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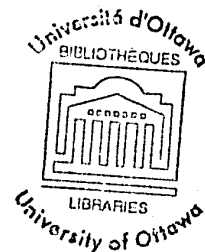
DEVELOPMENT AND APPLICATION OF A QUANTITATIVE VIRULENCE
ASSAY FOR *HAEMOPHILUS DUCREYI* IN AN IN VIVO MODEL OF INFECTION

A thesis submitted to the School
of Graduate Studies
University of Ottawa

In partial fulfilment of the requirement for the degree of
Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

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ABSTRACT

A temperature-dependant rabbit model of *Haemophilus ducreyi* infection was used as a quantitative virulence assay to evaluate the effect of host factors upon ulcerative cutaneous disease production. New Zealand White rabbits underwent inoculation with *H. ducreyi* strain #35000 either after iron loading, dexamethasone immunosuppression, prior infection or immunization. Chancroid-like disease was scored for severity, time, culture of lesions, and serologic response. In primary infections, culture-positive ulcerative lesions were consistently produced at and above 10^5 colony forming units inocula. Iron and dexamethasone treatment increased lesion severity and duration of culture positivity. Infection of previously infected animals produced sterile lesions of greater size and higher cumulative disease score at 10^5 colony forming units inocula. Infection of immunized animals produced sterile lesions of lesser severity at 10^5 colony forming units, with protection from ulcer formation.

Efficacy of ceftriaxone treatment was tested in naive control and iron loaded rabbits. Antibiotic was injected as a single intramuscular dose of 0.1 mg/Kg and 5 mg/Kg, four days following inoculation with *Haemophilus ducreyi* #35000. In naive control rabbits, antibiotic treatment at each dose sterilized the lesions within 24 hours with attenuation of disease effect. In naive iron loaded rabbits lesions were

sterilized later, on day 10 with 0.1 mg/Kg ceftriaxone and on day 5 with 5 mg/Kg ceftriaxone. Virulence scores corroborated the lessened microbiologic efficacy of antibiotic treatment.

We conclude that quantitative assay of infection and disease in iron loaded animals, with relative prolongation of disease effect and culture positivity of lesions may be a sensitive model in which to comparatively measure virulence related to bacterial factors. As well, limitations of efficacy or synergy of antibiotic treatments may be evaluated. We conclude that prior antigenic exposure and immune response through infection or immunization attenuates subsequent homologous infection. Disease produced in re-infection is amplified, while disease produced in inoculation after immunization is attenuated at lower inocula. This suggests that while there is inducible immunity to *H. ducreyi* infection and disease, the disease effect may be in part related to host inflammatory response to bacterial antigen or effect of bacterial toxin. This system of measurement of virulence may be useful towards understanding pathogenesis, and identifying strategy for vaccine development in chancroid.

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LIST OF ABBREVIATIONS

CAP	Chocolate agar plate
CFU	Colony forming unit
GUD	Genital ulcer disease
HIV	Human immunodeficiency virus
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
Mabs	Monoclonal antibodies
OM	Outer membrane
OMP	Outer membrane protein
PMNL	Polymorphonuclear leucocytes
SMX	Sulfamethoxazole
STD	Sexually transmitted disease
TMP	Trimethoprim

INTRODUCTION

Haemophilus ducreyi

Historical background

Haemophilus ducreyi was identified as the causative agent of chancroid in 1889 by Auguste Ducrey. Ducrey reported this based upon the technique of repeated serial autoinoculation of the skin of the forearm of patients with purulent material from their own genital ulcers. These findings supported the idea that there existed different etiological agents for syphilis and chancroid, both sexually transmitted genital ulcer diseases, since repeated autoinoculation did not produce serial ulceration in syphilis. In each of his patients, Ducrey found a single microorganism by Gram's stain in the ulcer exudate following the fifth or sixth serial reinoculation. He described the organism as a short, compact, streptobacillary rod, approximately 1.5 by 0.5 μm , with rounded ends. However, Ducrey was unable to grow the microorganism on artificial media. Between 1895 and 1900 several investigators were able to grow *H. ducreyi* on artificial media that, at the time, consisted of macerated human skin and agar (Albritton 1989, Morse 1989 and Kampmeien 1982).

Because of difficulties encountered in isolating the organism by culture and because of the gradual disappearance of endemic chancroid in industrialised countries, interest in the bacterium waned. It was not until the late 1970s and

early 1980s that there was renewed interest in characterizing the organism following recognition of several urban outbreaks of chancroid in North America and sporadic cases in Europe, Asia and Africa (Albritton 1989, Blackmore 1985, Hammond 1980, and Jessamine et al. 1990). During an urban epidemic of chancroid in Canada a selective agar for the primary isolation of *H. ducreyi* was developed. The medium consisted of gonococcal agar with bovine haemoglobin and 1% isovitalex (Lubwana et al. 1986 and Hammond et al. 1978).

Growth and morphology

H. ducreyi is a fastidious organism with very limited biochemical activity. The classic biochemical activities of *H. ducreyi* are reduction of nitrate and production of alkaline phosphatase. *H. ducreyi* is characteristically catalase negative, oxidase positive and is inert in most traditional biochemical tests. *H. ducreyi* is assaccharolytic: due to its lack of glycosidase activity it is unable to metabolize polysaccharides (Morse 1989 and Kilian 1976).

H. ducreyi is presently grown on chocolate agar plates (CAP) composed of GC agar base, 2% haemoglobin and 5% fetal bovine serum. Vancomycin is added to this medium for selection, when isolating the microorganism from a chancroidal ulcer. Recently, in vitro inhibitory activity of vancomycin on *H. ducreyi* has been reported (Clarridge et al. 1989). Characteristic and unique colonies are small, nonmucoid,

yellow-grey, semi-opaque and can be pushed intact across the agar surface. *H. ducreyi* colonies grow best at 33°C, pH 6.5-7.0 and 5% CO₂. The size and appearance of colonies vary depending on the growth medium and length of incubation. Colonies are generally pinpoint in size at 24 hours and increase to 1 to 2 mm in 48 hours. Viability of the organism in subculture diminishes after 48 hours. When *H. ducreyi* is grown in broth cultures, the incubation period is approximately 16 hours and clumping in suspension is observed by Gram's stain. Microscopically, *H. ducreyi* from plates or ulcer swabs appear as Gram negative bacilli that grow in chains and have characteristic "railroad tracks" or "schools of fish" arrangements (Albritton 1989 and Morse 1989).

Iron requirement and taxonomy

According to the present definition of the genus, the requirement of X or V factor for the growth of a Gram-negative rod or coccobacillus would place that organism within the genus *Haemophilus*. *Haemophilus ducreyi* was classified in the genus *Haemophilus* based upon its obligate requirement for haem as a growth factor. Furthermore, the guanine plus cytosine content was determined to be 0.38 mole fraction which, with the haem requirement, was considered sufficient for inclusion of the organism in the genus *Haemophilus*. However, DNA-DNA hybridization experiments suggest that *H. ducreyi* is unrelated or only distantly related to other species in the genus.

There is less than 10% DNA homology between *H. ducreyi* and other *Haemophili* (Albritton 1985 and Kilian 1976). Other findings do not support the inclusion of *H. ducreyi* in the genus *Haemophilus*: It was determined that *H. ducreyi* possessed the isoprenoid structure types dimethylmenaquinone and menaquinone which represents a major physiological and chemotaxonomic difference between *H. ducreyi* and other *Haemophilus* species (Carlone 1988).

The minimum concentration of haem required to initiate growth of *H. ducreyi* is 25 to 50 $\mu\text{g/mL}$ which is much higher than the 1 to 10 $\mu\text{g/mL}$ required by the other haem-dependent *Haemophilus* species (Morse 1989). *H. ducreyi* can utilize haem or the haem-containing proteins, bovine haemoglobin, human haemoglobin and catalase but not cytochrome C_{111} , FeCl_3 , human lactoferrin and human transferrin as sources for iron. *H. ducreyi* does not produce iron chelators (Lee 1991). When grown under conditions of iron limitation, growth is very sparse and hazy and quantitative changes in protein composition and ultrastructure of *H. ducreyi* are observed (Abeck et al. 1990).

Haemophilus ducreyi virulence factors

The requirements for study of bacterial pathogenicity or virulence mechanisms are (1) quantitative growth of the microorganism; (2) the establishment of an animal model of infection produced by small inocula, presented in a route and

producing disease similar to that in natural human infection and disease; (3) measurable disease effect (Smith 1989). With only recent development of in vitro growth of the bacterium, the understanding of *H. ducreyi* pathogenicity is still poor.

In general, bacteria must adhere to host tissue or cells, evade killing, survive, replicate and produce disease to be pathogenic. Potential virulence factors include mechanisms of adhesion, resistance to or evasion of humoral and cellular killing mechanisms, histotoxicity or cytotoxicity, and mediators or targets of the host inflammatory response. As disease is a condition of the host and virulence is the capacity of a pathogen to produce disease, then a pathogen's virulence must be a function of both host and pathogen factors (Mims 1990).

In view of the known importance of cell wall components as determinants of pathogenicity or virulence in other species of bacteria, work was done to determine the nature, structure and function of *H. ducreyi* cell-wall components. Bacterial envelope components such as lipooligosaccharide (LOS) (Desjardins 1992), capsular polysaccharide, outer membrane proteins, pili and toxins are often implicated in the pathogenicity of a microorganism. Odumeru *et al.* (1984) studied the susceptibility of "virulent" and "avirulent" strains of *H. ducreyi* to the bactericidal activity of normal human serum and to phagocytosis and killing by human polymorphonuclear leucocytes (PMNL). Virulence of a strain

was defined by the production of cutaneous lesions in an intradermal rabbit model of infection. It was shown that "virulent" strains were resistant to the complement-mediated lethal action of normal human and rabbit sera, whereas "avirulent" strains were susceptible. In addition, "virulent" strains were resistant to phagocytosis and killing by human PMNL, in contrast to "avirulent" strains. Furthermore, the bactericidal effect of human serum was inhibited by the LOS of serum sensitive strains but not by the LOS of serum-resistant strains (Odumeru 1985). Based on these results, a possible role for LOS in disease mechanism of *H. ducreyi* was suggested. Conclusions were not certain due to limitations of the animal model.

LPS is a major component of the outer membrane (OM) of Gram-negative bacteria, and consists of three components: Lipid A, core oligosaccharide and o-antigenic carbohydrate side chains. LPS molecules which lack O-antigenic side chains are referred to as rough LPS or LOS which is the case for *H. ducreyi*. *H. ducreyi* exhibits inter-strain variation in the composition of its LOS. Although there are no qualitative differences in the LOS of virulent and avirulent *H. ducreyi*, the total glucose:KDO (2-Keto-3-deoxy-octulosonic acid) ratio of the LOS of virulent strains exceeds that of avirulent strains. Thus, differences in the serum-sensitivity of strains may be associated with the amount or nature of carbohydrate present in the core region of the LPS (Johnson et

al. 1988, Odumeru et al. 1987 and Abeck et al. 1987). Furthermore, it was demonstrated by the rabbit intradermal test, that LOS may play an important role in the pathogenesis of chancroid since intradermal injections of *H. ducreyi* LOS produces inflammatory skin lesions resembling those caused by live and dead microorganisms (Campagnari et al. 1991).

Intradermal injection of *H. ducreyi* LOS in rabbits produces skin lesions that resemble those caused by live and heat-killed organisms. Injections of *N. gonorrhoeae* LOS will produce similar lesions (Tuffrey et al. 1990 and Campagnari et al. 1990). The apparently non-specific and equivalent toxicity of *H. ducreyi* and *N. gonorrhoeae* LOS may be due to structural similarities between their LOS. It was observed that 29 of 30 *H. ducreyi* strains possess LOS epitopes that are also seen in 98% of *N. gonorrhoeae* strains tested (Campagnari et al. 1990). The gonococcal LOS epitopes are sialated in vivo, resulting in LOS structures that mimic mature human erythrocyte antigens. The expression of these LOS epitopes by pathogens of diverse genera may be a mechanism by which the organism evade host immune responses. Therefore, *H. ducreyi* LOS may play a dual role in the pathogenesis of chancroid by allowing the organism to escape host defenses while recruiting inflammatory cells that result in skin necrosis (Campagnari et al. 1991 and Apicella et al 1990).

Haemophilus ducreyi cell wall is also characterized by the presence of outer membrane proteins (OMP). Apart from

their potential value in epidemiological studies (Johnson et al. 1988 and Saunders et al. 1986), OMP of *H. ducreyi* may also be related to pathogenicity, since studies with other bacteria indicate that surface antigens can determine virulence (Abeck et al.). *H. ducreyi* possess a 29K, a 40K and a 62K OMP which are antigenically conserved among strains demonstrated by the production of cross-reactive monoclonal antibodies in experimental animals (Hansen and Loftus 1984, Schalla et al. 1986 and Abeck et al. 1988). These molecules could possibly be involved in mediating adherence of the bacteria to host cells or affect the interaction of the organism with host defence mechanisms such as antibodies or phagocytic leucocytes (Albritton 1989, Johnson et al. 1988, Spinola et al. 1992, Morse 1989 and Abeck et al. 1988).

Other bacterial components generally involved in pathogenic mechanisms are pili. Pili can function as adhesins, allowing bacteria to colonize epithelial surfaces and permit initiation of infection. Pili are expressed by human non-enteric Gram-negative pathogens including non-typable and encapsulated *H. influenza*, *Neisseria meningitidis* and *N. gonorrhoea*. Pili have recently been isolated from *H. ducreyi* strains (Spinola et al. 1990). However, the relationship between the presence of pili and the pathogenesis of *H. ducreyi* infections still remains unclear.

H. ducreyi is thought to penetrate the normal skin through minor abrasions, but essential factors for adherence

and the mechanisms by which the organism produces tissue necrosis are unknown. Both humans and animals mount a humoral immune response to infection with *H. ducreyi*, but this response might not be protective since natural reinfection and recurrent disease is common, and repeated autoinoculations producing serial lesions was observed in earlier human studies of *H. ducreyi* (Morse 1989). A cellular immune response to infection with *H. ducreyi* has not been reported but is highly suspected (Albritton 1989).

Chancroid

Clinical presentation

H. ducreyi is the causative agent of chancroid, a genital ulcer disease (GUD) that is sexually transmitted. The incubation period of the disease varies between 3 to 10 days. At first, a tender papule appears which will ulcerate within 12 to 24 hours. Characteristically, necrotic ulcers are painful, irregular, superficial, and sharply demarcated with undermined borders. There are clinical variants of chancroidal ulcers: (1) dwarf chancroid which is a small ulcer with or without associated inguinal lymphadenopathy; (2) classic chancroid which is characterized by multiple satellite ulcers caused by autoinoculation; (3) giant chancroid which begins as a characteristic ulcer extends locally, and is associated with lymphadenopathic abscess and (4) phagedenic chancroid, the most disabling type, characterized by

widespread local necrosis and extensive destruction of the external genitalia (McCarley et al. 1988).

The disease is sometimes associated with lymphadenitis, or bubo formation (Ronald and Plummer 1989). This suppurative and necrotic lymphadenitis is also seen in other Gram-negative infections such as plague. The chancroidal bubo is characteristic and may be a useful clue to the diagnosis. It occurs in 25-60% of cases, is usually unilateral, painful and spherical, with marked redness of the overlying skin (Hand 1989).

In humans, when the disease is not treated, various outcomes are possible. Ulcers in men persist for 3 to 8 weeks with a mean duration of 5 weeks. In women, ulcers may persist for 4 to 6 months. Progressive destruction of genital tissue can lead to urethral fistulas, cicatrization of tissue with sexual dysfunction, and chronic draining inguinal abscesses. Spread has not been described (Ronald 1989).

Histologic sections have shown that chancroidal lesions consist of three zones. The surface zone at the base of the ulcer is shallow and is made up of polymorphonuclear (PMN) leucocytes, fibrin, red blood cells and necrotic tissues. Below this is a wider layer of edematous tissue in which endothelial cells in various stages of proliferation outnumber all other cellular components. There is inflamed tissue with numerous prominent small dilated vessels and strands of endothelial cells which approach the surface. At the junction

with the superficial zone, these vessels degenerate and are infiltrated with neutrophils. Some may be thrombosed. The third, deep zone merges with the middle zone and shows a fairly dense infiltrate of plasma cells and lesser numbers of lymphocytes. These features, taken individually, are not characteristic, but when found together permit a presumptive histologic diagnosis of chancroid (Freinkel 1987).

The disease is much more painful, and more commonly diagnosed in men than women. The lower female incidence may be due to underdiagnosis, and also due to the human ecology of this disease. Prostitution appears a constant epidemiologic feature of chancroid, thus the male-to-female case ratio in outbreaks may approach 10:1 (Ronald and Albritton 1989). Chancroid in women is often subclinical because of the slow progression of the ulcers, the lesser visibility of lesions on female genitalia and the minimal reported discomfort of ulcers in some women. Among women, the most common sites for infection are the labia majora, the forchette, the perineum, the medial aspects of the thighs, the clitoris and around the anus, in descending order of frequency (Ronald 1989). Women are noted to have multiple lesions (Hand 1989).

Two thirds of infected men have classical painful chancroidal ulcers. In men, chancroid presents in one of six sites: In uncircumcised men one half of all ulcers occur on the prepuce. Other sites for genital ulcers in males are the coronal sulcus, the frenulum, the glans and meatus and the

penile shaft. *H. ducreyi* can also cause purulent urethritis (Kunimoto 1988). Male circumcision may have an important influence on susceptibility to infection (Simonsen et al. 1988). It is believed that in the uncircumcised, delicacy and moisture of the mucosal preputial epithelium, and underlying glans facilitates infection, whereas the circumcised are relatively resistant to infection due to keratinization of the epithelium of the exposed glans. Men frequently have single lesions (Hand 1989).

Epidemiology

Chancroid is endemic to developing countries where genital ulceration is a commonly encountered problem in clinics for sexually transmitted diseases (STD). In many cities of developing countries, large female prostitute populations develop as a result of impoverishment of women. These women often have little if any access to health care. With their clientele, they form a dynamic high-prevalence epidemiologic reservoir for STDs (Ronald and Albritton 1990). In Kenya, *H. ducreyi* infection causes approximately 60% of male and female genital ulcers; Sexual contact in prostitution are the probable source of infection in 57% of men with chancroid (Plummer et al. 1983, Plummer et al. and Fast et al. 1984).

In contrast, in industrialized societies, chancroid appears more in the context of outbreaks and sustained

prostitution associated epidemics. Epidemics have occurred in cities of the United States and Canada (McCarley 1988 and Ronald 1989). In developed countries, STDs are generally less associated with urban prostitution. However, chancroid outbreaks have consistently been associated with prostitution, particularly in the setting of urban poverty and illicit drug use.

Diagnosis

It is difficult to determine the historical presence of chancroid in ancient times, because the infection can be confused with other sexually transmitted GUDs such as syphilis, herpes simplex and lymphogranuloma venereum. Differential diagnosis of chancroid on clinical grounds can be difficult and inaccurate. A positive Gram's stained smear of pus aspirated from a bubo is most reliable for diagnostic purposes. Smears and culture of ulcer exudate may also be considered diagnostically specific. It is important to exclude syphilis in every case of suspected chancroid, since the two diseases may be confused and since some chancroid patients may have simultaneous primary syphilis (Abeck et al. 1992). Dark-field examinations of exudate material for *Treponema pallidum* and serologic tests for syphilis should be performed (Hand 1989, Clarridge et al. 1990, Ronald and Plummer 1985 and Borchardt et al. 1970). *H. ducreyi* is a fastidious organism and, despite improved culture methods,

isolation rates from genital ulcers and buboes can still be lower than 60% (Finn et al. 1990) in the best of hands. Use of vancomycin in selective media may somewhat inhibit bacterial growth of *H. ducreyi* due to its recent reported sensitivity to the antibiotic (Clarridge et al. 1989). Culture of ulcers, and especially buboes have low sensitivity in clinical diagnosis.

Histologic techniques can also be used in diagnosis of GUDs. Most biopsies of ulcerative lesions of the genitalia are performed for the exclusion of malignancy and the diagnosis of a sexually transmitted disease is often fortuitous. These lesions show a prominent plasma infiltrate, but it is the relative admixture of leucocytes together with the architectural changes that direct the histopathologist towards the diagnosis. Therefore, ulcers with the three characteristic zones described earlier represent a presumptive evidence of chancroid (Freinkel 1987). However, biopsies of these genital lesions are usually not done because they are painful (Hammond et al. 1978) and more noxious than a trial of therapy.

In 1984, Hansen and Loftus raised monoclonal antibodies (Mabs) against *H. ducreyi*. These were shown by radioimmunoassay, to detect *H. ducreyi* in smears from ulcers caused by the pathogen in experimentally-infected animals (Hansen and Loftus 1984). Furthermore, it was demonstrated that rabbit serum and murine monoclonal antibodies raised

against a strain of *Haemophilus ducreyi* react with a single polypeptide band on immunoblot of molecular weight 29,000 in the OM fraction of *H. ducreyi* (Finn et al. 1990). Finally, due to extensive cross-reactivity between *H. ducreyi* and other *Haemophilus* species, a competition ELISA must be performed in order for hybridoma screening to select these cultures that produce specific Mabs for subsequent cloning (Odumeru 1989).

Another diagnostic tool available is the use of DNA probes. DNA probes are able to detect *H. ducreyi* strains in rabbit lesion exudate, while only a few are recoverable by culture (Parsons 1989). Ribosomal DNA and RNA probes are also reliable and specific for *H. ducreyi* and are thus good diagnostic tools (Sarafian et al. 1991 and Rossau et al. 1991).

Experimental enzyme immunoassays (EIA/ELISA) were done to detect serum IgG antibodies to *H. ducreyi*. Serum IgG antibody to *H. ducreyi* was reproducibly detected in the majority of Kenyan patients with chancroid and in approximately half of such patients from Thailand. A reliable serodiagnostic test may help to elucidate whether cases of clinical chancroid that are negative by culture for *H. ducreyi* are indeed caused by *H. ducreyi* (Museyi et al. 1988 and Desjardins et al. 1992).

Treatment

Most reported studies have used the agar dilution method to test the antimicrobial susceptibility of *H. ducreyi*. There

are geographic differences in the susceptibility of *H. ducreyi* to various antibiotics (Morse 1989). The emergence of β -lactamase producing strains of *H. ducreyi* limits the treatment of chancroid with penicillin or other β -lactamase-susceptible antibiotics. However, treatment of chancroid with amoxicillin plus clavulanic acid has yielded cure rates of over 90% in Kenyan patients (Plummer et al. 1983).

Other antimicrobials used in the treatment of chancroid include quinolones or spectinomycin given in a single dose and sulfonamides with trimethoprim or thiamphenicol given in multiple doses. The results obtained following tetracycline therapy have varied from good to poor (Dangor 1990).

Since the emergence of plasmid mediated resistance to penicillin, tetracycline (Albritton 1984) and sulfonamides, erythromycin given 500mg three times daily for 7 days has become established as the treatment of choice for chancroid (Morse 1989). The major disadvantage in using this is the need for multiple daily doses and extended treatment periods (Boyd 1989) resulting in patient non-compliance and increased cost.

Based on in vitro susceptibility testing, one of the most active drugs against *H. ducreyi* is ceftriaxone. Ceftriaxone is a novel third-generation cephalosporin with a serum half life between 7-9 hours, which distinguishes it from most other third-generation cephalosporins which have half-lives ranging from 0.9 to 2.8 hours (Sauxeral 1989). Extensive in vitro

testing has demonstrated ceftriaxone's broad spectrum of activity against many Gram-positive and Gram-negative organisms and its stability against most plasmid-mediated β -lactamase (LeSaux et al. 1989 and Neu et al. 1981). Ceftriaxone is one therapeutic response to the emerging problem of resistant STD pathogens. Its long half-life permits single-dose therapy or a once-daily injection schedule. Ceftriaxone is an excellent drug for the single-dose treatment of gonococcal urethritis and gonococcal ophthalmia neonatorum; furthermore, it has been shown to be effective against experimentally induced syphilis in rabbits (Bowmer et al. 1987 and Johnson et al. 1982). Ceftriaxone is effective against chancroid at an adult dose of 250mg intramuscularly. It is the treatment of choice for genital ulcer disease due to *H. ducreyi*, with a curative rate of 97% and more (LeSaux and Ronald 1989).

Chancroid and other STDs

The clinical diagnosis of genital ulceration is frequently erroneous. As many as 50% of ulcers will be incorrectly categorized if laboratory confirmation of the etiology is not accomplished. The recent advances in techniques for the culture of *H. ducreyi* now permit an exact diagnosis and should routinely be used in western societies (Chapel et al. 1977). In a study done in Atlanta, 33 patients presented with "chancroidal ulcers" and when cultured, herpes

simplex was isolated from 16 of the 33 patients. *H. ducreyi* was only isolated from one of the patients. Therefore, culture for herpes simplex is essential to exclude herpes in patients with chancroidal ulcers (Salzman et al. 1984).

Chancroid can also mimic granuloma inguinale and some patients with primary syphilis present with lesions that can readily be confused with the classic chancroid ulcer. Conversely, with chancroid, when induration is present, a syphilitic cause or coinfection should be suspected.

Dual infections with herpes simplex and *H. ducreyi* occur in Kenya in approximately 3% of patients (Ronald 1989). It is believed that this represents reactivation of herpes simplex in a patient who has recently acquired chancroid. Dual infections with *Treponema pallidum* and *H. ducreyi* occur in 2 to 3% of patients. All patients with genital ulcer disease should routinely have darkfield examination and serial serology to exclude *T. pallidum* infection, regardless of the presence of other etiologic agents of genital ulcer disease (Ronald 1989).

Two or more pathogens may coinfect an individual and result in a complex or undifferentiated clinical syndrome. For example, *Chlamydia trachomatis* and *N. gonorrhoeae* are frequently cultured concurrently from patients with mucopurulent cervicitis, pelvic inflammatory disease, and urethritis (Odumeru et al. 1987).

Chancroid and the human immunodeficiency virus (HIV)

Because HIV can be sexually transmitted, some of the observed association between HIV and other sexually transmitted diseases can be attributed to a shared risk of exposure and shared modes of transmission (Cameron and Padian 1990). Control and prevention of chancroid is very important because of the role it plays in the dissemination of HIV (Ronald et al. 1989, Boyd 1989 and Aral and Holmes 1991). Various observations support a complex bidirectional interaction between HIV and other sexually transmitted diseases with respect to infectivity and susceptibility in sexual transmission, and to virulence. It was demonstrated that the risk of acquiring HIV-1 was much higher among men who acquired GUD. Thus, the transmission rate of HIV is greatly increased when a man is exposed to a women infected by both GUD and HIV; it is thought that the ulcer raises the infectivity of an HIV-1 infected woman by increasing virus shedding in the genital tract. This could possibly be mediated by the recruitment and activation of HIV-1-infected macrophages and lymphocytes to the disrupted epithelial surface in the local inflammatory response, and direct contact of infectious exudate and blood with the genital epithelium of the susceptible sexual partner (Cameron et al. 1989 and Cameron and Padian 1990). In fact, studies were done that demonstrated the isolation of human immunodeficiency virus from chancroidal genital ulcers in both men and women (Plummer

et al. 1990 and Kreiss et al. 1989).

Retrospective studies done in a Nairobi cohort of female prostitutes indicated that age, duration of prostitution, use of oral contraceptives, and current GUD were associated with HIV-1 infection (Plummer et al. 1991). A study of men who had acquired an STD from a known cohort of prostitutes indicated that uncircumcised men were more than twice as likely to be HIV-1 seropositive than those who were circumcised (Simonsen et al. 1988). Also, a previous history of GUD was more frequent in the seropositive than the seronegative men (Simonsen et al. 1988 and Jessamine and Ronald 1990).

It is also believed that an interaction between HIV infection and the virulence of *H. ducreyi* exists. In fact, an increased incidence and prevalence of chancroid is observed among HIV infected patients (Simonsen et al. 1988 and Cameron et al. 1991). Increased prevalence of chancroidal GUD is seen in women with HIV, and with HIV immunodeficiency (Cameron et al. 1991). Furthermore, there is decreased effectiveness of antibiotic treatment for genital ulcer disease among HIV infected patients. A single dose of sulfonamide-trimethoprim or quinolone is effective in 94% without and 64% with HIV infection (Cameron et al. 1988). This HIV associated microbiologic and clinical treatment failure is corroborated for therapy with single dose fleroxacin (McDonald et al. 1989), ceftriaxone (Tyndall et al. 1991), and with 5 and 7 days erythromycin (Ronald et al. 1992). Thus HIV infection

seems to alter the virulence of *H. ducreyi*.

Thus, one can see an epidemiologic and biologic positive feedback setting emerging in which GUD enhances HIV incidence, and HIV increases GUD prevalence. Therefore, the development of control programs designed to limit the spread of HIV must consider the control of GUD particularly (Cameron and Padian 1990, Jessamine and Ronald 1990 and Wasserheit 1992).

Animal models of *H. ducreyi* infection

One must consider three important factors when developing an animal model of infection relevant to human disease: (1) the disease produced in the chosen animal species must be similar to the natural human disease; (2) the inoculum used to produce disease in an animal must be low, as in the human disease and (3) the route of infection must be similar (Smith 1989). The early limited understanding of *H. ducreyi* pathogenicity is due to prior lack of culture and lack of appropriate biological assays for assessing its virulence. One assay involves the production of cutaneous ulcerative lesions in an animal model of infection.

Early investigators were able to produce lesions in rabbits and monkeys following the direct inoculation of *H. ducreyi* into the skin. Guinea pigs, mice, cats, goats and sheep were all refractory to inoculation. Nevertheless, a suitable animal model is desirable to understand the virulence of *H. ducreyi* and the pathogenesis of chancroid (Morse 1989).

Although human volunteers and many animal species have been tried in the past, the classical test of virulence for *Haemophilus ducreyi* is the rabbit intradermal test (Morse 1990). Earlier investigators have demonstrated the ability of *H. ducreyi* to produce lesions in rabbits after intradermal injection (Feiner et al. 1945 and Kaplan et al. 1956), and this test has been used to define strains of *H. ducreyi* as virulent or avirulent based upon their ability to form necrotic, ulcerative skin lesions (Odumeru et al. 1984, Odumeru et al. 1985 and Odumeru et al. 1987). This does not represent an appropriate animal model of human disease since high inocula (10^9 CFU) injected intradermally are required to produce lesions which do not resemble the typical lesions observed in human disease (Campagnari et al. 1991). The use of mice also proved inadequate; although the lesions produced on inoculation with *H. ducreyi* were similar to chancroid, 10^7 CFU of *H. ducreyi* were required. The mouse model lacked specificity since ulcers would result from intracutaneous inoculation of live or dead bacteria (Tuffrey et al. 1988 and Tuffrey et al. 1990).

The kinetics of IgM and IgG antibody synthesis following intradermal infection in rabbits, was determined to be the same as in other bacterial infections. Antibodies of the IgM class were mainly directed against antigens with relative molecular masses of 79,000, 62,000, 55,000, 49,000 and 26,000 Da. Antibodies of the IgG class were directed against these

as well as against many other antigens with relative molecular weights of between 115,000 and 16,000 Da (Morse 1989).

A temperature dependent model of infection was developed by Purcell et al. in 1991. It was demonstrated that typical chancroidal ulcers could be consistently produced at 10^5 CFU of *H. ducreyi* if rabbits were housed at low ambient temperature (15-17°C). No lesions were produced in animals housed at room temperature, and the lesions produced were shown to be dependent on viability and replication of the microorganisms after intradermal injection on the shaved backs of rabbits.

Objectives and Hypotheses

The aim of this work was to develop and evaluate a standard scoring system of infection and disease as a quantitative virulence assay, and to measure changes in virulence related to manipulations of the host (iron loading, dexamethasone immunosuppression, prior infection, immunization and treatment with ceftriaxone) and manipulations of the pathogen using the temperature dependent rabbit model. We hypothesized that: (1) A quantitative scoring system amenable to statistical comparison demonstrating consistency and replicability would permit distinguishing host factors influencing virulence; (2) This quantitative virulence assay would permit evaluation of inducible immunity and therapeutic treatments in the model, relevant to human disease; (3) This

quantitative virulence assay measures an inflammatory response to bacterial antigen or bacterial histotoxicity in infection, which is responsible for disease production. Virulence in this animal model of infection was measured according to chosen quantitative parameters: (1) size of inocula; (2) onset and duration of lesions; (3) heirarchically scored inflammatory characteristics; (4) size of lesions; (5) culture positivity of lesions; and (6) kinetics of serologic antibody response.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Haemophilus ducreyi strain #35000

Haemophilus ducreyi strain #35000 was isolated from a chancroid outbreak in Winnipeg (Hammond et al. 1978) and was obtained from A.R. Ronald of the University of Manitoba. The microorganism was grown in a broth culture (APPENDIX) composed of 40% Mueller Hinton broth (CANLAB, Montreal, Quebec), 40% Alpha Minimal Essential Medium (MEM) (Gibco Laboratories, Life Technologies Inc., Grand Island, NY) and 20% Fetal bovine serum (Gibco Laboratories). The starting 50 mL broth cultures were inoculated with a suspension of approximately 4×10^8 CFU *H. ducreyi* from chocolate agar plates (CAP) (APPENDIX) incubated 48 hours at 33°C with 5% CO₂. The starting concentration of the broth was 8×10^6 CFU/mL. The broth cultures were incubated at 33°C with shaking for 16 hours which corresponded to mid-to-late log phase. The final concentration was $1-2 \times 10^8$ CFU/mL. Cultures were aliquoted and mixed with 10% DMSO (Dimethyl Sulfoxide, Fisher Scientific Limited, Ontario, Canada) and stored at -70°C until further use.

Haemophilus ducreyi strain #v1157

Haemophilus ducreyi strain #v1157 was obtained from A. R. Ronald from an outbreak in Seattle, Washington. The

microorganism was grown in 40% Mueller Hinton broth, 40% Alpha MEM and 20% Fetal bovine serum. Cultures were incubated at 33°C with shaking for 20 hours. Cultures were aliquoted and mixed with 10% DMSO and stored at -70°C until further use.

Haemophilus ducreyi growth curve

In order to determine *H. ducreyi* growth kinetics and maximum colony forming units (CFU) per mL, a growth curve was constructed. Broth cultures were grown at 33°C with shaking and sampled every 4 hours between 0 and 24 hours inclusive and then at 36 and 48 hours. Optical densities were determined on these samples at 600nm wavelength. CFUs were calculated by spreading culture aliquots on CAP and incubating plates at 33°C, 5% CO₂ for 48 hours. As well Gram's stain were done on all samples. It is important to note that measurement of CFU/mL underestimates bacteria/mL due to aggregation of the bacteria in broth cultures.

Inoculation of experimental animals

Once *H. ducreyi* #35000 growth curve was constructed, peak growth determined, and quantitation of suspended organisms made, animals could be inoculated with known quantities of microorganisms. Male New Zealand White (NZW) rabbits (2.5-3.0 Kg) were obtained from the National Research Council of Canada. The animals were housed in an air conditioned room with ambient temperature maintained at 12.5 ± 1.5 °C and were

given one week to adjust to their new environment before manipulations. Rabbits were shaved across the back and a phlebotomy was performed on a marginal ear vein four days prior to bacterial inoculation. Thereafter, their backs were shaved on a daily basis, or whenever required, to expose skin to ambient air temperature.

H. ducreyi broth cultures grown for 16 hours at 33°C with shaking were used for inoculation into rabbits. The cultures were centrifuged for 10 minutes at 2000xg in an Omnifuge RT centrifuge (Baxter, CANLAB division, Pointe Claire, Quebec) washed with sterile Mueller Hinton broth. Five suspensions in serial ten fold dilutions were made in sterile Mueller Hinton broth such that 0.1 mL inocula ranged from 10^7 to 10^3 CFU. Titre of inocula was confirmed prior to intraepithelial injection by spreading 1 and 10 μ L aliquots on CAP. Plates were incubated at 33°C, 5% CO₂ for 48 hours.

Each animal received intraepithelial injections of 0.1 mL of each dilution in triplicate on its back for a total of 15 injections per rabbit. 1 mL syringes with 26 gauge 3/8 inch tuberculin needles (Fisher Scientific) were used for inoculation. Rabbits were observed daily for 28 days and the diameters of the lesions produced were measured with a Vernier direct reading caliper. Measurements of diameters were done from one indurated extremity of the lesion to the other. Photographs of the rabbits backs were taken on day 7 post inoculation.

Scoring system

The scoring system used in this work was adapted to our objectives from the infection model of Purcell et al. (1991). Lesions were numerically scored hierarchically for severity from 0 to 4: 0= no lesion formation; 1= erythema; 2= induration; 3= suppuration; 4= ulceration. Erythema was defined as redness at the site of inoculation, induration as palpable firm elevation or nodule formation of the lesion, suppuration as pustule formation, fluctuence or drainage from a nodule, and ulceration as visible epithelial disruption.

Recovery of *H. ducreyi* from lesions

Bacteria were recovered from lesions by transepithelial injection of 0.1 mL or less of sterile PBS into the base of the lesions, and aspirating injected material into the syringe. The contents of the syringes were spread onto CAP and incubated for a maximum of four days or until *H. ducreyi* colonies were visible. *H. ducreyi* recovery was confirmed by characteristic appearance on Gram's stain and by characteristic morphology of cohesive colonies on CAP. Lesions were cultured on day four and every second day thereafter until no bacteria were recovered on two sequential attempts.

Host manipulations

Control rabbits

Four control rabbits were given *H. ducreyi* as described on page 26. These rabbits were 2.5 to 3.0 Kg in weight, and acclimatised to the experimental housing conditions.

Iron loading of rabbits

Four rabbits were given intramuscular injections of Iron Dextran (Dexafer 100mg/mL; Austin Laboratories, Joliette, Quebec) one week prior to bacterial inoculation. The total dose administered equalled 750 mg/Kg, and was equally divided to each hind leg. Blood samples were obtained before and after iron treatment. The rabbits were then inoculated with *H. ducreyi* strain #35000 as described on page 26.

Dexamethasone treatment of rabbits

Four rabbits were given subcutaneous injections of 2mg of dexamethasone (Azium 2mg/mL; from Shering, Pointe-Claire, Quebec) twice a week for three weeks prior to bacterial inoculation. Treatment was continued throughout the experiment, to produce and maintain chronic immunosuppression and anti-inflammatory effect.

Dexamethasone treatment plus iron loading of rabbits

Three rabbits were given dexamethasone as above plus iron dextran as above during the third week of the dexamethasone

treatment. Rabbits were then inoculated as on page 26.

Re-infection of rabbits

Four rabbits previously used as naive controls infected with *H. ducreyi* #35000 were re-infected, in the same manner described on page 26, eight weeks after primary infection which was six weeks after peak IgG antibody response.

Immunization of rabbits

Four rabbits were immunized with intramuscular injection of 10^9 CFU of *H. ducreyi* strain #35000 coupled with Freund's complete adjuvant (Gibco Laboratories) and of 10^9 CFU of *H. ducreyi* strain #35000 coupled with Freund's incomplete adjuvant (Gibco Laboratories) (APPENDIX). Injections were given in sequence four weeks apart. Four weeks following the booster injection, which corresponded to the serologic response plateau, the rabbits were inoculated with *H. ducreyi* strain #35000 as described on page 26.

Manipulations of the pathogen

Inoculation of heat killed pathogen

A 16 hour broth culture of *Haemophilus ducreyi* strain #35000 was heat killed prior to inoculation into rabbits. The culture was centrifuged for 10 minutes at 2000xg, washed, resuspended and 10 fold serial diluted in sterile Mueller Hinton broth. Aliquots of these dilutions were spread on CAP

for a viability check and the bacteria were then heat killed in a 56°C water bath for one hour. The heat killed inoculum was then injected into a rabbit.

Inoculation with *Haemophilus ducreyi* strain v1157

H. ducreyi strain v1157 was used for inoculation into two naive rabbits. The broth cultures were grown for 20 hours with shaking at 33°C. The inocula were then prepared as for *H. ducreyi* strain #35000 and injected in two rabbits (see page 26). Scoring and observations were done in the same manner described previously. These two rabbits were then re-infected with strain v1157 as described for re-infection experiments with *H. ducreyi* strain #35000 (see page 30).

Antibiotic experiments

Naive rabbits

Four naive rabbits were infected as described on page 26. Lesions were cultured at days 1, 2, 3, and 4. On day four, two rabbits received a single intramuscular injection of ceftriaxone (Rocephin, Hoffman-La Roche, Mississauga, Ontario). One rabbit received an injection of 0.1 mg/Kg and the other was given 5 mg/Kg of the antibiotic (APPENDIX). The two other rabbits did not receive any antibiotics and served as parallel controls.

Phlebotomies were performed every 2 hours after antibiotic treatment for 6 hours and at 24 and 36 hours. The

blood was centrifuged, the sera collected, aliquoted and stored at -70°C for further use.

The lesions were cultured in the four rabbits every 24 hours after day 4 until all were negative for *Haemophilus ducreyi* on two successive attempts. These rabbits were subsequently re-infected 8 weeks following primary infection. However, before re-infection, these rabbits were given an intramuscular injection of 5 mg/Kg ceftriaxone one one prior to inoculation. These rabbits were inoculated as described on page 26.

Iron loaded naive rabbits

Four naive rabbits were treated with iron dextran as described on page 29 one week prior to bacterial inoculation. On day 4 post bacterial inoculation, one rabbit received 0.1 mg/Kg ceftriaxone and another was given 5 mg/Kg. The other two rabbits did not receive any antibiotic treatment and served as parallel controls.

Phlebotomies were performed every 2 hours following antibiotic treatment for 6 hours and at 24 and 36 hours. The blood was centrifuged, the sera collected, aliquoted and stored at -70°C for further use.

The lesions were cultured in the four rabbits every 24 hours after day 4 until all were culture negative for *H. ducreyi*.

Enzyme linked immunosorbent assay (ELISA)

Antigen preparation and coating of plates

H. ducreyi strain #35000 was grown on CAP for 48 hours, at 33°C and 5% CO₂. The colonies were scraped off with a cotton swab and suspended in 10 mL PBS; centrifugation was done at 2000xg for 10 minutes. The final pellet was resuspended in 10 mL PBS containing 1% SDS (APPENDIX). The suspension was sonicated three times for 30 seconds with a pause of 10-15 seconds at maximum amplitude using an ice water bath. The suspension was mixed on a rotator for 2 hours at room temperature and then centrifuged at 50,000xg at 4°C for 90 minutes. The protein concentration of the supernatant was determined by Biorad protein assay. The antigen was stored in aliquots at -70°C.

Ninety-six well round-bottom plates (Nunc Polysorb U96), were coated with *H. ducreyi* antigen suspended in 0.1 M carbonate buffer pH 9.6 (APPENDIX), incubated for 2 hours at 37°C and washed 3 times with washing buffer (PBS, 0.1% tween 80). Plates were wrapped in foil and stored at 4°C for a maximum of two weeks before ELISA.

IgG and IgM ELISAs of rabbit sera

200 µL/well of each test serum was applied, to wells of a ninety-six well round-bottom plate, at 1:200 and seven times two fold serially diluted in PBS (1% NBCS, 0.11% tween 80), incubated for 30 minutes at 37°C and washed 5 times with

washing buffer. 200 μ L of a 1:4000 dilution of peroxidase conjugated goat anti-rabbit IgG (Tago Burlingame, CA) diluted in PBS (5% NBCS, 0.1% tween 80) was added to each well. With peroxidase conjugated goat anti-rabbit IgM (Tago Burlingame, CA), 1:1000 dilution was used. The plates were incubated for 30 minutes at 37°C then washed 5 times with washing buffer. Bound conjugated antibody was detected by adding 100 μ L of 0.36 mM ABTS (2,2'-azino-bis (3-ethyl-benthiozoline-6-sulphonate) (Boehringer Mannheim), and 0.03% H₂O₂ dissolved in citrate buffer (APPENDIX). The plates were kept at 20°C for 25 minutes and read spectrophotometrically at 405 nm. Rabbits were bled once a week during four weeks. The blood was centrifuged at 2000xg for 10 minutes and the serum was collected, aliquoted and stored at -70°C.

Each plate included one positive and one negative serially diluted control rabbit serum. Results were adjusted for plate to plate variation by expressing reactivity at 1:400 dilution of test sera as a ratio of test serum to plate negative control optical density plus 0.044 for IgG and 0.006 for IgM values (Desjardins et al. 1992).

Optimal antigen concentration and dilutions of peroxidase conjugated goat anti rabbit IgG/IgM were experimentally determined. 5 μ g/mL antigen, 1:4000 anti-IgG and 1:1000 anti-IgM were found to be optimal concentrations for rabbit sera ELISAs.

Statistical analysis

Standard parametric and non-parametric two-tailed statistical tests were to be used to compare disease severity and serologic response between experimental groups. Student's T test is used for p values reported in comparison of means, and Fisher's exact test for discrete variables.

Animal care

All experimental procedures involving animals meet with approval of the University of Ottawa Animal Care Committee, protocol number MI-53.

RESULTS

Haemophilus ducreyi quantitation

In order to work on an quantitative animal model of infection, one must first establish quantitative measurement for the pathogen in growth and presentation to the host. Standard growth curves were established for both *H. ducreyi* #35000 and v1157. In both cases, the CFU curve closely followed the optical density plot until peak CFU was reached after which CFUs rapidly decreased to eventually reach a value of zero and ODs decreased slightly and stabilized at 16 hours and 20 hours for *H. ducreyi* v1157 (Figures 1 and 2). Inocula were quantitated by dilution of known suspensions, and confirmed by colony counts. Variation in inocula was 0.26 ± 0.04 logs from the desired CFU dosage.

Optimization of IgG and IgM ELISAs for rabbit sera

An ELISA used for measuring IgG and IgM levels in human sera was used and had to be optimized for rabbit serum. 96 well plates were coated with 5, 10 and 20 μ g of *H. ducreyi* strain #35000 antigen. As well, different dilutions of peroxidase conjugated goat anti-rabbit IgG/IgM were tested (1:1000, 1:2000, 1:4000 and 1:8000). This was tested on both control negative and control positive sera.

It was established that 5 μ g was the best amount of antigen to use with a conjugate dilution of 1:4000 for

FIGURE 1

Growth curve of *Haemophilus ducreyi* strain #35000
expressed in optical density and colony forming units.

Open circles - growth in optical densities
Closed circles - growth in colony forming units

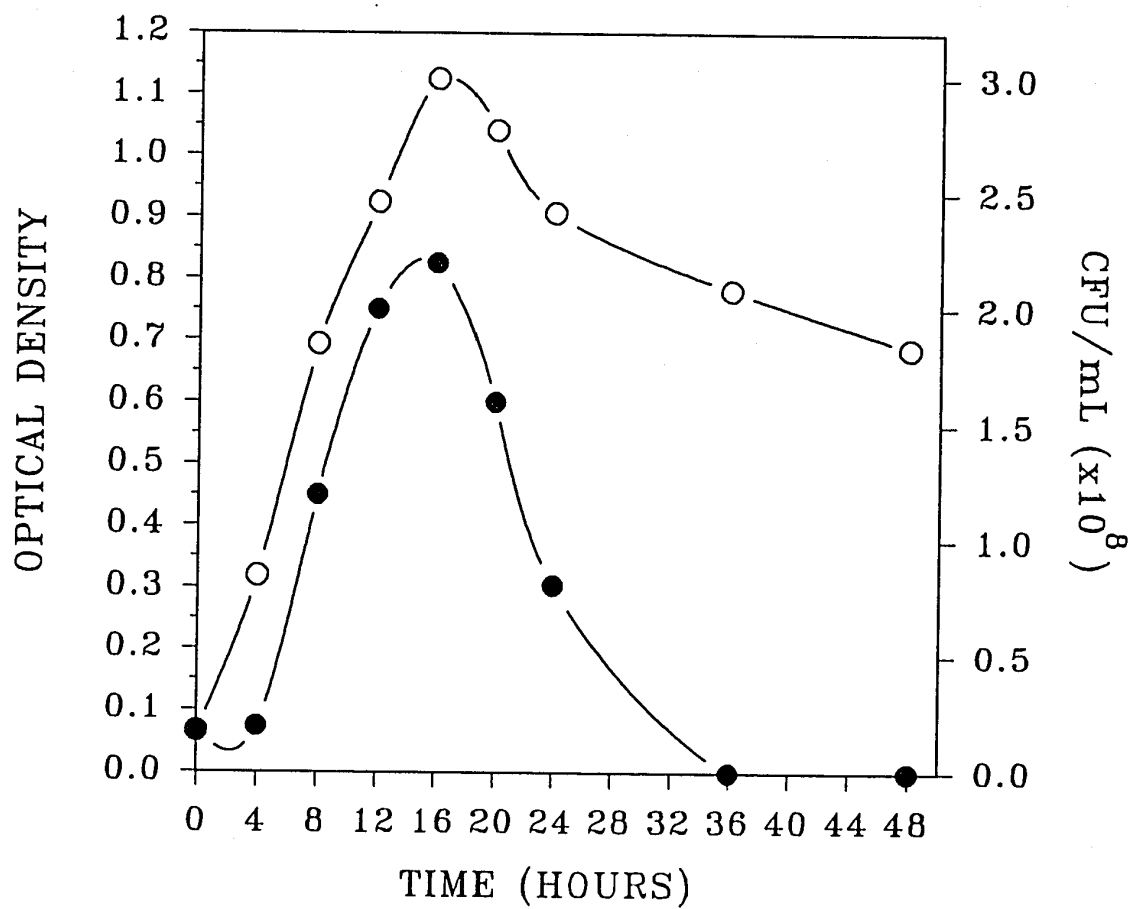
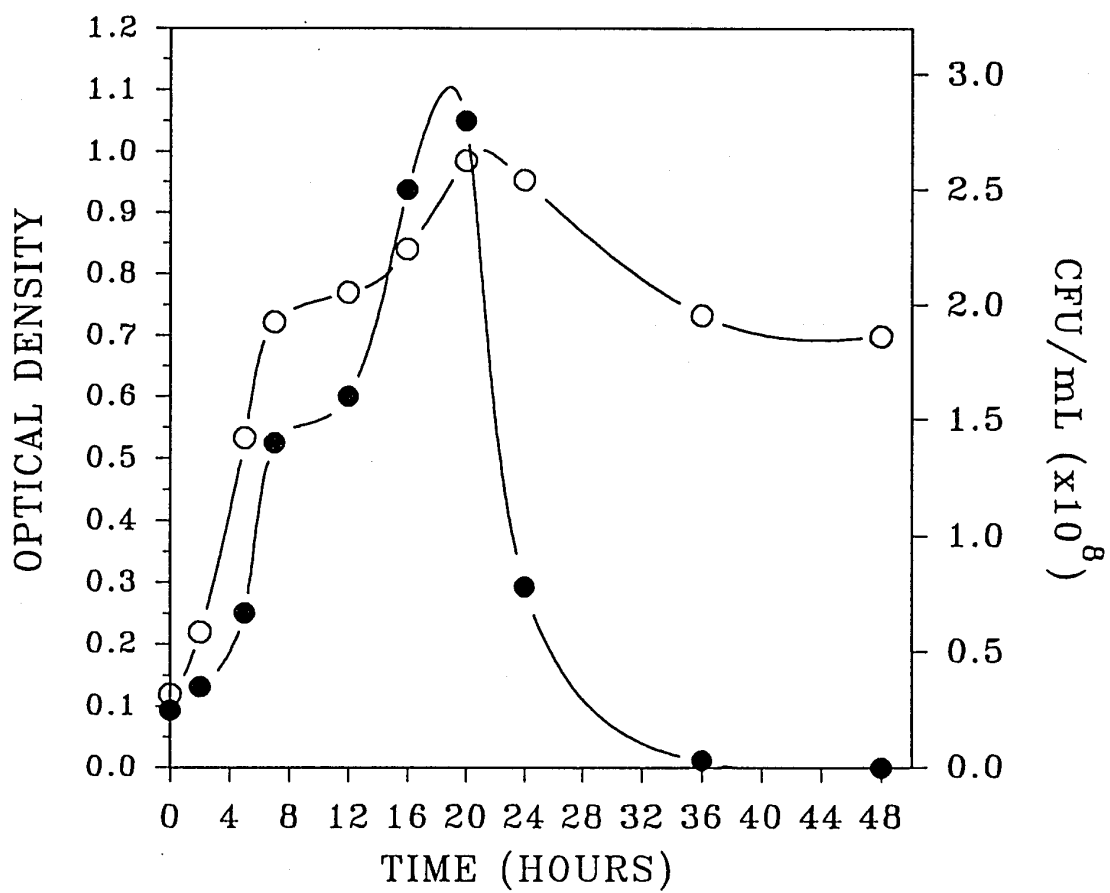


FIGURE 2

Growth curve of *Haemophilus ducreyi* strain #v1157
expressed in optical density and colony forming units.

Open circles - growth in optical densities
Closed circles - growth in colony forming units



anti-IgG and 1:1000 for anti-IgM for separation of control positive and negative sera. In both cases the serum dilution used to calculate ratios was 1:400 (Figures 3 and 4).

Intraepithelial rabbit assay: Host manipulations

Control animals

The highest inoculum was determined to be $4.0 \pm 1.4 \times 10^7$ CFU of *H. ducreyi* #35000. Results obtained for the control animals confirmed Purcell's observation (Purcell et al. 1991). Ulcers were consistently produced at 10^7 , 10^6 and 10^5 CFU (Figure 5). 24 hours after bacterial inoculation, each site was indurated at 10^7 , 10^6 and 10^5 CFU. Epithelium ulcerated on day 3 at 10^7 , 10^6 and 10^5 CFU. At 10^6 and 10^5 disease duration was 10 days and at 10^7 ulcer duration was approximately 13 days. At 10^4 and 10^3 peak scores of 2 and 1 respectively were recorded. Lesions were culture positive during week one and culture negative thereafter at the three higher inocula (Table 1). Size of lesions was also observed to be dose dependent. Timing and score of disease is illustrated in figure 6. Kinetics of IgM and IgG responses obtained were typical of a primary infection: Peak IgM response developed in the first week and peak IgG response developed in the second week and remained elevated. Peak IgM coincided with culture negativity and peak IgG with healing or decrease in lesion score and size (Figure 6).

FIGURE 3

Standard curve for IgG ELISA at 5 μ g antigen and 1:4000 conjugate dilution in rabbit control positive and control negative sera.

Open circles - control negative sera
Closed circles - control positive sera

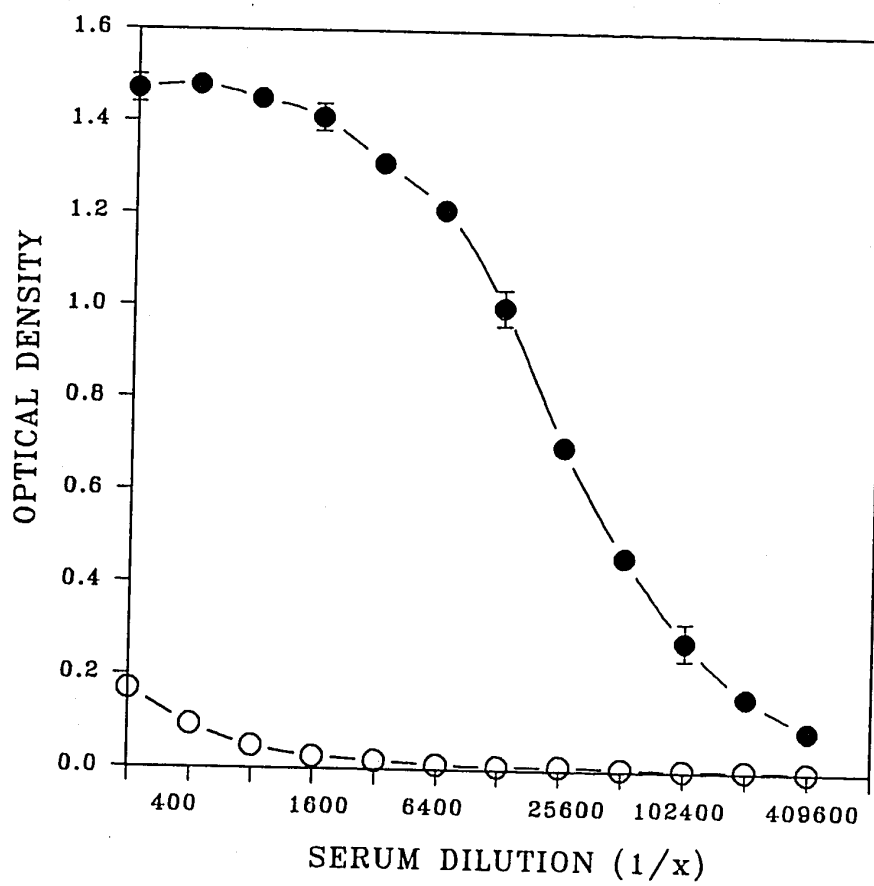


FIGURE 4

Standard curve for IgM ELISA at 5 μ g antigen and 1:1000 conjugate dilution in rabbit control positive and control negative sera.

Open circles - control negative sera
Closed circles - control positive sera

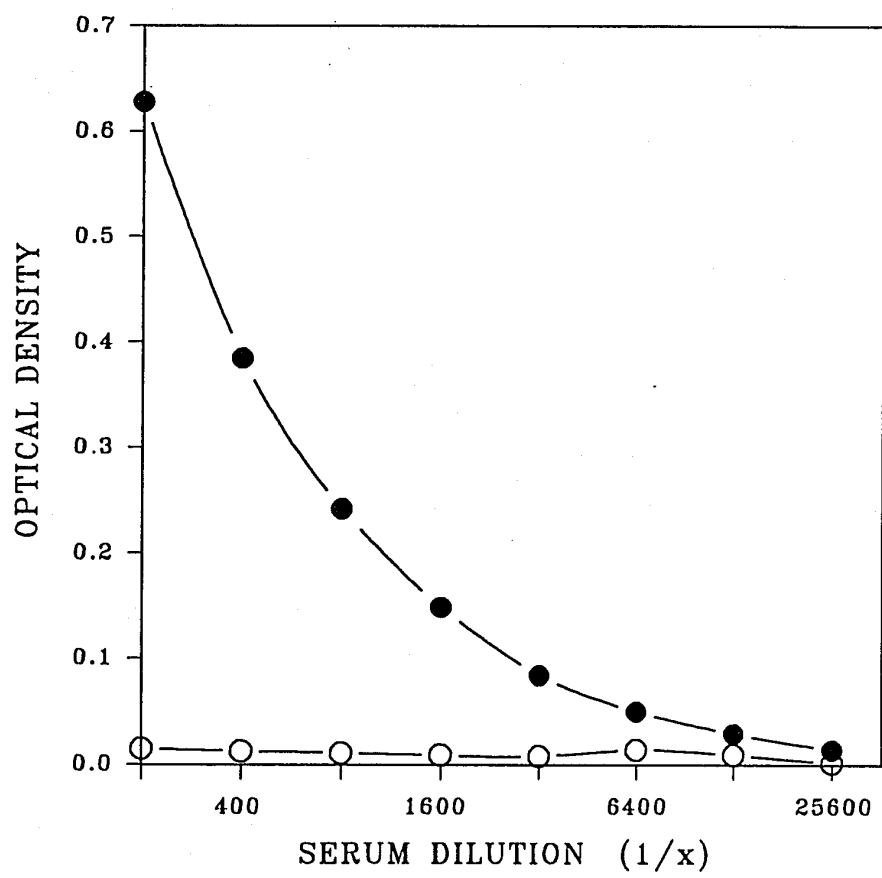


FIGURE 5

Photograph of rabbit lesions at day 7 post inoculation in a naive control rabbit. Arrows indicate inoculation sites and size of inocula (CFU/site).

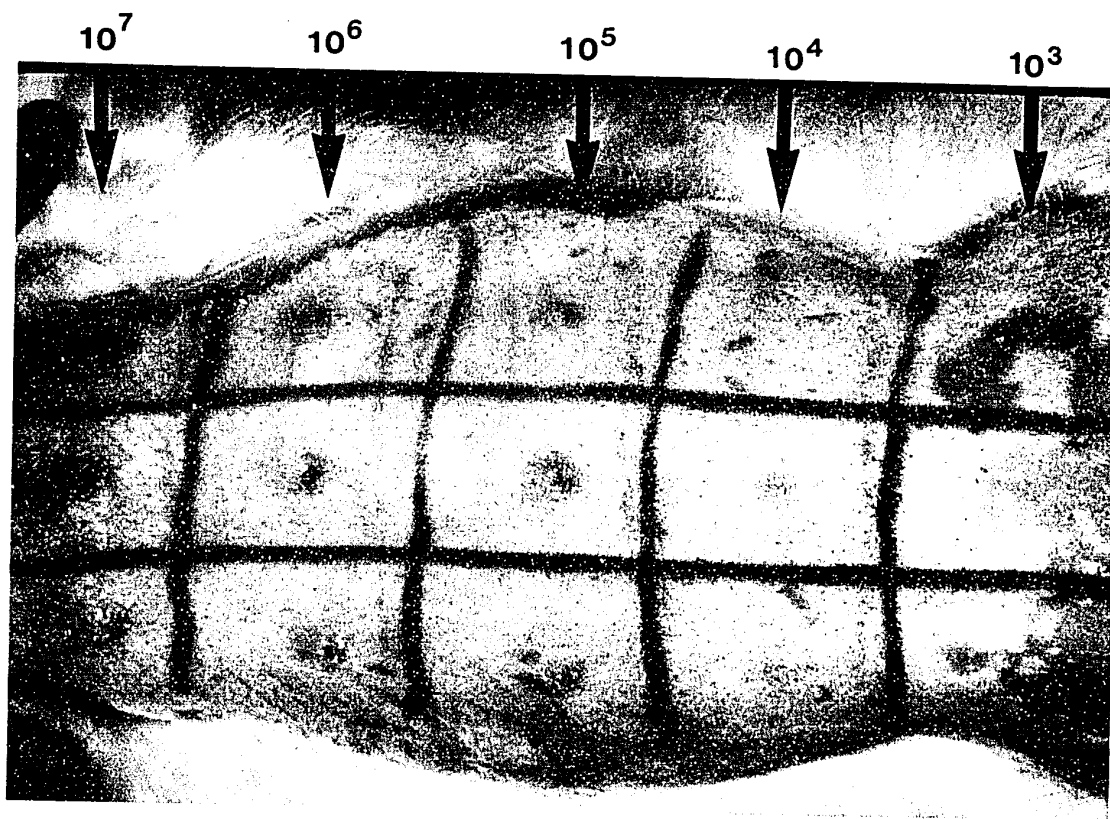


TABLE 1

Variations in infection and disease produced by titration of *H. ducreyi* in naive control, iron loaded, dexamethasone treated or iron loaded and dexamethasone treated rabbits. Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate in each group.

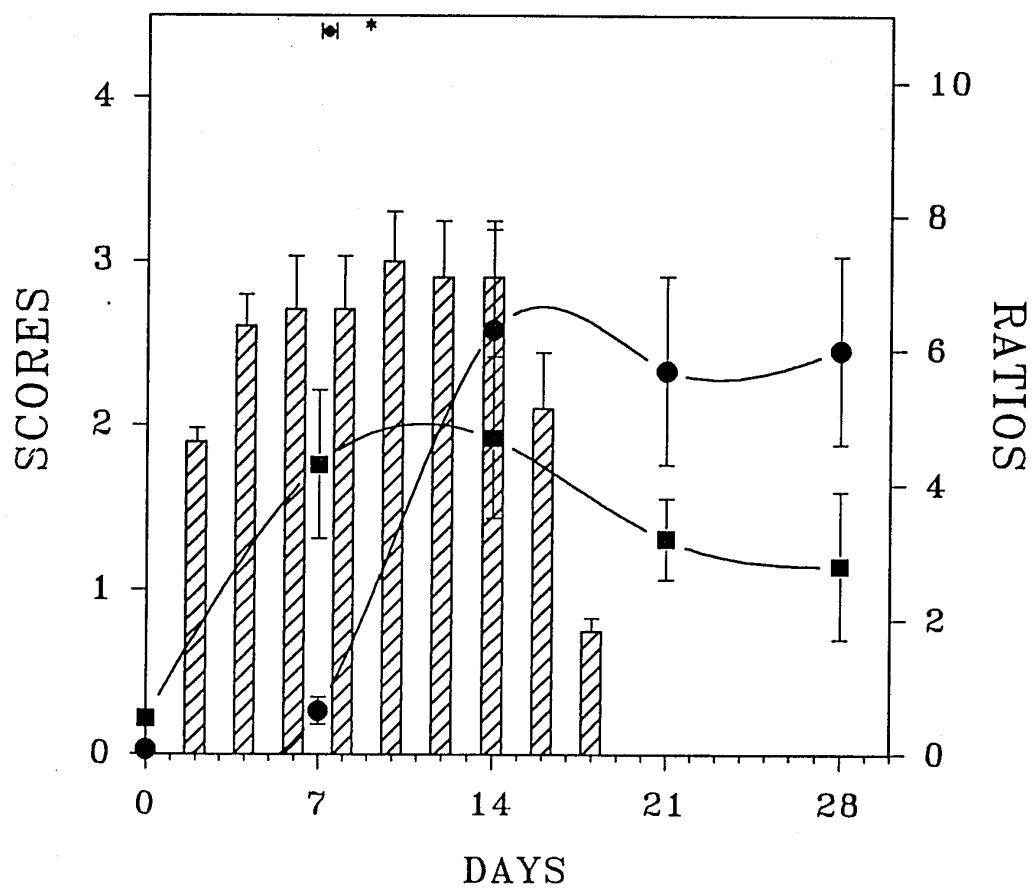
- * Dexamethasone treated rabbits
- ** Dexamethasone treated and iron loaded rabbits; these rabbits had to be sacrificed at day 7 due to a systemic illness.
- a: Significantly different from control ($p < 0.05$)
M.U.P.I.: Minimum ulcer producing inoculum.

PARAMETERS	ANIMALS			
	CONTROL	IRON	DEXA.*	IRON+DEXA**
# ANIMALS	6	5	4	3
# LESIONS	18	15	12	9
M.U.P.I. (CFU)	10^5	10^5	10^5	10^4
<u>10^5 CFU</u>				
ONSET (DAYS)	3 ± 0	2.5 ± 0.3	6.2 ± 0.3^a	4.7 ± 0.9
ULCER DURATION (DAYS)	10.7 ± 0.6	11.6 ± 0.8	12.7 ± 1.3^a	N/A
INDURATION PEAK SIZE (mm)	10.2 ± 1.1	13.8 ± 0.9^a	10 ± 0.6	11.4 ± 1.7
LAST CULTURE POSITIVE DAY	7.3 ± 0.3	12.1 ± 0.4^a	17 ± 2.1^a	N/A
PEAK SCORE	4 ± 0	4 ± 0	4 ± 0	4 ± 0
CUMULATIVE SCORE	39.9 ± 5.6	50.9 ± 1.6^a	54.6 ± 8.8^a	N/A
10^4 CFU PEAK SCORE	2.1 ± 0.1	2.2 ± 0.2	1.8 ± 0.08	4 ± 0^a
10^3 CFU PEAK SCORE	1 ± 0	1.3 ± 0.2	0.7 ± 0.2	1 ± 0

FIGURE 6

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for six naive control rabbits.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - Standard error



Iron loaded animals

The highest inoculum was determined to be $2.1 \pm 0.95 \times 10^7$ CFU of *H. ducreyi* #35000. Ulcers consistently appeared at 10^7 , 10^6 and 10^5 CFU (Figure 7). Disease onset was more rapid compared to controls; pustules appeared as early as 24 hours post inoculation at the three highest inocula. Ulcers at 10^7 , 10^6 and 10^5 CFU were culture positive until day 14 post inoculation and were culture negative thereafter ($p < 0.05$, Table 1). The lesions were approximately 35% larger than those obtained for the control animals ($p < 0.05$, Table 1 and Figure 7). Lesions resolved at approximately day 10 which is similar to controls (Table 1). At 10^4 and 10^3 CFU a peak score of 2 and 1 were obtained respectively. IgG and IgM responses followed similar patterns to controls, although slightly higher optical densities peaking later were observed. Peak IgM and IgG responses followed culture negativity and coincided with healing or decrease in lesion score and size (Figure 8).

Dexamethasone treated animals

The highest inoculum was determined to be $1.8 \pm 0.4 \times 10^7$ CFU of *H. ducreyi* #35000. Ulcers consistently appeared at 10^7 , 10^6 and 10^5 CFU (Figure 9). Onset of disease was between day 4 and 5 at 10^7 and 10^6 CFU and was delayed until day 6, twice as long as control animals, at 10^5 CFU ($p < 0.05$, Table 1). At 10^4 and 10^3 CFU, peak scores of 2 and 1 were obtained

FIGURE 7

Photograph of rabbit lesions at day 7 post inoculation in a naive iron loaded rabbit. Arrows indicate inoculation sites and size of inocula (CFU/site).

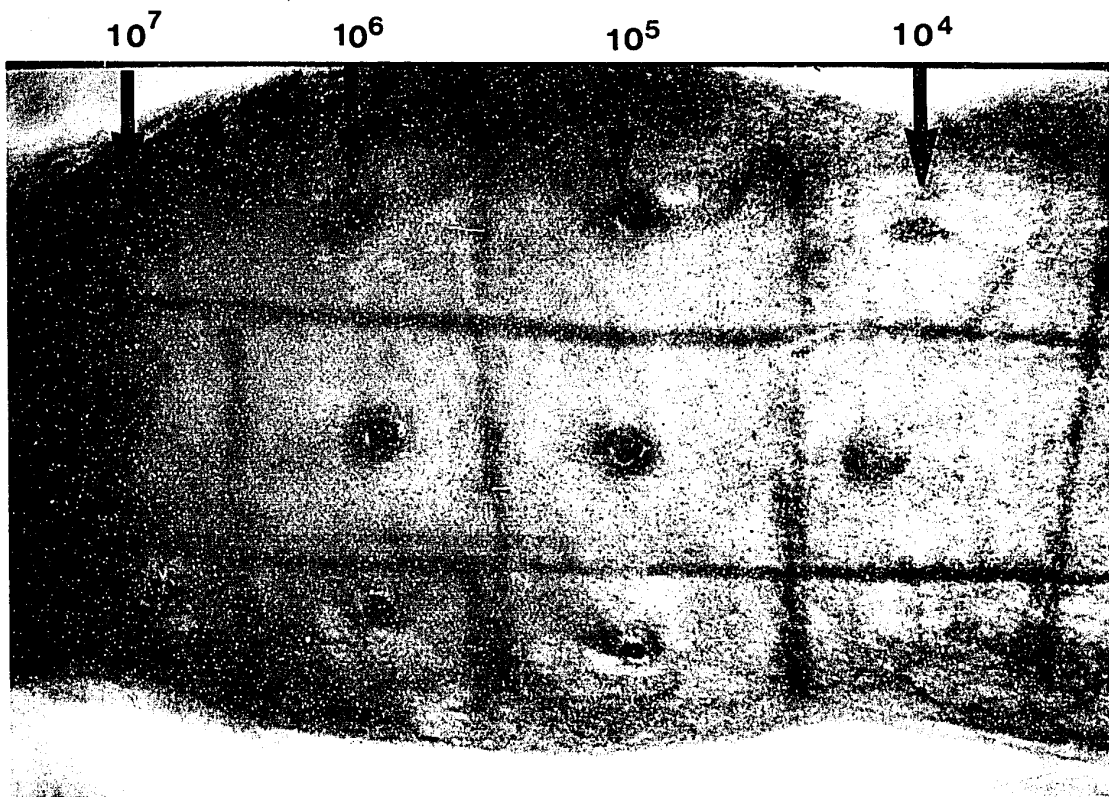


FIGURE 8

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for five iron loaded naive rabbits.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error

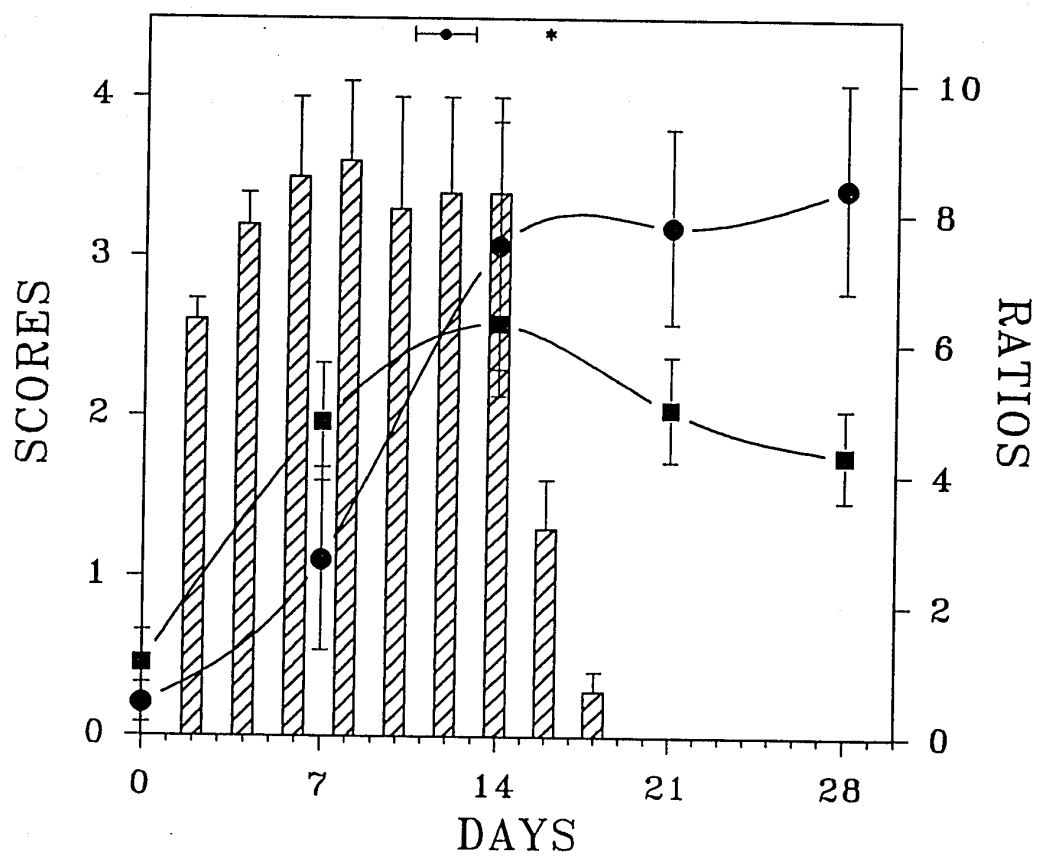
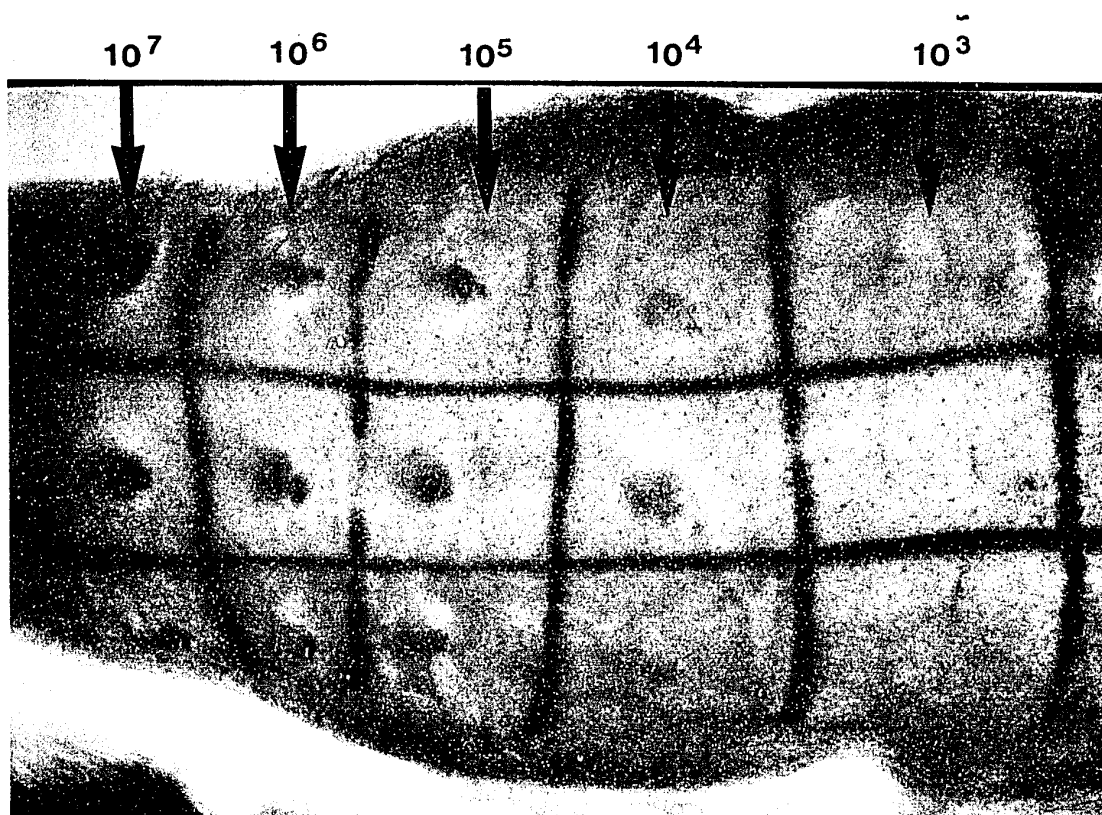


FIGURE 9

Photograph of rabbit lesions at day 7 post inoculation in a dexamethasone treated naive rabbit. Arrows indicate inoculation sites and size of inocula (CFU/site).



respectively. By day 3 lesions had resolved at 10^4 CFU and by day 2 lesions had resolved at 10^3 CFU. Distinct from controls, IgM response in dexamethasone treated rabbits peaked one week later and remained higher following peak response. IgG levels rose throughout 28 days observation (Figure 10).

Iron loaded and dexamethasone treated animals

The highest inoculum was determined to be $1.4 \pm 0 \times 10^7$ CFU of *H. ducreyi* #35000. Rabbits that received both iron and dexamethasone treatments produced ulcers consistently at 10^7 , 10^6 , 10^5 and 10^4 CFU (Figure 11), to give a minimum ulcer producing inoculum one log below controls. By day 3 the lesions at the two highest inoculations had ulcerated. Disease production was delayed until day 4 or 5 at 10^5 CFU, which is later than control and iron loaded rabbits and earlier than dexamethasone treated rabbits. Ulceration at 10^4 CFU occurred on day 6. Lesion size was similar to controls at 10^5 CFU (Table 1). Lesions were culture positive until day 7. These rabbits had to be sacrificed on day 7 due to systemic illness. IgG and IgM kinetics were not measured for these rabbits.

Re-infected animals

The highest inoculum was determined to be $2 \pm 0 \times 10^7$ CFU of *H. ducreyi* #35000. In the re-infected animals, ulcers were consistently obtained at 10^7 , 10^6 and 10^5 CFU and the lesions

FIGURE 10

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for four dexamethasone treated naive rabbits.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error

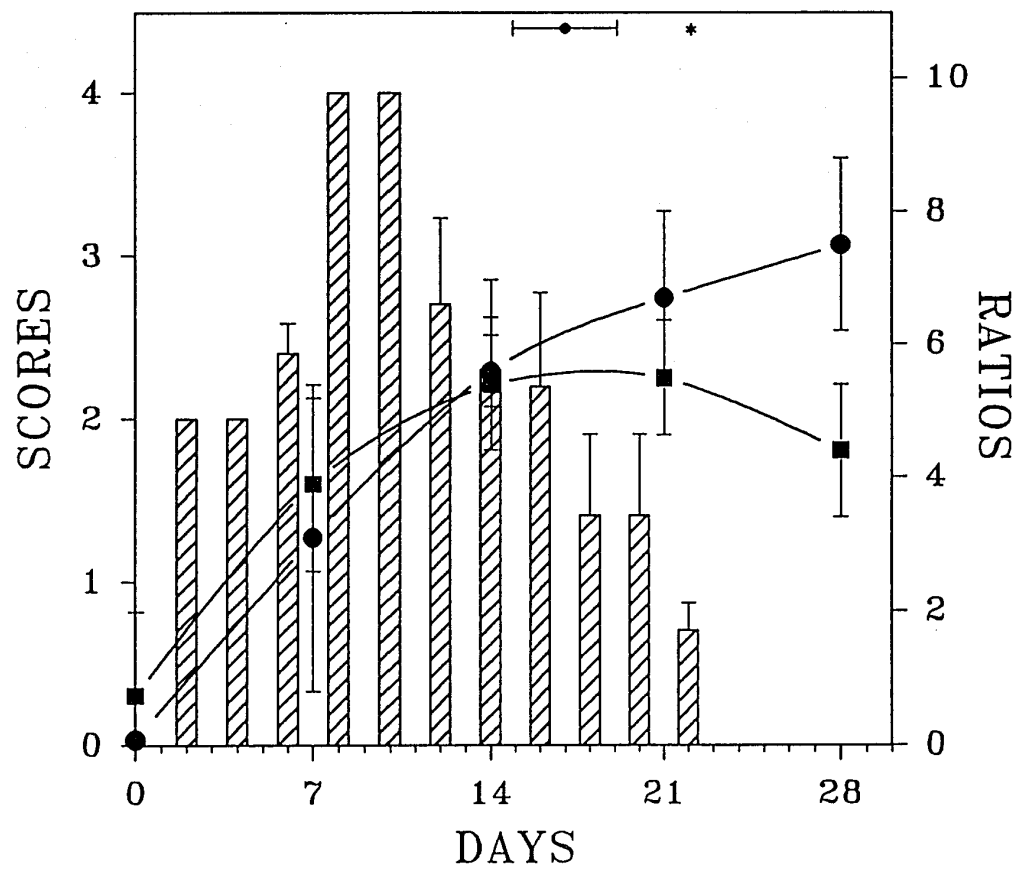
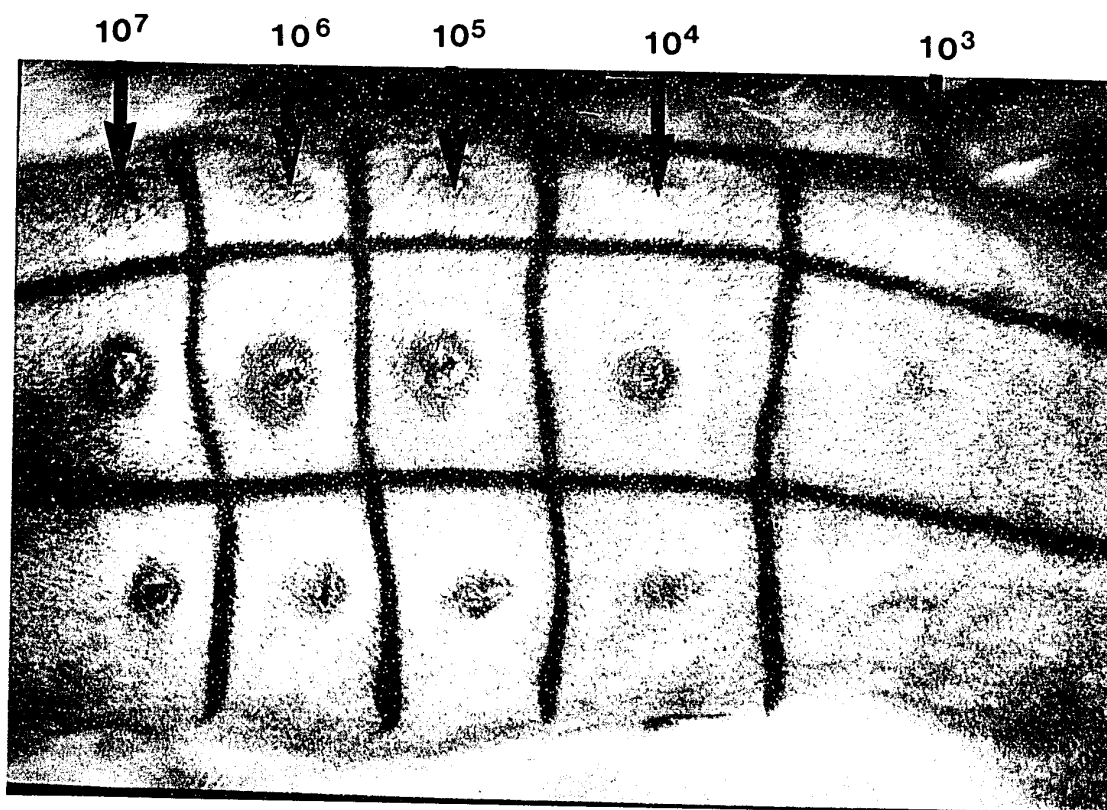


FIGURE 11

Photograph of rabbit lesions at day 7 post inoculation in a naive rabbit treated with both iron and dexamethasone. Arrows indicate inoculation sites and size of inocula (CFU/site).



were approximately 60% larger than the controls ($p < 0.05$, Figure 12 and Table 2). The disease effect produced in these animals was significantly different from controls in several parameters: disease was longer in duration and the cumulative score was elevated. When cultured, the lesions were found to be sterile. At 10^4 and 10^3 CFU, peak scores of 2 were obtained (Table 2). During this secondary infection, IgM peaked earlier at day 7 and then dropped while remaining higher than controls, and IgG levels remained higher than in primary infection of controls throughout the experiment (Figure 13).

Immunized animals

The highest inoculum was determined to be $3.2 \pm 0.2 \times 10^7$ CFU of *H. ducreyi* #35000. Ulcers were consistently obtained at 10^7 CFU and inconsistently at 10^6 CFU (Figure 14). The non-ulcerative indurated lesions produced at 10^5 were 35% larger than control but 15% smaller than those produced in re-infection ($p < 0.05$, Table 2) and, when cultured, all lesions were found to be sterile. A peak score of 2 was obtained at 10^5 and 10^4 CFU and a peak score of 1 was recorded at 10^3 CFU (Table 2). IgM and IgG levels were significantly higher in immunized rabbits compared to controls and re-infected animals at baseline and throughout observation (Figure 15).

FIGURE 12

Photograph of rabbit lesions at day 7 post inoculation in a re-infected rabbit. Arrows indicate inoculation sites and size of inocula (CFU/site).

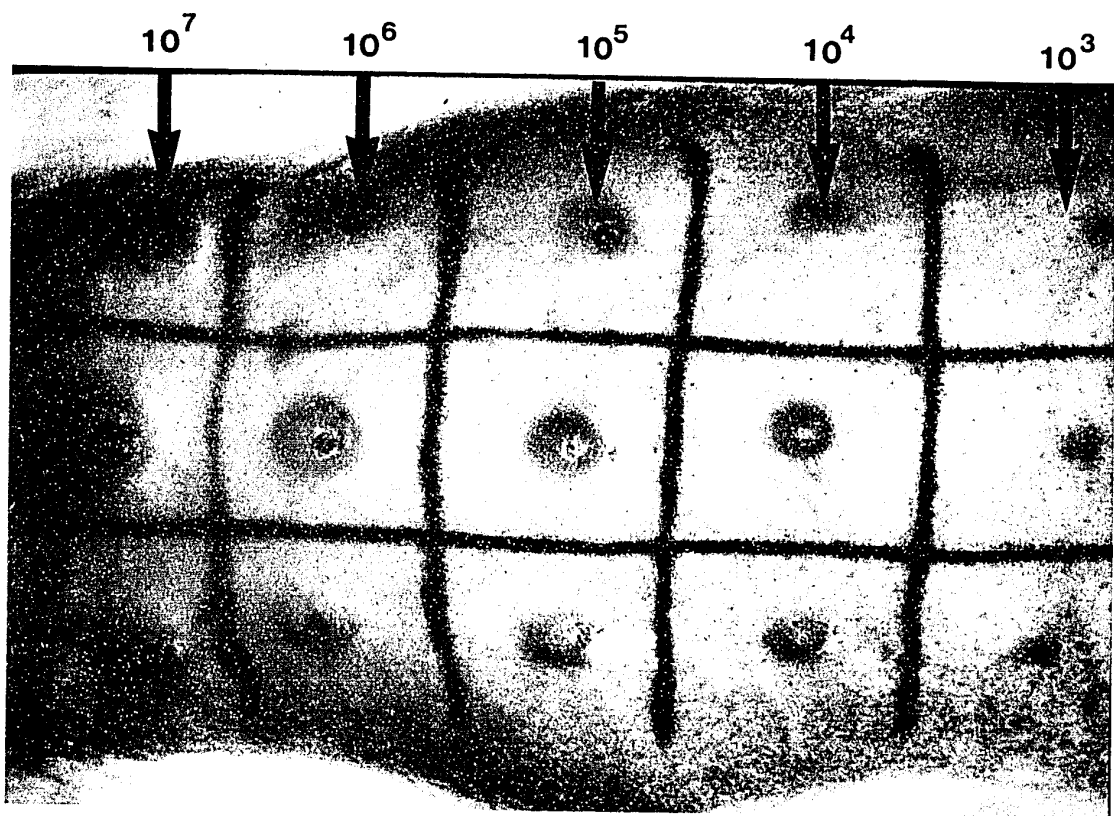


TABLE 2

Variations in lesion production by titration of *H. ducreyi* in previously infected and immunized rabbits compared to naive controls. Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate in each group.

a: Significantly different from control ($p < 0.05$)
M.U.P.I.: Minimum ulcer producing inoculum

PARAMETERS	ANIMALS		
	CONTROL	RE-INFECTION	IMMUNIZATION
# ANIMALS	6	4	4
# LESIONS	18	12	12
M.U.P.I. (CFU)	10^5	10^5	10^6
10^5 CFU			
ONSET (DAYS)	3 ± 0	3.5 ± 1.5	NO ULCER
ULCER DURATION (DAYS)	10.7 ± 0.6	19.5 ± 0.5^a	NO ULCER
INDURATION PEAK SIZE (mm)	10.2 ± 1.1	16.2 ± 0.2^a	13.7 ± 0.8^a
LAST CULTURE POSITIVE DAY	7.3 ± 0.3	STERILE	STERILE
PEAK SCORE	4 ± 0	4 ± 0	2 ± 0^a
CUMULATIVE SCORE	39.9 ± 5.6	59 ± 8.0^a	20.1 ± 2.9^a
10^4 CFU PEAK SCORE	2.1 ± 0.1	2 ± 0	1.9 ± 0.07
10^3 CFU PEAK SCORE	1 ± 0	2 ± 0^a	1 ± 0

FIGURE 13

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for four re-infected rabbits.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error

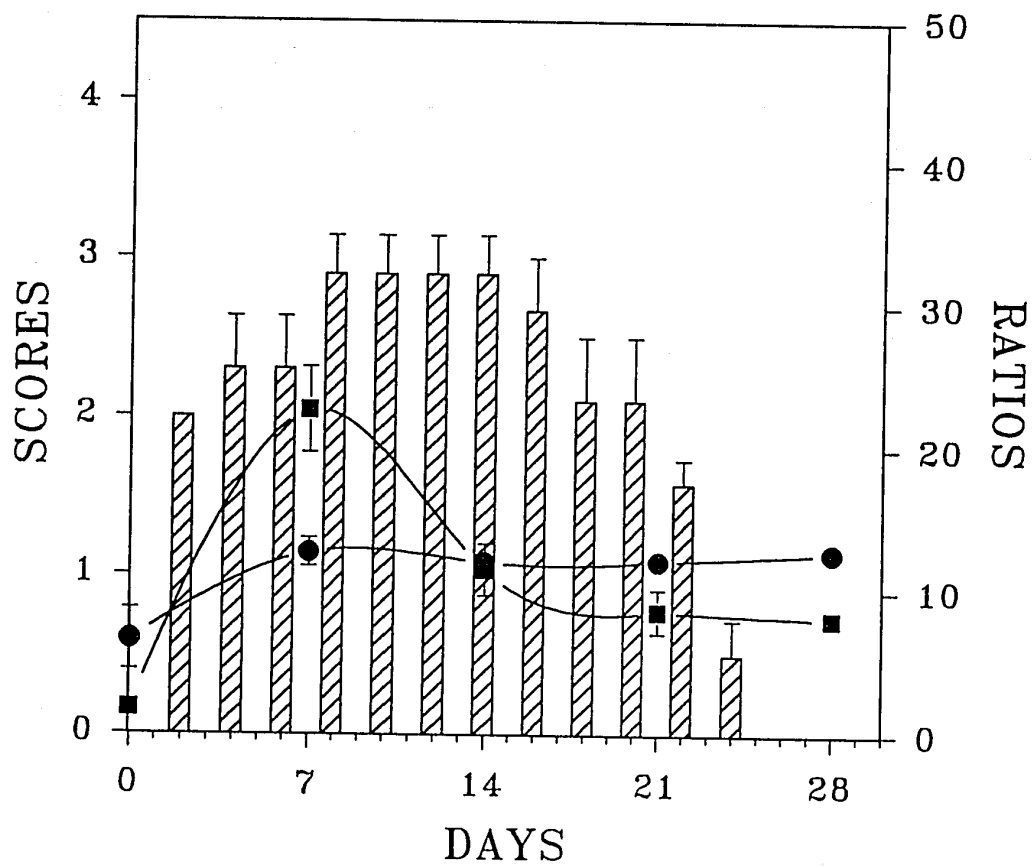


FIGURE 14

Photograph of rabbit lesions at day 7 post inoculation in an immunized rabbit. Arrows indicate inoculation sites and size of inocula (CFU/site).

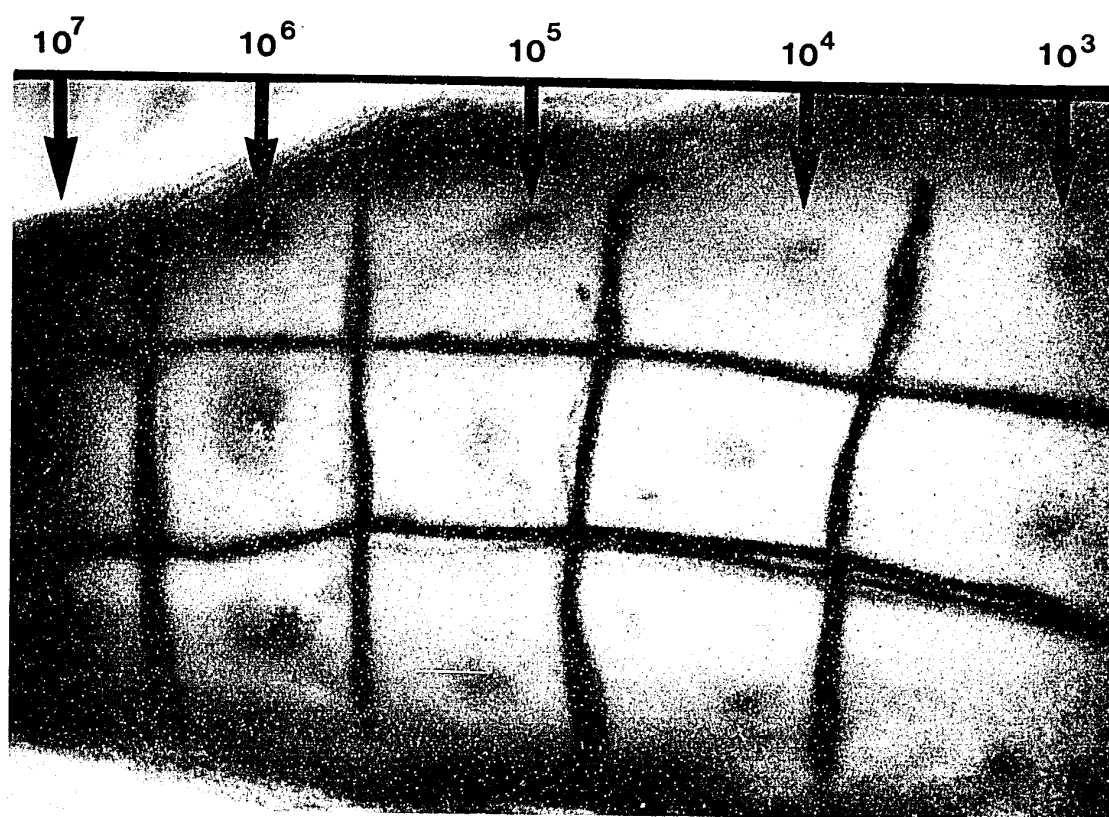
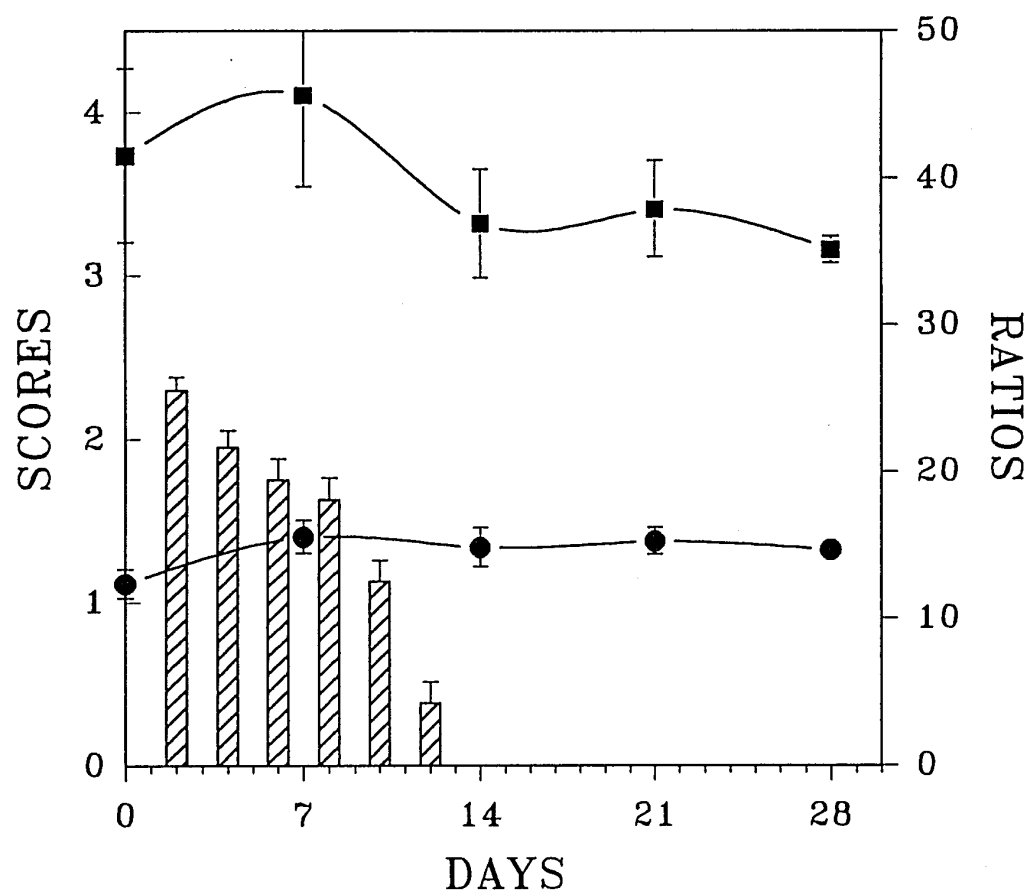


FIGURE 15

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for four immunized rabbits.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error



Intraepithelial rabbit assay: Manipulations of pathogen

Inoculation of heat killed pathogen

The highest inoculum was determined to be $2.5 \pm 0 \times 10^7$ CFU of *H. ducreyi* #35000. 24 hours post inoculation and later, no lesions were visible except for marks made by the injecting needle. This rabbit was also infected with live bacteria in parallel and therefore the antibody response elicited by the dead inocula is not known.

Inoculations with *H. ducreyi* strain #v1157

The highest inoculum was determined to be $4.0 \pm 0 \times 10^7$ CFU of *H. ducreyi* #v1157. No ulcers developed at 10^7 and 10^6 CFU at which levels peak score was 3 ± 0 . Lesions lasted 2 days and started to heal, and had resolved by day 4 for 10^7 CFU and day 3 for 10^6 CFU. Peak scores of 2 ± 0 were recorded at 10^5 and 10^4 CFU. All lesions were healed by day 5 and were all sterile from day 4. Peak IgM was observed at week 1 and IgG response peaked at 4 weeks (Figure 16). Disease effect is greater in naive rabbits infected with *H. ducreyi* #35000 (Table 3).

In rabbits undergoing homologous re-infection with *H. ducreyi* #v1157, no ulcers were produced at 10^7 , 10^6 and 10^5 CFU, however, the disease effect was increased and a peak score of 3 ± 0 was obtained for these three inocula. A score of 2 ± 0 was recorded at 10^4 and 10^3 CFU, and all lesions were sterile. During this secondary infection, IgM peaked earlier

TABLE 3

Comparisons in lesion production by titration of *H. ducreyi* strains #35000 and v1157 in naive and previously infected rabbits.

* Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate.

M.U.P.I.: Minimum ulcer producing inoculum.

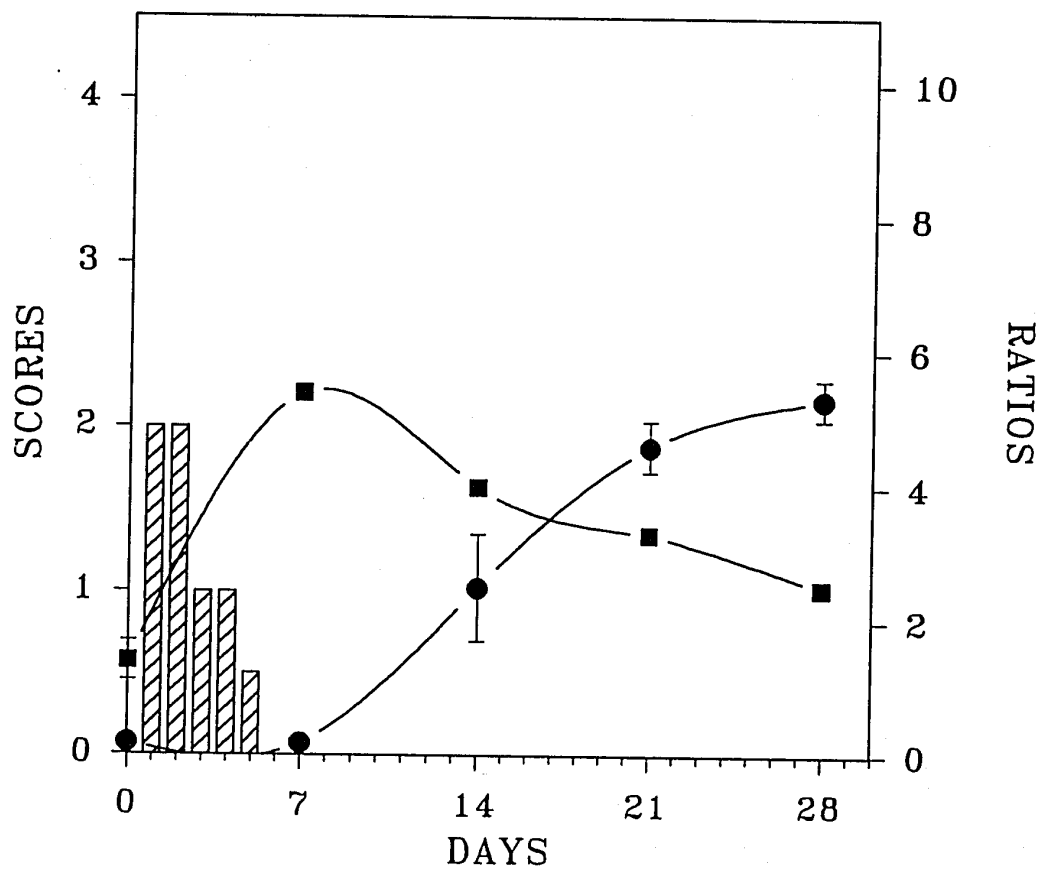
a: Significantly different from homologous control primary infection ($p < 0.05$)

PARAMETERS	PRIMARY INFECTION		SECONDARY INFECTION	
	#35000*	V1157*	#35000*	V1157*
# ANIMALS	6	2	4	2
# LESIONS	18	6	12	6
M.U.P.I. (CFU)	10^5	NO ULCER	10^5	10^7
10^5 CFU				
ONSET (DAYS)	3 ± 0	NO ULCER	3.5 ± 1.5	NO ULCER
ULCER DURATION (DAYS)	10.7 ± 0.6	NO ULCER	19.5 ± 0.5^a	NO ULCER
INDURATION PEAK SIZE (mm)	10.2 ± 1.1	NIL	16.2 ± 0.2^a	12 ± 0^a
LAST CULTURE POSITIVE DAY	7.3 ± 0.3	STERILE	STERILE	STERILE
PEAK SCORE	4 ± 0	2 ± 0	4 ± 0	3 ± 0^a
CUMULATIVE SCORE	39.9 ± 5.6	8 ± 0	59 ± 8.0^a	22.5 ± 3.5^a
10^4 CFU				
PEAK SCORE	2.1 ± 0.1	2 ± 0	2 ± 0	2 ± 0
10^3 CFU				
PEAK SCORE	1 ± 0	1 ± 0	2 ± 0^a	2 ± 0^a

FIGURE 16

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology in two rabbits infected with *H. ducreyi* #v1157.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Error bars - standard error



at day 7 and decreased while remaining higher than controls. IgG levels remained significantly higher than controls throughout the experiment (Figure 17).

Antibiotic experiments

Naive control rabbits

The highest inoculum was determined to be $5 \pm 0 \times 10^7$ CFU of *H. ducreyi* #35000. In all four rabbits ulcers appeared at 10^7 , 10^6 and 10^5 CFU on day 3 approximately. All lesions were culture positive on days 1, 2, 3, and 4 prior to antibiotic treatment. Overall duration of ulcerative disease did not change with ceftriaxone treatment. Complete resolution of disease was 18 days for control animals, 14 days and 12 days for rabbits treated with 0.1 mg/Kg and 5 mg/Kg ceftriaxone respectively. Another significant difference between rabbits receiving ceftriaxone and those receiving no treatment lies in the last culture positivity of the lesions. Antibiotic treatment significantly decreased duration of culture positivity of the lesions by approximately 40% (Table 4). Mean scores, IgG and IgM responses of naive rabbits receiving no ceftriaxone were incorporated in figure 6. The rabbit treated with 0.1 mg/Kg of ceftriaxone had an IgG response similar to that of controls but a higher IgM response on day 14 (Figure 18). IgM and IgG responses in rabbits treated with 5 mg/Kg ceftriaxone follow the same pattern as controls (Figure 19).

FIGURE 17

Disease score at 10^5 CFU *H. ducreyi* inoculation and serology in rabbits re-infected with *H. ducreyi* #v1157.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Error bars - standard error

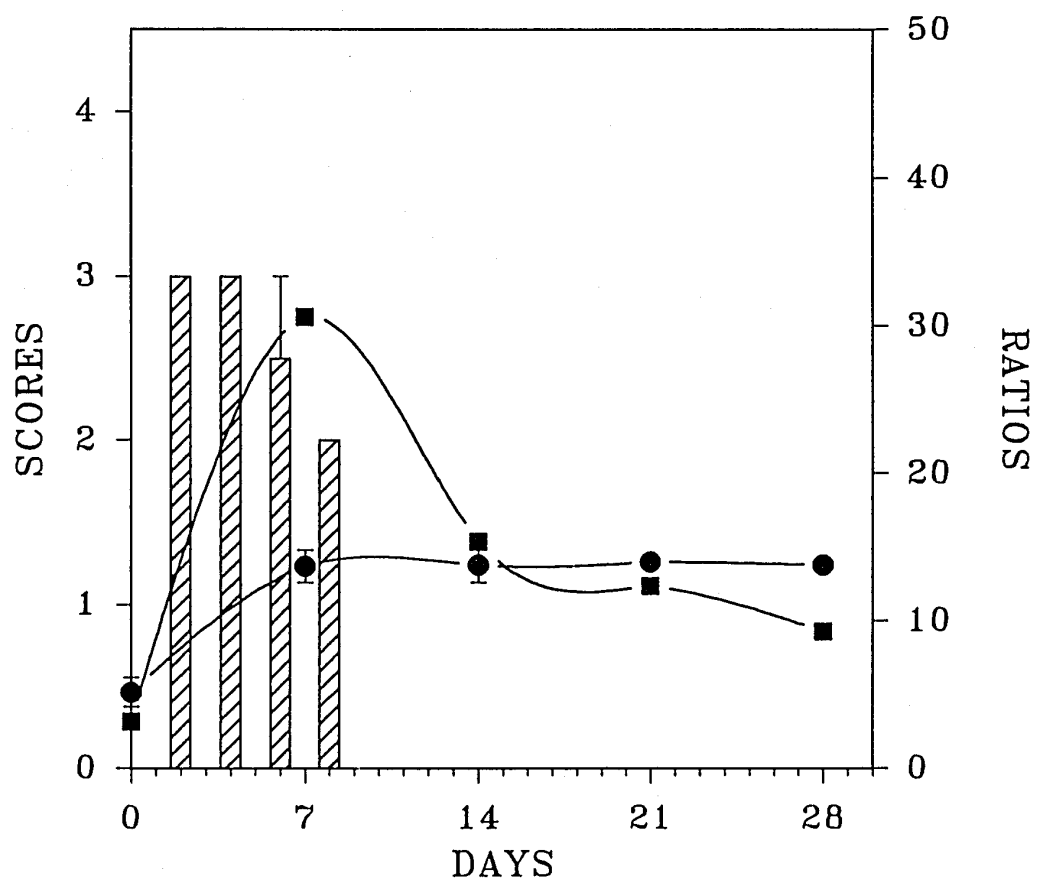


TABLE 4

Variations in lesion production by titration of *H. ducreyi* in naive rabbits treated with ceftriaxone 4 days post inoculation.

* Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate.

M.U.P.I.: Minimum ulcer producing inoculum.

a: Significantly different from control ($p < 0.05$)

PARAMETERS	CEFTRIAXONE (mg/Kg)		
	0*	0.1*	5*
# ANIMALS	6	1	1
# LESIONS	18	3	3
M.U.P.I. (CFU)	10^5	10^5	10^5
<u>10^5 CFU</u>			
ULCER DURATION (DAYS)	10.7 ± 0.6	9 ± 0	8 ± 0
INDURATION PEAK SIZE (mm)	10.2 ± 1.1	10.3 ± 0.7	9.8 ± 1.3
LAST CULTURE POSITIVE DAY	7.3 ± 0.3	4 ± 0^a	4 ± 0^a
CUMULATIVE SCORE	39.9 ± 5.6	41 ± 0	38 ± 0
10^4 CFU PEAK SCORE	2.1 ± 0.1	1.5 ± 0	1.5 ± 0
10^3 CFU PEAK SCORE	1.0 ± 0	1 ± 0	1.0 ± 0

FIGURE 18

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for one naive control rabbit receiving 0.1 mg/Kg ceftriaxone on day 4 post inoculation.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error

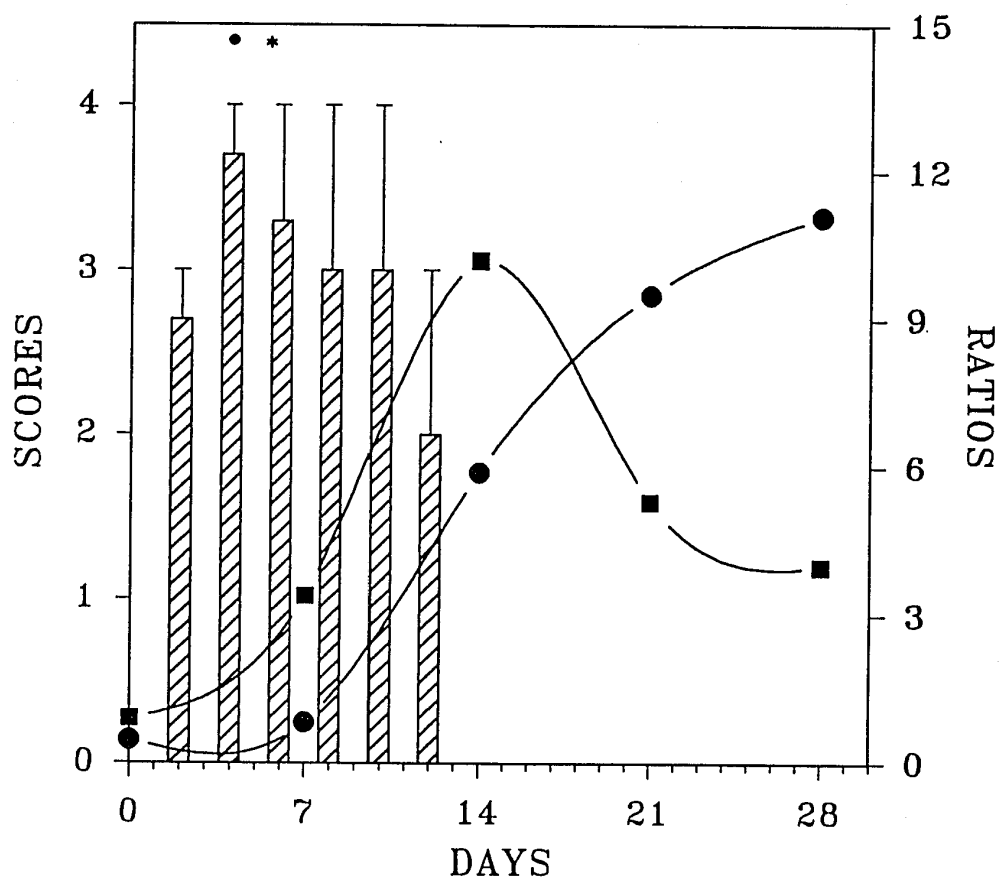
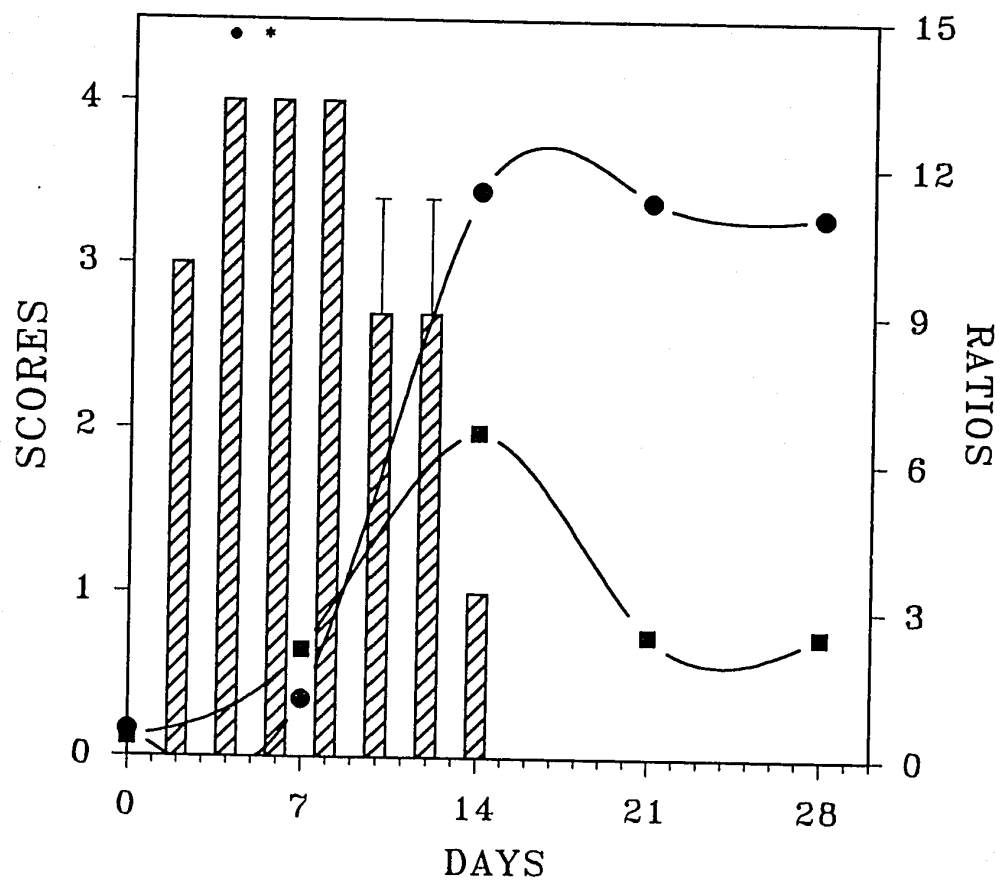


FIGURE 19

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for one naive control rabbit receiving 5 mg/Kg ceftriaxone on day 4 post inoculation.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterisk - last culture positive day
Error bars - standard error



Eight weeks following their primary infection, control rabbits were re-infected as previously described. One hour prior to re-infection, these rabbits were treated with 5 mg/Kg ceftriaxone intramuscularly. No ulcers were produced in these animals and indurated lesions were sterile from day 1. When compared to re-infected rabbits receiving no treatment with antibiotics one can see a significant difference in disease effect; the cumulative score is decreased by almost 90% when the rabbits are pre-treated with ceftriaxone (table 5).

Naive iron loaded rabbits

Results obtained for the two control iron loaded rabbits were incorporated into figure 8. The highest inoculum was confirmed at $5 \pm 0 \times 10^7$ CFU of *H. ducreyi* #35000. Ulcers were consistently produced at 10^7 , 10^6 and 10^5 CFU. All ulcers were culture positive on days 1, 2, 3, and 4 prior to antibiotic treatment. Duration of culture positivity was decreased by 60% in the rabbit treated with 5 mg/Kg ceftriaxone. Treatment with 0.1 mg/Kg ceftriaxone did not significantly affect culture positivity of the lesions (Table 6). Treatment with both doses of ceftriaxone yielded similar kinetics of serologic responses. However, these responses were significantly higher for IgM and slightly lower for IgG compared to controls (Figure 8, 20 and 21).

TABLE 5

Variations in lesion production by titration of *H. ducreyi* in re-infected rabbits and in re-infected rabbits receiving 5 mg/Kg ceftriaxone one hour prior to inoculation.

* Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate.

M.U.P.I.: Minimum ulcer producing inoculum

a: Significantly different from re-infected rabbits receiving no ceftriaxone treatment ($p < 0.05$)

PARAMETERS	RE-INFECTION*	RE-INFECTION + CEFTRIAXONE*
# ANIMALS	4	4
# LESIONS	12	12
M.U.P.I. (CFU)	10^5	$> 10^7$
<u>10^5 CFU</u>		
INDURATION	10.2 ± 1.1	10.1 ± 0.9
PEAK SIZE (mm)		
LAST CULTURE POSITIVE DAY	STERILE	STERILE
CUMULATIVE SCORE	59 ± 8.0	4.9 ± 1.2^a
10^4 CFU	2 ± 0	1 ± 0^a
PEAK SCORE		
10^3 CFU	2 ± 0	1 ± 0^a
PEAK SCORE		

TABLE 6

Variations in lesion production by titration of *Haemophilus ducreyi* in iron loaded naive rabbits treated with ceftriaxone 4 days post inoculation.

* Numeric values are expressed as the means $\pm$ standard deviation for rabbits inoculated in triplicate.

M.U.P.I.: Minimum ulcer producing inoculum.

a: Significantly different from control ($p < 0.05$)

PARAMETERS	CEFTRIAXONE (mg/Kg)		
	0*	0.1*	5*
# ANIMALS	5	1	1
# LESIONS	15	3	3
M.U.P.I. (CFU)	10 ⁵	10 ⁵	10 ⁵
<u>10⁵ CFU</u>			
ULCER	11.6±0.8	7±0 ^a	5±0 ^a
DURATION			
(DAYS)			
INDURATION	13.8±0.9	10.8±1.4	11.1±0.6
PEAK SIZE (mm)			
LAST CULTURE	12.1±0.4	10±0	5±0 ^a
POSITIVE DAY			
CUMULATIVE	50.9±1.6	34±0 ^a	28±0 ^a
SCORE			
10 ⁴ CFU	2.2±0.2	2±0	1±0
PEAK SCORE			
10 ³ CFU	1.3±0.2	0	0
PEAK SCORE			

FIGURE 20

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for one naive iron loaded rabbit receiving 0.1 mg/Kg ceftriaxone on day 4 post inoculation.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterix - last culture positive day

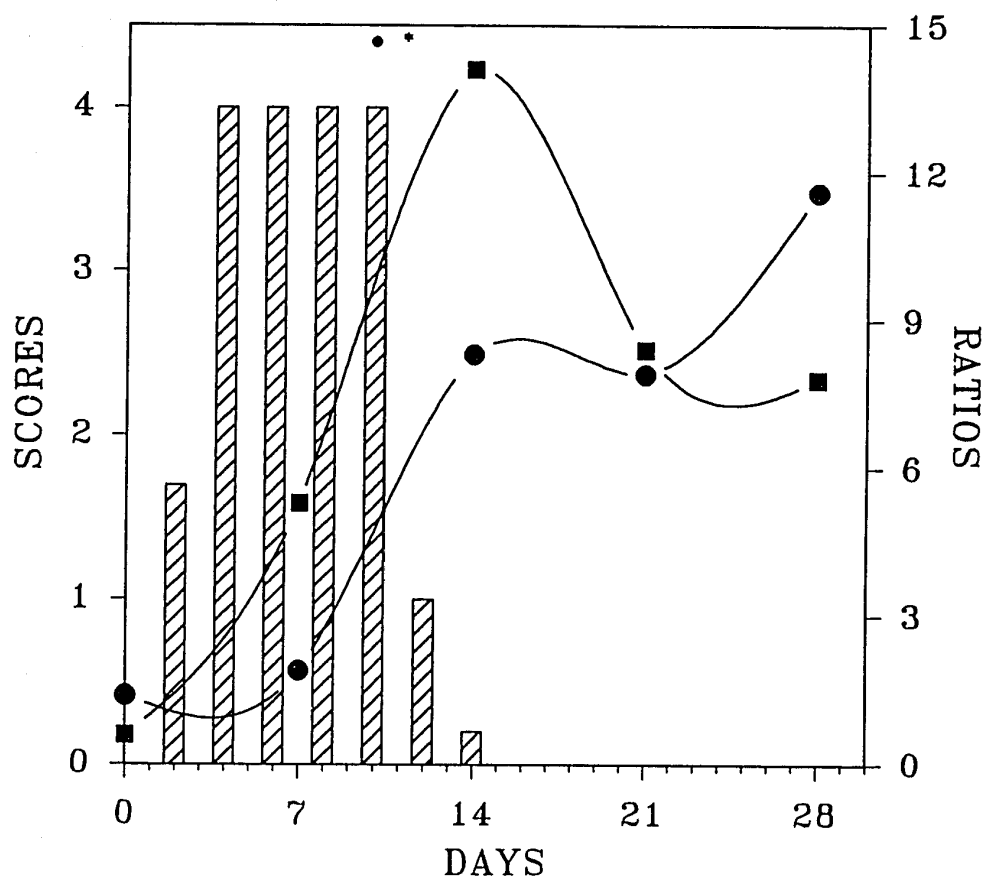
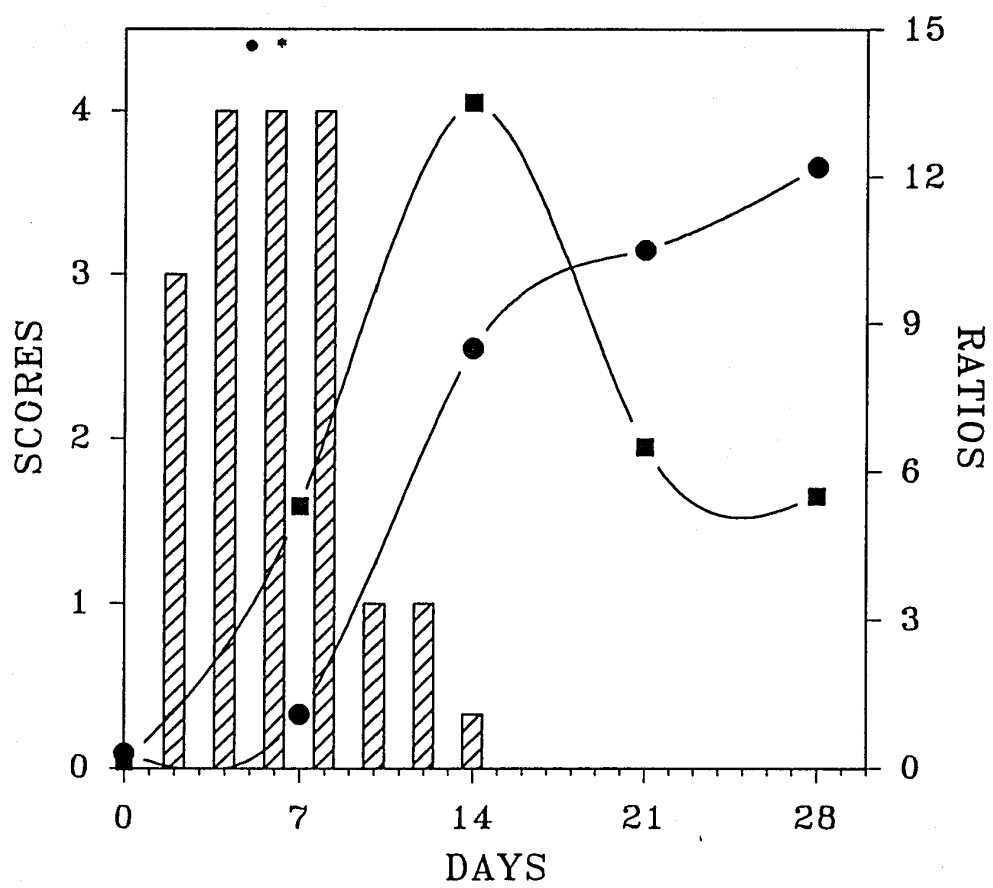


FIGURE 21

Disease scores at 10^5 CFU *H. ducreyi* inoculation and serology for one naive iron loaded rabbit receiving 5 mg/Kg ceftriaxone on day 4 post inoculation.

Bars - mean scores
Closed squares - IgM response
Closed circles - IgG response
Asterix - last culture positive day



DISCUSSION

Animal models for *Haemophilus ducreyi* infection

Various tools are required to study pathogenesis of an infectious disease. One of these is the development of an assay for measuring the virulence of the pathogen, which is its ability to produce a specific disease in a particular host. A relevant and useful animal model of infection must have certain desirable traits: (1) The disease produced in an animal host must resemble the natural human disease; (2) The size of inoculum producing the disease must be low enough to approach that in nature; (3) The route of inoculation must be similar to that of the natural infection; (4) The model must differentiate between virulent and avirulent strains of the pathogen and; (5) The animal model must be reproducible and measurable i.e. a quantifiable disease effect and comparative statistical methods are required (Smith 1989). Trouble arises, however, when animal models for human diseases are of questionable validity because of unnatural routes of infection and differences in syndromes produced.

Subsequent to the discovery of *Haemophilus ducreyi* as the etiologic agent of chancroid, numerous attempts were made to produce the disease in an animal model for this infection. Most investigations focused on a rabbit model of *H. ducreyi* infection, although recent attempts were made to develop a mouse model of infection. Early rabbit models of infection

were not adequate because they did not fulfil any of the requirements which define a good animal model of disease. The disease effect produced in these animal models did not resemble that of the natural human infection, and the inoculum used to produce disease was either high (10^9 CFU) or unmeasured (Odumeru et al. 1984, Odumeru et al. 1985, Odumeru et al. 1987, Feiner 1945 and Kaplan 1956). Although the disease effect produced in the mouse model was similar to the natural human infection, the inoculum size was too high: 10^7 CFU were required to produce disease (Tuffrey et al. 1988 and Tuffrey et al. 1990). As well, disease was not specific, in fact it was independent of infection as skin ulceration was produced by killed inocula.

An important difference between these models of infection and the natural infection lies in the route of inoculation. The portal of entry of *H. ducreyi* is presumably a break in the epithelium. It is around this break in the epithelium that the first pathological changes, in the form of edematous and swollen epithelial cells with an infiltration of polymorphonuclear leucocytes, are observed (Morse 1989). The rabbits and mice used in earlier investigations were all inoculated intradermally which in bypassing the epithelium avoids the natural portal of entry of *H. ducreyi* (Feiner 1945, Kaplan 1956, Odumeru et al. 1984, Odumeru et al. 1985, Odumeru et al. 1987, Tuffrey et al. 1988, and Tuffrey et al. 1990).

Finally, although these models appeared to be

reproducible, they were not quantified and could, therefore, not be used as an in vivo virulence assay. These models were nevertheless used to define "virulent" and "avirulent" strains of *H. ducreyi* based upon the ability to produce skin lesions in the animal host (Odumeru et al. 1984).

Purcell et al. (1991) developed a rabbit model of *H. ducreyi* infection which differed from other models by being temperature dependent. It was demonstrated that housing rabbits at low ambient temperature permitted skin infection and disease at low inoculum (10^5 CFU) that resembled the natural disease and that was dependent upon viability of the inoculum. This corroborated the temperature dependence of in vitro growth. Although this model was promising, it still lacked certain requirements. Even though this model was shown to be reproducible, it was not quantified in a manner permitting statistical comparisons of various parameters of virulence. Thus the temperature dependent rabbit model was a model of infection, not a quantitative virulence assay appropriate for study of immunopathogenesis, through comparative virulence, and inducible immunity.

Intraepithelial rabbit model of *H. ducreyi* infection

The temperature dependent rabbit infection model developed for measurement of disease in this study possesses the qualities of a good animal model of disease. The disease effect produced resembles the natural infection, the minimum

disease-producing inoculum was consistently 10^5 CFU, and the route of inoculation was intraepithelial, not intradermal as in previous studies. This animal model of disease is amenable to application because of its biological sensitivity, consistency and reproducibility. It is quantifiable according to relevant chosen parameters for measurement and statistical comparison of virulence. The scoring system used is reliable and makes quantitation of several relevant disease parameters possible. A major parameter of infection that showed replicability was duration of culture positivity of the lesions; this is easily quantifiable. In control rabbits lesions remained culture positive until approximately day 7. Furthermore, infection with heat killed inoculum did not result in the production of disease which suggests that bacterial antigenic or toxigenic content is not sufficient in itself to promote lesion development. However, because of the possibility that heat labile toxins or their toxic products may be destroyed by heat other controls should be sought. Hansen et al. (1991), when they proposed the temperature dependent rabbit model, pre-treated rabbits with ceftriaxone one hour prior to primary infection. No disease resulted. Similarly, in the present study, rabbits pre-treated with ceftriaxone one hour prior to secondary infection did not develop ulcers. Therefore, we can conclude that viable organisms are required to produce disease.

The validity of this animal model was also demonstrated

by the IgM and IgG responses observed. The IgM response was dominant in the first week of the infection whereas IgG predominated after the first week and remained elevated. These observations are typical of a primary infection with other microorganisms. Concerning virulence and the pathogenesis of chancroid, sterilization of lesions coincidental with peak antibody response and convalescence suggest relevance of inducible immunity in the natural course of this infection. The animal model of infection used here as a replicable in vivo quantitative virulence assay, will be useful in evaluating potential antibiotic treatments, pathogenesis, and inducible immunity to *H. ducreyi* infection relevant to human chancroid.

This quantitative assay which permits statistical comparison of virulence, can be applied to various situations. The temperature dependent rabbit disease assay was used to (1) Demonstrate virulence modification according to host factors; (2) Compare measurement of virulence between strains; (3) Study immunopathogenesis in re-infection experiments; and (4) Demonstrate inducible immunity.

Virulence modifications according to host factors

Modifying given host factors changed virulence of *H. ducreyi*. Iron is a bacterial growth factor, and was expected to promote infection without a major impact upon immune response. Dexamethasone is a potent immunosuppressant and

antiinflammatory agent which would be expected to attenuate disease produced by inflammation, and delay convalescence dependent upon immune clearance of infection. Expected changes occurred in each case which further demonstrated the validity and relevance of this in vivo virulence assay.

Observations in iron loaded animals

Prior treatment of rabbits with iron-dextran significantly increased the disease effect; lesions are increased in size and severity and the survival of the microorganism was significantly prolonged. Nevertheless, antibody peak responses, healing and convalescence were not delayed. Increased virulence related to iron overload may be related to iron as an important bacterial growth factor particularly for *Haemophilus ducreyi* which has a large requirement for iron when grown in vitro (Morse 1989 and Lee 1991).

Iron is an important growth factor for many bacteria, and vertebrate animals have developed an elaborate and efficient stratagem to withhold growth-essential iron from microbial invaders while retaining their own access to the metal. Iron stores are primarily located in two tissues, the reticuloendothelial system and the hepatic parenchyma. Iron stores are also present in macrophages. During an active infection, plasma iron levels are decreased and concomitantly accumulate in liver and spleen for short-term sequestration.

The release of iron from macrophages has been observed to be blocked in rodents injected with pathogenic microorganisms (Bothwell et al. 1979 and Weinberg 1984).

It has been demonstrated on numerous occasions that added iron enhances the ability of designated microbial strains to grow in host fluids, cells or tissues (Weinberg 1984). This was observed in the present study: loading a rabbit with 750 mg/Kg iron dextran significantly enhanced the ability of *H. ducreyi* to grow in vivo and produce disease. Although the most obvious and most frequently demonstrated mechanism whereby excess iron enhances infection is that of serving as a nutrient for the invading pathogen, other modes of action have been proposed to contribute to the weakening of host defense (Weinberg 1984). For example, in in vitro studies, iron in amounts greater or equal to 125 μM was shown to inhibit the phagocytic capacity of monocytes (Van Asbeck et al. 1982); iron may inhibit monocytes by suppressing the phagocytosis-associated metabolic burst as well as by inactivating peroxide (Kaplan and Basford 1979). Concentrations of iron between 10 and 100 μM can suppress the expression of T-cell determinants and the normal migration of B and T cell lymphocytes from the blood into the lymph nodes and spleen (Nishiya et al. 1979). Inasmuch as iron-binding proteins like lactoferrin and ferritin regulate bone marrow cell differentiation and immunological capability, iron overload could have a modulating effect on these vital host-

defense functions (De Sousa 1983).

Therefore, one could explain the longer duration of *H. ducreyi* infection in iron loaded rabbits by both a modulation of the host defense system and an excess of iron as a growth factor in the host (Lee 1991). Effects of iron loading on B and T lymphocytes, as well as the alteration in virulence itself, might account for the higher IgM response observed in iron loaded rabbits.

Single oral dose antibiotic therapies for human chancroid is a practical advantage in treatment of cases. The production of measurable disease and persistent culture positivity over a period of two weeks in iron loaded rabbits may offer a more sensitive model in which to study limitations and synergy of antibiotic treatments.

Observations in dexamethasone treated animals

Prior and continued treatment of rabbits with dexamethasone increased the virulence of *H. ducreyi*. The onset of disease was delayed until the end of the first week, survival of the microorganisms in the lesions was considerably extended, and convalescence was delayed along with the peak and increased level of antibody response.

Dexamethasone is a steroid which has many antiinflammatory and modulatory effects on the immune system which in the end leads to complex immunosuppression (Flower 1989). Dexamethasone inhibits the ability of the synthetic

chemotaxin N-formyl-methionine-leucine-phenylalanine to increase human granulocyte adherence to endothelium. It also substantially inhibits release of lactoferrin by cytochalasin-B treated neutrophils but fails to prevent augmented neutrophil aggregation and adherence to endothelial cells promoted by plasma concentrations of lactoferrin or following addition of rabbit or human lactoferrin in vitro (Butterfield and Gleich 1989). Dexamethasone also has inhibitory effects on anti-IgE-mediated histamine release (Schleimer 1989), production of inflammatory mediators in monocytes and macrophages (Guyre and Munck 1989) and on phospholipid hydrolysis and prostaglandin synthesis (Flower 1989).

Despite a vast amount of research, the mechanism of action of this corticosteroid remains unclear. It is known to suppress both cellular and humoral immune responses at a degree proportional to the concentration of compound at the site of inflammation. To prevent inflammation, dexamethasone blocks the permeability of the capillary endothelium reducing the leakage of fluid and the transport of proteins into the area of injury. The exudation of macrophages and other cells is also inhibited, and lysosomal membranes are stabilized. The reticulo-endothelial clearance of antibody-coated cells is also hindered, and prostaglandin production suppressed by the inhibition of arachidonic-acid release from membrane phospholipids. Dexamethasone reduces the number of circulating lymphocytes as well, particularly T lymphocytes

(Brooks et al. 1986).

In humans, clinical and epidemiologic evidence suggests that *H. ducreyi* has increased virulence in HIV associated immunodeficiency (Cameron et al. 1988 and Cameron et al. 1991). Significantly prolonged infection in the presence of immunosuppressive dexamethasone treatment corroborates the importance of immunocompetence in the resolution of *H. ducreyi* infection. Slightly delayed onset of ulcerative lesions in dexamethasone immunosuppressed rabbits suggests that the inflammatory response of the host may also be an important component of pathogenesis, or ulcer production. Significant delay in healing of lesions validates the relevance of infection to disease, and immunocompetence to convalescence.

Rabbits treated with both iron dextran and dexamethasone suffer from a disease greater than in rabbits treated with dexamethasone only but lesser than rabbits loaded with iron. Disease onset in animals treated with both compounds was delayed, perhaps due to presence of dexamethasone but the lesions were much larger in these animals than in those treated only with the steroid. However, as stated above, both iron and dexamethasone may cause modulation of the host immune system. The overall effect of immunosuppression may have been responsible for the systemic illness observed in animals treated with both compounds, whether it may have been related to the experimental infection or not.

Thus, examination of the statistical comparison of

expected modifications of virulence by iron overload and by dexamethasone immunosuppression, validates the infection model as relevant to disease, and validates our system of measurement of virulence as a useful assay.

Re-infection experiments

In humans repeated episodes of chancroid are common (Ronald 1989). Re-infections in the same host, and production of satellite lesions by autoinoculation during the course of a single episode of chancroid suggest that immunity may be incomplete, delayed in onset, transient or highly specific.

Experiments done by others on rabbits, concluded that the animals were not immune to re-infection with live bacteria and when re-infected with dead *H. ducreyi*, these animals developed a skin hypersensitivity to killed organisms. It was also observed that re-infection resulted in an enhanced reaction, characterized by a large erythematous zone surrounding the intensely red lesions (Feiner et al. 1945 and Kaplan et al. 1956).

Our results reported here confirm these earlier observations. Paradoxically, although re-infection failed to establish culture positive lesions, the lesions themselves were significantly of longer duration and size, and were as frequently ulcerating. Re-infection in the temperature-dependent rabbit model does not prevent the production of ulcers at high inocula and causes increased disease effect.

However, these experiments suggest that some degree of inducible immunity to infection itself exists in this animal model. Production of disease upon re-infection suggests that disease production in this model may be related to inflammation by the host, as well as to the infection per se.

Immunization experiments

Although re-infection experiments suggested that immunity associated with prior infection may be non-protective, vaccine development may still be feasible. "Artificial" immunization may confer disease protection not conferred by primary infection. In this study, rabbits which had been previously exposed to *H. ducreyi* #35000 via intramuscular injections of bacteria with adjuvant to produce strong serologic response were challenged after "vaccination". Inoculation of immunized rabbits produced sterile inflammatory lesions which did not ulcerate at 10^5 CFU inocula, as in primary infections of antigen-naive rabbits. These lesions resolved significantly faster than in naive controls, and in re-infected rabbits. This suggests that inducible immunity to infection and resistance to disease exists in this animal model. The specificity and nature of this inducible immunity still remain to be elucidated.

Inoculation with *Haemophilus ducreyi* strain #v1157

Strains of *H. ducreyi* have been classified as either

virulent or avirulent depending on their ability to form skin lesions in the rabbit intradermal test (Odumeru 1984). *H. ducreyi* #35000 is considered a virulent strain whereas *H. ducreyi* #v1157 can be considered as a lesser virulent strain of the pathogen. Both in vivo and in vitro differences are seen between these two strains. *H. ducreyi* #v1157 was shown to grow at a slower rate than *H. ducreyi* #35000 in vitro. *H. ducreyi* #v1157 reaches its peak growth after 20 hours in an incubated broth culture. *H. ducreyi* #35000 reaches its peak growth in 16 hours. CFU at peak growth are approximately the same for both strains. When growth is compared in vivo, strain #35000 causes a much more severe disease effect than strain #v1157; in fact, a peak score of 2 is obtained and no ulcers are formed in a naive rabbit infected with *H. ducreyi* #v1157, compared to peak score of 4 for *H. ducreyi* #35000. The slower rate of growth in vitro of *H. ducreyi* #v1157 may be related to its lesser virulence in this model. Host defenses are more able to cope with a slower growing pathogen than a faster one (Smith 1989); this could explain the lesser severity in the disease caused by *H. ducreyi* #v1157. Alternate explanations may be identifiable among biological tests on panels of "virulent" and "avirulent" strains in this model (Smith 1989). The measurable difference in disease and the failure to establish measurable infection corroborates the relevance of infection to disease in this model, as well as the validity of quantitatible measurement.

Re-infection of rabbits with strain #v1157 produced a similar effect as observed for strain #35000; the disease effect is amplified upon homologous re-infection with strain #v1157. Lesions remained sterile, but were larger than those observed for the primary infection. The peak score in re-infection increases to 3 compared to 2 for the primary infection. This again suggests that the disease effect observed upon re-infection may be more related to an augmented inflammatory response, than to infection established by *H. ducreyi* #v1157.

Antibiotic experiments

Naive rabbits

Many compounds have been used in the past in the treatment of chancroid. Single oral dose therapies are much favoured over long term regimens for efficacious therapeutic and effective disease control efforts based upon therapy. Furthermore, compounds which can eliminate more than one STD at a time are also important. Because of the emergence of plasmid-mediated resistance to various antibiotics, namely the penicillins and sulfonamides, the search for alternate treatment regimens for chancroid is necessary.

Trimethoprim-sulfamethoxazole (TMP-SMX) has been advocated as the treatment of choice for chancroid (Taylor et al. 1985). However, higher rates of failure of this combination are arising due to resistance of *H. ducreyi* to

sulfonamides (Plummer et al. 1983). Erythromycin in dosages of 500 mg three or four times daily for seven to 10 days is one of the few other treatment regimens, apart from ceftriaxone, that has proven to be effective worldwide.

Treatment failure rates of several antibiotic therapies are higher among HIV infected individuals including TMP-SMX, quinolone, ceftriaxone and erythromycin (Cameron et al. 1988, Mc Donald et al. 1989, Tyndall et al. 1991, and Ronald et al. 1992). The importance of single dose or short course therapies remains, and has great relevance in HIV co-infection for control of chancroid not to mention HIV transmission. In vitro antimicrobial susceptibility testing is laborious and unstandardised. In vitro antibiotic synergy and antagonism are often not relevant to in vivo conditions. An in vivo antibiotic susceptibility assay, particularly one which can identify the threshold of efficacy is important. One can study potential antibiotic combinations which through antibacterial synergy may be used to re-establish single-dose combination therapy for efficacious treatment of human chancroid in the presence of HIV co-infection.

Ceftriaxone is a parenteral cephalosporin which displays activity against Gram-positive and Gram-negative bacteria as well as enhanced stability against various types of β -lactamases. The pharmacokinetic characteristics of ceftriaxone have been defined in normal healthy human subjects after administration of single and multiple intravenous doses.

The elimination half life of ceftriaxone was shown to range from 5.9 to 8.8 hours, which is considerably longer than the half life range (0.6-3.0 hours) reported for other experimental and marketed cephalosporins (Patel et al. 1982, Patel et al. 1981 and Richards et al. 1984).

Ceftriaxone was shown to be 100% successful for the treatment of chancroid in Thailand and may be considered an important alternative to erythromycin because it can be given as a single dose. It is also effective for the treatment of penicillinase-producing *Neisseria gonorrhoeae* at the same or lower doses (Taylor et al. 1985, Hadsfield et al. 1983 and Le Saux and Ronald 1989). Ceftriaxone is also very effective at treating syphilis (Johnson et al. 1982 and Le Saux and Ronald 1989). Ceftriaxone was shown to be effective against experimentally induced syphilis in rabbits. Treatment with ceftriaxone was initiated after 7 to 10 days, when lesions were culture positive. The 50% curative dose for ceftriaxone was found to be 0.96 mg/Kg in rabbits infected with *T. pallidum* (Johnson et al. 1982).

In this study, naive rabbits were given 0.1 mg/Kg and 5 mg/Kg ceftriaxone 4 days post-inoculation; control rabbits receiving no antibiotic were tested in parallel. Within 24 hours no viable bacteria could be recovered from the lesions in rabbits treated with either dose of ceftriaxone. Therefore, ceftriaxone seems to be more effective at eliminating *Haemophilus ducreyi* than *T. pallidum* from rabbit

lesions. The healing process is also accelerated with antibiotic treatment, as measured in our quantitative assay.

The presumed mechanism of action of β -lactam antibiotics such as ceftriaxone is inhibition of peptidoglycan synthesis. The resulting disruption of cellular function is manifested as blebbing of the cell outer envelope, which is peripheral to and surrounds the peptidoglycan layer. In the case of the spirochetes, blebbing of the outer envelope usually precedes cell death (Johnson *et al.* 1982).

Iron loaded rabbits

Administering ceftriaxone to iron loaded rabbits as a single regimen was less effective at clearing the bacteria than it was for naive control rabbits. Although 0.1 mg/Kg ceftriaxone was enough to sterilize lesions in 24 hours in control rabbits, 10 days were required for the same dose to sterilize the lesions in iron loaded rabbits. 5 mg/Kg ceftriaxone sterilized the lesions in 48 hours compared to 24 hours for controls.

Iron loading a rabbit, or dexamethasone treatment is not analagous to human HIV associated immunodeficiency. However, the impact of a virulence-modifying host manipulation, with reduction of microbiologic and clinical efficacy of otherwise efficacious antibiotic treatment to the threshold of efficacy, is particularly useful. This assay may be used to compare in vivo relative efficacy of antibiotic therapies, and identify

in vivo synergy of combinations which may be highly relevant to human chancroid treatment in the setting of HIV co-infection. The objective of such future experiments would be to direct the re-development of effective new agents or combination treatments amenable to single-dose and short-course therapy.

Conclusions

In summary, the objective of applying a system of measurement to an animal model of infection producing a quantitative virulence assay was achieved. Through statistical comparison of virulence, we measured the impact of host factors on infection and disease. Results have validated the assay as a measurement of infection and disease, and shown the existence of inducible immunity in this model. Besides applicability of the assay to vaccine development strategy in future studies, we have shown that this assay will be applicable as an in vivo antibiotic susceptibility assay, to the urgent need for more effective treatment of chancroid in persons with HIV. As increased virulence lends itself to comparative measurement, iron loading may be of value in this assay. Both prevention and treatment of chancroid are highly relevant to STD and HIV public health internationally.

This virulence assay may be applicable as a fundamental component of future studies on pathogenesis. Caution is required in application of an animal infection to modelling of

molecular pathogenesis in humans.

Relevance of a specific bacterial or host factor in human disease must be sought to support its study in an animal model. Otherwise, confident interpretation of findings beyond the animal model is not possible. Nevertheless, with care in posing questions and experimental design, this virulence assay may be useful in understanding aspects of human chancroid pathogenesis.

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APPENDIX

Haemophilus ducreyi broth culture:

Mix 22 g Mueller Hinton broth with 1 L dd H₂O. Heat with frequent agitation and boil for one minute to completely dissolve powder. Autoclave.

Mueller Hinton broth	20 mL
Alpha Minimal essential medium	20 mL
Fetal bovine serum	2 mL

Haemoglobin solution:

Mix 10 g haemoglobin (dried bovine hemoglobin, Becton Dickinson, Cockeysville, M.D.) with 15 mL dd H₂O to make a paste then add rest of 500 mL of water until solution is mixed well. Autoclave.

GC agar base solution:

Mix 36 g GC agar base (Becton Dickinson, Cokeysville, M.D.) with 500 mL dd H₂O. Heat with frequent agitation and boil for one minute to completely dissolve powder. Autoclave.

Chocolate agar plates (CAP):

GC agar base solution	500 mL
Fetal bovine serum	50 mL
2% haemoglobin solution	500 mL
Isovitalex	10 mL

The sterile haemoglobin solution and GC agar medium were cooled to 50-60°C. The fetal bovine serum and the isovitallex were then aseptically added to the cooled GC medium, and then the sterile haemoglobin solution added to the medium. Gentle agitation would then produce a uniform medium which could be dispensed into petri plates.

Injections with Freund's complete/incomplete adjuvant:

A 16 hour *H. ducreyi* #35000 broth culture was grown. 10 mL of culture were spun down and resuspended in *H. ducreyi* broth.

0.5 mL of suspension plus 0.5 mL of adjuvant were emulsified with a 3 way stop-cock. 0.5 mL are injected intramuscularly into a rabbit.

Ceftriaxone preparation:

1 g of ceftriasone was mixed with 9.6 mL dd H₂O to obtain 0.1 mg/mL. Two 1:10 dilutions were made. 0.25 mL of 1 mg/mL solution was injected intramuscularly to obtain a 0.1 mg/Kg dose. 1.25 mL of 10 mg/mL solution was injected intramuscularly to obtain a 5 mg/Kg dose.

PBS plus 1% SDS:

1 g sodium dodecyl sulfate (BioRad Laboratories, Richmond, C.A.) was mixed in 100 mL PBS to obtain a 1% solution.

0.1 M carbonate buffer:

3.18 g Na₂CO₃ (BDH Chemicals, Toronto, Ontario) were mixed in 300 mL dd H₂O.

2.52 g NaHCO₃ (Baker Chemicals, Philipsburg, NJ) were mixed in 300 mL dd H₂O.

Approximately 100 mL of Na₂CO₃ solution was combined with approximately 175 mL NaHCO₃ and, if necessary, the pH was adjusted to 9.6.

Citrate buffer:

Citric acid (Fisher)	1.11 g
Na ₂ HPO ₄ (BDH)	0.117 g
dd H ₂ O	80 mL

pH to 4.25 exactly with 1M NaOH then dilute to 100 ml with dd H₂O.

1 M NaOH:

Mix 4 g NaOH (AnalaR) with 100 mL dd H₂O.

Substrate solution for EIA:

Citrate buffer	10 mL
H ₂ O ₂ (Fisher)	20 µL
ABTS	4 mg
dd H ₂ O	10 mL