinal lymphadenitis were used (Korting *et al.* 1993). The results from these studies may support the possibility that there is recruitment and activation of T lymphocytes in human chancroidal lesions and are consistent with a role for CMI in resolution of human disease.

6. Pilus vaccination in other models

Experimental vaccination against other Gram negative mucosal pathogens with pilus preparations have been described for veterinary and human vaccine trials. These include *N. gonorrhoea*, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and uropathogenic *E. coli* (UPEC). Since pili represent an important virulence factor for *N. gonorrhoeae*, several attempts have been made to develop a protective pilus vaccine, with little success. Although pilus specific anti-sera inhibited *N. gonorrhoeae* attachment to cell cultured *in vitro* and preliminary human trials held promise, large scale pilus vaccine field trials failed to demonstrate significant protection against disease (Boslego *et al.* 1991, McChesney *et al.* 1982, Tramont *et al.* 1981 and Brinton *et al.* 1978). Failure to induce protective immunity was attributed to several factors, including heterogeneity of infecting strains, induction of poor or un-protective mucosal immunity by parental administration, and most importantly, the remarkable antigenic variability of gonococcal pili.

Although results of the gonococcal pilus vaccine trials were disappointing, some success has been achieved with other pilus vaccines. Successful immunization against

Colibacillosis was achieved by parenteral immunization of dams with purified pili from virulent ETEC strains (Acres *et al.* 1979). Offspring who suckled on an immunized mother were also passively protected against disease. In the reversible intestinal tie adult rabbit diarrhea (RITARD) model, oral immunization of rabbits with purified ETEC pili significantly protected rabbits against infectious challenge (De la Cabada *et al.* 1981). In human ETEC vaccine trials, oral immunization of volunteers with purified ETEC pili failed to induce mucosal immunity or to protect against disease, which was attributed to the loss of immunogenicity by denaturation of the pili by gastric secretion (Evans *et al.* 1984). Parenteral and oral immunization with inhibitors of gastric secretion induced high titers of intestinal IgA in half of the volunteers, whereas direct administration of the pilus vaccine into the gastrointestinal tract was more efficient at inducing secretory IgA than parenteral and oral immunization (Evans *et al.* 1984 and Levine 1994).

Juvenile rabbits are susceptible to a diarrheal disease caused by an EPEC-like strain, with similar pathogenesis and virulence to the EPEC strains causing human disease. Pili from the rabbit strain, when delivered into the gastrointestinal tract by incorporation into microspheres, successfully protected rabbits from infectious challenge (Levine *et al.* 1994). Several candidate vaccines for urethritis associated with UPEC strains have been tested in mouse and primate models. Immunization of animals with purified UPEC pili administered intramuscularly in FCA and FIA was successful in protecting animals against infection.

7. Conclusions

Because of the well established link between HIV transmission and genital ulcer

disease, measures to control chancroid could have a significant impact on the epidemiology of HIV. The objective of demonstrating the feasibility of vaccination for chancroid in an animal model was achieved. I first standardized the performance of a *H. ducreyi* antibody ELISA, both for the measurement of serologic responses to immunizing antigens and for application as a sensitive sero-epidemiologic tool. The assay performance was found to be time-dependent and maintained in HIV seropositive individuals. The usefulness of such an assay may extend beyond the simple measurement of the kinetics of antibody responses to candidate vaccine antigens. It may best be applied for the identification of chancroid core groups such as prostitutes targeted for chancroid control programs, and for the evaluation of such control measures.

Previous work with the chancroid temperature-dependent rabbit model demonstrated that cell envelope extracts provide protective immunity against homologous and heterologous strains. I have extended this work by testing three purified antigens, LOS, an uncharacterized carbohydrate extract and a pilus preparation. Of the three, only the last succeeded in protecting rabbits from infection and disease with both homologous and heterologous strains. The immune system of the rabbit has distinctive features and poorly characterized mechanisms, in comparison with the human immune response. I also used the rabbit model to evaluate the immune responses to pilus preparation immunization. This may or may not permit inference to natural human chancroid, vaccine feasibility or mechanisms of potential inducible immunity, but activation of the cellular arm of immunity in this setting suggests that an inducible protective CMI for human disease is feasible for vaccine development. In this rabbit model, infection in naive controls was associated with the early

recruitment of a CD4 negative subset in the absence of sterilization or re-epithelialization, but as seen in rabbits vaccinated with a pilus preparation, healing was preceded by the recruitment of CD4 T lymphocytes, in a manner analogous to DTH responses. Characterization of immune responses associated with protective immunity in this temperature-dependent rabbit model of infection may provide useful information for the development of vaccines for human chancroid.

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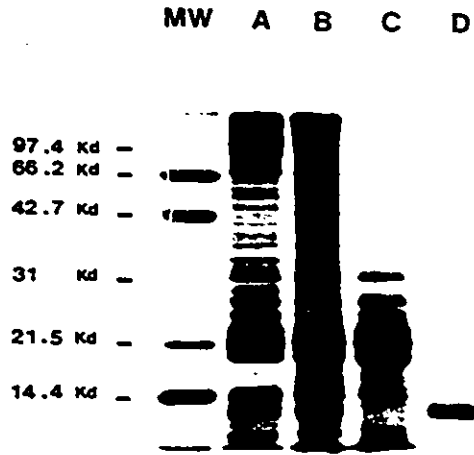
APPENDICES

Appendix 1

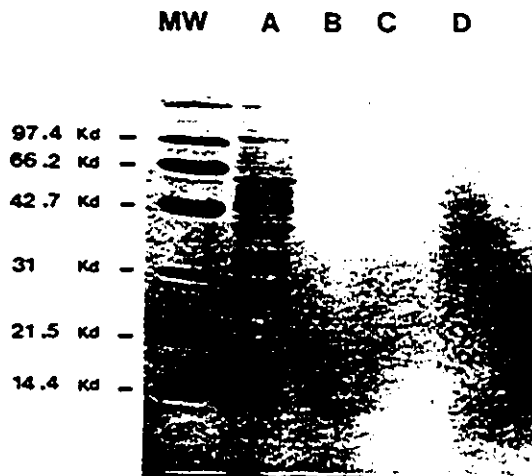
Figure 31

Silver stain (A) and Coomassie blue stain (B) of a 14% acrylamide SDS-PAGE with 2% urea. MW: Low molecular mass standards; Lane A: Crude soluble bacterial extract of *H. ducreyi* strain 35000; Lane B: Crude LOS extract prior to ultra-centrifugation; Lane C: supernatant of ultra-centrifugation product consisting of the uncharacterized carbohydrate extract; Lane D: Pellet of purified LOS extract.

A)



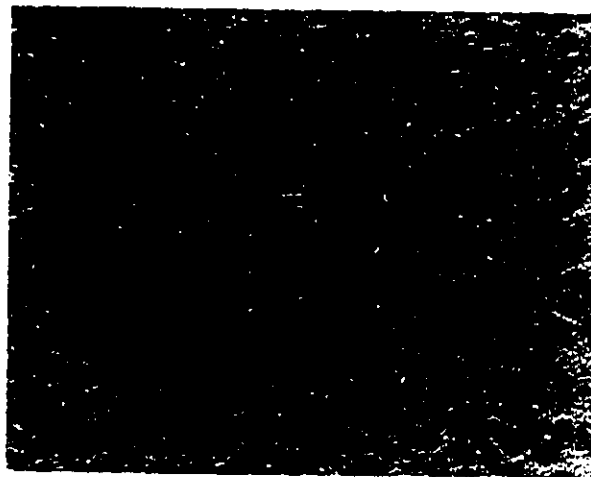
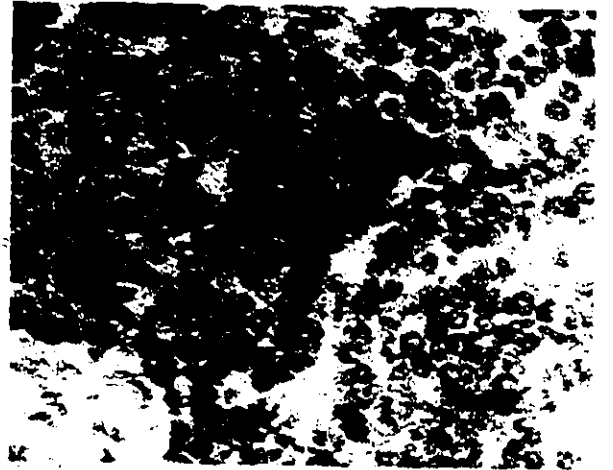
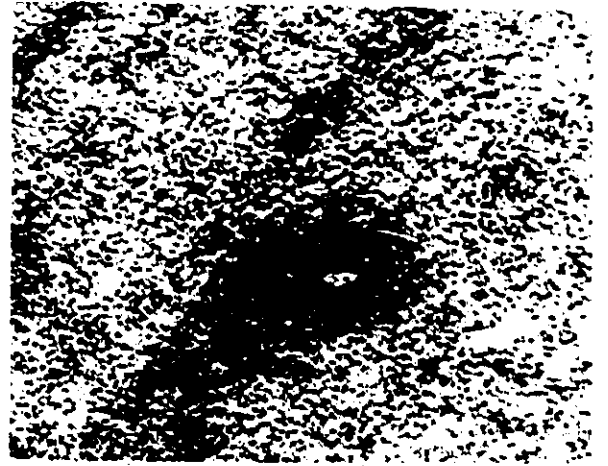
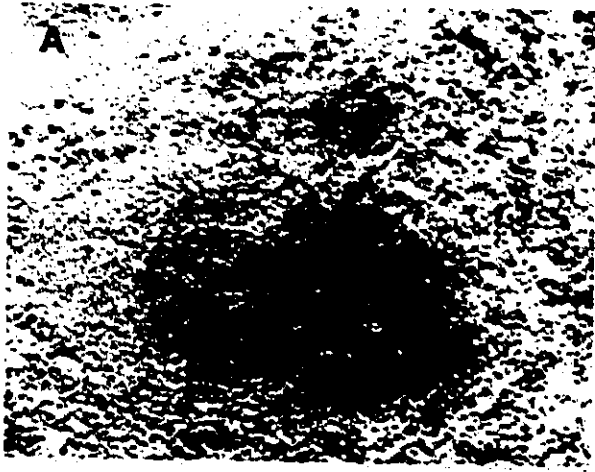
B)



Appendix 2

Figure 32

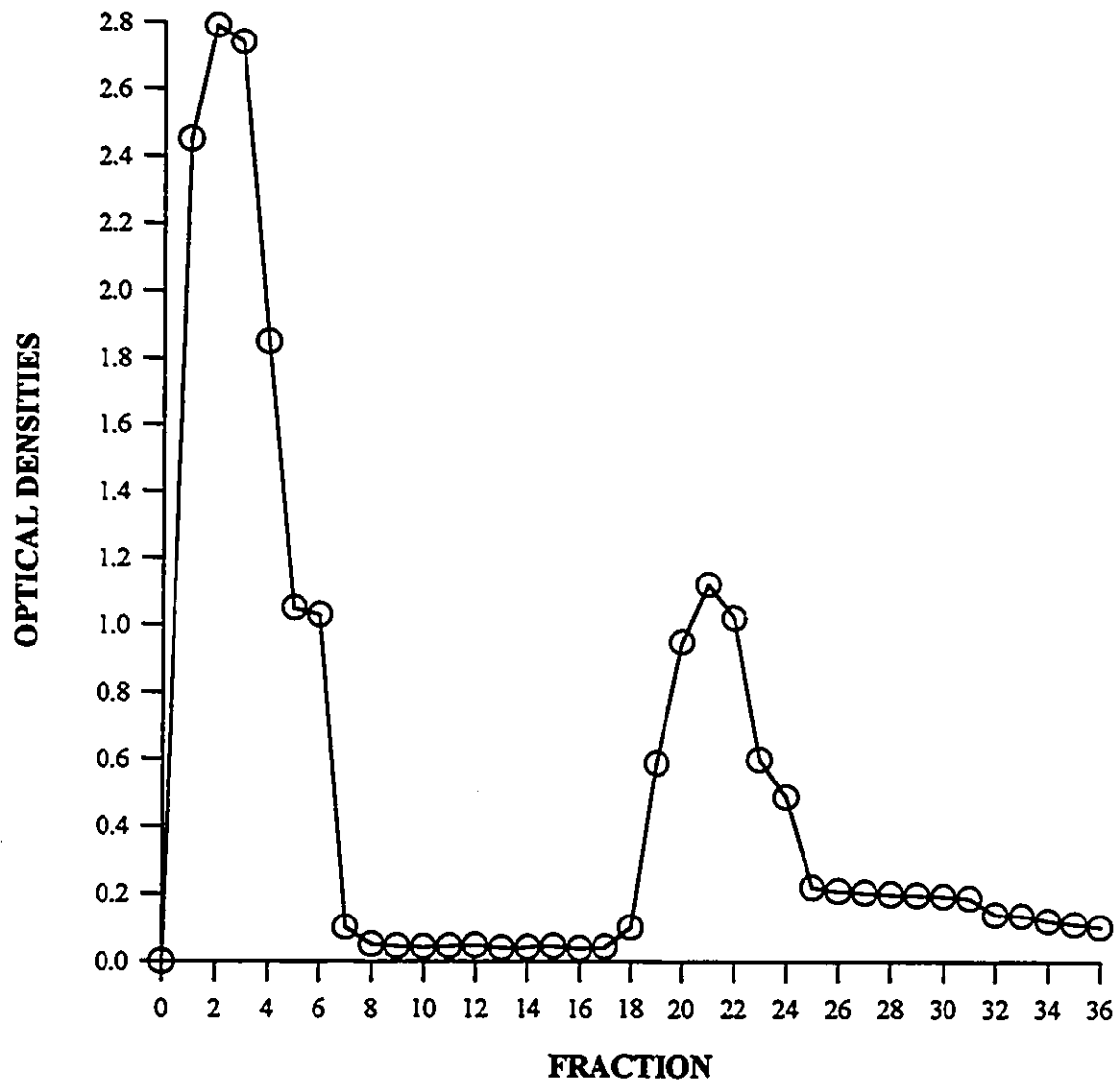
Immunophenotyping of T lymphocytes in spleens from normal naive rabbits. Low power view of spleen sections (x4) stained for the expression of the CD5 (A) and CD4 (B) T lymphocyte markers. High power magnification (40x) of CD5 positive (C) and CD4 positive (D) spleen lymphocytes. Negative control of spleen section stained with normal mouse sera (E).



Appendix 3

Figure 33

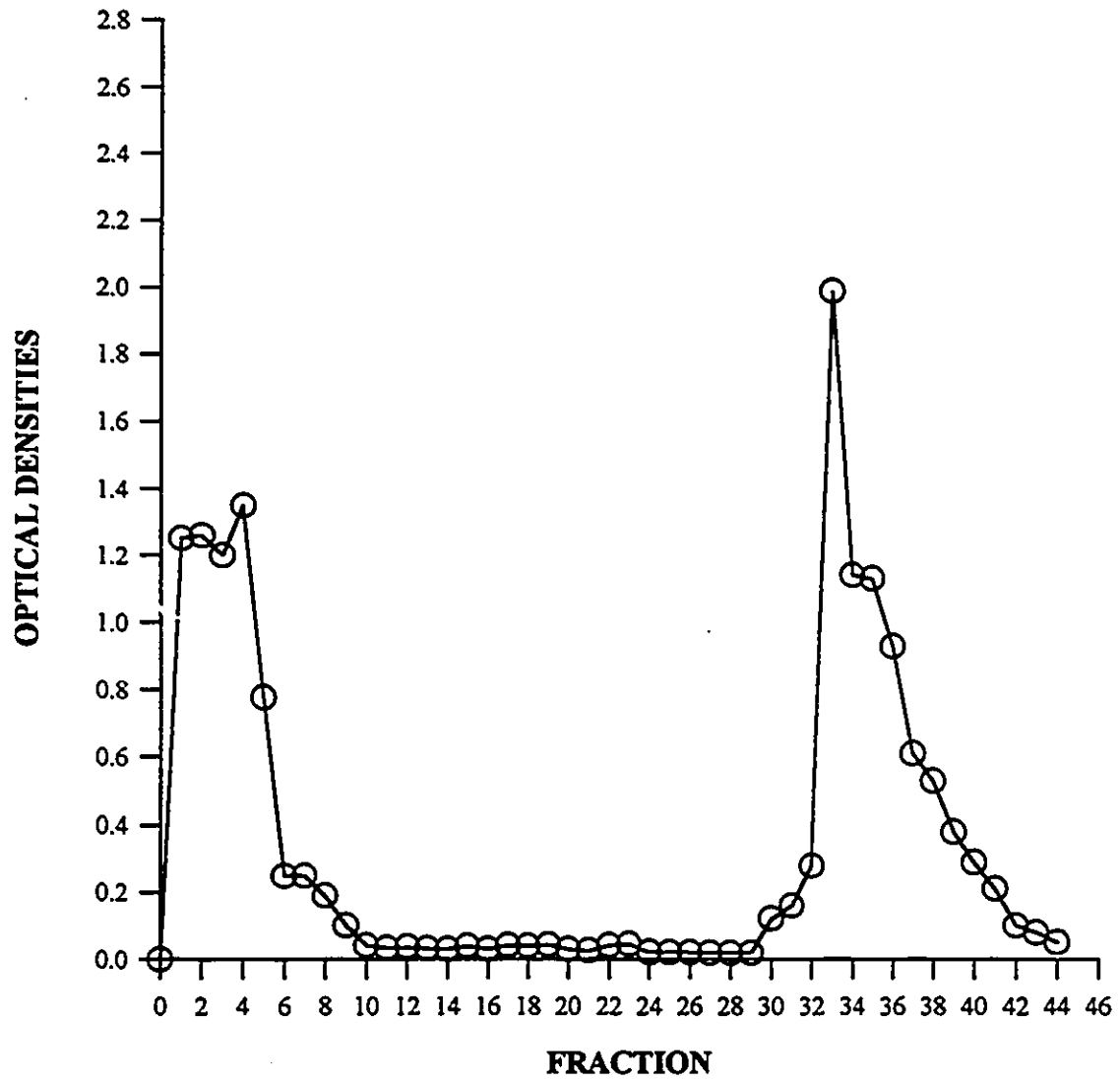
Protein G affinity chromatography purification of polyclonal IgG from sera of normal unimmunized rabbits. Fractions 18 to 25 correspond to polyclonal IgG.



Appendix 4

Figure 34

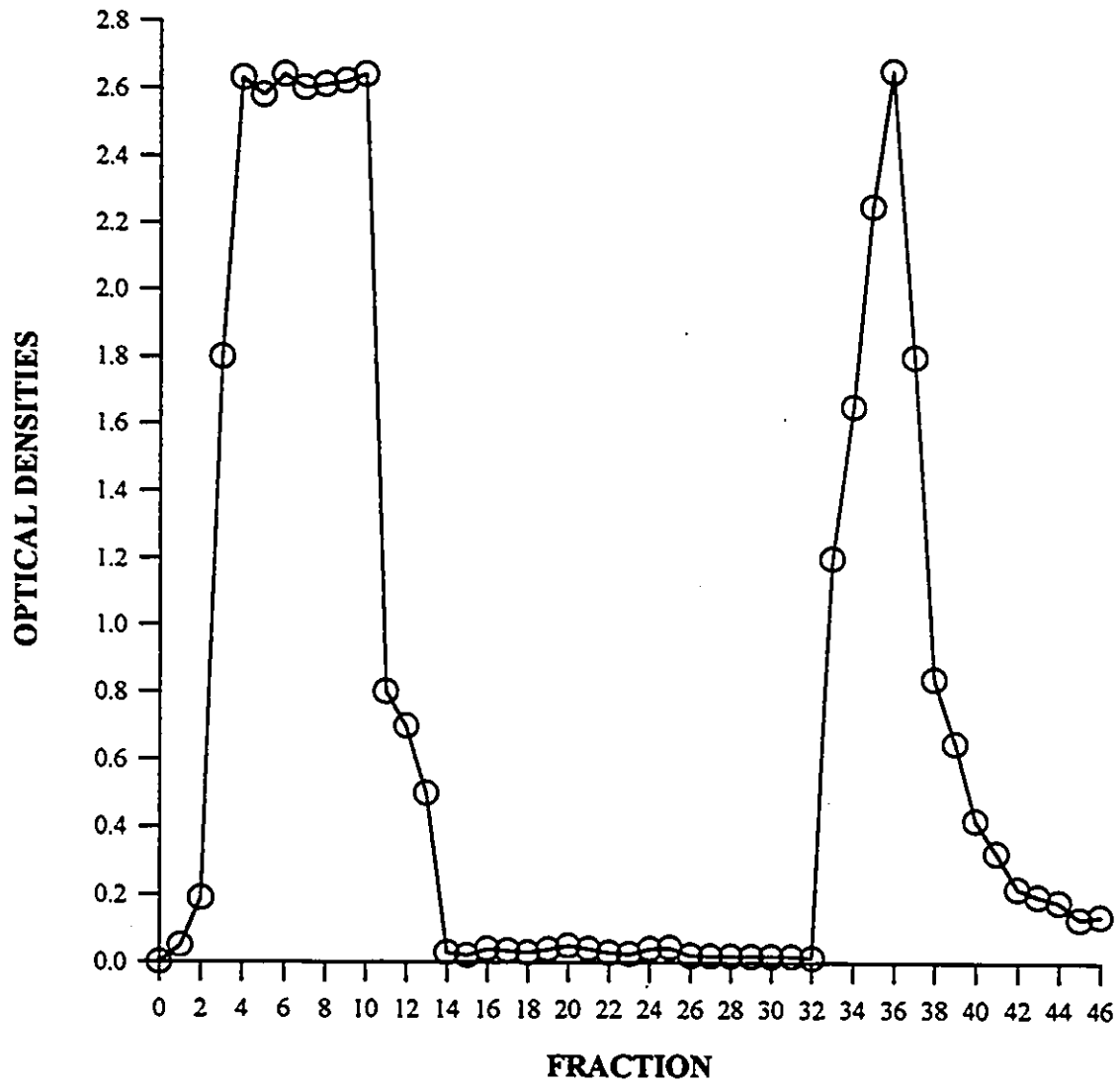
Protein G affinity chromatography purification of polyclonal IgG from sera of rabbits immunized with 10^8 CFU/ml of heat killed whole cell lysate. Fractions 30 to 41 correspond to purified polyclonal IgG.



Appendix 5

Figure 35

Protein G affinity chromatography purification of polyclonal IgG from sera of rabbits immunized with 100 μ g of the pilus preparation from *H. ducreyi* 35000. Fractions 33 to 42 correspond to purified polyclonal IgG.



Appendix 6

Figure 36

List of publications and published abstracts and presentations.

Publications:

Desjardins, M., LG Filion, DW Cameron. 1995. Inhibition EIA for human antibody to *Haemophilus ducreyi*: Antigenicity of Lipooligosaccharide. In preparation.

Desjardins, M., LG Filion, S Robertson, L Kobylinski, DW Cameron. 1995. Evaluation of humoral and cell mediated immunity to *Haemophilus ducreyi* infection in pilus preparation immunized rabbits. Submitted Infect Immun.

Desjardins, M., LG Filion, S Robertson, DW Cameron. 1995. Inducible immunity with a pilus preparation booster vaccination in an animal model of *Haemophilus ducreyi* infection and disease. Infect Imm 63: 2012-2020.

Kauri, T, I Ahonkhai, M Desjardins, DJ Kushner. 1993. Histidase activity of the halophilic archaeacterium, *Haloferax volcanii*. Microbios 30:245-249.

Desjardins, M., CE Thompson, LG Filion, JO Ndinya-Achola, FA Plummer, AR Ronald, P Piot, DW Cameron. 1992. Standardization of an enzyme immunoassay for human antibody to *Haemophilus ducreyi*. J Clin Microbio. 30:2019-2024.

Papers and posters presented at meetings:

Abrahams MC, M Desjardins, LG Filion, and GE Garber. 1995. Inducible immunity to *Trichomonas vaginalis* in a mouse model of infection. 95th General Meeting of the American Society for Microbiology.

Cameron DW, M Desjardins, A Viques, C Major, and G Herrera. 1995. Field evaluation of *Haemophilus ducreyi* antibody ELISA for chancroid sero-epidemiology. Fifth annual Conference on HIV/AIDS Research. Winnipeg, Manitoba.

Desjardins M, LG Filion, S Robertson, L Kobylinski, and DW Cameron. 1995. Evaluation of humoral and cell mediated inducible immunity to *Haemophilus ducreyi* in an animal model of chancroid. International Symposium on Sexually Transmitted Disease Research. New Orleans, Louisiana.

Desjardins M, S Robertson, L Kobylinski, LG Filion, and DW Cameron. 1995. Characterization of the cellular infiltrate of chancroidal lesions in pilin vaccinated rabbits. 95th General Meeting of the American Society for Microbiology.

Abrahams MC, M Desjardins, LG Filion, and GE Garber. 1994. The role of immunization with *Trichomonas vaginalis* in a mouse model of infection. Royal College of Physicians and Surgeons.

Abrahams MC, M Desjardins, LG Filion, and GE Garber. 1994. Immunogenicity of *Trichomonas vaginalis* in a mouse model of infection. 94th General Meeting of the American Society for Microbiology.

Desjardins M, LG Filion, and DW Cameron. 1994. Inducible immunity to *Haemophilus ducreyi*. 94th General Meeting of the American Society for Microbiology.

Desjardins M, LG Filion, and DW Cameron. 1993. Immunogenicity and inducible disease protection by Lipooligosaccharide of *Haemophilus ducreyi* in a quantitative virulence assay. 93rd General Meeting of the American Society for Microbiology.

Cameron DW, M Meloche, M Desjardins, LG Filion. 1993. Inducible immunity to *Haemophilus ducreyi* infection in an in vivo quantitative virulence assay. Royal College of Physician and Surgeons. Vancouver, Canada.

Desjardins M, LG Filion, DW Cameron. 1992. Inhibition Enzyme immunoassay for human antibody to *Haemophilus ducreyi*: Antigenicity of Lipooligosaccharide and effect of HIV on relative antibody affinity. 92nd General Meeting of the American Society for Microbiology.

Cameron DW, LG Filion, CE Thompson, M Desjardins, JO Ndinya-Achola, AR Ronald, FA Plummer, and P Piot. 1991. Effect of HIV infection on the human serologic response to natural *Haemophilus ducreyi* infection in acute chancroid. Seventh International Conference on AIDS Florence Italy.

Desjardins M, LG Filion, GE Garber, CE Thompson, and DW Cameron. 1991. Standardization of an enzyme immunoassay for detection of IgG and IgM antibody against *Haemophilus ducreyi*: Time, HIV infection and performance. 91st General Meeting of the American Society for Microbiology.