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IN VITRO ANTIMICROBIAL INTERACTIONS AND *IN VIVO* SYNERGY OF
CEFTRIAZONE AND STREPTOMYCIN AGAINST *HAEMOPHILUS DUCREYI*

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

By
Josée Roy



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ABSTRACT

Haemophilus ducreyi is the etiologic agent of chancroid, a classic sexually transmitted genital ulcer disease. In endemic areas, HIV and chancroid interact in a complex, bidirectional epidemiologic and biologic manner so as to amplify the prevalence of each other. Chancroid outbreaks can be controlled with the help of efficacious antibiotic treatment. The ideal treatment for chancroid should be effective, inexpensive, well-tolerated and for reasons of compliance, capable of being administered as a single dose. Single doses of ceftriaxone, azithromycin and fleroxacin have been used successfully. However, because of HIV associated immune disease, an unacceptable failure rate of over 20% has been observed with concurrent HIV infections, and this, in the absence of *H. ducreyi* drug resistance.

In this study, two-drug combinations of ceftriaxone, streptomycin, azithromycin and rifabutin were assayed *in vitro* by the checkerboard agar dilution technique for their activity against *H. ducreyi* strains proven susceptible by limiting agar dilution minimum inhibitory concentration determination. The ceftriaxone-streptomycin combination proved to be supra-additive in activity with a fractional inhibitory concentration of 0.63. The *in vivo* comparative activity of each drug and of the combination was tested in the temperature-dependent rabbit model of infection. Rabbits, grid-inoculated in triplicate with titred inocula from 10^6 to 10^2 colony forming units or with a single inoculum level of 10^4 CFU were treated with single intramuscular injections of subcurative doses of 0.05 mg/kg ceftriaxone, 10 mg/kg streptomycin, and with a combination of 0.05 mg/kg ceftriaxone + 10 mg/kg streptomycin. Controls which received no antibiotic treatment were also used. *In vivo* activity was

measured by the decrease in the duration of culture positivity of lesions, as well as by the decrease in lesion diameter and score. The combination exhibited significant activity while no therapeutic response to the single agents was observed. Lesions in the combination were culture positive for 2.60 days compared to the lesions in the controls and individual antibiotics that stayed positive for 7.00-7.50 days post-treatment. 5.80 days were required for the lesions in the combination to be reduced to half their size before treatment, compared to 8.25 to 9.67 days for the other groups. Finally, 6.60 days were required for the lesions to reepithelialize (to reach a score of 2) when the combination of antibiotics was administered, while 9.38 to 11.67 days was the time needed for the same process to occur in the control and individual antibiotic groups.

We conclude that a supra-additive effect observed between ceftriaxone and streptomycin *in vitro* by the checkerboard technique was corroborated by a synergistic effect observed between the same antimicrobial agents in the temperature-dependent rabbit model of infection. This combination may be evaluated as a single-dose in the treatment of chancroid in all patients, particularly those with HIV coinfection.

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I wish to thank my parents from the bottom of my heart for supporting me in all of my decisions and for believing in me.

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*This thesis is dedicated to my parents Rose and Hector, my brother Gilbert, and
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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CA	Chocolate agar
CFU	Colony forming unit
CPE	Cytopathic effect
CVA	Coenzymes-vitamins-amino acids
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
FIC	Fractional inhibitory concentration
GUD	Genital ulcer disease
h	Hours
HFH	Human foreskin fibroblasts
HIV	Human immunodeficiency virus
LOS	Lipooligosaccharide
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
MUPI	Minimum ulcer producing inoculum
OMP	Outer membrane protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

INTRODUCTION

I. *Haemophilus ducreyi*

1. Historical background

Haemophilus ducreyi was recognized as the cause of soft chancre or chancroid in 1889 by Auguste Ducrey. By repetitive serial cutaneous inoculation of organisms obtained from a cleansed soft chancre or from an intact bubo, he showed that the same organism was recovered within tissues or from the secretion of lesions. He described a bacillus with rounded ends that grew in chains especially in tissue but that could also appear as single cells in secretions. Bassereau, a pupil of Ricord had concluded in 1852 that two kinds of chancres existed: a firm chancre and a soft chancre. Exudate from the latter could be mechanically re-inoculated in the patient to form lesions, while this wasn't true for the former syphilitic chancres. Thus, Ducrey's work confirmed that the soft chancre was the result of infection with an organism other than the one causing syphilis (Albritton 1989; Morse 1989; Kampmeier 1982).

Ducrey was unable to culture the causative agent of chancroid on artificial media. However between 1895 and 1900 several investigators succeeded in this task by using media consisting of macerated human skin and agar. In 1900, Bezancon et al. isolated the organism on blood agar alone and were able to reinoculate it in humans to produce soft chancres. Nevertheless, persisting difficulty in isolating *H. ducreyi* prompted many efforts in the development of other diagnostic methods. The late 1960's and early 1970's marked a decrease of interest in chancroid and *H. ducreyi*, that was to be renewed in the late 1970's and

early 1980's. This is when isolates became available from several chancroid outbreaks in North America and sporadic cases in Europe, Asia and Africa (Albritton 1989). Subsequently, there has been an explosive increase of interest in *H. ducreyi* and chancroid due to its association and interaction with HIV (Cameron and Padian 1990).

2. Taxonomy and growth requirements

H. ducreyi is classified in the genus *Haemophilus* because of its requirement for the X factor (haem) for growth. The need for X or/and V (nicotinamide adenine dinucleotide) factors for growth places an organism within the genus *Haemophilus* according to the present definition, although this is no longer restricted to members of the genus *Haemophilus*, but rather to the *Pasteurellaceae* family. Its guanine-plus-cytosine content although, classifies it as part of the *Haemophilus* genus (Morse 1989; Albritton 1989). In 1988, *H. ducreyi* was found to possess the structure types demethylmenaquinone and menaquinone. The presence of these represents a major physiological and chemotaxonomic difference between this organism and other species of the same genus (Carlone et al. 1988). Furthermore, using DNA-DNA hybridization (Casin et al. 1985), different *Haemophilus* species were shown to be 0 to 6% related to *H. ducreyi*.

More evidence corroborating the unrelatedness of *H. ducreyi* to other *Haemophilus* species include its possibly unique mode of iron acquisition and unique haemoglobin-binding protein (Elkins 1995; Lee 1991). *H. ducreyi* possesses an obligate requirement for haem at concentrations of 25 to 50 µg/mL, which is much higher than the quantities required by other haem-dependent *Haemophilus* species (Morse 1989). The pathogen can only obtain its iron

from haem compounds, free or bound to serum carrier proteins, and does so in a way that may be unique amongst *Haemophilus* species. It produces no siderophores (low molecular weight iron-chelators that solubilise and bind iron prior to its internalisation) and possesses no functional receptors for iron-binding glycoproteins such as lactoferrin and transferrin (Lee 1991). However, it has a 100 kDa, surface-exposed, haemoglobin-binding outer membrane protein whose expression is regulated by haem and shares no homologies with other proteins of the *Haemophilus* genus (Elkins 1995). Considering all these differences between *H. ducreyi* and other members of its genus, it would seem that a new taxonomic assignment is needed for the organism. A consensus likely awaits further developments in knowledge of *H. ducreyi* or change in taxonomy.

3. Basic structure

H. ducreyi appears as small, nonmucoid, yellow-grey, semiopaque, adherent colonies on solid media. Because of adherence of cells within the colony, this latter can be pushed intact across the agar surface. The organism is a Gram negative pleomorphic, streptobacillus 1.2 to 1.5 μm in length, 0.5 μm in width and with rounded ends. In liquid culture or tissue the cells appear as characteristic parallel chains best described as “railroad tracks”, while on solid agar they form “schools of fish” and “fingerprints” (Albritton 1989). In liquid culture large agglomerations of cells can also be observed and these adhere to mammalian cells when co-cultured with them (Alfa et al. 1995).

4. Virulence factors and pathogenesis

The pathogenicity of *H. ducreyi* is poorly understood, but by identifying its virulence factors, it may become more clear. In other species of bacteria, cell wall components are known to be important determinants of pathogenicity, and some of these have been examined in *H. ducreyi* (Johnson et al. 1988). Electron microscopy of *H. ducreyi* reveals the typical appearance of a Gram negative cell wall with a cytoplasmic membrane surrounded by a rugose membrane. The bacterium also possesses a micro-capsule, a discontinuous distribution of exocellular material which can be stabilized by polyvalent rabbit antiserum (Albritton 1989; Johnson et al. 1988). Pili were isolated from all strains examined by Castellazo et al. (1992) and most could be examined by transmission electron microscopy, appearing as fine and tangled appendages.

Bacterial adherence is an important step in initiation of infection and is usually a specific event between eukaryotic receptors and bacterial adhesins (Brentjens et al. 1994). *H. ducreyi* adheres to fibroblasts, several carcinoma cell lines and keratinocytes, all of human genital origin. It also binds to extracellular matrix proteins of bovine and human origin (Totten et al. 1994a; Brentjens et al. 1994; Lammel et al. 1993; Alfa et al. 1993; Lagergård et al. 1993; Abeck et al. 1992). It is unclear if virulence as determined in the temperature-dependent rabbit model of infection (Purcell et al. 1991) dictates the degree of attachment to cells, as mixed results have been obtained (Lammel et al. 1993; Alfa et al. 1993). It is also unknown if pili present at the surface of *H. ducreyi* cells are responsible for attachment. Adhesins are a common and important aspect of most pathogenic bacteria that fall in two broad categories: pili and other surface proteins (Finlay and Falkow 1989). Pili in *H. ducreyi*

were described by Castellazo et al. (1992) but other groups have failed to reproduce these findings. However, Alfa et al. (1993) described a fibrillar matrix that could possibly correlate to the mesh of pili previously described. Additionally, Lagergård et al. (1993) was able to purify the 24 kDa protein identified by Spinola and coworkers (1990) as the pilin monomer.

The outer membrane protein profile of *H. ducreyi* is typical of Gram negative bacteria with one to several proteins predominating (Morse 1989). The surface-exposed major outer membrane protein (MOMP) whose molecular weight was defined to be between 39 to 42 kDa (Odumeru et al. 1983; Abeck et al. 1988b) was recently studied by Spinola et al. (1993), who suggested that it possessed different conformations accounting for the range in molecular weights. The MOMP was also found to belong to the Omp-A family of proteins and subsequently determined to be structurally and antigenically related to the Omp-A proteins of members of *Pasteurellaceae*. Because it can be modified by heat contains cysteine residues and is cationic at a pH of 8.0, it may not be a classic porin protein. This antigenically conserved protein in the outer membrane of *H. ducreyi* may be responsible for stabilizing the membrane and conferring serum resistance on the organism. OmpA may alternatively bind antibodies that block serum bactericidal activity (Spinola et al. 1993).

Another protein that has been characterised, is the 18 kDa surface-exposed protein possessing an epitope that is conserved in the *Pasteurellaceae* family. Because it is conserved it may be important in bacterial survival on mucosal surfaces *in vivo* (Spinola et al. 1992). A 28 kDa outer membrane protein was cloned by Stewart et al. (1992) and found to be common to all *H. ducreyi* strains examined. Four other polypeptides (A-D) were characterized and found to be exclusive to *H. ducreyi*. They are not outer membrane proteins

but may nevertheless have an active role in host-parasite interaction. The C and D polypeptides are soluble and may thus act as secreted or released virulence factors (Alfa et al. 1992).

Lipooligosaccharides (LOS) are one of the main constituents of the outer cell envelope of Gram-negative bacteria. Purified LOS from *H. ducreyi*, were found to cause skin lesion formation when tested in the classical (non temperature-dependent) rabbit model and the mouse model (Campagnari et al. 1991; Tuffrey et al. 1990). Virulent and avirulent strains as defined by the classical rabbit model were shown to differ in the complement-mediated bactericidal effect of human and rabbit serum. Virulent strains were resistant to the bactericidal action of serum while avirulent strains were not resistant (Odumeru et al. 1984). Differences in the core polysaccharide of LOS have also been documented. The total glucose:KDO ratio of the LOS of virulent strains exceeds that of the avirulent strains. In the same study, differences in the electrophoretic mobility of the LOS of the two types of strains were revealed by SDS-PAGE analysis (Odumeru et al. 1987). The LOS of most *H. ducreyi* strains express an epitope which is also found on the LOS of many *Neisseria gonorrhoeae* and *Neisseria meningitidis* strains. In addition, the epitope is immunochemically similar to the terminal tetrasaccharide of paragloboside, a glycosphingolipid precursor of the major human blood group antigen. This suggests that through molecular antigenic mimicry *H. ducreyi* may be able to evade certain host immune defences (Trees and Morse 1995). Campagnari et al. (1994) recently used pyocin to select for pyocin-resistant strains. These strains exhibited a truncated LOS structure which lacked the terminal lactosamine and were no longer recognized by the monoclonal antibody 3F11 which had been used to define the

terminal LOS epitope. *N. gonorrhoeae* can modify its LOS structure by the addition of sialic acid thereby rendering it more serum resistant. There was an absence of sialic acid in the LOS preparations of the *H. ducreyi* mutant strains whereas it was present in the *H. ducreyi* original strain. This suggests that the terminal lactosamine structure may serve as the acceptor site for sialylation (Campagnari et al. 1994). The presence of the epitope is unclear but its presence on microbial surfaces could mask the pathogen on the mucosal surface and blunt the host immune response. Alternatively, the region may function as an adherence factor that would mediate short-range attachment to and uptake by human cells (Campagnari et al. 1994; Melaugh et al. 1992). As discussed above, the factor(s) responsible for cellular attachment have not yet been elucidated. Mechanisms of *H. ducreyi* internalization are also unknown (Lagergård et al. 1993; Lammel et al. 1993).

Cell-free extracts of different strains of *H. ducreyi* have been shown to exhibit *in vitro* cytotoxic activity against human epithelial cells leading to cell death (Purven and Lagergård 1992). In contrast, Hollyer et al. (1994) found no diffusible cytotoxin (exotoxin) activity to occur on human foreskin fibroblasts (HFF). In fact, they showed cytopathic effect (CPE) to be solely contact-dependent in nature. In a follow-up study Lagergård et al. (1993) also showed that CPE and cell death occurs by cytotoxin-producing bacteria bound to the epithelial cells. They maintain that adherence enhanced CPE that was regularly seen with just the diffusible cytotoxin. They suggest that adherence may constitute a first step in the pathogenic mechanism of *H. ducreyi* followed by cytotoxin action on the cells (Purven and Lagergård 1992). Another group hypothesized that LOS or membrane blebs have a possible role in CPE (Hollyer et al. 1994). A hemolytic activity that appears to differ from the

cytotoxic activity described above, was identified by Palmer et al (1994). Horse erythrocyte cells are the preferred target while human, sheep and rabbit cells are relatively poor targets. The haemolytic activity may prove to be toxic against human cells such as lymphocytes, macrophages, neutrophils, epithelial cells and cell lines (Palmer et al. 1994).

Alfa et al. (1995) set out to identify *in vitro* virulence-associated properties of *H. ducreyi*. Most strains studied had the ability to cause CPE in HFF and to form microcolonies on HFF monolayers. The ability to adhere to those same cells was also examined. Strains found to be deficient in one or more of these three phenotypic characteristics failed to produce lesions in the temperature-dependent rabbit model while strains positive for all three categories did. These characteristics may prove useful in predicting the virulence potential of other *H. ducreyi* strains and may help in efforts to determine the molecular basis of the virulence of the pathogen (Alfa et al. 1995).

5. Immunology

There is a paucity of information regarding the mechanisms of the immunologic response to *H. ducreyi* (Dejardins et al. 1995). Studies have suggested that no protective immunity develops during the course of infection (Albritton 1989). However, infection was protective to a certain degree in the temperature-dependent rabbit model as proven when rabbits were rechallenged with homologous and heterologous strains (Hansen et al. 1994). Protection was seen when the animals were immunized with *H. ducreyi* cell envelopes or a pilus preparation prior to infection (Desjardins et al. 1995; Hansen et al. 1994). In the rabbit, circulating antibodies can be detected as soon as one week post-infection, and serologic titres

and reactivity plateau at around 6 weeks to be sustained beyond 8 weeks (Marc Desjardins, personal communication). However, histologic characteristics of lesions in the temperature dependent rabbit model and in human experimental infections suggest that there is a cellular immune response that may be more important than humoral immunity (Lagergård 1995, Desjardins et al. 1995; Hansen et al. 1994; Spinola et al. 1994).

II. Chancroid

1. **Clinical and histopathological features**

After direct person to person contact, *H. ducreyi* enters the body through a break in the integrity of the epithelium. The first symptoms of disease usually appear between 4 and 7 days post-infection. The initial lesion is present around the break and is a small inflammatory papule surrounded by a narrow erythematous zone. This rapidly becomes pustular and ruptures to form a painful, sharply circumscribed ulcer with ragged undermined edges. The floor of the ulcer is irregular and has a granular appearance. The lesions are shallow and vary in size from 1 to 20 mm in diameter. They are very vascular and bleed easily on scraping (Morse 1989; Clarridge 1990). Ulcers usually resolve in the second or third week, but can persist longer. They are preponderantly localized on mucosal and moist parts of the genitals. Males usually have single ulcers more commonly found on the preputial orifice, the mucous surface of the prepuce, the frenulum and the coronal sulcus. Lesions are less common on the glans, the shaft of the penis or the anus. In women, ulcers are usually multiple and occur mostly at the entrance to the vagina. They can also be found on the labia, the fourchette, the vestibule and the clitoris. Painful, unilateral, inguinal lymphadenopathy

(bubo) occurs in up to 50% of patients and may become fluctuant and rupture, resulting in a draining abscess. This is less common in women probably because lymphatic drainage from the posterior portion of the vagina and the cervix is to the sacral nodes (Lagergård 1995; Mroczkowski and Martin 1994; Clarridge et al. 1990; Morse 1989).

Several varieties of chancroid have been described. Classic chancroid refers to infection with multiple ulcers each measuring 0.3 to 2 cm. These may be formed by autoinoculation and occur in 40% of infected men and 80% of infected woman. Giant ulcers are formed when several smaller ulcers merge. The transient chancroid is typical of rapidly resolving ulcers followed by acute regional lymphadenitis with suppuration in 10 to 20 days. Others include the follicular type that originates in a hair follicle, the dwarf chancroid, the papular chancroid which starts as a papule, becomes ulcerated and then raised around the edges and finally the phagedenic chancre which becomes large and destructive with widespread necrosis of tissue due to necrotizing secondary infection (Morse 1989).

Histologically the ulcer can be described as having three zones. A superficial zone is made up of necrotic tissue with red cells, some fibrin and degenerated polymorphonuclear granulocytes (PMNs). This forms the base of the ulcer. Microorganisms are present between cells and inside neutrophils. The second zone consists of edematous inflamed tissue with small dilated vessels which are infiltrated with PMNs found at the junction with the superficial zones. No microorganisms are present here. A deep zone with a fairly dense infiltrate of plasma cells, some lymphocytes and some infiltration of PMNs at the periphery, is present. (Lagergård 1995).

2. Diagnosis and other genital ulcer diseases

Despite the distinctive features of “classic” chancroid, the disease can be atypical and mixed infections are possible. It is therefore important to use diagnostic tools to rule out the possibility of other causative agents. Chancroid can be confused with the lesions of herpes simplex virus, syphilis, lymphogranuloma venereum and granuloma inguinale (donovanosis). This is especially true in industrialized nations with little clinical familiarity and rarity of chancroid. In developing countries where the disease is endemic, and is the most common GUD, predictive values of chancroid disease are much higher (Joseph and Rosen 1994, Mroczkowski and Martin 1994 and Jessamine and Ronald 1990).

The diagnosis of chancroid has recently been improved with the development of a selective culture medium. With this method of diagnosis, bacteria are recovered from the chancroidal lesions and plated out on selective media. The use of two separate types of culture media simultaneously has increased the isolation rate from 56-70% to 81% (Joseph and Rosen 1994). The first medium most commonly utilized consists of gonococcal agar base with 1% bovine haemoglobin and 5% fetal bovine serum. The second frequently used medium is Mueller-Hinton agar base with 5% chocolate horse blood. Both media are supplemented with 1% coenzymes-vitamins-amino acids (CVA) enrichment and 3µg/mL of vancomycin to inhibit Gram positive bacteria (Tyndall et al. 1994; Plourde et al. 1992). Some laboratories use heart infusion agar as the second medium and add 1% IsoVitalX (a vitamin and amino acid enrichment) as a supplement instead of CVA (Mroczkowski and Martin 1994). Nevertheless, because of the requirement for specialized culture and specimen transport needs, low recovery in many inexpert settings can occur.

Gram staining of exudates are of little value because of contamination with other Gram negative rods. Histologic sections of the lesions can strongly suggest the diagnosis but the biopsy is painful and often not practical in sexually transmitted disease clinics. Culture results can be confirmed by biochemical tests. Tests for alkaline phosphatase, β -lactamase, nitrate reductase and oxidase are typically positive. The test for catalase is usually negative (Joseph and Rosen 1994; Mroczkowski and Martin 1994; Goens et al. 1994).

New identification methods for *H. ducreyi* developed in the past 5 to 10 years show potential in improving the laboratory diagnosis of *H. ducreyi* infections. Indirect immunofluorescence using a monoclonal antibody reactive with the outer membrane constituents of *H. ducreyi* has been described but is not sensitive enough for clinical use (Schalla et al. 1986). Karim et al. (1989) developed a monoclonal antibody as an immunofluorescence reagent. It showed up to 93% sensitivity in detecting culture positive cases of chancroid in patients but since *H. ducreyi* could also be detected in culture-negative cases, the final sensitivity is of 63%. Johnson et al. (1994) tested the polymerase chain reaction as a method of detection of *H. ducreyi*. PCR was positive for 62% of the culture positive specimens but was also positive for 49% of the culture-negative specimens recovered from patients. By comparing positive results obtained using regular techniques to positive results obtained with PCR, the investigators concluded that PCR was giving true results. Also, they suggest that the presence of Taq DNA polymerase inhibitors in the nucleic acid extracts may have been responsible for the failure at detecting *H. ducreyi* in all of the culture-positive specimens. Orle et al. (1994) described a multiplex PCR where *H. ducreyi*, *T. pallidum* and HSV types 1 and 2 were simultaneously amplified. Specimens recovered from

the lesions of patients determined to be culture-positive or -negative by selective culture media were utilised in the study. Most culture-positive specimens were PCR positive but some culture negative specimens also were. By doing PCR using a different target gene, it was confirmed that the latter were not false-positive results. This PCR assay could greatly improve the diagnosis of chancroid and other genital ulcer diseases.

3. Epidemiology

The underlying cause of genital ulcer disease (GUD) is usually a microorganism that has been acquired through sexual interaction. GUD is more common in tropical countries than in temperate parts of the world. The proportion of patients with sexually transmitted diseases presenting with ulcers ranges from 10 to 30 % in developing countries compared with 2 to 5% in western Europe and North America. In the former, chancroid is the most common form and is endemic to such countries as South Africa, Kenya and Thailand. In the latter, herpes simplex virus is encountered most often. Syphilis is also common to both, but constitutes a higher proportion of cases in the developed world. Lymphogranuloma venereum and granuloma inguinale (donovanosis) are rare forms of GUD in industrialized nations but are endemic in some regions of Central and West Africa and Southeast Asia (Mroczkowski and Martin 1994; Jessamine and Ronald 1990).

Prostitution is an important risk factor for GUD in both settings. In Canada an urban outbreak occurred from 1975 to 1977. The few cases that were diagnosed in the years following, were imported and could be linked back to foreign contact with a prostitute (Hammond et al. 1978). In the United States, chancroid is assuming more importance and

may in fact be endemic now in south Florida and New York City. Outbreaks have occurred in such urban centres as New Orleans, Louisiana and Houston, Texas (Joseph and Rosen 1994; Jessamine and Ronald 1990). Recent increases in chancroid and syphilis have been associated with cocaine abuse, more specifically with crack, the most common form of the drug. Crack produces a relatively short-lived high so that heavy users require multiple daily doses. Many women resort to sexual acts in exchange for the drug or for money used for the purchase of the drug (Mroczkowski and Martin 1994).

Another risk factor for the acquisition of a GUD is male circumcision. The uncircumcised state increases the risk for acquisition of chancroid three-fold (Cameron et al. 1989). This may relate to the provision of warm and nutritious environment required by *H. ducreyi*. Another explanation may be that this region is the most easily traumatized during sexual intercourse. Small breaks in the subpreputial non-keratinized mucosal epithelium would serve as a portal of entry for the organism (Jessamine and Ronald 1990).

In both tropical and temperate areas, men account for the majority of chancroid cases. The male to female ratio is at least 4:1 and as high as 10:1. Female prostitution accounts for much of this ratio. This may also be explained by the fact that women are not as aware of the disease as men because their lesions are usually smaller, discrete and thus not as visible. The clinical impression is that they are also less painful. Women also have less access to health care, through disproportional poverty. This would lead to underreporting of chancroid for this sex and women would thus serve as a reservoir of the disease (Mroczkowski and Martin 1994; Jessamine and Ronald 1990).

Because of the insensitive culture methods and the reliance of the clinical picture as

a standard of diagnosis, knowledge on the epidemiology of chancroid is limited. This is also due in part to the absence of suitable markers needed to differentiate strains of *H. ducreyi* (Morse 1989). In the last decade, strains have been characterized phenotypically by various methods.

Outer membrane protein (OMP) profiles were done on 105 *H. ducreyi* isolates and seven different electrophoretic patterns were observed (Odumeru et al. 1983). However, strains isolated from an outbreak in Winnipeg between July 1975 and February 1977 belonged to the same subtype. Characterization of OMPs of *H. ducreyi* in Southeast Asia and Nairobi classified the strains in three different membrane protein pattern groups at the most. Thus, this typing method does not discriminate sufficiently between strains (Sarafian et al. 1991; Morse 1989).

Sloutmans et al. (1985) used an indirect immunofluorescence assay to divide 16 strains of *H. ducreyi* into types I to IX. This method is not ideal because it requires the identification of type strains and the production of type-specific antisera (Sarafian et al. 1991).

The API-ZYM system was used by VanDyck and Piot (1987) to determine the enzyme profile of 200 strains of *H. ducreyi* isolated from a variety of geographical locations. Not enough discrimination was established with only three biovars identified.

Korting et al. (1988) used 14 lectins and observed 20 agglutination patterns among 43 *H. ducreyi* strains isolated from different geographic areas. The stability of the carbohydrate cell surface components that bind these lectins has not been demonstrated. Additionally, the fact that *H. ducreyi* agglutinates spontaneously when suspended in buffer or medium makes the utility of this method uncertain (Sarafian et al. 1991).

By digesting *H. ducreyi* DNA with different restriction enzymes and hybridizing it to *E. coli* rRNA, Sarafian et al. (1991) was able to identify 12 distinct ribotypes among 44 clinical isolates from different parts of the world. This system is highly reproducible and reliable and may prove useful as an epidemiological tool.

4. GUD, chancroid and HIV

The role of GUD as a risk factor for HIV transmission has been repeatedly suggested. The histology of chancroidal lesions supports the hypothesis that they may serve as a portal of entry and of exit for HIV. The inflammatory nature of the lesion may permit enhanced viral multiplication in immune target cells and may lead to cell death with release of free infectious virus. HIV has been recovered from genital ulcer exudates (Augenbraun and McCormack 1994; Jessamine et al. 1990; Kreiss et al. 1989).

Studies in Nairobi, Kenya showed that in men, a history of GUD was associated with HIV seropositivity. This interaction was much higher in uncircumcised men. Regular contact with prostitutes was also an independent risk factor. In women, independent risk factors for HIV included oral contraceptive use, *Chlamydia trachomatis* infection and more importantly, GUD. Over 60% of HIV antibody seroconverting women experienced at least one episode of GUD in the period prior to seroconversion. Additionally, women concurrently infected with HIV had a greater frequency of ulcer episodes per month compared to those who remained seronegative (Cameron and Padian 1990; Jessamine et al. 1990; Jessamine and Ronald 1990).

Therapeutic evidence that HIV immune disease increases the virulence of pathogens

has been demonstrated. Single-dose regimens of trimethoprim-sulfonamide, fleroxacin, ceftriaxone and azithromycin have all had >20% failure rates and delayed healing of lesions has been reported (Behets et al. 1995; Tyndall et al. 1994; Tyndall et al. 1993; Cameron and Padian 1990; MacDonald et al. 1989).

A complex bidirectional interaction between HIV and GUD exists with respect to transmission and virulence. A cycle of amplification is emerging in which GUD enhances HIV transmission, HIV increases GUD frequency and the lack of male circumcision augments the transmission of both. Efforts toward the prevention and control of GUD should be considered important in the control and prevention of HIV transmission (Augenbraum and McCormack 1994, Cameron and Padian 1990; Jessamine and Ronald 1990).

5. Virulence of *H. ducreyi* and animal models

Animal models are important in many studies such as those dealing with pathogenicity, virulence, potential vaccines and antibiotic activity. Suitable animal models are ones where the disease produced is similar to that seen in humans, "natural" routes of infection are used, the pathogen can be propagated and quantified and the disease effect and immune response can be quantified (Smith 1989).

Earlier studies on animal models for *H. ducreyi* made use of guinea pigs, mice, cats, goats and sheep. These were all refractory to inoculation but other studies involving rabbits and monkeys were successful in inducing lesions. All of these early studies are difficult to evaluate since the virulence of the organism and the antibody status of the animals were unknown (Morse 1989).

The classical test of virulence for *H. ducreyi* is the rabbit intradermal test (Dienst 1948; Feiner and Mortara 1945). Strains have been defined as virulent if intradermal inoculation into a rabbit produced necrotic lesions. The inoculum size required for the formation of ulcers was high, at least 10^8 CFU (Odumeru et al. 1987 and Odumeru et al. 1984). In addition, purified LOS from *H. ducreyi* and other Gram negative bacteria was found to induce ulcers (Campagnari et al. 1991). Antibodies of the IgM class were directed against antigens with molecular masses of 79, 62, 55, 49 and 26 kDa. IgG antibodies were directed against the same ones as well as to numerous antigens with molecular masses between 11 and 16 kDa.

A mouse model was developed by Tuffrey et al. (1988) where 10^7 CFU of *H. ducreyi* injected intradermally produced ulcerative lesions after 24 h. However, these lesions could also be produced by heat-killed cells and with purified LOS.

Recently, a primate model for chancroid was developed by Totten et al. (1994b). Adult pig-tailed macaques were infected with 10^7 - 10^8 CFU of *H. ducreyi*. Males were inoculated on the exposed surface of the foreskin while females were inoculated adjacent to the vaginal opening. Ulcerative lesions in the males appeared at 7-13 days post-infection and *H. ducreyi* could be recovered from them for up to 20 days. The lesions were clinically and histologically similar to the ones seen in human infections. However, pustular or ulcerative lesions failed to develop in females. Heat-killed *H. ducreyi* did not lead to the formation of lesions in males, and pretreatment with ceftriaxone was preventative. Antibodies were developed to *H. ducreyi* against antigens of 12, 17, 27, 39 and 60 kDa. Even though this primate holds potential as an animal model for chancroid, it has obvious disadvantages. In

first place, primates are very costly and secondly, the inoculum is probably unnaturally large.

The temperature-dependent rabbit model of infection (Purcell et al. 1991) addressed the problem of high inocula. The animals were housed at 15-17°C, thereby reducing their skin temperature to one more appropriate for *H. ducreyi* growth. At 10⁵ CFU, there was consistent production of necrotic lesions. At the same inoculum, heat-killed organisms did not give rise to lesions, which indicates that the LOS content alone was insufficient to promote lesion development. Additionally, the presence of a bactericidal antimicrobial prevented lesion development. This suggests that the bacteria must replicate to produce a lesion. The fact that viable organisms could be recovered for up to 2 weeks post-challenge also strongly indicates this.

Meloche et al. (1992) developed a quantitative virulence assay for the temperature-dependent rabbit model of infection. Changes in *H. ducreyi* virulence after immunization, iron loading, dexamethasone immunosuppression, and prior infection could be measured. The alterations were determined by several quantitative parameters: number of days ulcers were positive, lesion size, lesion score, peak lesion size and minimum ulcer producing inoculum. Desjardins et al. (1995) used the same assay to show that a pilus preparation induces homologous and heterologous strain protection in the temperature-dependent rabbit model of infection.

6. Antimicrobial treatment and resistance

Sulfonamides have been used to treat chancroid since 1938. In the 1960s, sulfonamide resistant strains were first reported. Resistance is now prevalent throughout the

world. A 4.9 MDa non-conjugative plasmid (Su^r) encodes for the resistance to this drug and is 79% related to the enteric streptomycin-sulfonamide resistance plasmid RSF1010. This type of plasmid encodes for a dihydropteroate synthase that can function normally in the presence of high concentrations of sulfonamides. It is a type II enzyme. A 3.1 MDa plasmid also encodes for sulfonamide resistance as well as for streptomycin and kanamycin resistance (Morse 1989).

Trimethoprim in combination with sulfonamide has been used successfully to treat chancroid. In spite of a high prevalence of sulfonamide resistant strains, the combination with trimethoprim still remained effective. However, a failure rate of about 30% was observed with trimethoprim-sulfametrole single doses in patients concurrently infected with HIV (Cameron et al. 1988). Furthermore, the increasing resistance to trimethoprim dramatically decreased the effect of short-term and long-term doses of trimethoprim-sulfonamide combinations, so that they are no longer predictably effective. The mechanism of trimethoprim resistance is unknown. In other organisms, a plasmid that specifies a trimethoprim-resistant dihydrofolate reductase or a mutation in a chromosomal gene encoding thymidylate synthase is responsible for the resistance (Knapp et al. 1993; Plourde et al. 1992; Morse 1989).

Rifampin is highly active against *H. ducreyi* (MIC = 0.004-1 µg/mL) and has been used successfully in combination with trimethoprim (Morse 1989). Rifabutin is another drug of the same class that demonstrates low MICs (0.004-0.016 µg/mL) (Farmitalia Carlo Erba 1993). However, the use of rifamycin/ansamycin for the treatment of non-mycobacterial infections in tuberculosis endemic areas is controversial because resistance to these drugs

usually develops very rapidly and this would pose a danger in areas where tuberculosis and leprosy rates are high (Schmid 1986). Although short-course regimens are unlikely to influence the susceptibility of *Mycobacterium tuberculosis*, in the last 5 years no reports of these drugs as a treatment for chancroid have been published (Abeck et al. 1988a). No rifampin-resistant isolates have been reported (Morse 1989).

Chloramphenicol has been used in a few studies but the perception of the potential for hematologic complications has precluded its routine use. Resistant isolates contain a 34 MDa conjugative plasmid encoding a chloramphenicol acetyltransferase (CAT). It also contains a tetracycline resistance determinant. The plasmid shares 70 to 80% homology with pR1234, a chloramphenicol-tetracycline *H. influenzae* CAT plasmid. The CAT enzyme is molecularly related to the enteric type II and *H. influenzae* CAT enzymes (Morse 1989).

Aminoglycosides such as streptomycin, kanamycin and gentamicin have been used in chancroid treatment. Streptomycin resistance has been reported in Singapore, Amsterdam and France but *in vitro* susceptibility values were only reported for the French isolates (Morse 1989). In contrast, a report from Korea at around the same time found streptomycin to be one of the best treatments (Fitzpatrick et al. 1981). A high frequency of kanamycin and gentamicin resistance has been reported from Amsterdam and also from Thailand for the former. Streptomycin and kanamycin resistance is associated with a 3.1 MDa plasmid which can also encode resistance to sulfonamides. An aminoglycoside phosphotransferase is detectable in these strains but the mechanisms of resistance are unknown (Willson et al. 1989; Morse 1989).

Tetracycline was used for many years as a treatment of choice against chancroid but

is no longer recommended because of widespread plasmid and chromosomally-mediated resistance (Knapp et al. 1993; McNicol and Ronald 1984). One of the plasmids, of 34 MDa was described above; the other is of 30 MDa. They are not homologous. The 30 MDa plasmid is conjugative and unlike the 34 MDa one, does not mobilize the ampicillin resistance (Amp^r) plasmid. It appears to be related to a tetracycline resistance plasmid found in *H. influenzae*. Strains demonstrating phenotypic resistance contain two copies of a chromosomal TetM gene encoding streptococcal tetracycline resistance. TetM has been described in many urogenital pathogens including *Mycoplasma hominis*, *Gardnerella vaginalis* and *N. gonorrhoeae*. This suggests that these organisms participate in or draw upon a common genetic pool (Morse 1989).

The β -lactams, penicillin and ampicillin were once useful drugs showing high activity against *H. ducreyi*. Now, most isolates test positive for β -lactamase activity. This TEM-type enzyme is encoded by plasmids of three common sizes, 7.0, 5.7 and 3.2 MDa, all of which are nonconjugative. The 7.0 Amp^r plasmid is homologous with the 4.4 MDa gonococcal Amp^r plasmid and contains the complete transposable ampicillin-resistance sequence, TnA. In contrast, the *N. gonorrhoeae* Amp^r only contains 40% of the TnA sequence. The *H. ducreyi* 5.7 Amp^r plasmid is homologous with the 3.2 MDa gonococcal Amp^r plasmid and contains the complete TnA sequence while the 3.2 Amp^r plasmid is identical to it. This latter carries about 40% of the TnA sequence. (Morse 1989; McNicol and Ronald 1984).

A larger plasmid of 23.5 MDa that is often isolated with the nonconjugative Amp^r and Su^r smaller plasmids, possesses a mobilizing activity. Its mechanisms are unclear but it has been shown to mobilize small R-plasmid to *E. coli*, *H. parainfluenzae* and *N. gonorrhoeae*.

This latter can have a 24.5 MDa phenotypically cryptic plasmid that has the same mobilizing activity as the *H. ducreyi* 23.5 MDa plasmid. Despite similarities, hybridization studies showed the two to be distinct (Schmid 1990; Morse 1989; McNicol and Ronald 1984).

Amoxicillin in combination with clavulanic acid has shown good activity against many strains of *H. ducreyi* (Morse 1989). However, two recent reports suggest that the bacterium is acquiring resistance to this combination (Motley et al. 1992; Knapp et al. 1993). The factors responsible for resistance have not been identified.

A 250 mg single intramuscular dose of the third-generation cephalosporin, ceftriaxone, has proven effective against chancroid (LeSaux and Ronald 1989; Bowmer et al. 1987). Failure of treatment has been associated with HIV positivity in many cases and also to unexplained factors. Tyndall et al. (1993) found a >25% failure rate in a HIV-negative group and a 40% failure rate in a HIV-positive group. The results of the HIV-negative group are difficult to explain since ceftriaxone is resistant to β -lactamase activity and in this study remained susceptible *in vitro*. Primary HIV infection without antibody seropositivity, evidenced by the high seroconversion rate measured in men with chancroid in that setting might account for some of this. A recent study in New Orleans (Martin et al. 1995) proved ceftriaxone to be very effective against *H. ducreyi*. Other new drugs from this group that may prove useful are cefotaxime, ceftazidime, cefetamet, cefetrame, ceftizoxime and cefodizime (Schmid 1990).

Antibiotics of the macrolide/azalide group show good activity against *H. ducreyi*. The macrolide erythromycin has been effective as a 500 mg oral dose four times a day for 7 days. Most strains are more susceptible to azithromycin, the new prototype antibiotic of the azalides

and it can be administered as a single 1 g dose. Unfortunately, a recent study by Tyndall et al. (1994) showed a >20% failure rate in HIV-seropositive patients.

The new fluoroquinolones hold much promise as a treatment for chancroid. Single doses of ciprofloxacin (500 mg) have been very successful as have been single-dose therapies with 200-400 mg fleroxacin. HIV infection has been associated with delayed healing and with treatment failure (Plourde et al. 1992; MacDonald et al. 1989; Schmid 1990).

Current recommended regimens for the treatment of chancroid are azithromycin in a 1 g single-oral dose, ceftriaxone in a 250 mg single-intramuscular-dose or erythromycin 500 mg orally four times a day for 7 days. Alternative regimens are amoxicillin 500 mg, plus clavulanic acid, 125 mg, orally three times a day for 7 days or ciprofloxacin 500 mg orally two times a day for 3 days (Cameron 1995). Reese and Betts (1993) also report streptomycin as an alternate treatment.

7. Antimicrobial combinations

Antimicrobial combinations can provide broad-spectrum empiric coverage for seriously ill patients or can be used to improve outcomes of treatment against specific microorganisms. For both situations, an impaired host or enhanced bacterial virulence may justify or increase the need for antimicrobial combinations. Thus, improved outcomes in infections can be achieved by combining two or more antibiotics (Eliopoulos and Moellering Jr. 1991).

It is important that the antimicrobials that are being combined interact positively in order to serve as a potential treatment. When the antibiotic activity is measured *in vitro*, four

different results may be observed. These are synergy, additivity, autonomy and antagonism.

Synergy defines a positive interaction and occurs when “the combined effect of the drugs being examined is significantly *greater than* the expected result - based on their independent effects when the drugs are used separately” (Eliopoulos and Moellering Jr. 1991). Antagonism is a negative interaction that occurs when “the combined effect of the drugs being examined is significantly *less than* their independent effects when they are tested separately” (Eliopoulos and Moellering Jr. 1991). When no significant interaction is observed between the antimicrobial being tested, the result can be described as additivity or autonomy. With additivity it is assumed that “the result observed with more than one drug should be the sum of the separate effects of the drugs being tested if those drugs do not interact with one another” (Eliopoulos and Moellering Jr. 1991). Autonomy (indifference) suggests that “the combined effect of drugs that do not interact with one another should be simply the effect of the more (most) active drug alone” (Eliopoulos and Moellering Jr. 1991).

Synergy *in vivo* can be demonstrated by the use of animal models by administering graded doses of the combined agents and the single agents. It can be defined by using PD_{50} values (the protective dose that enable 50% of the animals to survive), or by simply noting a response to the agents combined where none existed with the single agents (Cleeland and Squires 1991).

8. Objectives

An ideal treatment regimen for chancroid should preferably be effective, well-tolerated, inexpensive and capable of being administered as a single dose (Abeck et al 1988a).

Some of these criteria will help to ensure patient compliance and thus increase effectiveness of the treatment. As stated above, a high rate of treatment failures has been reported when using single dose treatments in HIV seropositive patients. If the spread of chancroid in developing countries where it is endemic cannot be controlled, the transmission of HIV will increase. A single-dose treatment of greater activity may help to improve chancroid treatment efficacy in HIV coinfection. This can often be achieved in some infections by combining two or more antibiotics.

The objectives of this project were:

- 1) To test a panel of antimicrobial agents for their individual and combined activities by the *in vitro* checkerboard agar dilution technique against three strains of *H. ducreyi*.
- 2) To assess the activity of the antibiotics possessing the most positive interaction (as defined by *in vitro* testing) in the temperature-dependent rabbit model of chancroid. The activity and efficacy of single drug or combination treatment were to be compared in this *in vivo* quantitative virulence assay.
- 3) To corroborate the *in vitro* results with the *in vivo* results.

MATERIALS AND METHODS

I. In vitro antimicrobial testing

1. Bacterial strains and culture conditions

***Haemophilus ducreyi*, strains 35000, RO-40 and RO-34**

Haemophilus ducreyi, strain 35000 was originally isolated from an outbreak in Winnipeg, Manitoba in 1975 (Hammond et al., 1978a) and was obtained from A.R. Ronald at the University of Manitoba. RO-40 and RO-34 are clinical isolates collected by D.W. Cameron at the Nairobi City Commission Dermatovenerology Clinic in Nairobi, Kenya in 1987. All strains were stocked in sheep's red blood cells (Cederlane) and grown on chocolate agar (CA) plates (APPENDIX) supplemented with 1% IsoVitaleX (Becton Dickinson Microbiology Systems, Cockeysville, Md) and 5% fetal bovine serum [FBS (Gibco-BRL, Life Technologies, Inc. Grand Island, NY)]. Plates were incubated for 48 h or 24 h at 33°C in 5% CO₂ and high humidity.

***Escherichia coli*, strain ATCC 25922 and *Staphylococcus aureus*, strain ATCC 29213**

Reference strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were grown on CA plates or in Mueller-Hinton broth supplemented with 1% IsoVitaleX. Plates were incubated for 24 h at 37°C in 5% CO₂ and broth cultures were incubated for 2 hours at 37°C in an environmental shaker.

Antimicrobial susceptibility testing

Antimicrobial testing was done on CA plates supplemented with 1% gonococcal (GC) supplement (Difco Laboratories, Detroit, Mich.). Plates were incubated for 48 h at 33°C in 5% CO₂ and high humidity.

2. Antimicrobial agents.

All strains were tested for their susceptibility to ceftriaxone (Sigma Chemical Company, St Louis, Mo), streptomycin (Sigma Chemical Company, St Louis, Mo), azithromycin (Pfizer, Pointe-Claire-Dorval, Québec, Canada) and rifabutin (Farmitalia Carlo Erba, Nerviano, Italy). All stock solutions were prepared in water. The final stock concentrations for ceftriaxone, azithromycin and rifabutin were 1280 µg/mL and 20480 µg/mL for streptomycin. Azithromycin and rifabutin were initially dissolved in methanol (<2% of the final volume) because of their lack of solubility in water (APPENDIX). The solutions were sterilized with a Millipore 0.22 µm filter and then stored at -70°C.

3. Minimum inhibitory concentration (MIC) determination

MIC's for ceftriaxone, streptomycin, azithromycin and rifabutin against *H. ducreyi* strains 35000, RO-40 and RO-34 were determined using the agar dilution technique (National Committee for Clinical Laboratory Standards 1993; Slaney et al. 1990; Hammond et al. 1978b). All antimicrobial agents were diluted in water and incorporated into CA supplemented with 1% GC supplement, to yield the appropriate 2-fold dilution series. Plates were poured in duplicate for each drug concentration and two plates without antibiotics

served as controls. The range of concentrations tested were 0.001 to 8 $\mu\text{g/mL}$ for ceftriaxone, 0.063 to 512 $\mu\text{g/mL}$ for streptomycin, 0.00005 to 4 $\mu\text{g/mL}$ for azithromycin and 0.002 to 2 $\mu\text{g/mL}$ for rifabutin. Plates were used the same day or were stored at 4°C for use the next day.

H. ducreyi strains were grown on CA plates for 48 h then subcultured to fresh plates that were incubated for 24 h. The plate growth was suspended in Mueller-Hinton broth with 1% IsoVitaleX and allowed to stand to sediment for at least 15 min. at room temperature. The optical density of each supernatant was adjusted to that of a 0.5 McFarland barium sulfate standard (APPENDIX) to obtain a suspension of approximately 1 to 2×10^8 CFU/mL. A few colonies of the reference strains were selected from plates grown for 24 h at 37°C and were inoculated into 4 mL of Mueller-Hinton broth supplemented with 1% IsoVitaleX. This suspension was incubated at 37°C for approximately 2 h in an environmental shaker. Optical densities were adjusted as described above. All suspensions were diluted 1:10 and 2 μL (approximately 10^4 CFU) were spot-inoculated onto the plates with a standardized pipet. MICs were defined as the lowest concentration of an antibiotic that completely inhibited growth (disregarding a single colony) after 48 h at 33°C in 5% CO_2 and high humidity. Assays were repeated 9 more times using serial concentrations narrowed down to the region of interest as part of the checkerboard agar dilution technique for FIC index determinations.

4. Fractional inhibitory concentration (FIC) index determination

FIC indices for *H. ducreyi*, strains 35000, RO-40 and RO-34 when exposed to varying

concentrations of all two-drug combinations of ceftriaxone, azithromycin, rifabutin and streptomycin were determined by using the checkerboard agar dilution technique (Eliopoulos 1991; Greenwood 1989) (APPENDIX). Plates and inocula were prepared as described above. The range of concentrations four doubling dilutions below the MIC and the range of concentrations two doubling dilutions above the MIC were tested for each antibiotic. All concentration combinations of the two drugs were tested. The assays were performed in duplicate in three separate experiments. In the two subsequent experiments only the concentration combinations in the range of interest were tested.

II. *In vivo* antimicrobial testing

1. Bacterial strains and culture conditions

H. ducreyi, strain 35000 was grown on CA plates and also in broth consisting of a mixture of Mueller-Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md) and α -minimal essential medium (Gibco-BRL, Life Technologies, Inc., Grand Island, NY) in a 1:1 ratio (vol/vol) supplemented with 17% fetal bovine serum (FBS). Plates were incubated at 33°C for 48 h with 5% CO₂ and high humidity while broth cultures were incubated for 12 h at 33°C in an environmental shaker.

2. Animals

Male New Zealand White rabbits (2.5 to 3 kg) were purchased from Charles River Canada and housed in an 11.7-m² room. A temperature of 14 ± 1°C was maintained with a Thermo Air plus air conditioning unit (Desjardins et al. 1995). The backs of the rabbits were

shaved prior to infection and thereafter were shaved when necessary. All rabbits were kept under identical conditions for the duration of the experiments.

3. Infection of experimental animals

Broth-grown *H. ducreyi*, strains 35000 and RO-40 were harvested at the late mid-log phase by centrifugation at 3,000xg for 10 min in an Omnifuge RT centrifuge (Baxter, CANLAB division, Pointe Claire, Québec). Pellets were washed once with phosphate buffered saline (pH 7.2) (APPENDIX) and resuspended in Mueller-Hinton broth. 1 mL syringes with 26 gauge, 3/8 inch tuberculin needles were prepared from which 100 µL was collected, diluted and plated out in duplicate on CA plates in order to determine the CFU counts for each needle. Plates were incubated at 33°C with 5% CO² and high humidity for 48 h.

The animals were infected using two different techniques. In the first technique, serial dilutions of the *H. ducreyi* broth, from 10⁷ to 10³ CFU/mL were prepared, put in the syringes and injected intraepithelially in triplicate in 100 µl doses, for a total of 15 injections, into the shaved backs of each animal to give final inocula of 10⁶ to 10² CFU. In the second technique, 10⁴ CFU were injected into the shaved backs of the rabbits in 15 locations in a grid-like fashion.

For a period of 21 days starting from the day after infection, the transverse lesion diameters were measured with a Vernier direct reading caliper and the severity of the lesions was scored (0-Nil, 1-Erythema, 2-Induration, 3-Suppuration, 4-Ulceration). After the lesions fell below 2 mm they were not measured thereafter. For rabbits receiving the range of inocula

(technique #1), these manipulations were done on two of the lesions for each inoculum size. For rabbits receiving only 10^4 CFU (technique #2), the manipulations were done on eight of the lesions. Some of the lesions were cultured by lateral injection of 0.1 mL of phosphate buffered saline (pH 7.2), in order to recover *H. ducreyi* along with the cell debris. This was accomplished on the one remaining lesion of three in each of the 10^6 - 10^4 CFU groups (technique #1) or on seven lesions (technique #2). This was repeated each day until the aspirates from the lesions were shown to be sterile for four consecutive days. Aspirates were inoculated onto CA plates, incubated at 33°C and after 48 h examined for evidence of colonies morphologically typical of *H. ducreyi*. The push test (because of adhesion between cells of *H. ducreyi*, the colonies can be pushed intact across the plate), Gram staining and examination of bacterial morphology under the microscope were also utilized in identification.

4. Control rabbits

Control rabbits were infected with *H. ducreyi* with the two different techniques as described above. They received no antibiotic treatment.

5. Determination of virulence and minimum ulcer producing inoculum (MUPI)

Using the serial dilution inoculation technique (#1), the virulence and minimum ulcer producing inoculum for *H. ducreyi*, strains 35000 and RO-40 were determined. In the temperature-dependent rabbit model of infection devised by Purcell et al. (1991) that we have utilized, virulence is determined by the consistent production of ulcerative lesions from which live *H. ducreyi* cells can be recovered. As outlined above, the animals were infected and

lesions were measured, scored and cultured.

6. Individual streptomycin and ceftriaxone antibiotic treatment.

H. ducreyi, strain 35000 was grown and harvested as described above. Syringes were prepared and the CFU concentrations were determined individually also as described above. The two different inoculation techniques were utilised. When all lesions at 10^4 CFU had ulcerated, i.e. at 96 h after infection, a total of 11 rabbits were treated with single-doses of ceftriaxone (0.05, 0.1 or 5 mg/kg), and a total of 13 rabbits with single-doses of streptomycin (2.5, 5, 10 or 15 mg/kg) by intramuscular injection in the hind leg.

7. Combination streptomycin and ceftriaxone treatment

Rabbits infected with *H. ducreyi*, strain 35000, were administered a combination of streptomycin and ceftriaxone 96 h after infection. Each drug was injected i.m. in a different leg. 4 rabbits received combinations of 5 mg/kg of streptomycin + 0.05 mg/kg of ceftriaxone and 5 rabbits received 10 mg/kg of streptomycin + 0.05 mg/kg of ceftriaxone.

8. Iron loading

Some of the animals were administered 600 mg/kg of iron dextran (Dexafer 100 mg/mL; Austin Laboratories, Joliette, Québec) by the intramuscular route, one week prior to inoculation. This was done over the period of one week, spacing out the injections so that they were not administered every day. A maximum volume of 2 mL per leg per day was given.

9. Statistical analysis.

In vitro - The ANOVA was used to compare the FIC indices between strains. Where normality failed, the Kruskal-Wallis ANOVA on ranks was also performed.

In vivo - Analysis was done on the data derived from serial measures for days 0 (treatment day) to 17 at 10^4 CFU. The lesion sizes were expressed as a percentage of day 0 lesion size so that all lesions were 100% on day 0. For each rabbit, for all 17 days, the medians of the lesion size percentages and of the lesion scores from non-manipulated lesions were calculated. The mean time in days for a 50% decrease in lesion size and the mean time in days to reach a score of 2 were compared between groups. For each rabbit, the median of the culture positivity data was calculated. The mean time in days of culture positivity duration was compared between groups. The Student's t-test was used in all comparisons. When equal variance failed, the Mann-Whitney Rank Sum Test was also done. For each rabbit the median of the inoculum size, was calculated and the means of the groups were compared by ANOVA with the Student-Newman-Keuls test (multiple all pairwise comparison method). The analysis for comparing the 10 mg/kg streptomycin group, the 0.05 mg/kg ceftriaxone group and the combination was done in two different ways. In the first, the results of the animals in technique #1 and technique #2 were combined. Only data from the lesions at 10^4 CFU were utilized from the first technique. To make sure that the results were not being influenced by the two different techniques employed, the analysis was also performed on the results obtained for the animals from technique #2 only. Non-ulcerative lesions were not considered in the analysis.

RESULTS

1. Determination of virulence and minimum ulcer producing inoculum

The virulence of *H. ducreyi*, strain 35000 was determined by infecting rabbits with serial inocula of 10^6 to 10^2 CFU (technique #1) and also with just 10^4 CFU (technique #2). Live *H. ducreyi* was recovered from the lesions produced at 10^6 to 10^4 CFU for up to 15 days after infection. It is also at those inocula that ulcerative lesions were consistently formed. Thus, the MUPI was 10^4 CFU. At 10^4 CFU, for 40 rabbits, 372 lesions were observed. 352 out of the 372 lesions (94.6 %) ulcerated. The MUPI observed for strain RO-40 was also 10^4 CFU. Only one rabbit was tested but all three lesions produced at 10^4 CFU ulcerated. The lesion that was cultured was culture positive for 8 days after ulceration.

2. Minimum inhibitory concentrations

The MICs for ceftriaxone, streptomycin, azithromycin and rifabutin against *H. ducreyi* strains 35000, RO-40 and RO-34 were determined using the agar dilution technique. The three strains were tested for their susceptibility to the different antimicrobial agents to determine if inter-strain variability existed among them. The MICs did not differ between strains and were 0.002, 8, 0.008-0.016 and 0.016-0.032 $\mu\text{g/mL}$ for ceftriaxone, streptomycin, azithromycin and rifabutin, respectively (Table 1). Documented MICs for the reference strains were only available for ceftriaxone and azithromycin.

S. aureus ATCC 29213 and *E. coli* ATCC 25922 were utilized. The MICs fell within the "accepted" ranges except for azithromycin against *S. aureus*. The MIC obtained was 2

while the range is documented to be 0.25-1. This may be due to the fact that all the strains had to be tested on supplemented CA instead of on the regular Mueller-Hinton agar.

3. Fractional inhibitory concentration indices

The susceptibility of *H. ducreyi*, strains 35000, RO-40 and RO-34 to all two-drug combinations of ceftriaxone, streptomycin, azithromycin and rifabutin was tested. The isobologram illustrates these results in the form of a graphic (Figure 1). The highest FIC index was of 1.5 for the ceftriaxone-azithromycin and streptomycin-azithromycin combinations. Slight differences, although not statistically significant were observed between strains in all the rifabutin combinations with RO-40 being the most susceptible in two of three combinations (Table 2).

4. Iron loading

Seven rabbits were iron loaded with 600 mg/kg of iron dextran in the first experiments conducted. Table 3 indicates which rabbits in each group were iron loaded. No apparent differences existed in terms of length of culture positivity and lesion size between the iron loaded and non-iron loaded rabbits (Table 4). The only difference was that ulcers were sometimes observed at 10^3 CFU in the iron loaded animals while this was not seen in the non-iron loaded ones. At this point we decided to undergo all future experiments without the iron-loading.

Table 1

Minimum inhibitory concentrations in $\mu\text{g/mL}$ for ceftriaxone, streptomycin, azithromycin and rifabutin against *Haemophilus ducreyi*, strains, 35000, RO-40 and RO-34.

MIC ($\mu\text{g/mL}$)				
Strain (n = 10)	Ceftriaxone	Streptomycin	Azithromycin	Rifabutin
35000	0.002	8	0.008-0.016	0.016-0.032
RO-040	0.002	8	0.008-0.016	0.016
RO-034	0.002	8	0.008-0.016	0.016-0.032
Reference (n = 3)				
<i>E. coli</i> ATCC 25922	0.063	-	-	-
<i>S. aureus</i> ATCC 29213	4	-	2	-

Table 2

Fractional inhibitory concentration indices for all two-drug combinations of ceftriaxone, streptomycin, azithromycin and rifabutin against *Haemophilus ducreyi*, strains 35000, RO-40 and RO-34.

- a Mean FIC index \pm standard deviation. Calculation of FIC index is in APPENDIX.
- b C=Ceftriaxone, S=Streptomycin, A=Azithromycin, R=Rifabutin
- c Comparative evaluation with RO-40 and RO-34 (ANOVA)
- d The all pairwise multiple comparison procedure (Student-Newman-Keuls Method) showed no differences between the groups.
- e Normality failed. $P=0.14$ with the Kruskal-Wallis ANOVA on ranks.

FIC index^a

Strain	C+S ^b (n = 3)	C+A (n = 3)	C+R (n = 3)	S+A (n = 3)	S+R (n = 3)	A+R (n = 3)
35000	0.63 ± 0	1.50 ± 0	1.33 ± 0.29 (P = 0.05) ^{cA}	1.50 ± 0	0.92 ± 0.14 (P = 0.08) ^{cB}	0.75 ± 0.21 (P = 0.22) ^c
RO-040	0.63 ± 0	1.50 ± 0	0.92 ± 0.14	1.50 ± 0	0.67 ± 0.70	0.69 ± 0.11
RO-034	0.63 ± 0	1.50 ± 0	0.83 ± 0.14	1.50 ± 0	0.92 ± 0.14	1.08 ± 0.38

Figure 1

Isobologram demonstrating the supra-additive effect of the ceftriaxone-streptomycin combination. The threshold of additivity is represented by the dotted line. Axes are in FICs.

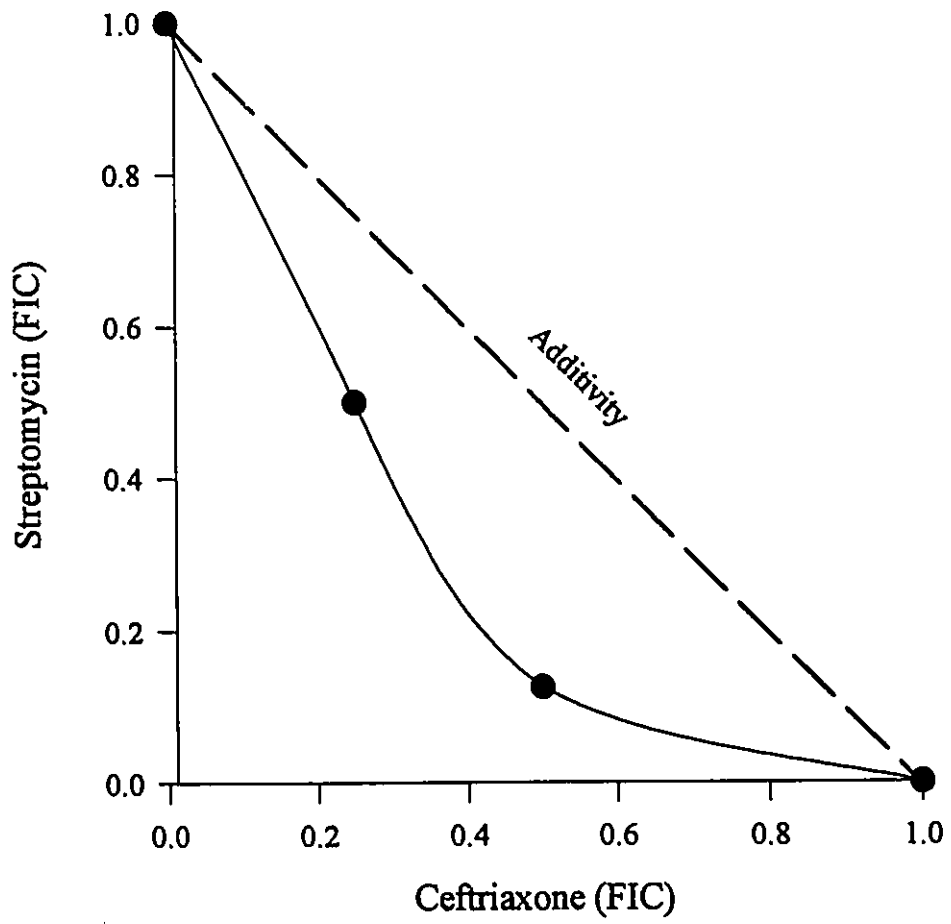


Table 3

List of all rabbits with treatment received and techniques applied.

- a** CF=Ceftriaxone, SM=Streptomycin
Numbers indicate the dosages in mg/kg

Number of rabbits

Treatment groups	Technique #1		Technique #2	Total
	Iron	No iron		
Control (no treatment)	2	3	3	8
0.05 CF ^a	-	2	4	6
0.1 CF	-	1	1	2
5 CF	-	-	3	3
2.5 SM ^a	1	2	-	3
5 SM	1	2	1	4
10 SM	1	-	4	5
15 SM	1	-	-	1
0.05 CF + 5 SM	-	2	1	3
0.05 CF + 10 SM	-	-	5	5

Table 4

Lesion history of control iron loaded rabbits and control non iron loaded rabbits at 10^4 CFU.

- a** Data are means \pm standard deviations, of median values for each rabbit.
- b** In the control group that received no iron, 3 rabbits were inoculated using technique #1 and 3 were inoculated using technique #2. See table 3.

Animal group	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in day 0 lesion size	Time (days) to reduction in lesion score from 4 to 2
Control ^b - No iron (n=6)	3.23 ± 2.25	8.17 ± 1.60	9.33 ± 1.21	11.17 ± 2.23
Control - Iron (n=2)	3.19 ± 0.59	6.50 ± 0.71	8.00 ± 2.83	8.50 ± 3.54

5. Control rabbits

Control rabbits received no antibiotics. For technique #1, the lesions were culture positive for 8.00 ± 1.87 days. By day 8.60 ± 1.67 , the lesions were half the size as of those of day 0. Finally, by 9.80 ± 2.77 days the score had been reduced to 2. The results were very similar when the lesions from the second technique were analysed. The lesions were culture positive for 7.33 ± 1.15 days, they were reduced by 50% by 9.67 ± 1.53 days and had reached a score of 2 by 11.67 ± 2.31 days (Table 5). Table 3 indicates how many rabbits were included in each technique.

6. Ceftriaxone treatment

Meloche (unpublished results) showed that a dose of 0.1 mg/kg of ceftriaxone was sufficient to sterilize the aspirate from rabbit lesions after 48 h. When the rabbits were iron loaded, 5 mg/kg were required. These two concentrations were therefore tested along with a lower one of 0.05 mg/kg. A dose of 5 mg/kg sterilized the lesions 0.33 ± 0.58 hours after administration. At 0.1 mg/kg, 5 ± 0 days were required while at 0.05 mg/kg 8.33 ± 3.20 days were needed. This last group was not significantly different from the control while the groups where the rabbits were treated with 0.1 mg/kg and 5 mg/kg, were (Table 6 and Figure 2). The lesions of the rabbits treated with 5 m/kg of ceftriaxone healed significantly faster than the lesions of the control rabbits as shown by lesion diameters (Table 6, Figures 3, 4 and 5). The time required for the lesions to reach a score of 2 differed between these two groups but fell just above the level of significance ($P=0.056$). The rate of healing at the other concentrations (0.1 mg/kg and 0.05 mg/kg) was similar to that of the controls (Table 6,

Table 5

Lesion history of controls inoculated with technique #1 and the controls inoculated with technique #2.

- a** Data are means \pm standard deviations, of median values for each rabbit.
- b** See table 4 for numbers of iron loaded rabbits in each group.

Animal group	Inoculum size ^a (CFU) x 10 ⁶	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2
Technique 1 ^b Control (n=5)	2.76 ± 1.67	8.00 ± 1.87	8.60 ± 1.67	9.80 ± 2.77
Technique 2 Control (n=3)	3.99 ± 2.48	7.33 ± 1.15	9.67 ± 1.53	11.67 ± 2.31

Table 6

Lesion history of the controls and the rabbits treated with 0.05, 0.1 and 5 mg/kg of ceftriaxone, at 10^4 CFU.

- a Data are means \pm standard deviations, of median values for each rabbit.
- b See table 4 for techniques used for each group.
- c CF=Ceftriaxone, SM= Streptomycin
- d Comparative evaluation of the inoculum sizes between all the groups using an ANOVA (P=1.00).
- e Comparative evaluation of the group vs the control group (t-test)

Animal group	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2
Control ^b (n=8)	3.22 ± 1.92 ^d	7.75 ± 1.58	9.00 ± 1.60	10.50 ± 2.62
0.05 mg/kg CF ^f (n=6)	3.10 ± 1.94	8.33 ± 3.20 (P = 0.66) ^e	7.83 ± 1.60 (P = 0.20) ^e	9.00 ± 2.45 (P = 0.30) ^e
0.1 mg/kg CF (n=2)	3.35 ± 2.33	5.00 ± 0.00 (P < 0.05) ^e	7.00 ± 1.41 (P = 0.15) ^e	6.50 ± 1.54 (P = 0.07) ^e
5 mg/kg CF (n=3)	3.02 ± 1.42	0.33 ± 0.58 (P < 0.0001) ^e	6.00 ± 1.00 (P < 0.05) ^e	7.00 ± 1.00 (P = 0.06) ^e

Figure 2

Mean length of culture positivity for the control, 0.05, 0.1 and 5 mg/kg ceftriaxone dose groups, at 10^4 CFU. Error bars are standard error.

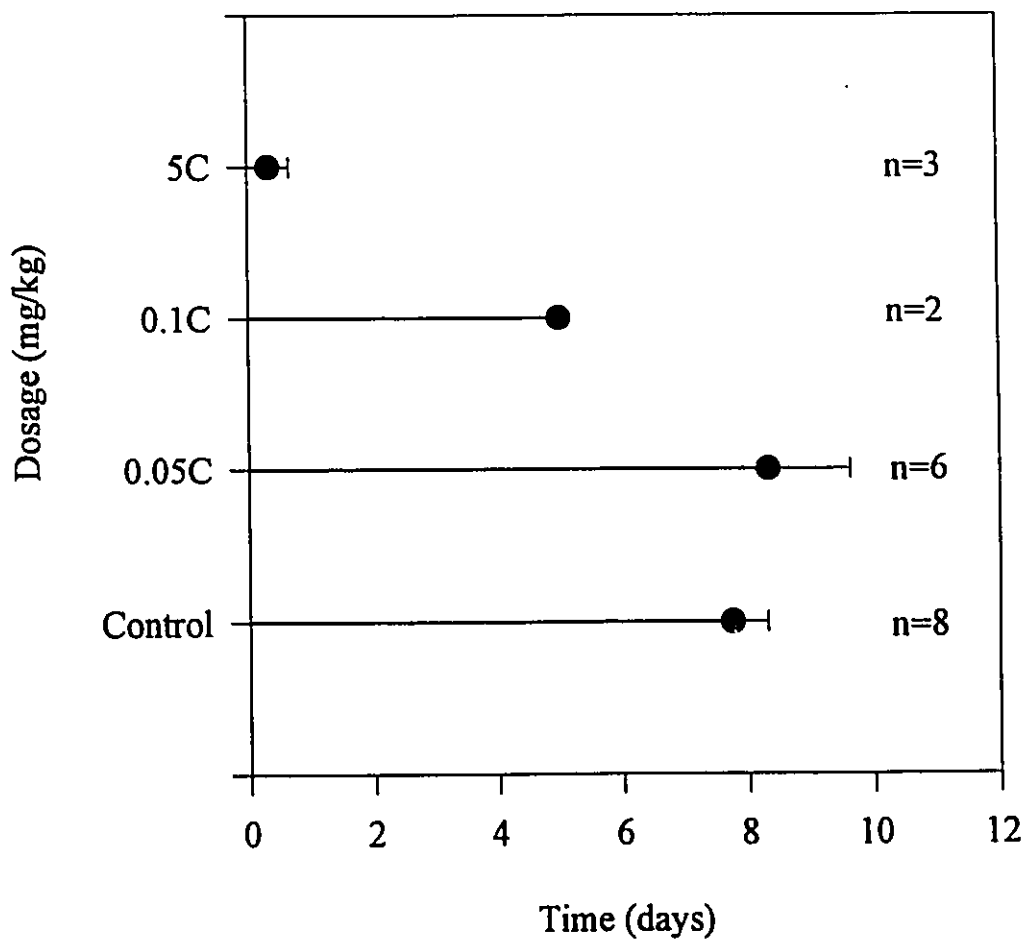


Figure 3

Course of disease for the controls and the 0.05, 0.1 and 5 mg/kg ceftriaxone dose groups at 10^4 CFU.

Bars indicate the mean % of the baseline lesion size (Day 0) remaining. The line plot indicates the mean scores while the scatter plot represents the mean of the last culture positive day.

Empty bars and empty circles - Controls
Diagonally hatched bars and full squares - 0.05 m/kg ceftriaxone
Vertically hatched bars and full circles - 0.1 mg/kg ceftriaxone
Horizontally hatched bars and triangles - 5 mg/kg ceftriaxone
Error bars are standard error.

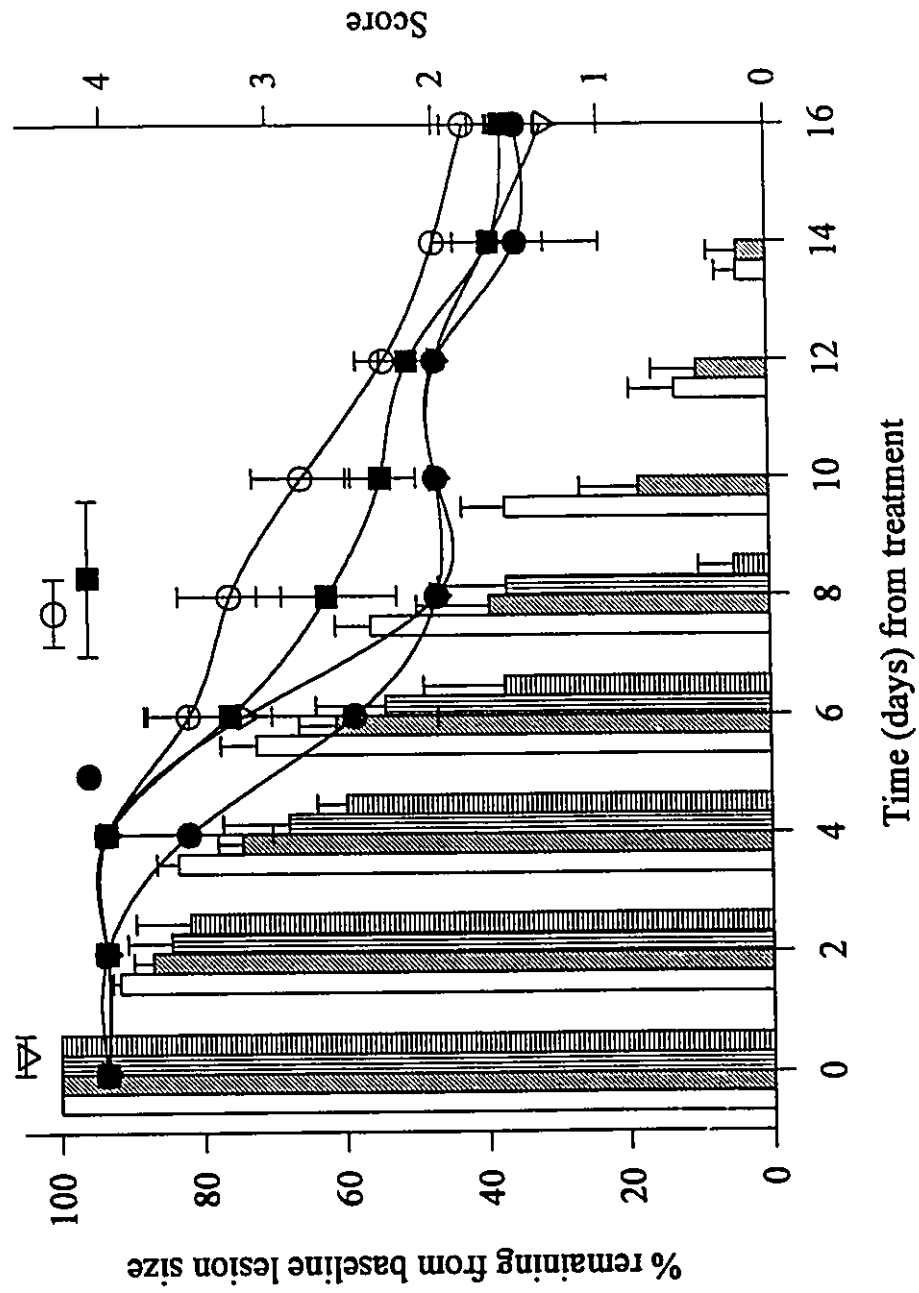
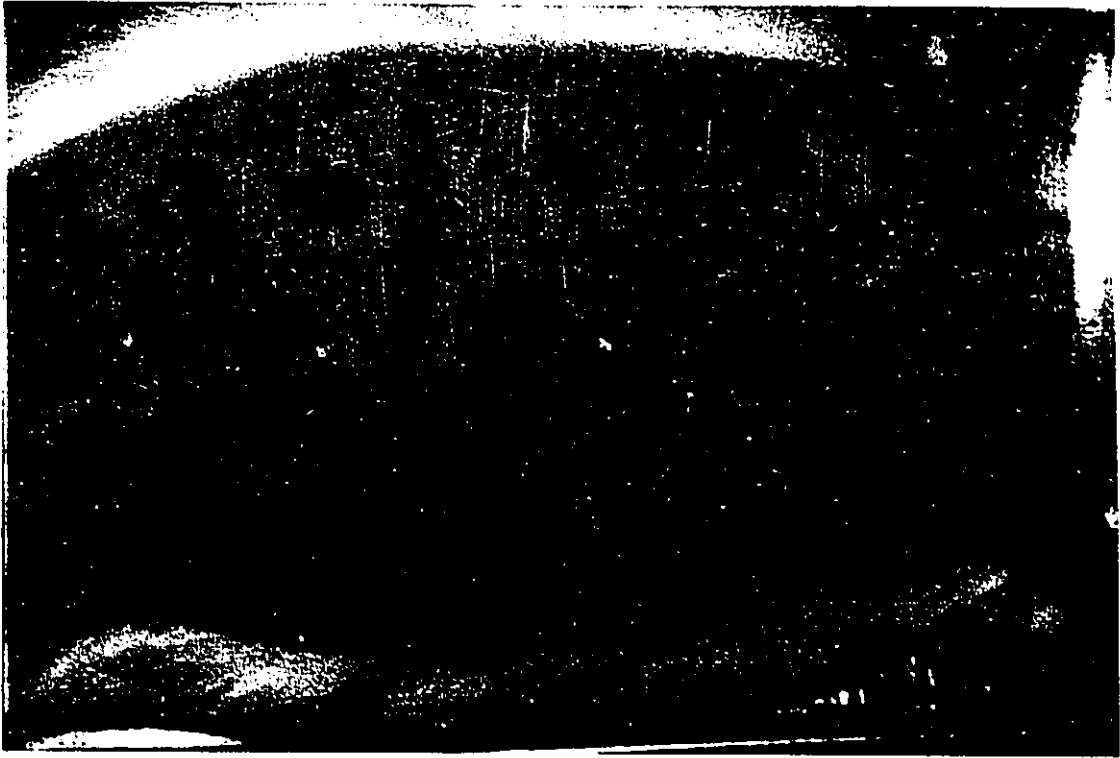


Figure 4

Appearance of lesions at day 0 at an inoculum of 10^4 CFU for a control (A) and a rabbit receiving 5 mg/kg of ceftriaxone (B).

A



B

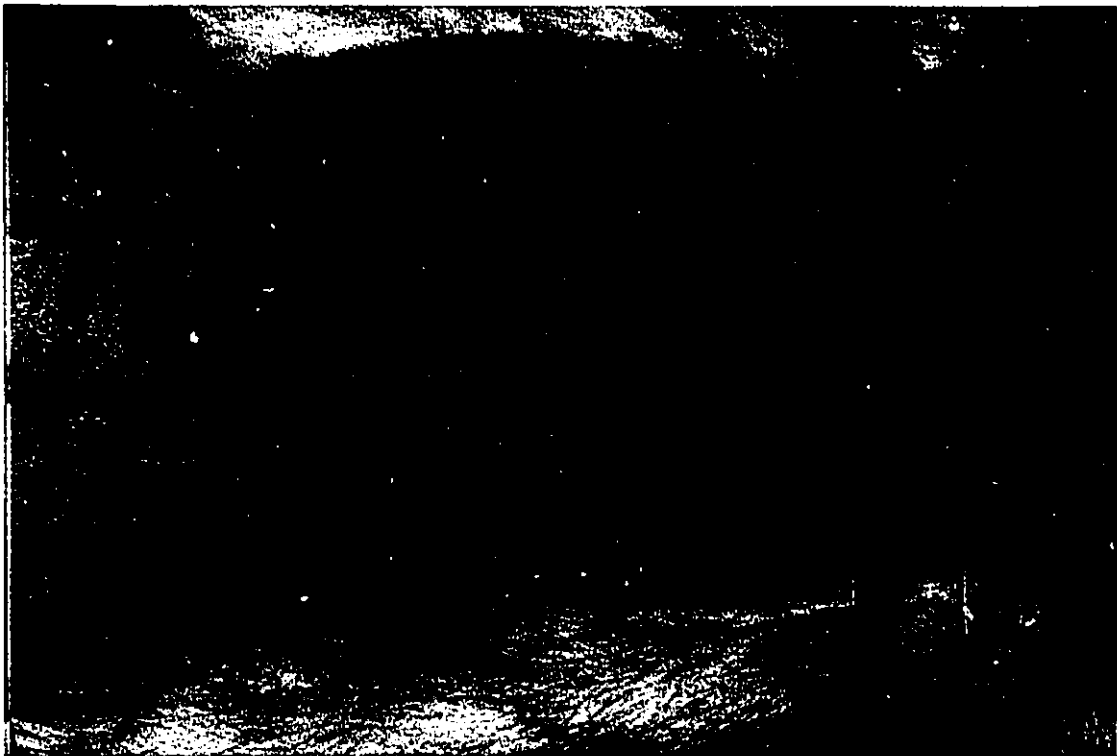


Figure 5

Appearance of lesions at day 8 at an inoculum of 10^4 CFU for a control (A) and at day 10 for a rabbit receiving 5 mg/kg of ceftriaxone (B).

A



B



Figure 3).

7. Streptomycin treatment

At concentrations of 2.5, 5 and 10 mg/kg of streptomycin, the lesion aspirates became sterile between 7 and 8.5 days. This was similar to the results obtained for the control lesions where culture positivity lasted for 7.75 ± 1.58 days. No significant differences existed between the aforementioned groups and the controls (Table 7 and Figure 6). One rabbit tested with 15 mg/kg of streptomycin showed a decrease in the number of culture positive days after treatment (Table 7 and Figure 6). The rate of healing of lesions for all groups as measured by lesion diameter and score was similar. The 2.5, 5 and 10 mg/kg groups were not significantly different from the controls (Table 7 and Figure 7).

Since the concentration of 15 mg/kg was not repeated, statistical analysis couldn't be performed. The results for 15 mg/kg of streptomycin in a single rabbit were similar to those observed with 0.1 mg/kg of ceftriaxone. The subtherapeutic effect of streptomycin at lower doses ensured that additive therapeutic activity could be detected by comparison of single-drug treatment with 2-drug treatment at these doses.

8. Ceftriaxone and streptomycin combination

When 0.05 mg/kg of ceftriaxone was combined to 5 mg/kg of streptomycin, no significant differences from the control were observed. Eight days post-treatment were required for sterilization of the lesion aspirates on the rabbits of this group. (Table 8 and Figure 8). Approximately 9 days were required for the lesions to be reduced by 50% and

Table 7

Lesion history of the controls and the rabbits treated with 2.5, 5, 10 and 15 mg/kg of streptomycin, at an inoculum of 10^4 CFU.

- a Data are means \pm standard deviations, of median values for each rabbit.
- b See Table 4 for inoculation techniques used for each group.
- c SM=Streptomycin
- d Comparative evaluation of the inoculum sizes between all the groups using an ANOVA (P=0.16).
- e Comparative evaluation of the group vs the control group (t-test).
- f Equal variance test failed. P=0.81 with the Mann-Whitney Rank Sum Test.

Animal group ^b	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2 ^c
Control (n=8)	3.22 ± 1.92 ^d	7.75 ± 1.58	9.00 ± 1.60	10.50 ± 2.62
2.5 mg/kg SM ^e (n=3)	2.73 ± 1.51	8.33 ± 1.53 (P = 0.60) ^e	8.00 ± 2.00 (P = 0.41) ^e	9.67 ± 2.31 (P = 0.64) ^e
5 mg/kg SM (n=4)	3.21 ± 2.01	7.54 ± 2.98 (P = 0.72) ^{ef}	7.25 ± 1.50 (P = 0.10) ^e	8.75 ± 2.36 (P = 0.29) ^e
10 mg/kg SM (n=5)	5.36 ± 1.65	8.40 ± 2.70 (P = 0.59) ^e	8.60 ± 0.55 (P = 0.61) ^e	9.50 ± 1.54 (P = 0.46) ^e
15 mg/kg SM (n=1)	3.28	3.00	8.00	9.00

Figure 6

Length of culture positivity for the control, 2.5, 5, 10 and 15 mg/kg streptomycin dose groups, at 10^4 CFU. Error bars are standard error.

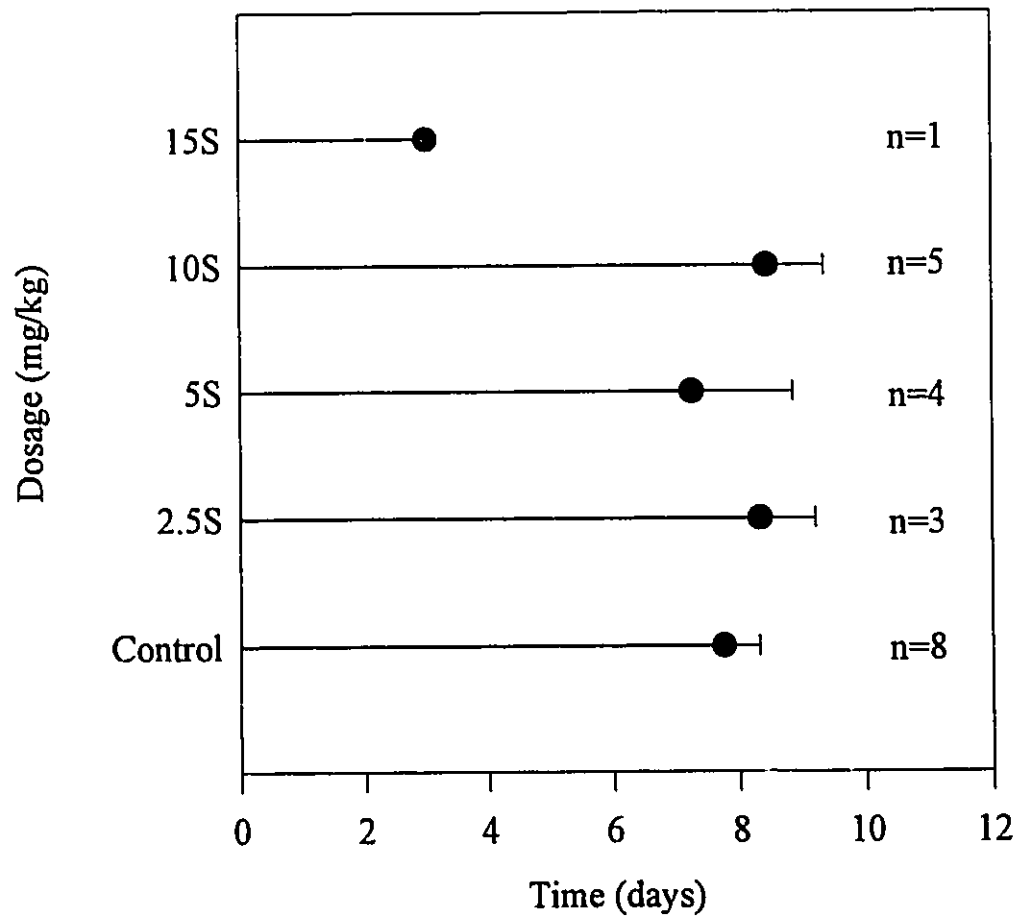


Figure 7

Course of disease for the controls and the 5, 10 and 15 mg/kg streptomycin dose groups at 10^4 CFU.

Bars indicate the mean % of the baseline lesion size (Day 0) remaining. The line plot indicates the mean scores while the scatter plot represents the mean of the last culture positive day.

Empty bars and empty circles - Controls
Diagonally hatched bars and full squares - 5 mg/kg streptomycin
Vertically hatched bars and full circles - 10 mg/kg streptomycin
Horizontally hatched bars and triangles - 15 mg/kg streptomycin
Error bars are standard error.

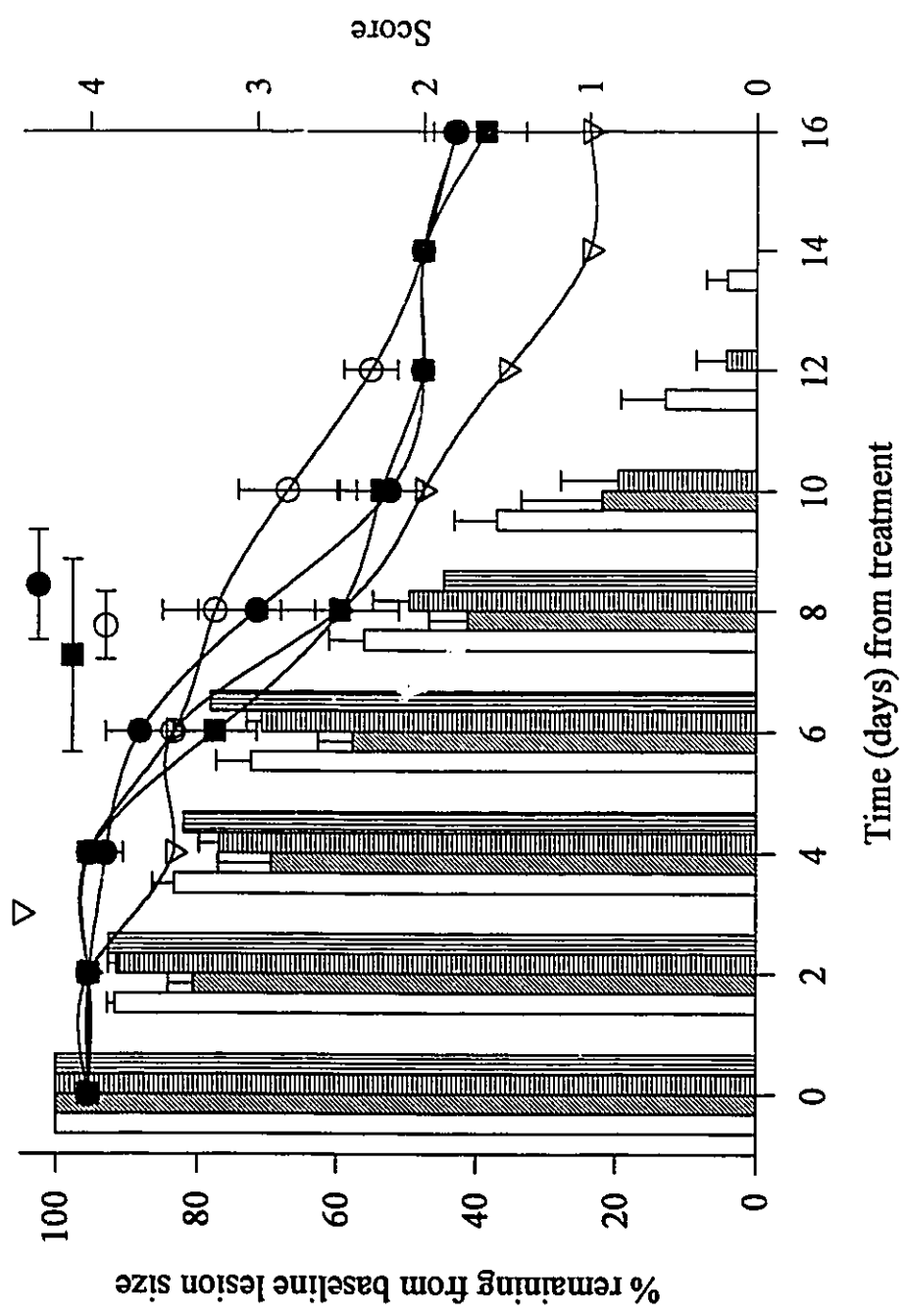


Table 8

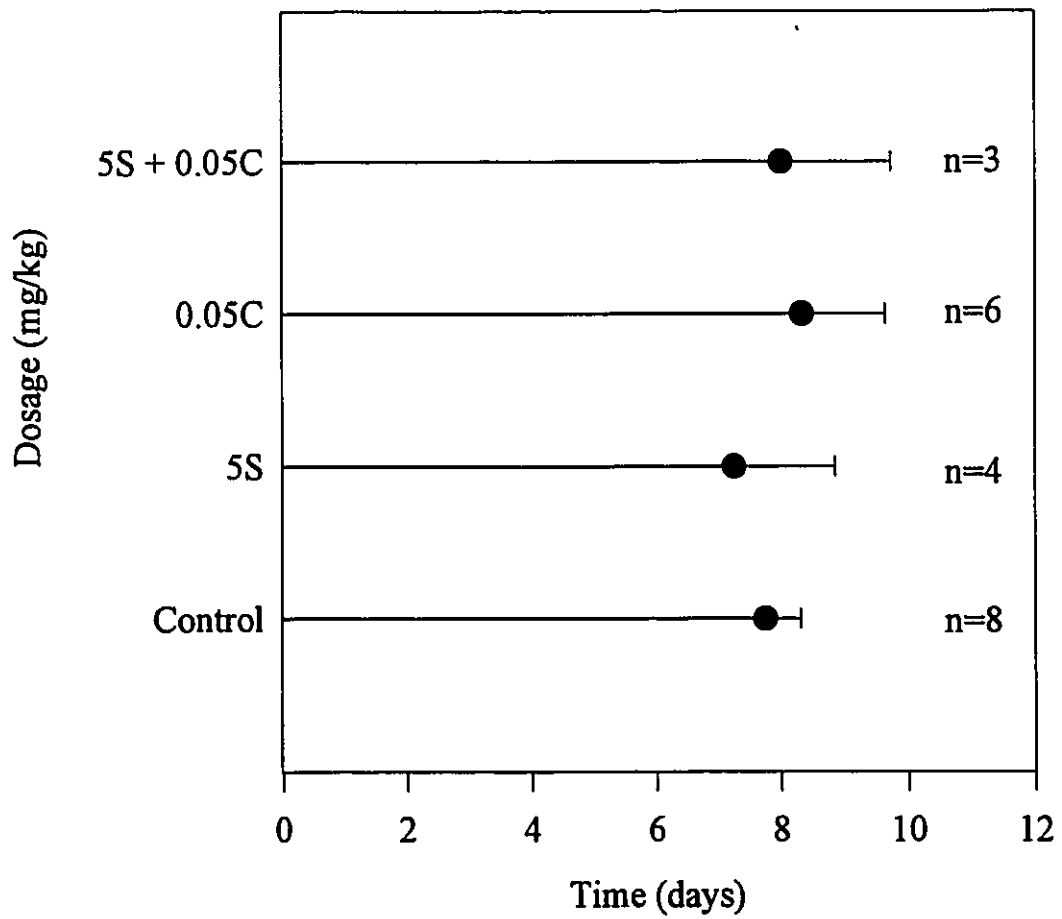
Lesion history of the controls and the rabbits treated with 0.05 mg/kg of ceftriaxone, 5mg/kg of streptomycin and a combination of 0.05 mg/kg of ceftriaxone + 5 mg/kg of streptomycin, at an inoculum of 10^4 CFU.

- a Data are means \pm standard deviations, of median values for each rabbit.
- b See Table 4 for inoculation techniques used in each group.
- c CF=Ceftriaxone, SM= Streptomycin
- d Comparative evaluation of the inoculum sizes between all the groups using an ANOVA (P=1.00).
- e Comparative evaluation of the group vs the control group (t-test).
- f Equal variance test failed. P=0.81 with the Mann-Whitney Rank Sum Test.
- g Equal variance test failed. P=0.92 with the Mann-Whitney Rank Sum Test.

Animal group ^b	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2
Control (n = 8)	3.22 ± 1.92 ^d	7.75 ± 1.58	9.00 ± 1.60	10.50 ± 2.62
0.05 mg/kg CF ^e (n=6)	3.10 ± 1.94	8.33 ± 3.20 (P = 0.66) ^g	7.83 ± 1.60 (P = 0.20) ^g	9.00 ± 2.45 (P = 0.30) ^g
5 mg/kg SM ^f (n=4)	3.21 ± 2.01	7.54 ± 2.98 (P = 0.72) ^{g,h}	7.25 ± 1.50 (P = 0.10) ^g	8.75 ± 2.36 (P = 0.29) ^g
0.05 mg/kg CF + 5 mg/kg SM (n=3)	2.90 ± 2.99	8.00 ± 3.00 (P = 0.86) ^{g,h}	9.33 ± 0.58 (P = 0.74) ^g	12.7 ± 1.53 (P = 0.22) ^g

Figure 8

Length of culture positivity for the controls, 0.05 mg/kg of ceftriaxone, 5 mg/kg of streptomycin and the combination of 0.05 mg/kg of ceftriaxone + 5 mg/kg of streptomycin dose groups, at an inoculum of 10^4 CFU. Error bars are standard error.



close to 13 days were needed for reepithelialization (score of 2) to occur (Table 8 and Figures 9, 10 and 11).

When the dose of streptomycin in the combination was increased to 10 mg/kg, the times to lesion aspirate sterilization and healing were significantly decreased. The lesion aspirates for the combination group were sterile after 2.60 ± 1.67 days (Table 9 and Figure 12). Not only was this number significantly different from the control but it was also different from the individual drugs in the combination (Table 9). 5.80 ± 0.84 days after treatment, the lesion size had decreased by 50%, making this treatment group significantly different from the control and from the individual concentrations of 0.05 mg/kg of ceftriaxone and 10 mg/kg of streptomycin. 6.60 ± 1.75 days were needed for reepithelialization of the lesions. This is different from the numbers seen with the control and also with the 10 mg/kg of streptomycin. A lesser difference existed with 0.05 mg/kg ceftriaxone which did not reach statistical significance perhaps due to small numbers (Table 9 and Figure 13). Figures 14-17 show the contrast in lesion size between the different groups.

When only the results obtained with technique #2 were analysed, basically the same results were obtained. The individual numbers for the control, 0.05 mg/kg ceftriaxone and 10 mg/kg streptomycin groups changed since a mix of both techniques had been used, while the combination results stayed the same because of the sole use of technique #2. (Table 10, Figures 18 and 19). The same significant differences existed between the groups.

Figure 9

Course of disease for the controls and the combination dose group of 0.05 mg/kg of ceftriaxone + 5 mg/kg of streptomycin at an inoculum of 10^4 CFU.

Bars indicate the mean % of the baseline lesion size (Day 0) remaining. The line plot indicates the mean scores while the scatter plot represents the mean of the last culture positive day.

**Empty bars and empty circles - Controls
Hatched bars and full squares - Combination
Error bars are standard error.**

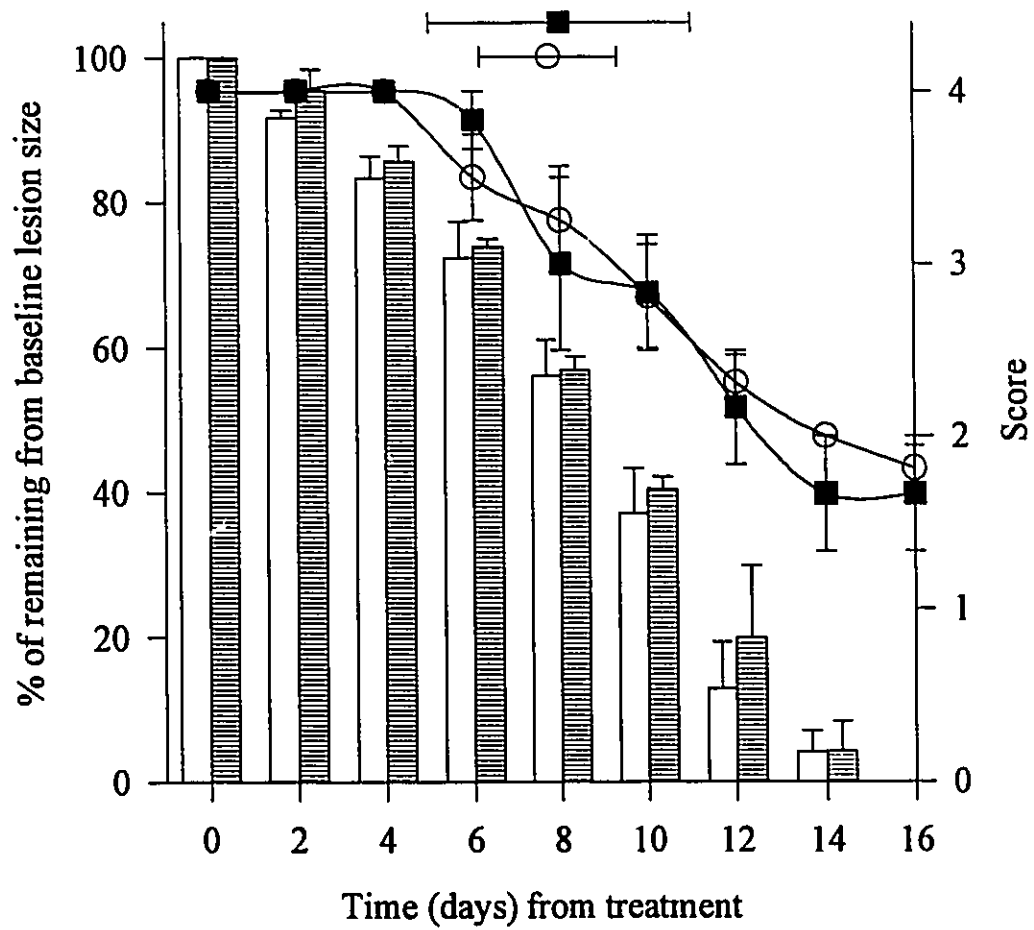
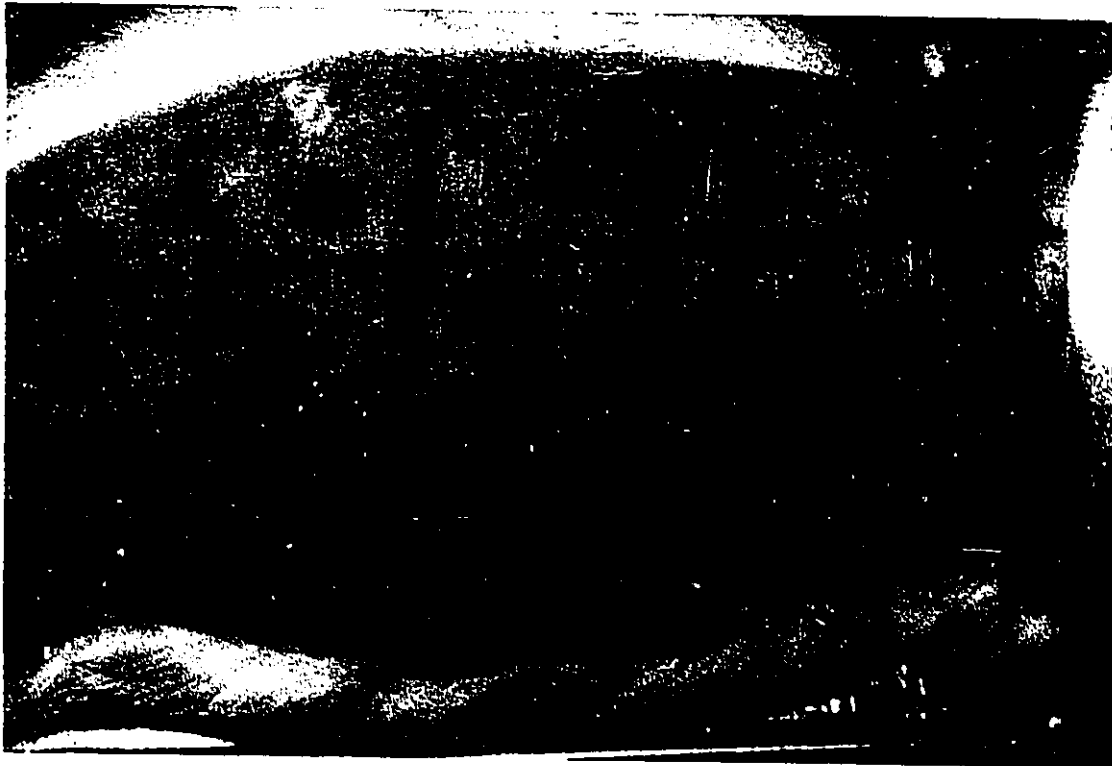


Figure 10

Appearance of lesions at day 0 for a control (A) and rabbit receiving the combination of 0.05 mg/kg of ceftriaxone + 5 mg/kg of streptomycin (B), at an inoculum of 10^4 CFU.

A



B

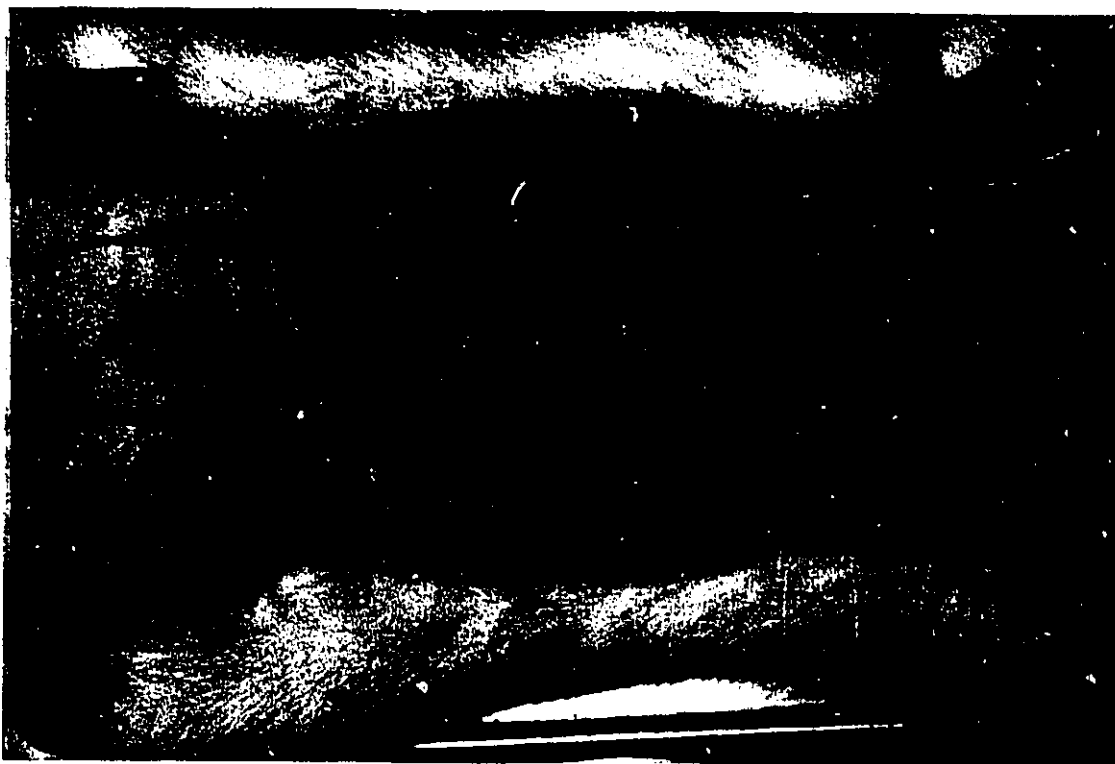


Figure 11

Appearance of lesions at day 8 for a control (A) and a rabbit receiving the combination of 0.05 mg/kg of ceftriaxone + 5 mg/kg of streptomycin (B), at an inoculation of 10^4 CFU.

A



B

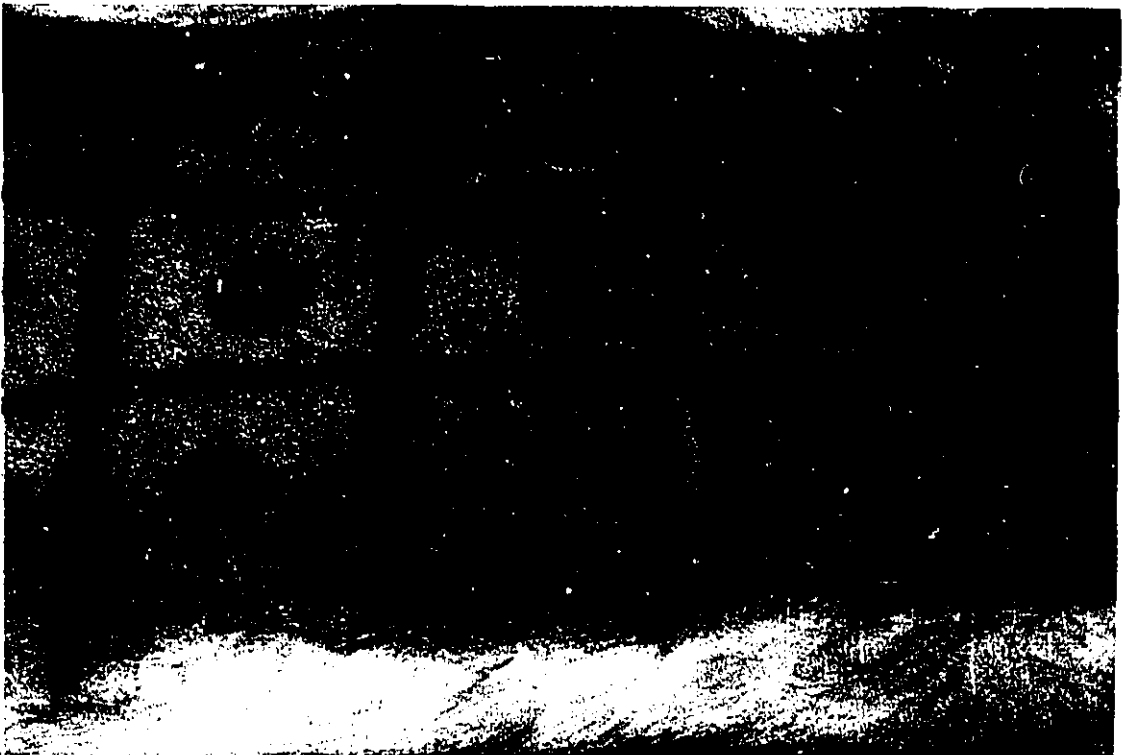


Table 9

Lesion history of the controls and the rabbits treated with 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and a combination of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin, at 10^4 CFU. Animals from the two inoculation techniques are included.

- a Data are means \pm standard deviations, of median values for each rabbit.
- b See Table 4 for inoculation techniques used for each group.
- c CF=Ceftriaxone, SM= Streptomycin
- d Comparative evaluation of the inoculum sizes between all the groups using an ANOVA (P=0.72).
- e Comparative evaluation of the group vs the control group (t-test).
- f Comparative evaluation of the group vs rabbits treated with 10 mg/kg of streptomycin (t-test).
- g Comparative evaluation of the group vs rabbits treated with 0.05 mg/kg of ceftriaxone (t-test).

Animal group ^b	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2
Control (n=8)	3.22 ± 1.92 ^d	7.75 ± 1.58	9.00 ± 1.60	10.50 ± 2.62
0.05 mg/kg CF ^e (n=6)	3.10 ± 1.94	8.33 ± 3.20 (P = 0.66) ^e	7.83 ± 1.60 (P = 0.20) ^e	9.00 ± 2.45 (P = 0.30) ^e
10 mg/kg SM ^f (n=5)	5.36 ± 1.65	8.40 ± 2.70 (P = 0.59) ^e	8.60 ± 0.55 (P = 0.21) ^e	9.50 ± 1.54 (P = 0.46) ^e
0.05 mg/kg CF + 10 mg/kg SM (n=5)	5.13 ± 2.47	2.60 ± 1.67 (P < 0.0005) ^f (P < 0.005) ^f (P < 0.01) ^g	5.80 ± 0.84 (P < 0.005) ^e (P < 0.0005) ^f (P < 0.05) ^g	6.60 ± 1.75 (P < 0.05) ^e (P < 0.05) ^f (P = 0.10) ^e

Figure 12

Length of culture positivity for the control, 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and the combination of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin dose groups, at an inoculum of 10^4 CFU. Animals from both inoculation techniques are included. Error bars are standard error.

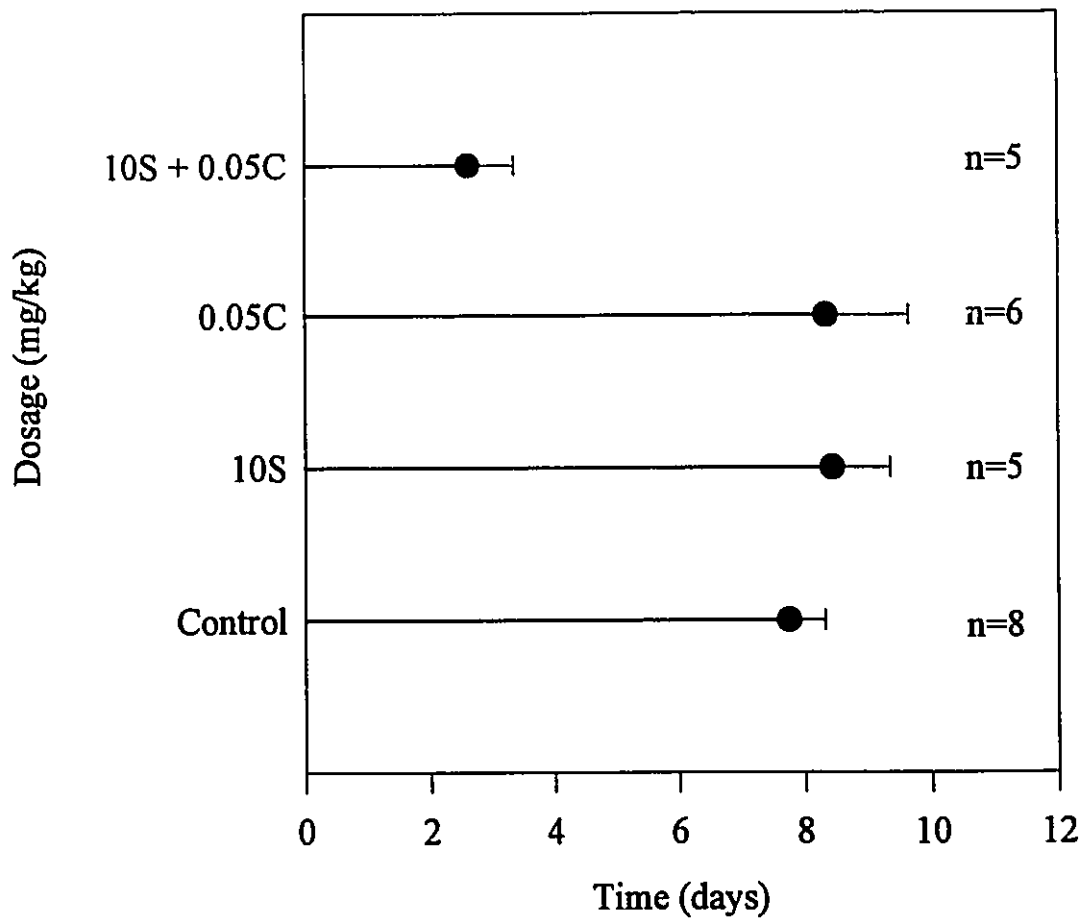


Figure 13

Course of disease for the controls, the dose groups of 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and the combination dose group of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin at 10^4 CFU.

Animals from both inoculation techniques are included. Bars indicate the mean % of the baseline lesion size (Day 0) remaining. The line plot indicates the mean scores while the scatter plot represents the mean of the last culture positive day.

Empty bars and empty circles - Control group
Diagonally hatched bars and full squares - 10 mg/kg of streptomycin
Vertically hatched bars and full circles - 0.05 mg/kg of ceftriaxone
Horizontally hatched bars and triangles - Combination.
Error bars are standard error.

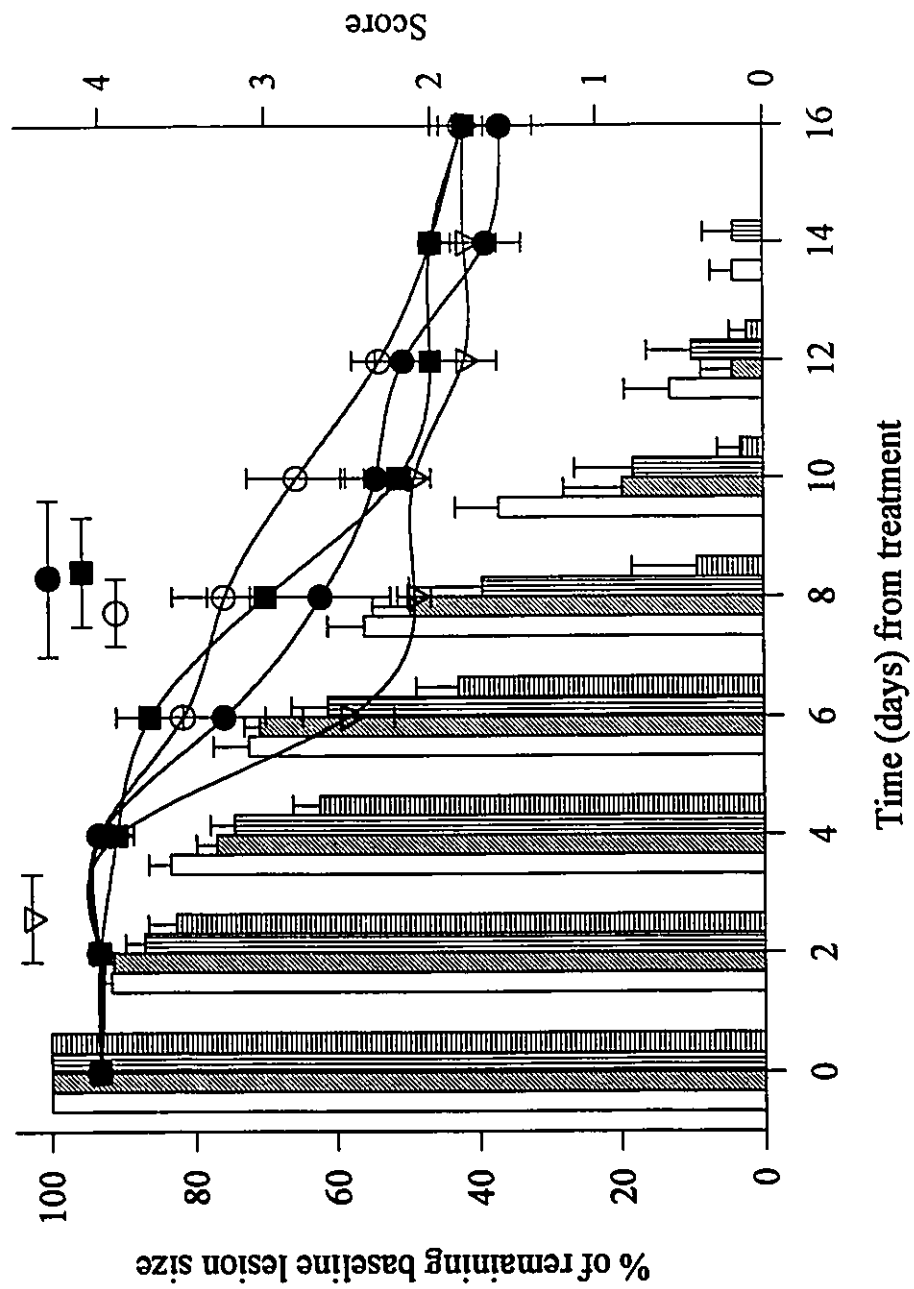


Figure 14

Appearance of lesions at days 0 (A) and 8 (B) for a control rabbit at an inoculum of 10^4 CFU.

A



B

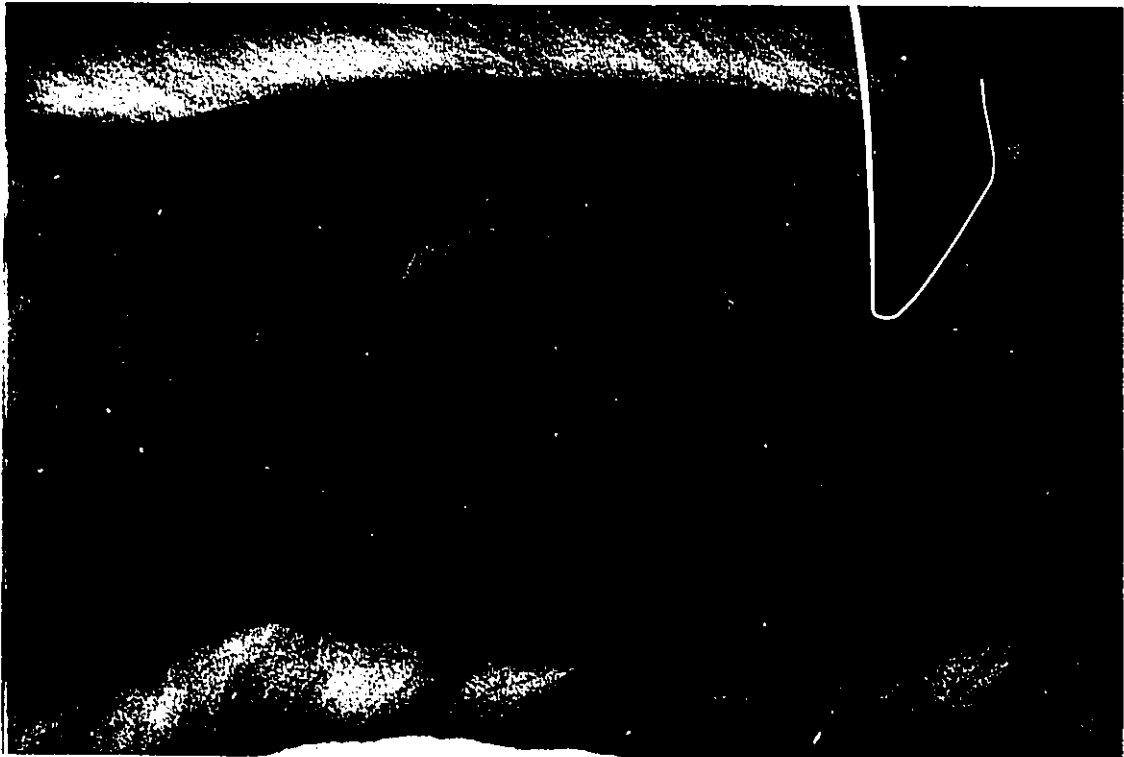


Figure 15

Appearance of lesions at days 0 (A) and 8 (B) on a rabbit treated with the combination of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin, at an inoculum of 10^4 CFU.

A



B



Figure 16

Appearance of lesions at days 0 (A) and 8 (B) on a rabbit treated with 0.05 mg/kg of ceftriaxone, at an inoculum of 10^4 CFU.

A



B

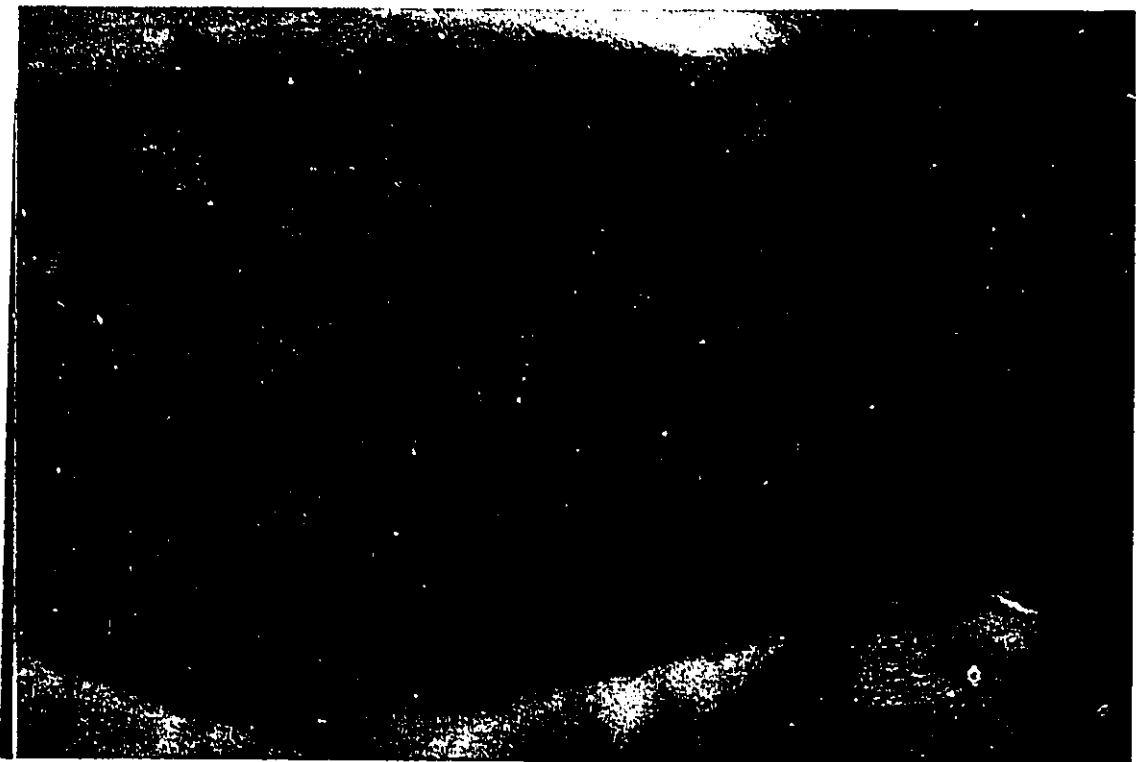


Figure 17

Appearance of lesions at days 0 (A) and 8 (B) on a rabbit treated with 10 mg/kg of streptomycin, at an inoculum of 10^4 CFU.

A



B

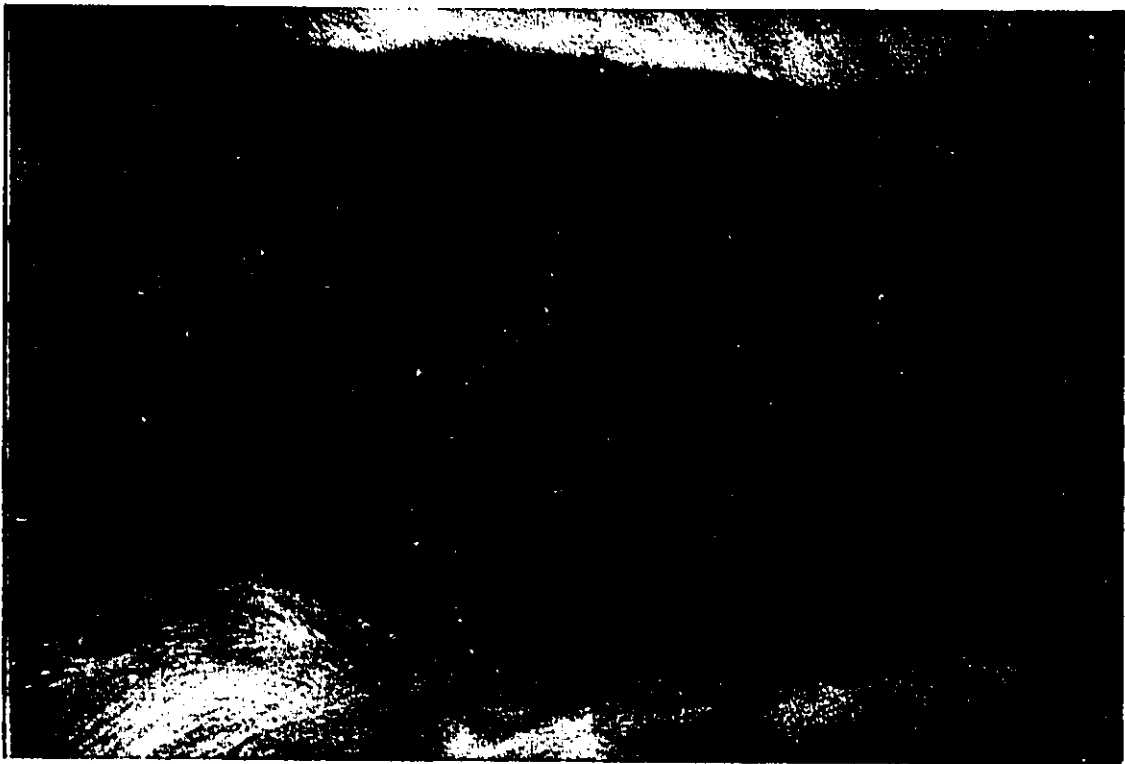


Table 10

Lesion history of the controls and the rabbits treated with 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and a combination of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin, at 10^4 CFU. Only animals inoculated using technique # 2 are included.

- a Data are means \pm standard deviations.
- b CF=Ceftriaxone, SM= Streptomycin
- c Comparative evaluation of the inoculum sizes between all the groups using an ANOVA (P=0.43).
- d Comparative evaluation of the group vs the control group (t-test).
- e Comparative evaluation of the group vs rabbits treated with 10 mg/kg of streptomycin (t-test).
- f Comparative evaluation of the group vs rabbits treated with 0.05 mg/kg of ceftriaxone (t-test).
- g Equal variance test failed. P=0.40 with the Mann-Whitney Rank Sum Test.
- h Equal variance test failed. P < 0.05 with the Mann-Whitney Rank Sum Test.

Animal group	Inoculum size ^a (CFU) x 10 ⁴	Number of culture positive days	Time (days) to 50% reduction in pre- treatment lesion size	Time (days) to reduction in lesion score from 4 to 2
Control (n=3)	3.99 ± 2.48 ^e	7.33 ± 1.15	9.67 ± 1.53	11.67 ± 2.31
0.05 mg/kg CF ^b (n=4)	3.78 ± 2.11	7.00 ± 2.71 (P = 0.85) ^d	8.25 ± 1.71 (P = 0.31) ^d	9.75 ± 2.75 (P = 0.40) ^d
10 mg/kg SM (n=4)	6.04 ± 0.74	7.50 ± 2.08 (P = 0.91) ^d	8.50 ± 0.58 ^g (P = 0.21) ^g	9.38 ± 1.75 (P = 0.19) ^d
0.05 mg/kg CF + 10 mg/kg SM (n=5)	5.13 ± 2.47	2.60 ± 1.67 (P < 0.01) ^d (P < 0.01) ^e (P < 0.05) ^f	5.80 ± 0.84 ^d (P < 0.005) ^d (P < 0.001) ^{g,h} (P < 0.05) ^f	6.60 ± 1.75 (P < 0.05) ^d (P < 0.05) ^e (P = 0.07) ^f

Figure 18

Length of culture positivity for the control, 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and the combination of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin dose groups, at 10^4 CFU. Only animals inoculated with technique #2 are included. Error bars are standard error.

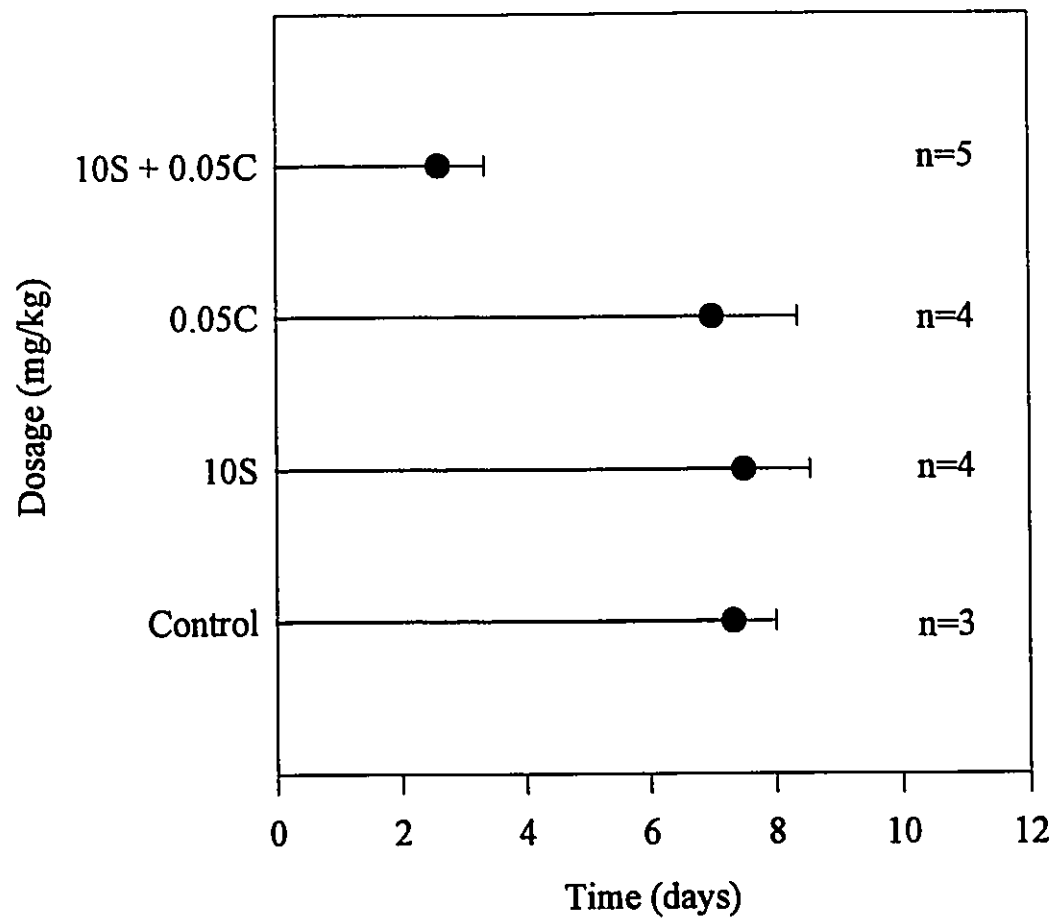
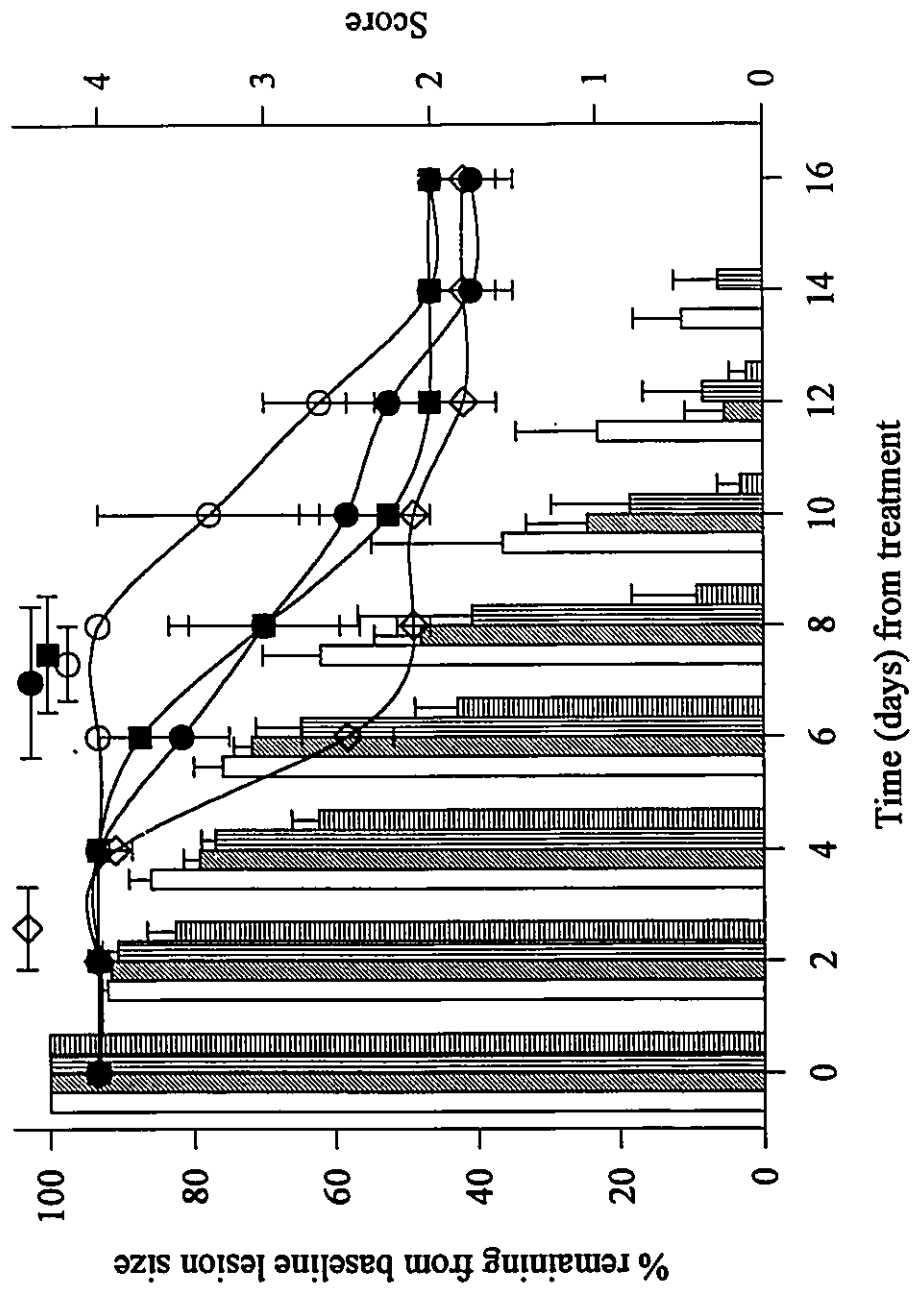


Figure 19

Course of disease for the controls, the dose groups of 0.05 mg/kg of ceftriaxone, 10 mg/kg of streptomycin and the combination dose group of 0.05 mg/kg of ceftriaxone + 10 mg/kg of streptomycin groups at 10^4 CFU.

Only animals inoculated with technique #2 are included. Bars indicate the mean % of the baseline lesion size (Day 0) remaining. The line plot indicates the mean scores while the scatter plot represents the mean of the last culture positive day.

Empty bars and circles - Control group
Diagonally hatched bars and full squares - 10 mg/kg of streptomycin
Vertically hatched bars and full circles - 0.05 mg/kg of ceftriaxone
Horizontally hatched bars and diamonds - Combination
Error bars are standard error.



DISCUSSION

1. Antimicrobial combinations

Four important rationales for the use of antimicrobial combinations exist in the presence of improved efficacy of combinations. The dosage of a potentially toxic antimicrobial can be decreased, for a successful clinical outcome at the same time. This may also be helpful in lowering the cost of an expensive treatment. Examples of potentially harmful antimicrobial drugs related to dose include 5-fluorocytosine and chloramphenicol which can suppress the marrow, and streptomycin which can be nephrotoxic and ototoxic with prolonged or repeated use.

Another use for antimicrobial combinations is in the treatment of documented or suspected polymicrobial infections. It may be necessary in some diseases to treat with agents active against each of the likely or major pathogens.

In third place, antibiotics are sometimes combined to prevent or delay the emergence of drug-resistant subpopulations of an organism. When two or more agents to which bacteria develop different modes of resistance are used together, the possibility that clones will emerge that are resistant to all antimicrobials is reduced. For example, the use of multiple drugs in the treatment of tuberculosis reduces the risk of selection or induction of resistant strains. Rifampin is often used with another antimicrobial because bacterial resistance to it can be rapidly induced if it is used alone. Aminoglycosides and β -lactams are often used together to address the predilection of some enteric bacilli to develop resistance to single drugs in treatment of infections.

Yet another advantage is the potential synergistic therapeutic effect the antimicrobials may exhibit when combined. Many synergistic combinations have been documented. At present there are four known mechanisms of antimicrobial interaction that result in synergy: 1) Sequential inhibition of a common biochemical pathway; 2) Inhibition of β -lactamase or decreased production of β -lactamase; 3) Sequential inhibition of cell wall synthesis; 4) The use of β -lactams or other agents acting on the cell wall to permit increased entry of aminoglycosides (Eliopoulos and Moellering Jr. 1991).

Some of these concepts have already been applied towards *H. ducreyi*. The combination of trimethoprim and a sulfonamide is synergistic because the drugs sequentially block two steps in the synthesis of folic acid. Together they also decrease the chances of bacterial resistance (Reese and Betts 1993). The combination was used successfully against *H. ducreyi* until individual resistance to the sulfonamides and trimethoprim rendered the combination unacceptable for treatment (Schmid 1989; Plourde et al. 1992).

Amoxicillin and clavulanic acid is another combination that has been useful against *H. ducreyi* because of their synergistic effect. Clavulanic acid is a β -lactamase inhibitor that thus protects amoxicillin (a β -lactam) from hydrolysis by the β -lactamase produced by the bacteria. They were successfully used together against the pathogen in question until resistant strains slowly prevailed (Fast et al. 1982; Todd and Benfield 1990; Motley et al. 1992).

Antagonism may result from the combination of two or more drugs. Combinations that yield such an interaction are: 1) Combinations of bacteriostatic agents with β -lactams; 2) Combinations of 50S subunit ribosomal inhibitors; 3) Combinations of aminoglycosides with bacteriostatic agents and 4) Combinations of β -lactams containing one agent that derepresses

β -lactamase production (Eliopoulos and Moellering Jr 1991). No antibiotic combinations for the treatment of chancroid have generated such results.

There is no paucity of effective drugs for the treatment of chancroid at the moment. Many antimicrobials such as ceftriaxone, azithromycin, fleroxacin, erythromycin and other new fluoroquinolones and cephalosporins possess great activity against *H. ducreyi* (Le Saux and Ronald 1989; Bowmer et al. 1987; Tyndall et al. 1994; MacDonald et al. 1989). An ideal treatment for chancroid should be effective, inexpensive, well-tolerated and capable of being administered as a single dose. These last two criteria help to ensure patient compliance and thus the effectiveness of the treatment (Abeck et al. 1988a). Ceftriaxone, azithromycin and fleroxacin are efficacious as single-dose treatments because of their long half-lives (Reese and Betts 1993). However, a >20% failure rate has been reported for these drugs in patients concurrently infected with HIV (MacDonald et al. 1989; Tyndall et al. 1993; Tyndall et al. 1994). In order to keep the treatments for chancroid efficacious for patients also infected with HIV, multiple doses must be administered. Patient compliance is apt to decrease in this situation, making the treatment less effective. An antibiotic combination may have an increased activity against *H. ducreyi*, thereby making single-dose treatments once again effective for *all* patients.

2. Antimicrobial agents

Four antimicrobial agents were chosen for testing against *H. ducreyi*. Ceftriaxone has a prolonged half-life of 6-8 hours, the longest of all currently available third-generation cephalosporins, and also has good tissue penetration. It is effective *in vitro* against *H.*

ducreyi with MICs ranging from ≤ 0.002 to $0.008 \mu\text{g/mL}$ (Wiedemann and Atkinson 1993; Le Saux and Ronald 1989; Bowmer et al. 1987). Ceftriaxone is active against β -lactamase producing strains and no resistance to it has been identified (Trees and Morse 1995). It is bactericidal and works by inhibiting bacterial cell wall synthesis (Reese and Betts 1993). When cephalosporins are combined with an aminoglycoside they are often synergistic against sensitive bacteria (Greenwood 1989; Eliopoulos and Moellering Jr. 1991).

Streptomycin is an aminoglycoside that was used up to the early 1980s as a treatment against chancroid. It is still recommended by Reese and Betts (1993) as an alternate agent. Although resistance has been documented in Amsterdam, Singapore and France in the early eighties (Morse 1989), at around the same time a report from Korea showed streptomycin to be one of the best treatments (Fitzpatrick et al. 1981). It is a bactericidal agent and acts by penetrating the cell wall and membrane to bind irreversibly to the 30S bacterial ribosomes. The synthesized proteins are abnormal and nonfunctional and bacterial death ensues (Reese and Betts 1993). It has a serum half-life of 2.5 hours which is fairly long compared to other drugs and has fairly good tissue penetration. (Gerding et al. 1991; Reese and Betts 1993).

Azithromycin is an azalide that possesses MICs between 0.002 and $0.06 \mu\text{g/mL}$ against *H. ducreyi* (Knapp et al. 1993) and has been used in a single dose to treat chancroid (Tyndall et al. 1994). It possesses a serum half-life of 19 to 24 hours and a tissue half-life of 2-3 days. The drug serum levels are low but are extremely elevated in tissues. It is bactericidal and inhibits protein synthesis, similarly to erythromycin, i.e. it binds in a reversible fashion to the 50S ribosomal subunits of susceptible organisms (Greenwood 1989; Reese and Betts 1993).

Rifabutin is a semi-synthetic ansamycin showing good *in vitro* activity against *H. ducreyi*

with an MIC of 0.004-0.016 µg/mL. Human studies are needed to study its potential as a treatment for chancroid (Abeck et al. 1988; Farmitalia Carlo Erba 1993). It has a long half-life of 35-40 hours and is bactericidal in two ways. First it can inhibit the activity of DNA-dependent RNA polymerase and secondly it can inhibit DNA synthesis (Farmitalia Carlo Erba 1993).

Not only have all the drugs tested in this project been used successfully to treat chancroid (except for rifabutin which has only been tested *in vitro*), but their mechanisms of action also presented potential for synergy if combined. Ceftriaxone and streptomycin fall in one of the categories of synergistic drugs. In fact, the effect of β -lactams combined with aminoglycosides against Enterobacteriaceae has been studied and has proven synergistic with the checkerboard broth microtiter technique (Eliopoulos and Moellering Jr. 1991; Jones and Packer 1982). Azithromycin and streptomycin both inhibit protein synthesis but by binding to the two different components of the bacterial ribosomes. This combination could thus likely have resulted in synergy through sequential inhibition of protein synthesis. The combination of the other drugs don't fall in one of the categories that describe synergy. However, they do not fall in the categories that describe antagonism either. In fact some antibiotic combinations have been known to interact synergistically by unknown mechanisms (Eliopoulos and Moellering Jr. 1991). Hence, all the drugs studied were tested in pairs to determine if positive interactions would arise between them.

3. *In vitro* antimicrobial testing

Determination of virulence

In selecting a panel of *H. ducreyi* strains for antimicrobial testing, it was important to select virulent strains for the sake of relevance to clinical situations although all isolates are from human infections. The most accepted method for virulence testing of *H. ducreyi* is the use of the rabbit model of chancroid (Purcell et al. 1991). *H. ducreyi* strain 35000 and RO-34 have already proven to be virulent in the temperature-dependent rabbit model of infection (Desjardins et al. 1995; Purcell et al. 1991). The virulence of strain 35000, was verified by the temperature-dependent rabbit model of infection. While other studies have shown 10^5 CFU to be the MUPI for strain 35000 (Alfa et al. 1995; Hansen et al. 1994; Purcell et al. 1991), a MUPI of 10^4 CFU was obtained here. The fact that these groups used plate-grown bacteria as opposed to broth-grown bacteria for infection may account for this difference. Their rabbits were also housed at a slightly higher temperature (15-17 °C) compared to ours (14±1°C), which may be more important. Strain RO-40 proved to be virulent with a MUPI of 10^4 CFU.

Minimum inhibitory concentrations

As stated above, strains 35000 and RO-34 are virulent as defined by the temperature-dependent rabbit model (Desjardins et al. 1995; Purcell et al. 1991). We found RO-40 to also be virulent in the same model. Strains 35000 (Winnipeg) and RO-34 (Nairobi) possess a different outer membrane profile (Desjardins et al. 1995) and strain RO-40 (Nairobi) also differs from these two (unpublished results). This was of importance since these strains

probably represent isolates that originate from different sources. Even though these strains were different, the MICs were the same for all four antimicrobial tested. MICs for all of the drugs that we tested have been documented and the results we obtained were similar to those previous reports (Dangor et al. 1990).

Fractional inhibitory concentration indices

The lowest FIC index obtained was of 0.063 ± 0 for the ceftriaxone-streptomycin combination (Table 2). A value of 1 represents additivity, a value of less than or equal to 0.5 synergy, and a value of more than 4 represents antagonism. These results suggest a combined activity that is at least additive, although not synergistic (Eliopoulos 1991; Greenwood 1989). The tests where FIC indices of 1.5 were obtained, were ones where the presence of a second antibiotic did not change the MIC in any of the combinations. By definition, as explained in the introduction, this therefore represents additivity or autonomy. The other antibiotic combinations reflected at least additivity or supra-additivity. Since ceftriaxone and streptomycin showed the best consistent positive interaction, we decided to undertake the *in vivo* experiments with those two drugs.

Methods in combination antimicrobial testing

Different *in vitro* methods can be used to assess the activity of antimicrobial combinations. The one used the most frequently is the checkerboard, so-called because of the pattern of tubes or microtiter wells formed by the multiple dilutions of the two drugs being test (Eliopoulos and Moellering 1991). The checkerboard can be done by the agar

dilution method as in this project or by the broth method. With the latter, the organism is inoculated in broth containing different concentrations of the antimicrobial being tested. The tubes are observed for turbidity after 16-20 h of incubation and MICs are determined (Amsterdam 1991).

Killing curves measure the microbicidal activity of the combination being tested. The advantage to this technique is that serial colony counts are measured at different points in time from the incubated tubes where the organism is present with the antimicrobial. This allows a more dynamic and broader picture of the way the antimicrobials interact with each other. It is however tedious and limits the number of antibiotic concentrations and combinations that can be tested (Eliopoulos and Moellering 1991).

The diffusion method is very easy to perform. Disks or strips of paper containing drugs A and B are arranged on a plate that has been inoculated with the organism to be tested. The pattern of growth around the disks or strips determine the interaction that exists between the two antimicrobial. The disadvantage is that FIC values cannot be obtained (Eliopoulos and Moellering 1991).

Because *H. ducreyi* is a fastidious organism that grows slowly and that requires special nutrients, routine susceptibility testing in liquid media may not be adequate. No studies to demonstrate that *H. ducreyi* can be tested accurately and reproducibly by the rapid standard methods mentioned above, have been done (Amsterdam 1991). The only *in vitro* antimicrobial test that has been established for *H. ducreyi* is the agar dilution technique (Trees and Morse 1995; Hammond et al. 1978b; Slaney et al. 1990).

In this study the agar dilution technique was used to determine the MICs of ceftriaxone,

streptomycin, azithromycin and rifabutin against *H. ducreyi* as well as to produce FIC indices for all two-drug combinations against the same organisms. The MICs were similar to previously published reports although the MIC of azithromycin against the ATCC 29213 *S. aureus* strain fell just outside the accepted range. This may be a result of the use of supplemented media. Combinations including rifabutin showed some degree of supra-additivity against some of the *H. ducreyi* strains. The lowest FIC index (0.63) was obtained for the ceftriaxone-streptomycin combination by the checkerboard technique. It was therefore decided that this supra-additive combination would be assessed *in vivo*. Since the checkerboard technique does not measure bactericidal activity and only assesses synergy at one point in time, correlation with *in vivo* testing may be inaccurate. Time-kill methodology in general appears to be a more sensitive way to evaluate antimicrobial activity than the MIC/FIC methods (Stratton 1991). A standardized time-kill methodology for *H. ducreyi* would thus certainly be of interest. All combinations in this study would have to be tested *in vivo* in order to determine for certain their true combined interactions.

4. *In vivo* antimicrobial testing

As discussed above, *in vitro* testing of drug combinations against microorganisms can often be inconsistent. For that reason, animal trials in relevant models of infection and disease are important to verify the drug interactions which may justify human clinical trials.

Iron is a limiting nutrient for cell growth, and high levels may predispose a subject to infection and cancer. It is a nutrient required for all bacterial life with the exception of lactic acid bacteria (Lauffer 1992). The body must protect itself against infections and invasions

and one way it does so is through the actions of the iron-withholding defence system (Weinberg 1984). Iron loading would hence be expected to lengthen disease or aggravate its severity. Meloche (unpublished results) observed a significant increase in lesion size and length of culture positivity in rabbits iron loaded with 750 mg/kg of iron dextran. Because of the observed stress on the rabbits induced by iron injections in Meloche's experiments, it was decided that a 20% lower dose of 600 mg/kg would be administered. The purpose was to increase disease severity and length of culture positivity in order to better measure the differences between groups such as between controls and rabbits treated with antibiotics. Even though the total iron concentration had been decreased from that used by Meloche, we still found it to be very taxing on the animals, with diarrhea and lethargy. No clear differences existed in our hands, between the iron loaded and non iron loaded rabbits, and infection persisted without iron loading for as long as in iron loading by Meloche. It was thus decided at this point to undergo all future experiments without the iron loading.

A range of concentrations of each drug, ceftriaxone and streptomycin were assessed in the temperature-dependent rabbit model of infection. This model is the most ideal one for studying chancroid because of such advantages as low inocula (10^4 CFU as demonstrated here) for consistent production of disease, the use of a "natural" route of infection, the resemblance to the human infection, the production of a measurable immune response to a primary infection with a microorganism and ample space for multiple lesions (Desjardins et al. 1995; Purcell et al. 1991). Also, the ability to quantify infection and disease is critical. For ceftriaxone, the concentrations tested were based on individual concentrations used in humans (250 mg) as was done by Meloche (unpublished results). For streptomycin, the same

correlation was made (1 g doses are used in humans) as well as a comparison with streptomycin doses used in combination with penicillin to treat enterococcal endocarditis in a rabbit model of infection where low and high doses of streptomycin were used (3.5 and 10 mg/kg respectively) (Henry et al. 1986).

The approach was to use subtherapeutic doses of each drug and then to combine these to assess synergy. This approach was taken instead of immediately combining the antibiotics in the same ratios as the ones at which the most favourable combination was seen *in vitro*, i.e. where the lowest FIC index was observed. The rationale for this is that there are numerous factors present *in vivo* but absent *in vitro* that may dictate the activity of the antimicrobial in such a way that not the same drug ratios may be needed. As well, comparison of subtherapeutic doses of antibiotic with combinations is needed to permit detection of any synergy in a quantitative virulence assay of limited dynamic range.

In vivo serum levels of an antibiotic are often important. The optimal use of β -lactams should emphasize achieving levels slightly above the MIC, for example, 4 to 8 times above it. This is because these agents possess a time-dependent bactericidal activity. In contrast, aminoglycosides have concentration-dependent bactericidal activity. This means the rates of killing increase with the increasing drug concentrations up to a point of maximum effect. These serum levels can however be misleading. Some of the antimicrobial can bind to serum binding proteins thus making their passage into lymph and the surrounding tissues difficult. Although 83-95% of ceftriaxone is bound to proteins it still penetrates tissues quite well. Nevertheless, it can be easy to overestimate the activity of a highly protein-bound agent. Streptomycin is only bound 30% to serum proteins (Gerding et al. 1991)

It is quite possible that these differences between ceftriaxone and streptomycin may alter the way they appear to occur together *in vitro* when put in an *in vivo* situation. In fact, a ratio of 1:200 (ceftriaxone:streptomycin) demonstrated a synergistic interaction *in vivo*. *In vitro*, a ratio of 1:1000 was shown to give similar results. The *in vivo* interaction seemed better than *in vitro* and was synergistic as defined by Cleeland and Squires (1991). They state that a combination is considered synergistic *in vivo* if one of three criteria is met, one being that “no response to the single agents is obtained, while the combination exhibits significant activity”. This synergy was evident when statistical analysis was done on the groups containing animals inoculated with the two different techniques and also on the group containing animals strictly inoculated with inocula of 10^4 CFU (technique #2). In both analyses, the days of culture positivity and the time to a 50% reduction in lesion size was significantly different between the combination and the control as well as between the combination and the individual agents. Also in both analyses, the time to a score of 2 was significantly different between the combination and the control and also between the combination and the individual 10 mg/kg of streptomycin, although not quite as different nor statistically significant between the combination and the individual 0.05 mg/kg of ceftriaxone. This lack of statistical significance might be related to small numbers of measures or relatively great variance. It may also be because of the slightly higher speed of lesion healing seen with the single dose of ceftriaxone than with the single dose of streptomycin or due to the generally lower effect of any treatment on the speed of lesion healing measured by reduction in score, and hence the ability to produce differences in this parameter. Nevertheless, more rapid lesion healing was significant in every other aspect and comparisons may have been more so

if slightly higher concentrations of each drug had been utilized. The 10 mg/kg streptomycin + 0.05 mg/kg ceftriaxone combination shows that the interaction of the two drugs was indeed at least as favourable *in vivo* as *in vitro*.

5. Conclusions

The supra-additive interaction demonstrated between ceftriaxone and streptomycin by the *in vitro* checkerboard agar dilution technique was corroborated by the synergistic interaction observed in the *in vivo* rabbit model of infection when a single dose was administered. Whether such clinical synergy would be observable in humans will have to be determined by clinical trials. The biggest concern is whether a single dose of the ceftriaxone-streptomycin combination would be more efficacious in treating chancroid in HIV positive immunosuppressed patients.

Since bactericidal activity of antimicrobials is important in treating infections where the host is immunosuppressed, ceftriaxone and streptomycin are ideal (Stratton IV 1991). Additionally, combination of drugs in immunosuppressed patients have been shown to dramatically improve the outcome in some instances. This was clearly evident in a study by Andriole (1971) where a 90% mortality rate by a *Pseudomonas* infection in immunosuppressed patients when treating with either carbenicillin or with gentamicin was reduced to 5% when the two drugs were combined. Other advantages that may be conferred by the combination of ceftriaxone and streptomycin in the treatment of chancroid, are the decrease of the development of bacterial resistance and lower costs of treatment because smaller concentrations of the drugs can be used.

Even though antibiotic resistance to streptomycin has been documented in a few locations, the 3.10 MDa plasmid responsible for resistance to streptomycin-kanamycin has not been reported lately. As part of a single dose combination of great efficacy, it is perhaps unlikely that streptomycin resistance would become prevalent. In fact, the trimethoprim sulfonamide combination proved very effective against *H. ducreyi* even though sulfonamide-resistant strains existed. Trimethoprim and sulfonamides were being used individually, thus resistance to the individual drug became prevalent. With the emergence of TMP resistance, the sulfonamide-coding plasmid quickly became disseminated and resistance to the combination widespread (Plourde et al. 1992). No resistance to ceftriaxone has yet been reported and if the ceftriaxone-streptomycin combination proved effective, the need to use it individually would diminish.

Both drugs are readily available in developing countries, and streptomycin is inexpensive. Ceftriaxone is well-tolerated and safe. Streptomycin can be ototoxic and nephrotoxic but only if used in high doses for long periods of time (Reese and Betts 1993).

We are hopeful that the combination of ceftriaxone and streptomycin will help in reducing the poor clinical outcome of chancroid treatment observed in patients concurrently infected with HIV. This improvement could play a major role in halting or slowing the transmission of HIV, through more effective treatment-based public health control programs for chancroid in populations at risk. Based on the results obtained, it is necessary that the efficacy of ceftriaxone and streptomycin be comparatively evaluated individually and in combination through clinical trials.

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APPENDIX

1. Chocorage agar plates.

3.6% w/v gonococcal agar

1% w/v haemoglobin

1% v/v Isovitalex

5% v/v fetal bovine serum

2. Formula for quantity of antibiotics to be used in making stocks.

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration } (\mu\text{g/mL})}{\text{Assay potency } (\mu\text{g/mg)}}$$

3. 0.5 McFarland barium sulfate standard

0.05 mL of 0.048M BaCl₂ (1.75% w/v BaCl₂•2H₂O) is added to 99.5 mL of
0.35N H₂SO₄ (1% v/v).

4. Calculation of the fractional inhibitory concentration index for combinations of two antimicrobials.

$$\frac{A}{\text{MIC}_A} + \frac{B}{\text{MIC}_B} = \text{FIC}_A + \text{FIC}_B = \text{FIC index}$$

“A” is the concentration of drug A in a tube that is the lowest inhibitory concentration in its row. MIC_A is the MIC of the organism to drug A alone. FIC_A is the fractional inhibitory concentration of drug A. “B”, MIC_B and FIC_B are defined in the same fashion

for drug B.

5. Phosphate buffered saline

0.01M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

0.01M Na_2HPO_4

9 g/L of NaCl

pH is adjusted to 7.2 - 7.3.