Characterization of the Cytopathic Effect of *Haemophilus ducreyi* on Human Foreskin Fibroblast (HFF) Cells *In Vitro*

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE (MSc.)

Department of Medical Microbiology
University of Manitoba
Winnipeg, Manitoba

ISBN 0-315-98969-6

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CHARACTERIZATION OF THE CYTOPATHIC EFFECT OF HAEMOPHILUS DUCREYI ON HUMAN FORESKIN FIBROBLAST (HFF) CELLS IN VITRO

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Little is known about the mechanisms by which Haemophilus ducreyi causes damage to the cells of its host. An in vitro cytopathic effect (CPE) on human foreskin fibroblasts (HFF) has recently been reported (10). The aim of this study was to characterize the cytopathic effect that H. ducreyi exerts on HFF cells in vitro. Using an adaptation of a tetrazolium-based cytotoxicity assay described by Scudiero et al (98,104), we have been able to confirm that the observed CPE is specific to H. ducreyi. Our data suggest that the CPE is not due to an excretable cytotoxin. Rather, the CPE was found to be mediated by H. ducreyi LOS, and was of two types: contact-dependant, and contact independent. In the contact dependant mechanism, H. ducreyi strains that were unable to attach to HFF cells in great numbers were not able to exert a CPE as well as H. ducreyi strains that were able to attach to HFF cells in large numbers. Thus the CPE observed using this approach was designated to be dependant on bacterial attachment to the HFF cells. In the contact indpendant mechanism, all strains of H. ducreyi tested were able to exert a CPE regardless of their capacity to attach to HFF cells. Purified lipooligosaccharide (LOS) from a variety of H. ducreyi strains was able to directly cause damage in amounts as low as 25 μg/well. It was also found that H. ducreyi actively shed LOS into the extracellular milieu. These results suggest that H. ducreyi are able to exert cell damage using a multi-step process of localization, growth and damage by extrusion of LOS onto the HFF cell surface.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Michelle Alfa for her supervision throughout this project. Though I may not have demonstrated it, I have come out of this project a far more confident scientist as a result of her patience and support.

I would also like to thank the members of my advisory committee, Dr. Pat McNicol, Dr. Grant McClarty, and Dr. Janice Dodd for their valuable advice and critical review of this manuscript.

Financial support throughout the project in the form of student stipends from the Department of Medical Microbiology and a graduate Fellowship form the University of Manitoba is gratefully acknowledged.

I would also like to gratefully thank my family for their support and understanding. Life would have been alot tougher without them.

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LIST OF ABBREVIATIONS

cfu	colony forming units
CPE	cytopathic effect
°C	degrees Celcius
DNA	deoxyribonucleic acid
g	grams
G+C	guanosine plus cytosine
h	hours
HFF	human foreskin fibroblasts
HIV	Human Immunodeficiency Virus
KDO	ketodeoxyoctulosoinc acid
KDa	kilodalton
LPS	lipopolysaccharide
LOS	lipooligosaccharide
LM	light microscopy
MDa	megadalton
µg	microgram
µl	microliter
µm	micrometer
mA	milliamps
mm	millimeter
ml	milliliter
mM	millimolar
M	molar
Mol%	molar percentage
nm	nanometers
%	percent
PBS	phosphate buffered saline
PC	phosphatidyl choline
RNA	ribonucleic acid
rpm	rotations per minute
SEM	scanning electron microscopy
SCM	serum containing tissue culture medium
SFM	serum free tissue culture medium
SUV	single unilammelar vescicles
SDS-PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
w/v	weight per volume
XTT	sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene - sulfonic acid hydrate

INTRODUCTION

The bacterium Haemophilus ducreyi has been known to be the etiological agent of chancroid for over a century (33,79), yet little is known of the pathogenesis of this organism. The hallmark of chancroid is characterized by a painful, soft, purulent ulcer that appears 4-10 days after infection (55). Globally, chancroid occurs primarily in non-industrialized countries (1,7,54,79). It has been shown that the incidence of chancroid in industrialized nations has been steadily increasing over the last decade (54,68,79,103).

Recently, the role of genital ulceration in Human Immunodeficiency Virus (HIV) transmission has been investigated. Reports have shown that genital ulceration can cause a 5-fold increase in the risk of HIV transmission in heterosexuals (53). The lack of a suitable vaccine either for H.ducreyi (7) or HIV (100) underscores the importance of understanding the mechanisms of ulcer formation by H.ducreyi, which is a major risk factor for HIV infection. In understanding the pathogenesis of H.ducreyi, alternate strategies for treatment of chancroid can be formulated.

The ability of H.ducreyi to damage a variety of cultured human cell lines in vitro has been recently described (10,92). Little is known as to the mechanisms by which H.ducreyi is able to damage these cell lines. The characterization of the cytopathic effect (CPE) seen with human foreskin fibroblast (HFF) cells can provide valuable information on possible components responsible for H.ducreyi-mediated cell damage.

I.) LITERATURE REVIEW

A.) Chancroid

1.0 - Characteristics

Chancroid, a sexually transmitted disease caused by the bacterium Haemophilus ducreyi, has been known for over a century (1,2,79). The hallmark of chancroid is characterized by its primary lesion, a small papule that turns into a painful, soft, purulent ulcer with ragged edges (35,55). The genital ulcers seen in chancroid commonly occur at the distal prepuce, the mucosal surface of the prepuce on the frenulum, the coronal sulcus and the glans in men. In women, ulcers are commonly found in the labia minora and labia majora, as well as in the area of the urethra and vagina (1,3,79). Though the disease is widely considered to be confined to the genitalia, a recent case report of pedal chancroid indicates that extragenital ulceration is possible (76).

Clinical symptoms of chancroid are generally not seen until 4-10 days after the initial infection (1,3,55,79). To date, the disease has not been shown to occur systemically, and in more than half of patients presenting with chancroid, the disease is confined to localized ulceration (3). In some patients, however, swelling of regional lymph nodes occurs 1-2 weeks after the appearance of the primary lesion (3,14).

Histologically, a typical chancroidal lesion is 2-3 mm in depth, with three distinct zones of inflammation. The first

(upper) zone of the lesion contains necrotic tissue, red blood cells, some fibrin, and degenerate polymorphonuclear lymphocytes. The second (middle) zone of the lesion is edematous in nature, and contains strands of endothelial cells that approach the surface of the ulcer, and prominent dilated vessels throughout. The third, deepest zone of the ulcer contains a fairly dense infiltrate of plasma cells, with a lesser number of lymphocytes (35). Gram-stained preparations of biopsies from chancroidal ulcers have revealed that H. ducreyi exists as small extracellular clumps in between cells (35,77).

1.1 - Occurrence

Until recently, chancroid was thought of as primarily a disease common in developing regions, such as Africa and Asia (79). In Africa, chancroid remains the primary cause of genital ulceration. Studies at one centre in the Gambia revealed that 52% of patients presenting with genital ulcers were diagnosed with chancroid (74). With increased immigration and international travel, outbreaks in countries such as Greenland (67), Canada (26,42), the United States (7) and the United Kingdom (68) have been reported, demonstrating that chancroid is not confined to developing nations or tropical climates (7,54).

The incidence of chancroid has been on the increase in North America, with greater than 2,000 cases per year being reported since 1985 (7). Though it was originally thought

that the number of reported cases of chancroid have been slowly decreasing since 1990, new areas continue to report outbreaks (7, 103). The actual incidence of chancroid across North America is suspected to be greater than previously thought, as evidence of underreporting of chancroid has been demonstrated (103). With the introduction of more reliable diagnostic procedures and educating physicians to be more "chancroid minded", the diagnosis and reporting of chancroid in North America should be more reliable in the future (7,79,103).

1.2 - Role in HIV Transmission

The interest in chancroid as a disease has risen significantly in the last decade or so (79,76), due partly to the increased incidence of chancroid in industrialized nations (7,79,103), and partly due to the recent report that chancroid can cause a five fold increase in the risk of HIV transmission amongst heterosexuals (53). Studies undertaken in Nairobi have demonstrated that 63% of males seropositive for HIV had a previous history of genital ulcer disease (109). Chancroid is the major cause of genital ulceration in the area (74), and a synergistic relationship between the dissemination of chancroid and HIV infection has been observed (109,40). Patients that were seropositive for HIV appeared to be more prone to genital ulceration or had more severe ulcers than HIV seronegative individuals (40).

It has been suggested that ulcers of patients presenting

with chancroid could serve as a portal of entry for the HIV virus, as an HIV seropositive individual with chancroid could transmit the HIV virus more efficiently. Furthermore, a person with chancroid exposed to an HIV seropositive individual would be infected with HIV easier than a person without chancroid (40,53,63). Studies on HIV seropositive men in Zimbabwe and their wives revealed that HIV positive men with a history of genital ulceration were more likely to have an HIV seropositive wife. In fact, 60 % of the wives of HIV seropositive men with a history of genital ulceration were themselves HIV positive (63).

Thus, chancroid plays a central role in the dissemination of HIV in the heterosexual population in Africa. Presently, it is estimated that approximately 13 million people worldwide are infected with the HIV virus. By the year 2000, the number of HIV positive individuals is expected to triple (52). In absence of an effective means to control the dissemination of HIV, further understanding is needed of the factors that make it easier for HIV to spread from host to host. Presently, there are no reliable vaccines for chancroid (7) or HIV (100). Therefore, greater understanding of the factors that facilitate the spread of HIV is needed, so that an effective means of controlling the spread of HIV can be found.

B.) *Haemophilus ducreyi*

1.0 - Chancroid and H. ducreyi

Though the disease has been in existence perhaps for centuries (79), chancroid was not identified as a distinct condition from other types of genital ulceration until the 1800's (15).

Probably the earliest differentiation between hard and soft genital ulcers was in ancient times. Hippocrates observed that certain genital ulcers were accompanied by buboes. The ancient physician Celsus further defined genital ulcers as those that were "dry and clean" and those that were "moist and purulent" (55).

Bassereau was the first modern scientist to differentiate the soft ulcers of chancroid from the hard ulcers of syphilis (15). The etiological agent of chancroid was not identified until 1889 when Auguste Ducrey published a report on the so-called "virus" of soft chancre (33). Although Ducrey was not able to culture the organism *in vitro*, he was able to culture the "virus" in his patients by inoculating the skin of their forearms with exudate from their own genital ulcers (79).

For the next century, the etiological agent of chancroid was most commonly referred to as "Ducrey's bacillus" (16). It was not until 1920 when the classification of bacterial types was undertaken by the Society of American Bacteriologists that the bacillus of Ducreyi was assigned to the genus *Haemophilus* (124). The present name, *Haemophilus ducreyi* appeared in the

first edition of Bergey's Manual in 1923 (58), and remains in the genus Haemophilus up to the present.

1.1 - Bacterial Characteristics

When grown on solid agar-based media, H. ducreyi exist as small non-mucoid colonies that are semi-opaque and yellow-gray in colour (7,112). The colonies themselves are tightly adherent, existing on agar plates as small colonies which can be pushed intact across the agar surface (7,79,113). This high degree of cell-cell adherence among H. ducreyi can be seen in biopsies of human lesions, where they exist as long chains known as the so-called "school of fish" morphology (35). Due to this high level of adherence between H. ducreyi, uniform suspensions of the bacterium are difficult to obtain (7,79).

Studies employing light and electron microscopy have given a better view of H. ducreyi colony morphology. Transmission electron microscopy (TEM) studies (7,54) have also shown that H. ducreyi possess a fibrillar matrix at their surface that appears to be intimately involved in adherence of H. ducreyi to its host cell (7,10,54). Scanning electron microscopy (SEM) (11) and light microscopy (LM) (10,107) studies on cultured cell lines infected with H. ducreyi have shown that H. ducreyi can grow to form large microcolonies. The exact role of attachment and microcolony formation by H. ducreyi is not fully understood. Recently, it has been observed that the ability of H. ducreyi to form microcolonies

varies with the virulence of the strain. Avirulent H. ducreyi strains did not form extensive microcolonies on the surface of cultured cells, whereas virulent H. ducreyi strains were able to form extensive microcolonies when allowed to grow on cultured cell monolayers (11). Thus, the colony characteristics of H. ducreyi may play a role in the ability of the organism to cause disease.

1.2 - Biochemical Characteristics

As is the case with many pathogens, H. ducreyi is a fastidious organism, which reflects its lack of sophisticated biosynthetic machinery (79). The fastidious nature of the organism hindered the development of a chemically defined growth medium. It was not until some 20 years after the initial discovery of the bacterium by Auguste Ducrey (33) that a blood agar based medium was developed (16). Previous culture methods for H. ducreyi involved inoculating the forearms of chancroid patients with exudates from their own genital ulcers (7,79).

Most studies devoted to H. ducreyi biochemistry were undertaken either for the purpose of differential diagnosis or epidemiological purposes (1,7,79). Probably the most distinctive biochemical features of H. ducreyi is its requirement for X (haemin) and V (NADH) factors for growth (8,79,43). In fact, it is the requirement for X and V factors that served as a basis for including H. ducreyi in the genus

Haemophilus (58). In terms of haemin supply, free haemin is not required, as H. ducreyi is able to obtain haemin from a variety of haem-containing molecules such as haemoglobin, myoglobin, and certain other haem proteins (8,43,64). Albumin has also been shown to be essential for the growth of H. ducreyi, however it has been proposed that albumin serves to adsorb components toxic to the organism rather than providing any direct nutritive value (65).

The knowledge of the organization and control of the biochemical pathways of H. ducreyi is minimal. Despite the apparent paucity of knowledge of the biochemistry of H. ducreyi, investigators have been able to identify certain consistent biochemical properties common to H. ducreyi. For instance, H. ducreyi have been found to have a broad range of phosphatase activity, including alkaline phosphatase, acid phosphatase, and phosophoamidase (57). The presence of cytochrome oxidase and absence of catalase amongst different H. ducreyi strains has also been demonstrated (58). Furthermore, nitrate reduction has been suggested as a consistent feature of H. ducreyi (58), although this observation has been disputed by other investigators (79,113). The apparent discrepancies between investigators regarding nitrate reduction by H. ducreyi may be due to the different types of substrates and test systems used (79).

H. ducreyi possesses a wide range of amino peptidase activity, although some of these aminopeptidases have been

shown to be strain-dependant (58,122). Esterase activity has also been reported by certain investigators, however there is variability among reports due to the assay systems used (58,79). To date, no phospholipase activities have been reported, although weak ß-haemolysis is seen after 4-5 days growth on Muller-Hinton blood agar (58). The ability of H. ducreyi to exhibit α-haemolysis on both rabbit and sheep blood agar has also been reported (31,79).

Recently, some attention has been paid to the ability of H. ducreyi to elaborate certain tissue degrading enzymes that could contribute to its pathogenesis. It was found that H. ducreyi was relatively inert with respect to extracellular enzyme production as no activity was detected for protease, elastase, lecithinase, lipase and collagenase (2). Recent studies have demonstrated that H. ducreyi was able to produce a haemolysin (87,116). Although haemolysin activity was demonstrated, the exact role of haemolysin production in the pathogenesis of H. ducreyi was not elucidated. Thus, haemolysin production by H. ducreyi remains an area in need of further study.

2.0 - Genetics of H.ducreyi

Despite the increased interest in H. ducreyi - related research in recent years, little is known of the genetics of this organism. Investigations into the genetic makeup of H. ducreyi have primarily addressed the mechanisms of antibiotic resistance that H. ducreyi possesses (79). It has only been

recently that investigators have been focusing on the chromosome of H. ducreyi. A large body of reports have been dedicated to the taxonomy of H. ducreyi. Studies into the use of specific DNA probes for the detection of H. ducreyi have also been undertaken in recent years (89,90). However, investigations into the structure and function of various chromosomal elements of H. ducreyi have been almost non-existent until recently.

2.1 - Taxonomy of H.ducreyi

Historically, H. ducreyi has always been included in the genus Haemophilus in the family Pasteurellaceae (58). The inclusion of H. ducreyi in the genus Haemophilus was based on the observations that H. ducreyi was phenotypically similar to other Haemophilus species (57). At the genetic level, however, H. ducreyi does not seem to exhibit the same degree of relatedness with other Haemophilus species as is seen at the phenotypic level.

DNA hybridization studies with other members of Haemophilus and the Pasteurellaceae suggest that there does not seem to be a great deal of DNA homology between H. ducreyi and other members of Haemophilus and the Pasteurellaceae. The apparent lack of DNA homology with other members of Haemophilus and the Pasteurellaceae led certain investigators to challenge the inclusion of H. ducreyi in the family Pasterellaceae (6,23). Further studies on the taxonomy of the Pasteurellaceae using DNA:rRNA hybridization techniques (28)

and 16s rRNA sequence data (99) revealed that there was enough relatedness between the genome of H. ducreyi and other pasteurellaceae to allow for the inclusion of H. ducreyi in the Pasteurellaceae. More thorough studies into the taxonomy of the Pasteurellaceae using 16s rRNA sequence data (30) suggest a more complex taxonomic picture. By comparing the sequences of 16s rRNA from 54 representative strains composing the Pasteurellaceae, investigators were able to divide the family into four distinct phylogenetic clusters that were not specific to any given genus. As a consequence, H. ducreyi was shown to be related to certain species of such genera as Pasteurella, Actinobacillus, and Haemophilus. Though the data presented in the study provided a clear view on the phylogenetic relationship between H. ducreyi and other Pasteurellaceae, the authors submitted that the phylogenetic branching of the Pasteurellaceae was too complex to make any objective divisions of the Pasteurellaceae into new genera. Thus, the taxonomic position of H. ducreyi requires further study.

2.2 - The Chromosome of H.ducreyi

Probably the earliest identification of a specific functional element on the H. ducreyi chromosome involved the characterization of the antibiotic resistance determinant Tet M (54). Analysis of the Tet M determinant revealed that there were two intact Tet M genes resident on the H. ducreyi chromosome. These Tet M sequences were also found to share a

great deal of homology to other Tet M sequences found in other pathogens such as Mycoplasma hominis (97), Ureaplasma urealytica (96), Gardnerella vaginalis (95), Streptococcus agalacticae (95), and Neisseria gonorrhoaeae (79).

There have been few attempts to localize genes on the chromosome of H.ducreyi. The paucity of chromosomal data is partly due to the lack of suitable genetic markers for study and an absence of adequate information on the genetic exchange mechanisms of this organism (54). The guanosine plus cytosine (G+C) content of the H.ducreyi chromosome was shown to be 38 to 39 mol% (23,57). Though not exhibiting much DNA homology with other members of Haemophilus and Pasteurellaceae, H. ducreyi exist as a highly homogeneous group with DNA homology values of 85% to 100% between strains (23).

Recently, three DNA sequences encoding for H. ducreyi specific proteins have been characterized from a lambda gt11 library (89,90). Though the function of these proteins was not immediately deduced, the DNA probes were proposed as being useful for the identification of H. ducreyi in clinical samples. It is only quite recently that a protein encoding gene from the H. ducreyi chromosome has been cloned, sequenced and expressed in a suitable system. Using H. ducreyi specific DNA probes developed earlier (89), researchers were able to characterize and express two open reading frames that produced proteins later shown to be homologous to the Gro EL and Gro ES heat shock proteins present in several bacterial pathogens.

Furthermore, the expressed proteins from the DNA fragments also exhibited homology with a eukaryotic 60 KDa Heat shock protein (90).

In order to advance knowledge of the pathogenic determinant(s) of H. ducreyi, more information regarding the H.ducreyi chromosome and its products is needed (7,54,79). Recent attempts at creating specific isogenic mutants have been described (45). Using electroporation to transform H. ducreyi with a plasmid shuttle vector (pLS88), investigators were able to effect allelic exchange between wild-type alleles and mutant alleles. Further investigation of these isogenic mutants may help to identify any gene products involved in the pathogenesis of H. ducreyi.

2.3 - The plasmids of H. ducreyi

Due to their clinical importance, the antibiotic resistance determinants of H. ducreyi have been widely studied. Although H. ducreyi does not seem to be extensively related to other members of Haemophilus and Pasteurellaeceae at the chromosomal level, the organism does seem to share a considerable gene pool with both Haemophilus and Pasteurellaceae as far as antibiotic resistance plasmids are concerned (7,54,70,79).

The genetic determinant of sulfonamide resistance in H. ducreyi has been shown to be mediated by a 4.9 MDa plasmid (5,54,70). Analysis of this plasmid has revealed a significant level of homology with the streptomycin -

sulfonamide resistance plasmid RSF1010 found in many enteric bacteria.

Plasmids encoding for Ampicillin resistance in H. ducreyi were first reported from an outbreak of chancroid in Winnipeg in 1970 (19). Analysis of the plasmid revealed that it was 5.7 MDa in size and coded for a TEM type-1 ß-lactamase (13). Later, studies involving ß-lactamase production in H. ducreyi strains revealed that another plasmid, 7.0 MDa in size, also mediated resistance to B-lactam drugs in H. ducreyi (20). Further analysis of the 5.7 and 7.0 MDa plasmids revealed a high degree of sequence homology with the 3.2 and 4.4 MDa ß-lactamase resistance plasmids in Neisseria gonorrhoaeae respectively. The only significant difference between the H. ducreyi plasmids and the N. gonorrhoaeae plasmids was that both H. ducreyi plasmids carried a complete TnA nucleotide sequence (46), whereas the N. gonorrhoaeae plasmids contained only 40 % of the TnA sequence (19,20). A third ampicillin resistance plasmid was found in H. ducreyi that was 3.6 MDa in size. Further analysis of this plasmid revealed that the 3.6 MDa plasmid was identical to the 3.2 MDa gonococcal plasmid (13).

Recently, studies of ß-lactamase producing strains of H. ducreyi from Thailand revealed the presence of a 5.4 kb plasmid (pTH126) that coded for a ROB-1 type ß-lactamase (73). Further analysis of the sequence revealed that the pTH126 plasmid found in H. ducreyi was similar to the ROB-1 ß-

lactamase plasmid pVM105 found in Actinobacillus pleuropneumoniae.

Tetracycline resistance in H. ducreyi has also been shown to possess a plasmid mediated component (9). Analysis of this plasmid revealed that the plasmid was 30 MDa in size and could be transferred between bacteria through conjugative matings. Investigations into the sequence of the 30 MDa plasmid revealed that it was related to certain tetracycline resistance plasmids in Haemophilus influenzae (9).

A plasmid encoding aminoglycoside resistance in H. ducreyi has also been described (101). Analysis of this plasmid revealed that the plasmid was 3.1 MDa in size and coded for resistance for kanamycin and streptomycin. Though the gene products of the plasmid were shown to be able to modify kanamycin, no modification of streptomycin was demonstrated. Further study of the plasmid suggested that although streptomycin modification was not explicitly demonstrated, resistance to streptomycin was nevertheless associated with the 3.1 MDa plasmid (101).

Recently, a conjugative plasmid with no apparent function has been described in H. ducreyi (29). The plasmid was shown to be 23.5 MDa in size and was able to mobilize small non-conjugative plasmids originally isolated in Haemophilus parainfluenzae and Neisseria gonorrhoaeae (71). To date, no transfer of H. ducreyi chromosomal elements using this plasmid has been described (7).

Thus, the ability of H. ducreyi to acquire genetic information from a variety of species and genera is well established (70,72,79). Further study in this area may be required to pinpoint the exact mechanisms of transfer between H. ducreyi and other bacteria so that the question of antimicrobial resistance in H.ducreyi can be fully understood.

C. Pathogenesis of H. ducreyi

1.0 - Entry of H. ducreyi Into Host

Presumably, H. ducreyi is thought to enter the host through a break in the integrity of the epithelium, as application of H. ducreyi on the skin of human volunteers required that the skin be scarified in order for a productive H. ducreyi infection to occur (31). Furthermore, studies in animals have also demonstrated that infection with H. ducreyi did not result when H. ducreyi were inoculated on to an intact epidermal surface. Rather, intradermal injection was required in order to initiate H. ducreyi infection in these models (21,119,120).

2.0 - Adherence of H. ducreyi to Host Cells

The adherence of a microbial pathogen to cells in the host is thought to be an important event in the establishment of an active infection for a wide range of pathogens (34). Recently the ability of H. ducreyi to attach to a number of cell types has been investigated.

The exact mechanism by which H. ducreyi is able to attach

to human cells is not fully understood, however it has been demonstrated that H. ducreyi can exhibit a certain degree of specificity in terms of which cell type the organism will bind to. For instance, H. ducreyi attach more readily to human keratinocytes (18), and human foreskin fibroblasts (HFF) than to endometrial carcinoma cells (HEC-1-B) or HeLa cells in vitro (60,118). Attachment was also shown to be temperature dependant, as H. ducreyi were able to attach at 35°C and 30°C (11,118), but not at 4°C (11)

Based on their ability to attach to human foreskin fibroblasts, three strain-specific patterns of attachment were recognized: Those that attached specifically to the fibroblasts, those that attached between the cells, and those that either attached poorly or not at all (118). Furthermore, the inability of certain H. ducreyi strains to attach and form adherent microcolonies may correlate with virulence, as it has been demonstrated that certain avirulent H. ducreyi strains are not able to attach to human foreskin fibroblasts as well as virulent strains (11).

Inhibition of H. ducreyi adherence to McCoy cells by cell-free culture supernatants of log-phase bacteria has been reported. The ability of the culture supernatants to inhibit H. ducreyi adherence was not reversed when the culture supernatants were heated to 70°C for 30 minutes, suggesting that a non-proteinaceous factor is involved (3).

Early data has suggested that H. ducreyi do not possess

pili (7,79). Recently, researchers have been able to isolate and characterize pili that were found in 10 out of 12 H. ducreyi strains that were freshly isolated from chancroidal lesions. The isolated pili consisted of monomers of pilin and were found to be approximately 24 KDa in size. Pilus expression was found to be stable upon multiple passage in vitro and the pili were morphologically distinct from pili found in N. gonorrhoeae (111). Using a particle agglutination test, the ability of H. ducreyi to bind extracellular matrix proteins such as fibronectin, collagen and gelatin has been demonstrated (4). The ability of H. ducreyi to bind such molecules may provide a means for the organism to colonize the host and elicit cell damage.

3.0 - Invasion of Host Cells

The ability to invade host cells is used by many pathogens in order to evade host defenses and multiply (34). Though in vivo data from human (35,77) and animal (21,91,120,121) studies suggest that H. ducreyi exist primarily in the extracellular milieu, in vitro assessments of invasion are conflicting. Studies undertaken with various epithelial cell lines suggest that upon attachment to host cells in vitro, H. ducreyi are able to gain entry into the cells (60,107). However, data published using human foreskin fibroblasts suggest that H. ducreyi remain outside the cells (10,11). One possible explanation is that the ability of H. ducreyi to invade host cells depends on the cell type.

Nevertheless, more studies are needed to resolve this question.

4.0 - Lipooligosaccharide

Lipopolysaccharide (LPS) is a major structural component of gram-negative bacteria. Structurally, LPS consists of three components: Lipid A, a core oligosaccharide that is connected to the lipid A, and an o-antigenic side chain connected to the core oligosaccharide (66). Two types of LPS exist: **smooth** LPS, which consists of all three components listed above, and **rough** LPS which lacks an o-antigenic side chain. Of these two types, H. ducreyi possesses the rough type of LPS (85). Lately, the term **lipooligosaccharide** (LOS) has been used to describe the LPS of H. ducreyi and other similar pathogens, due to its short chain of sugars that compose its core oligosaccharide (22,37).

Early studies found that the LOS of virulent strains of H. ducreyi was functionally different than LOS from avirulent H. ducreyi strains, and thus the LOS of H. ducreyi was associated with virulence of this organism (83,84). Investigations into the chemical composition of H. ducreyi LOS revealed that the total glycose:KDO (ketodeoxyoctulosonic acid) ratios of virulent H. ducreyi strains consistently exceeded that of avirulent strains. Thus, it is believed that the primary difference between the LOS of virulent and avirulent strains of H. ducreyi lies in the composition of their core oligosaccharide (85). Detailed structural

determinations of the H. ducreyi LOS have not been undertaken until quite recently (78). The LOS of H. ducreyi has been shown to express a common epitope with the LOS of N. gonorrhoeae, as both species of LOS are able to bind the monoclonal antibodies 3F11 and 6B4 (22). These monoclonal antibodies bind to epitopes that are similar in structure to the terminal oligosaccharides of paragloboside, a glycosphingolipid precursor to the human I and i erythrocyte antigens (75). The molecular structure of the LOS from H. ducreyi strain 35000 has been determined by Mass spectrometry (78). The data indicate that the oligosaccharide region consists of a nonasaccharide that is linked directly to a single KDO moiety which is phosphorylated (78). This KDO residue is in turn linked to the lipid A region, which has been shown to be similar to that of the lipid A region of Haemophilus influenzae strain I-69 Rd-/b+ (48).

5.0 - Antiphagocytic Activity

A fundamental feature of many pathogens is the ability to avoid phagocytosis by neutrophils, macrophages, and other phagocytic cells of the host (39). Studies comparing virulent and avirulent strains of H. ducreyi have shown that unlike their avirulent counterparts, virulent strains of H. ducreyi are resistant to phagocytosis and killing by human neutrophils in vitro (83,84).

The most common means utilized by bacteria to avoid phagocytosis is the presence of an antiphagocytic capsule

(81). The presence of such a capsule on H. ducreyi has not been consistently demonstrated. Electron microscopy of H. ducreyi strains stained with ruthenium red or Alcian blue revealed a discontinuous exocellular layer of material that could be stabilized into a continuous layer by polyvalent rabbit sera (7). Later studies by other groups failed to demonstrate any surface appendages (including capsules) on the surface of H. ducreyi (54). Such conflicting data are analagous to the earlier situation experienced by researchers involved in the study of the N. gonorrhoaeae capsule (27,49). Although the presence of a capsule was demonstrated by one group of researchers (7), no morphological differences were observed between capsules from both virulent and avirulent strains of H. ducreyi. Furthermore, the H. ducreyi capsule lacked the fine structure seen in the type b capsule found in H. influenzae (81). Although there seems to be a presence of an exocellular matrix of some sort on the surface of H. ducreyi, the specific role of this matrix in the pathogenesis of H. ducreyi has not been demonstrated.

6.0 - Excreted Cytotoxin(s)

Though the pathogenic changes seen in H. ducreyi infection are well characterized in humans (35,112), little is known of the mechanism(s) by which H. ducreyi is able to damage host cells. Although excretion of a cytotoxin can often be the major determinant of virulence for many pathogens, it is not necessarily the sole determinant of

pathogenesis (34,56). The identification of haemolysin-producing strains of H. ducreyi has been reported (87,116). Though it has been known for some time that H. ducreyi can exhibit a certain degree of haemolysis on blood agar (58), detailed studies of the nature and mechanism of haemolysis by H. ducreyi have only begun quite recently (87,117).

The presence of an excreted cytotoxin that is capable of damaging transformed human epithelial cells in vitro has also been reported (92). The cytotoxin has been purified and has been shown to be able to damage human epidermoid carcinoma (HEP-2) cells in vitro (93). However, a role that this cytotoxin can play in ulcer formation is unclear, since both cytotoxin-producing and cytotoxin negative strains of H. ducreyi are equally able to cause damage in animal models of H. ducreyi infection (61,62). Furthermore, it has been suggested that H. ducreyi are relatively inert with respect to the toxicity of their extracellular products (2). Using both plate and spectrophotometric assays, it has been demonstrated that H. ducreyi does not produce certain key tissue degrading enzymes into its environment such as protease, elastase, lecithinase, lipase, or collagenase. Furthermore, cell-free culture supernatants obtained from H. ducreyi have been shown not to exert damage on Vero cells or primary human keratinocytes (1,2). Thus the significance of any putative cytotoxin excreted by H. ducreyi remains to be further clarified.

6.1 - Cytopathic Effect of H. ducreyi

A cytopathic effect of H. ducreyi on human cells in vitro has been reported (10,92). Although a cytotoxin has been proposed as a possible mediator of this phenomenon (92), discrepancies exist amongst researchers as to the types of cells that are damaged (10) and the relevance of the putative H. ducreyi cytotoxin in the pathogenesis of the organism (61,62,92,93). Furthermore, the means by which H. ducreyi is able to exert this cytopathic effect is poorly understood.

7.0 - In Vivo Models of H. ducreyi Infection

7.1 - Studies in Humans

The use of humans to study H. ducreyi pathogenesis was first employed by Auguste Ducrey (33). Though Ducrey was not able to isolate the organism in pure culture, he was able to passage the bacterium in the forearms of chancroid patients. The use of biopsies from patients with chancroid proved essential to understanding the histopathology of H. ducreyi infection in the human host. Early investigations were limited to the questions ascertaining the ultrastructure of H. ducreyi in its natural setting (86). The data generated from these reports confirmed the gram negative morphology of H. ducreyi in vivo. Later reports investigating the ultrastructural morphology of H. ducreyi in the human ulcer revealed little new information about the interaction of H. ducreyi with host cells, other than the observation that H.

ducreyi existed primarily as extracellular bacteria (77).

Probably one of the most informative of the early studies of biopsies from chancroid lesions was done by Frienkel (35). In his study, Frienkel was able to characterize the three distinct zones of inflammation of a typical chancroidal lesion. Further evidence that H. ducreyi existed primarily as extracellular clumps was provided, as well as identification of different immune cell types present in the lesion. Though Frienkel observed cell types from both the cellular and humoral arms of the immune system, he did not speculate as to what type of immunity was involved in the lesion. Rather, the main objective of the study was to provide evidence for the usefulness of tissue biopsy as a method of diagnosis for chancroid (108).

7.2 - Studies in Animals

A suitable animal model of H. ducreyi pathogenesis could prove helpful in studying the role of H. ducreyi in lesion formation. Early attempts at defining an animal model of H. ducreyi infection revealed that rabbits and monkeys reliably produced ulcers when injected with H. ducreyi, whereas guinea pigs, sheep, mice cats and goats were refractory to H. ducreyi infection (79). The most widely used model to date has been the rabbit model of H. ducreyi infection. Using this model, researchers have been able to study the specificity and kinetics of antibody responses after direct challenge with H. ducreyi (82,102). Furthermore, the virulence of different

strains of H. ducreyi has been defined using the ability of a given strain to produce ulcers as an indicator (31,44). Though it has been reported recently that rabbit lesions do not histologically resemble lesions seen in humans, the rabbit model has been used to investigate the role that H. ducreyi LOS plays in lesion formation (21).

Recently, a mouse model ofH. ducreyi infection has been described (119,120) The mouse model has also been used to study the possible role ofH. ducreyi components in lesion formation (120). The data generated from both models demonstrate that live bacteria were not needed to induce ulceration. Furthermore, purifiedH. ducreyi LOS alone was able to induce lesion formation (21,120), indicating a central role for this component in lesion formation. However, ulceration was demonstrated in the mouse model for the avirulent H. ducreyi strain CIP542 (120), whereas this strain had failed to produce lesions in the rabbit (44). Later comparisons of the mouse and rabbit models revealed that CIP542 was able to produce lesions in both models (21). Though both the rabbit and mouse models have been presented as sound tools for the study of the pathogenesis of H. ducreyi, certain limitations exist in both systems. For instance, large innocula of bacteria (10^7-10^9 CFU) are required for reproducibility of ulcer formation (21,44,119,120). Moreover, the survival of bacteria in these systems is not great, as viable bacteria can only be isolated from ulcers within a

relatively short time (6-10 days). Furthermore, it was shown that the lesions spontaneously heal after 1-2 weeks (21,120), whereas humans are not able to successfully clear the infection (2). The histology of the lesions in these models has also been demonstrated not to exhibit a wide degree of similarity to human lesions (21).

The development of a temperature dependant rabbit model has recently been reported (91), in where rabbits were held at a reduced ambient temperature (15-17°C)(105). The housing of rabbits at reduced ambient temperatures was first described using the pathogen Treponema pallidum as a means of holding skin temperature of the animal at the optimal growth temperature of T. pallidum. Thus, by holding the rabbit at a reduced temperature, Purcell et al (91) demonstrated that the skin temperature of the rabbit would correspond with the optimal growth temperature of H. ducreyi (33°C), enhancing the bacteria's ability to replicate and cause damage. In using this approach, the temperature dependant rabbit model is able to overcome some of the apparent shortcomings of other animal models. Firstly, a smaller inoculum of bacteria (10^5-10^6 cfu) was needed to achieve reproducible ulceration. Secondly, H. ducreyi were able to survive for significantly longer times than seen with previous models, and only viable organisms were able to cause ulcers. However, the histopathology of the lesions did not totally resemble human ulcers.

The apparent discrepancies between different animal models of H. ducreyi infection suggest the need for further

studies to resolve the role of LOS and bacterial invasion in ulcer formation. Despite their apparent shortcomings, animal models can still be considered relevant provided their strengths and limitations are understood.

II.) MATERIALS AND METHODS

1.0 - Bacterial Strains

The *H. ducreyi* strains used included: 35000 (clinical isolate, Winnipeg, Canada), RO18 (clinical isolate, Kenya), A77 and CIP542 (obtained from the Institut Pasteur). All *H. ducreyi* isolates were kindly provided by Dr. A.R. Ronald. Other bacterial isolates included: *Haemophilus influenzae* type b (ATCC 10211) and *Neisseria meningitidis* type Y (Cadham Provincial Laboratory, Winnipeg, Manitoba). The *Haemophilus* and *Neisseria* strains were grown on chocolate agar consisting of Columbia agar (Becton Dickinson Microbiology Services, Cockeysville, MD) supplemented with XV supplement (PML, Tualatin, OR) and 10 mg/ml hemoglobin (Becton Dickinson). Cultures were incubated at 35°C in 5% CO_2 and high humidity. All bacterial strains were maintained as frozen stocks at -70°C in skim milk.

2.0 - Cell Culture

The human foreskin fibroblast (HFF) cell line that was used as the *in vitro* model is a non-transformed cell line obtained from pooled children's foreskins (10). HFF cells were cultivated using serum supplemented medium (SCM) consisting of: RPMI 1640 medium (ICN Biomedicals, Costa Mesa CA) supplemented with 1mM sodium pyruvate (ICN), 2mM L-glutamine (ICN), and 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York) in 75 cm^3 tissue culture flasks

(Corning, Corning, New York). Cells were harvested by treatment with a 0.5% trypsin solution in 0.53 M EDTA. All cell cultures were incubated at 35°C in 5% CO_2 and high humidity.

3.0 - SDS-PAGE Electrophoresis

Electrophoresis was done using the discontinuous gel system described by Laemmli (59). The concentration of the running gels was 12.5% and the concentration of the stacking gels was 4.5%. Gels were run at 20 mA for a total of 4 hours, and then they were stained either with Coomassie blue or according to the silver stain method of Tsai and Frasch (119). Once stained, gels were dried in a Biorad model 543 gel drying apparatus (Biorad, Richmond CA) under vacuum for 1h.

4.0 - Preparation of Bacterial Components

4.1 - Cell-Free Culture Supernatants

In order to determine if H. ducreyi produced either a constitutively secreted exotoxin or a HFF cell induced exotoxin, bacteria suspended in SCM were inoculated at a concentration of 1X 10^8 cfu/well into a 24 well tissue culture tray (Corning Corning, NY), or in SCM alone and incubated for 48h at 35°C in 5% CO_2 and high humidity. The supernatant fluid was then decanted and filtered through a 0.45 μm sterile filter (MSI, Westboro, MA). For both methods of supernatant generation, the H. ducreyi strains tested remained viable as evidenced by a 2-log increase in the colony forming unit (CFU) count. Culture supernatant (100 μl) was added to the first well and serially diluted 1:2.

4.2 - Whole Cell Lysate Preparations

Overnight cultures of *H. ducreyi* strains grown on chocolate agar were suspended in serum free medium (SFM) consisting of RPMI-1640 medium containing 2 mM L-glutamine and 1mM sodium pyruvate (ICN) to concentrations of 4.5 X 10^{10} cfu/ml for strain 35000, 3.4 X 10^{10} cfu/ml for strain RO18, 5.3 X 10^{10} cfu/ml for strain A77 and 6.2 X 10^{10} cfu/ml for strain CIP542. Each bacterial suspension was lysed separately using a French Press at 4°C. The whole cell lysate was centrifuged at 14,000 rpm for 5 min. in an Eppendorf 5415 C centrifuge (Eppendorf, Hamburg, Germany). The supernatant was used to represent soluble components of the bacteria. An insoluble pellet fraction was obtained by washing the pellet three times with sterile phosphate buffered saline (PBS), followed by resuspension in PBS. The protein concentration of the soluble and insoluble whole cell lysate was determined using a commercial Coomassie Brilliant Blue dye binding method according to the manufacturer's instructions (Biorad, Richmond, CA).

4.3 - Bacterial Lipooligosaccharide

Bacterial lipooligosaccharide was isolated using a modification of the phenol extraction method of Inzana (51). A total of 40 overnight cultures of *H. ducreyi* (approximately 1 g wet wt.) were harvested from chocolate agar plates and resuspended in 10 ml of wash buffer consisting of 0.01 M PBS (pH=7.5) with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The resuspended bacteria were mixed thoroughly using a Touch Mixer model 232

(Fisher Scientific, Ottawa, ON) and pelleted by centrifugation. The pelleted bacteria were then resuspended in 5 ml of sterile distilled water heated to a temperature of 65-70°C, and 5 ml of 1% Triton X-100 (Sigma, St.Louis, MO) was added. The mixture was then held at 65-70°C for 30 minutes with frequent mixing. The mixture was then transferred to a sterile 20 ml glass bottle and 10 ml of a 95% hot (65-70°C) phenol solution was added and incubated for 15 min. at 65-70°C with vigorous mixing every 2 min. The mixture was then transferred to a 30 mL Corex centrifuge tube (Corning, Corning New York), cooled to 10°C in an ice bath and centrifuged in a Beckman model J2-21 centrifuge (Beckman, Palo Alto, CA) using a Beckman JA.20 rotor at 10,000 g for 20 minutes. The top aqueous layer was then removed to a separate glass bottle and the bottom phenol phase was re-extracted in an equal volume of hot sterile distilled water, extracted, cooled to 10°C, and subjected to centrifugation as described previously. The top aqueous layer from this second extraction was pooled with the aqueous layer from the first extraction, heated to 65-70°C and re-extracted with an equal amount of hot phenol. The aqueous layer from this final step was cooled and pelleted by centrifugation as described previously. The resultant pellet was opaque and white in colour, and represented a crude preparation of bacterial LPS. These pellets were re-suspended in 0.01 M PBS (pH=7.4) to a volume of 250 μl and 5 M NaCl (Biorad,Richmond CA) was added to a final concentration of 0.5

M. Ten volumes of cold 99% ethanol were then added, and the mixture was kept at -20°C overnight. The LPS-ethanol mixture was then centrifuged as described previously, and a second re-extraction with cold ethanol was done. The final pellet was re-suspended in approximately 1-2 mL of 0.01 M PBS (pH=7.4) containing 6 mM Tris (Biorad, Richmond CA) and 150 mM of NaCl (Biorad, Richmond CA). The LPS was then dialysed against distilled water for 72h at 4°C to remove any residual phenol and low molecular weight bacterial products. LPS preparations were stored at -20°C until needed.

The purity of the LOS preparations was analyzed by running 1 μl/ lane net of LOS preparation per lane on a polyacrylamide gel as described in Materials and Methods, section 3.0 (SDS-PAGE Electrophoresis). Duplicate gels were then stained either with Commassie blue stain in order to visualize any possible protein contaminants, or silver stained using the method of Tsai and Frasch (118) to visualize the carbohydrate moieties of the LOS .

4.3.1 - Assay for LOS Carbohydrate Determination

The amount of LOS present in the final preparations was estimated by using the phenol-H_2SO_4 method of Dubois et al (32), which was originally developed as an assay for the quantitation of reducing sugars. Since H. ducreyi LOS contains carbohydrate moieties as a part of its structure (85), the phenol-H_2SO_4 assay was employed as a means to estimate the relative amounts of LOS in the preparations. Suspensions of purified LOS were diluted either 1:5 or 1:10 to

a final volume of 500 μl sterile distilled water in borosilicate glass culture tubes (Fisher, Ottawa ON), and 100 μl of a 5% solution (w/v) of phenol was added to each sample and thoroughly mixed. Upon mixing the LOS and phenol solutions together, 2 ml of concentrated H_2SO_4 was added to each tube and thoroughly mixed to homogeneity. The tubes were left to stand for 30 min. at room temperature to allow the colourimetric reaction to occur. All samples were read at an absorbance of 484 nm using a Spectronic 601 spectrophotometer (Milton Roy, Rochester NY). A standard curve was constructed using a commercial preparation of E.coli 0127:B8 LPS.

5.0 - Scanning Electron Microscopy

A 1.0 ml suspension of 1 X 10^5 cells / ml of HFF cells was inoculated onto glass coverslips placed in the wells of a 24-well tray (Corning, Corning New York). The monolayers were then infected with approximately 1 X 10^7 cfu of H.ducreyi per well. The infected monolayers were then incubated for 1, 4 and 24 h at 35°C with high humidity. At specified times, the monolayers were washed three times with serum free medium (SFM) consisting of: RPMI 1640 medium (ICN Biomedicals, Costa Mesa CA) supplemented with 1mM sodium pyruvate (ICN), and 2mM L-glutamine (ICN). The monolayers and adherent bacteria were then fixed for 10 min. at room temperature with a 2% (wt / vol) solution of gluteraldehyde (Polysciences, Wilmington PA). The monolayers were then washed a further three times in SC buffer containing 0.1 M sodium cacodylate and 0.01 M $CaCl_2$ at pH 7.4. Secondary fixation was achieved by incubating the

monolayers for 1 h at room temperature with a 1% solution (wt / vol) of OsO_4 (Polysciences, Wilmington PA) in SC buffer. The monolayers were then washed three times in fresh SC buffer and dehydrated through a graded series of ethanol. The coverslips were then critical point dried in CO_2 using a model SPC-1500 critical point drier (The Bomar Co., Tacoma WA) and sputter coated with gold on a Hummer V gold coating apparatus (Technics Tokyo, Japan). All samples were viewed using a JEOL model 35C scanning electron microscope (Jeol, Tokyo Japan) with an acceleration voltage of 15 kV.

6.0 - XTT Cytotoxicity Assays

The procedure employed is based on an assay developed by Scudiero et al (98,104) using the tetrazolium dye XTT, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene - sulfonic acid hydrate (Sigma, St. Louis, MO). In order to determine the conditions of the assay, HFF cells were seeded into 96 well trays (Corning, Corning NY) at a concentration of 2 X 10^4 cells/well and diluted serially 1:10. The HFF cells were then incubated overnight at 35°C with 5% CO_2 and high humidity, and either subjected to the XTT assay (as described below), or pulsed overnight with antibiotics before being subjected to the XTT assay. Live H. ducreyi were also tested in the same manner, with an initial inoculum of 1 X 10^7 cfu/well that was serially diluted 1:10.

The XTT assay was adapted to infection with live H. ducreyi using the following procedure: HFF cells were seeded

into 96-well trays (Corning, Corning, New York) at a concentration of 2 X 10^4 cells/well and incubated 24 to 48 h until monolayers were confluent. After exposure to either bacteria or cell fractions, all plates were washed three times in serum free meduim (SFM) then 100 μl of serum containing medium (SCM) was inoculated into each well. Colour development was achieved by adding 25 μl/well of a solution consisting of 100 mM PMS (Sigma, St. Louis MO) and 1 mg/ml XTT in SCM. The plates were then incubated at 35°C in 5% CO_2 for 4 h. Colour production was read at 450 nm with a reference wavelength of 650 nm on an Emax spectrophotometer (Molecular Devices, Menlo Park, CA.)

6.1 - Live and Gentamicin-Killed Bacterial Assays

Approximately 10^8 cfu of a 24 h culture of each bacterial strain was inoculated into the first well of a 96-well tray that contained confluent monolayers of HFF cells. The total volume in the first well was 200 μl. The bacterial suspension in the first well was then diluted serially 1/10. The plates were allowed to incubate for 24 h at 35°C in 5% CO_2 before being washed four times with SFM to remove non-adherent bacteria. SCM containing 200 μg/ml gentamicin (Sigma, St. Louis, MO), 100 μg/ml streptomycin (Gibco, Grand Island N.Y.), and 62.5 μg/ml of penicillin (Gibco, Grand Island N.Y.) was then added and incubated overnight. This antibiotic pulse killed any residual bacteria. The plates were then assayed for monolayer damage using the XTT assay described above. Control experiments using 10^8 cfu of H. ducreyi demonstrated

that this concentration of antibiotics was sufficient to kill all of the bacteria within 2 h. However, an overnight pulse was required to ensure that the killed bacteria were unable to reduce the XTT substrate.

6.2 - Assays of Cell Fractions

Whole cell lysate, insoluble pellets, soluble fractions, and cell-free culture supernatants were inoculated into the first well of a 96-well tray containing confluent HFF monolayers. The protein concentration in the first well was 0.5 mg/ml. All cell fractions inoculated into the first well were then serially diluted 1/2. The trays were incubated for 24-48 h and the damage to the HFF cells was assayed using the XTT assay.

6.3 - Regeneration of HFF Monolayers

To determine if HFF cells can recover from the adverse effects of H. ducreyi exposure, two sets of confluent monolayers were infected with H. ducreyi strains 35000 (7.4 X 10^7 cfu/well), RO18 (6.1 X 10^7 cfu/well), CIP542 (4.1 X 10^7 cfu/well), and A77 (5.4 X 10^7 cfu/well) and allowed to incubate for 24 h, allowing sufficient exposure for development of CPE. The control set was tested for monolayer viability using the XTT assay. The second set was treated with antibiotics (200 μg/ml gentamicin, 100 μg/ml streptomycin and 62.5 μg/ml penicillin), incubated for a further 72 h and then tested with the XTT assay. The prolonged incubation with antibiotics served to ensure that no viable bacteria remained, allowing the HFF cells an opportunity to regenerate.

6.4 - Attachment and CPE

To determine if attachment and microcolony growth was a factor in CPE, approximately 10^7 cfu were inoculated into the first well and serially diluted 1/10. Bacteria were allowed to attach for 4 h and subsequently washed four times in SFM. The monolayers were allowed to incubate for a further 20 hours, then assayed for cell damage using the XTT assay. A second set was treated in the same way, except that after 4 h exposure, the non-adherent bacteria were washed off and then SCM media was added and the monolayer was incubated for an additional 20 h. Cell damage was then monitored by the XTT assay.

6.5 - Effect of Lipooligosaccharide

Purified preparations of bacterial lipooligosaccharide (LOS) in distilled water from H. ducreyi strains 35000, RO18, A77 and CIP542 were added to sterile borosilicate glass tissue culture tubes and lyophilized overnight. The dried LOS was then resuspended to a concentration of 2.1 mg/ml in SCM tissue culture medium. The resuspended LOS was then dispersed in a Branson 1200 Bath sonicator (Branson Ultrasonics, Danbury CT) for a period of 10 minutes.

In order to assess the role of free versus bound LOS in LOS-mediated cell damage, preparations of LOS-containing liposomal single unilammelar vesicles (SUV's) in SCM supplemented with antibiotics were prepared by a modification of the method of Papahadjopolous (88). Briefly, chloroform solutions of phosphatidylcholine (PC) were mixed in a 1:2 mass

ratio with LOS preparations from H. ducreyi. The PC-LOS mixture was then dried first under a stream of N_2 gas in order to evaporate the chloroform. The samples were then freeze-dried overnight in a Lyph-Lock 6 freeze drier (Labconco). The SUV's were then prepared by suspending the LOS-PC mixture in a buffer of 6 mM Tris (Biorad, richmond CA) and 15 mM NaCl (Biorad, Richmond CA) and allowing the mixture to swell for 10 minutes. The aqueous dispersions were then sonicated for 45 min in a Branson 1200 bath sonicator (Branson Ultrasonics, Danbury, CT). After sonication, the SUV suspensions were subjected to centrifugation at 104,000 X g at 4°C for 60 min. in a Beckman TL-100 tabletop ultracentrifuge to remove large vesicles, undispersed PC and unincorporated LOS (88). The amount of LOS in the SUV preparation was determined by subjecting 100 μl aliquots of the LOS-SUV suspensions to the phenol-H_2SO_4 assay described in section 4.3.1.

In order to assess HFF cell damage, preparations of free LOS and LOS-containing SUV's were added to confluent HFF cell monolayers at an initial concentration of 100 μg/well and serially diluted 1:2. The monolayers were incubated for 24 h and cell damage was assayed using the XTT assay.

7.0 - Shedding of Lipooligosaccharides

To determine the level of LOS shedding exhibited by H. ducreyi, a time-course experiment that monitored the total amount of LOS carbohydrate in suspension was employed. A 1.5 ml suspension of bacteria (1 X 10^9 cfu / ml) in 0.01 M hepes

buffer (pH=7.4) (Sigma, St. Louis MO) was added to a 1.5 ml Eppendorf tube (Eppendorf, Hamburg FRG) and incubated at 35°C for 0, 1, 2, 6, and 24h. At each time frame, the suspensions were subjected to centrifugation at 14,000 rpm using an Eppendorf 5415C model centrifuge (Eppendorf, Hamburg FRG) to pellet the bacteria. The LOS and other components that were released by the bacteria was obtained by removing 1.0 ml of the supernatant. The amount of LOS in the supernatants was estimated by the phenol-H_2SO_4 assay (32) described in section 4.3.1 of Materials and methods. All experiments were done in triplicate.

Samples were then concentrated overnight by freeze drying in a Lyph-Lock 6 freeze dryer (Labconco). The sample was then resuspended in sample buffer to a final volume of 20 μl. The concentrated samples (20 μl/lane) were electrophoresed on a 12.5% SDS-PAGE gel as described in section 3.0 of Materials and Methods. Visualization of supernatant components was achieved. Visualization of supernatant products was achieved by either staining the gel with Coomassie blue stain or by the Silver stain method of Tsai and Frasch (119).

7.1 - Dot Blot Immunodetection of LOS

In order to confirm the presence of LOS in supernatants of H.ducreyi, a dot blot procedure using the mouse monoclonal antibody 2D2 (110) directed against H.ducreyi LOS was used (Provided by DR. I.W. MacLean, Dept. of Medical Microbiology, University of Manitoba).

Culture supernatants of H. ducreyi RO18, A77 or Neisseria

meningitidis obtained in the LOS shedding experiment described above were concentrated by lyophilization overnight in a Lyph Lock 6 freeze dryer (Labconco). The freeze dried supernatants were then resuspended in 50 μl of distilled water and applied to strips of nitrocellulose (Schleicher and Shuel, Keene NH) and allowed to dry at room temperature for 30 min. The nitrocellulose strips were then blocked by overnight incubation with 1% Bovine serum albumin (BSA) in Tris buffered saline (TBS) pH=7.5 with gentle agitation at 4°C. The following day, the blocking agent was removed and a solution of anti-LOS mouse monoclonal antibody 2D2 (1:500 dilution in TBS with 1% skim milk) was added and incubated overnight with gentle agitation at 4°C. The monoclonal antibody solution was then removed and the nitrocellulose strips were washed three times for 20 min. each at room temperature in TBS with 1% Tween-20 (TTBS) (Biorad, Richmond CA). After the final washing step, the TTBS was removed and a 1:1000 dilution of peroxidase conjugated goat anti-mouse antibody in TBS with 1% skim milk was added and allowed to mix at room temperature for 2 hours. Excess conjugated antibody was then removed by washing the nitrocellulose strips three time for 20 min. in TTBS at room temperature. Antigen-antibody complexes were visualized by preparing a peroxidase substrate a solution of 60 mg 4-chloro-1-napthol (Sigma, St. Louis MO) in 20 ml of 99% methanol (Biorad, Richmond CA). This solution was added to 100 ml of TBS supplemented with 60 μl of 30% hydrogen peroxide (Biorad, Richmond CA). The peroxidase substrate solution was

then added to the nitrocellulose strips and agitated at room temperature for a maximum of 30 min. Positive visualization of antibody-antigen complexes was demonstrated by the presence of dark purple blots on the nitrocellulose strips.

8.0 - Statistical Analysis

All data are presented as the mean of triplicate experiments ± one standard deviation from the mean. Variability of the XTT assay was examined by determining a 95% confidence interval of three separate experiments performed on different days. The confidence intervals were calculated for the *H. ducreyi* strain 35000 at a concentration of 10^6 cfu/well.

III.) RESULTS

1.0 - Scanning Electron Microscopy

In order to assess the interactions between HFF cells and H. ducreyi, scanning electron microscopy (SEM) was done on uninfected and infected monolayers. At 1h post infection, only occasional bacteria were found on the HFF cells for all strains tested (Fig 1 B-I). At 4h post infection, the bacterial morphology of virulent strains was noticeably different from that of avirulent strains, as the virulent strains 35000 and RO18 formed chains of bacteria (FIG 2 B,C,F,G) whereas chaining was not a feature of the avirulent strains A77 and CIP542 (FIG. 2 D,E,H,I). At 24h post infection, virulent strains 35000 (Fig 3 B,C) and RO18 (Fig 3 F,G) had formed large microcolonies that enveloped the HFF cell surface, whereas the avirulent strains A77 and CIP542 produced relatively fewer microcolonies that were relatively small compared to those formed by virulent H. ducreyi strains (Fig 3 D,E,H,I). At all time frames tested, H. influenzae was not found to significantly adhere to HFF cells.

2.0 - XTT Cytotoxicity Assays

It is difficult to reliably quantify HFF cell damage by visual methods. Also, the large number of adherent microcolonies (Fig 3 d,h) attest to the potential for bacterial cells to interfere with quantitative measurements. To characterize the cytotoxic effect of H. ducreyi on HFF cells, we adapted the XTT assay originally described by Scudiero et al (98,104). The relative ability of HFF cells and

Figure 1: Scanning Electron Microscopy (SEM) of HFF cells infected with H.ducreyi at 1h Post Infection.

HFF cells were grown on glass coverslips and infected with H.ducreyi 35000 (B,C), A77 (D,E), RO18 (F,G), and CIP542 (H,I) at a multiplicity of infection of 10:1. Uninfected monolayers (A) are included as a negative control. The monolayers were infected with the microorganisms for 1h, washed, fixed, dehydrated and coated with gold as described in section 5.0 of Materials and Methods.

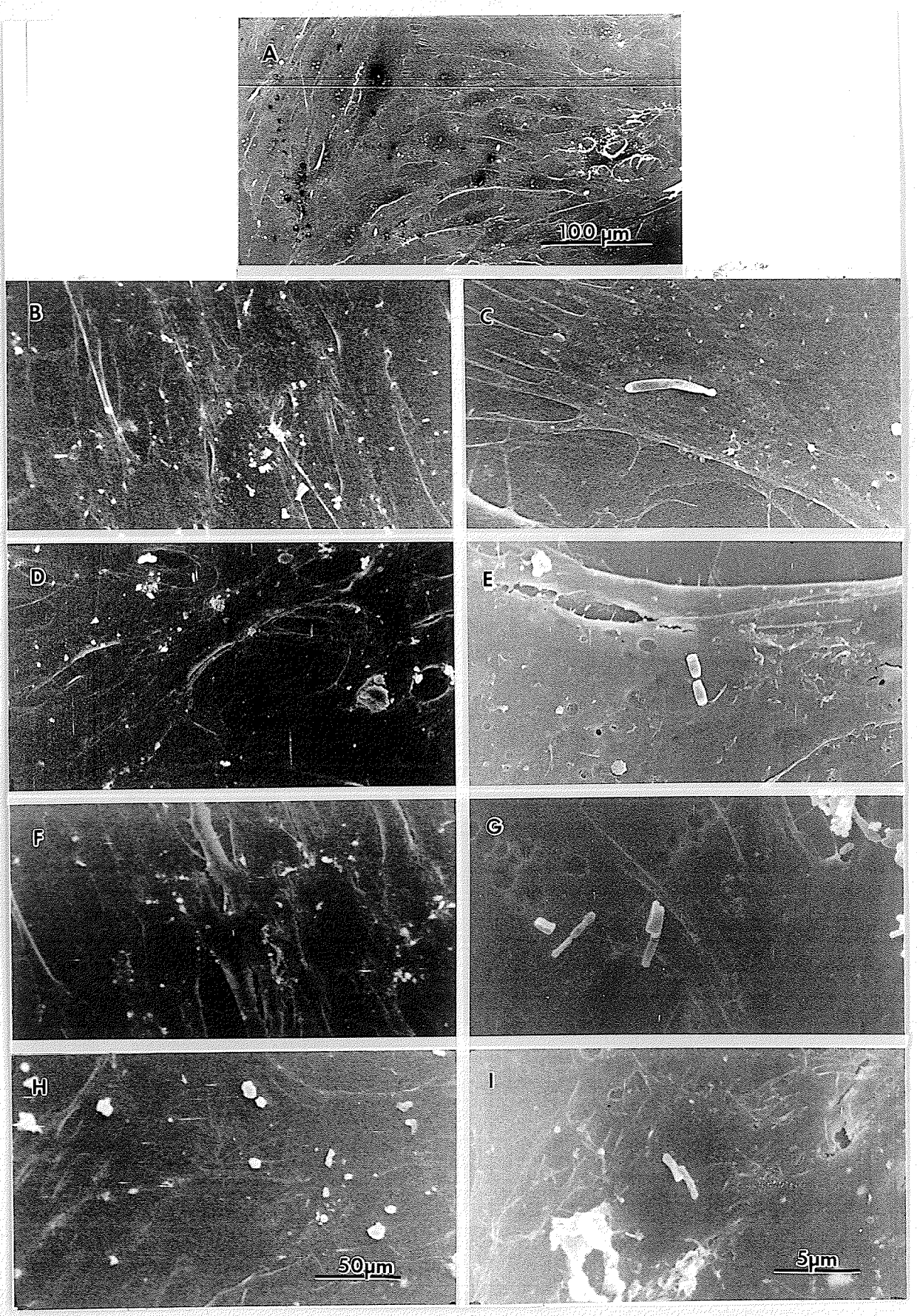

A
100 µm
B
C
D
E
F
G
H
50µm
I
5µm

Figure 2: Scanning Electron Microscopy (SEM) of HFF cells infected with H.ducreyi at 4h Post Infection.

HFF cells were grown on glass coverslips and infected with H.ducreyi 35000 (B,C), A77 (D,E), RO18 (F,G), and CIP542 (H,I) at a multiplicity of infection of 10:1. Uninfected monolayers (A) are included as a negative control. The monolayers were infected with the microorganisms for 4h, washed, fixed, dehydrated and coated with gold as described in section 5.0 of Materials and Methods.

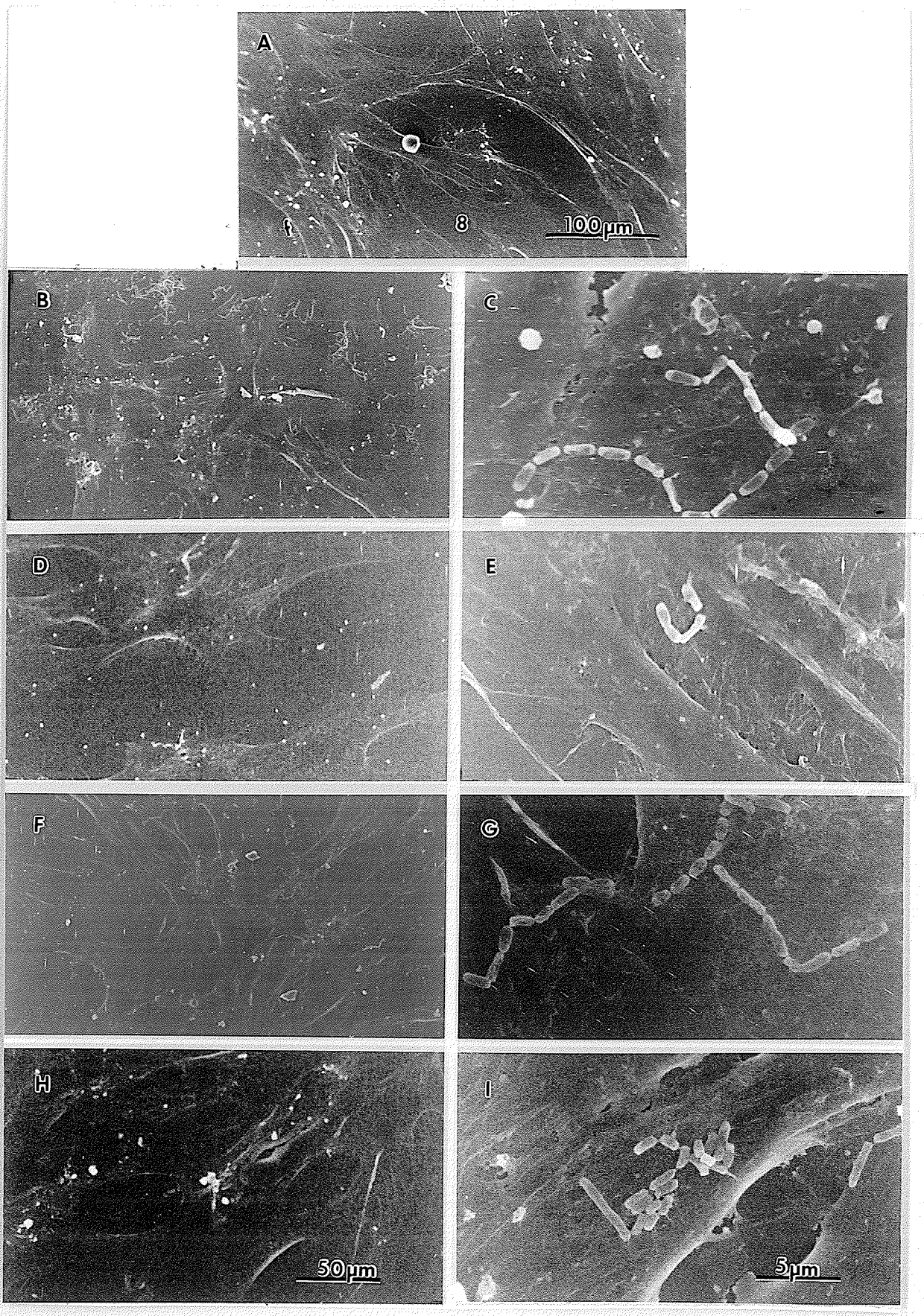
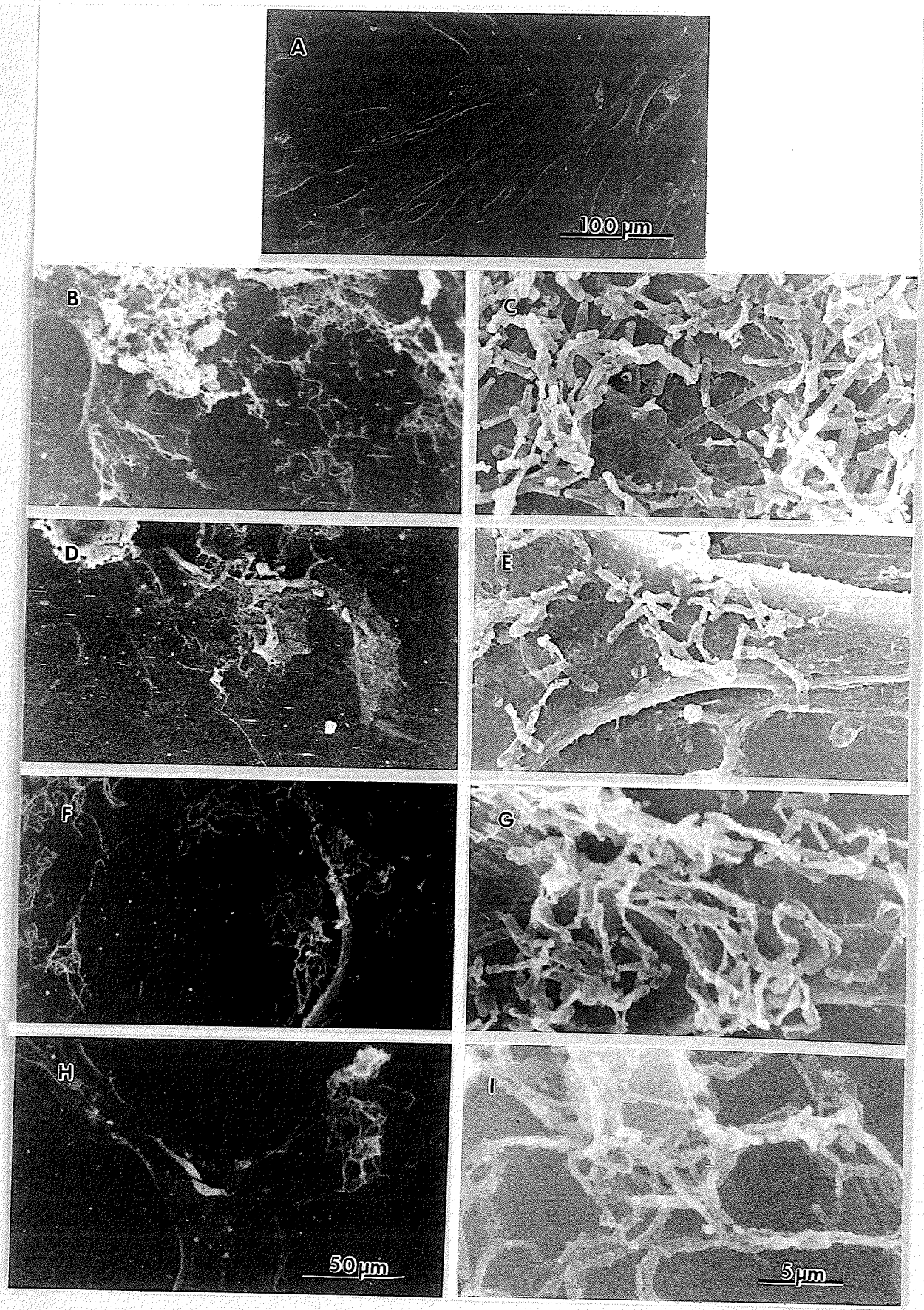

A
100μm
B
C
D
E
F
G
H
50μm
I
5μm

Figure 3: Scanning Electron Microscopy (SEM) of HFF cells infected with H.ducreyi at 24h Post Infection.

HFF cells were grown on glass coverslips and infected with H.ducreyi 35000 (B,C), A77 (D,E), RO18 (F,G), and CIP542 (H,I) at a multiplicity of infection of 10:1. Uninfected monolayers (A) are included as a negative control. The monolayers were infected with the microorganisms for 24h, washed, fixed, dehydrated and coated with gold as described in section 5.0 of Materials and Methods.


A
100 µm
B
C
D
E
F
G
H
50µm
I
5µm

H. ducreyi to reduce XTT to its coloured formazan product was examined. Both HFF cells and H. ducreyi were found to be able to reduce XTT (Table 1a). By introducing an overnight pulse of antibiotics, the formazan production of H. ducreyi was virtually eliminated (Table 1b), whereas the ability of HFF cells to produce the formazan product of XTT was only slightly inhibited (Table 1a). The XTT assay combined with an antibiotic pulse proved to be a reproducible measure of CPE (Fig 4). The 95% confidence interval for the CPE of H. ducreyi 35000 at 10^6 cfu/well was found to be 64.6 ± 3.286 % of the absorbance at 450/650 nm of uninfected control wells.

In order to ensure that the formazan end product of XTT reduction was due solely to HFF cells, unbound bacteria were washed off and then the monolayers were treated with a mixture of antibiotics as described in section 6.1 of Materials and Methods to kill any residual bound bacteria. An overnight pulse with antibiotics was sufficient to eliminate colour development due to bacterial reduction of XTT. When compared to wells without antibiotics, the antibiotic-pulsed wells gave absorbance values indicative of monolayer damage (Fig 5a), whereas wells without antibiotics showed colour development despite the low numbers of HFF cells seen by light microscopy. These results indicated that if the bacteria were not sufficiently killed by an antibiotic pulse, they would interfere with the XTT assay. As a result, all subsequent XTT assays included an antibiotic pulse.

Table 1: The Ability of HFF Cells and H.ducreyi 35000 to Reduce XTT to its Formazan Product

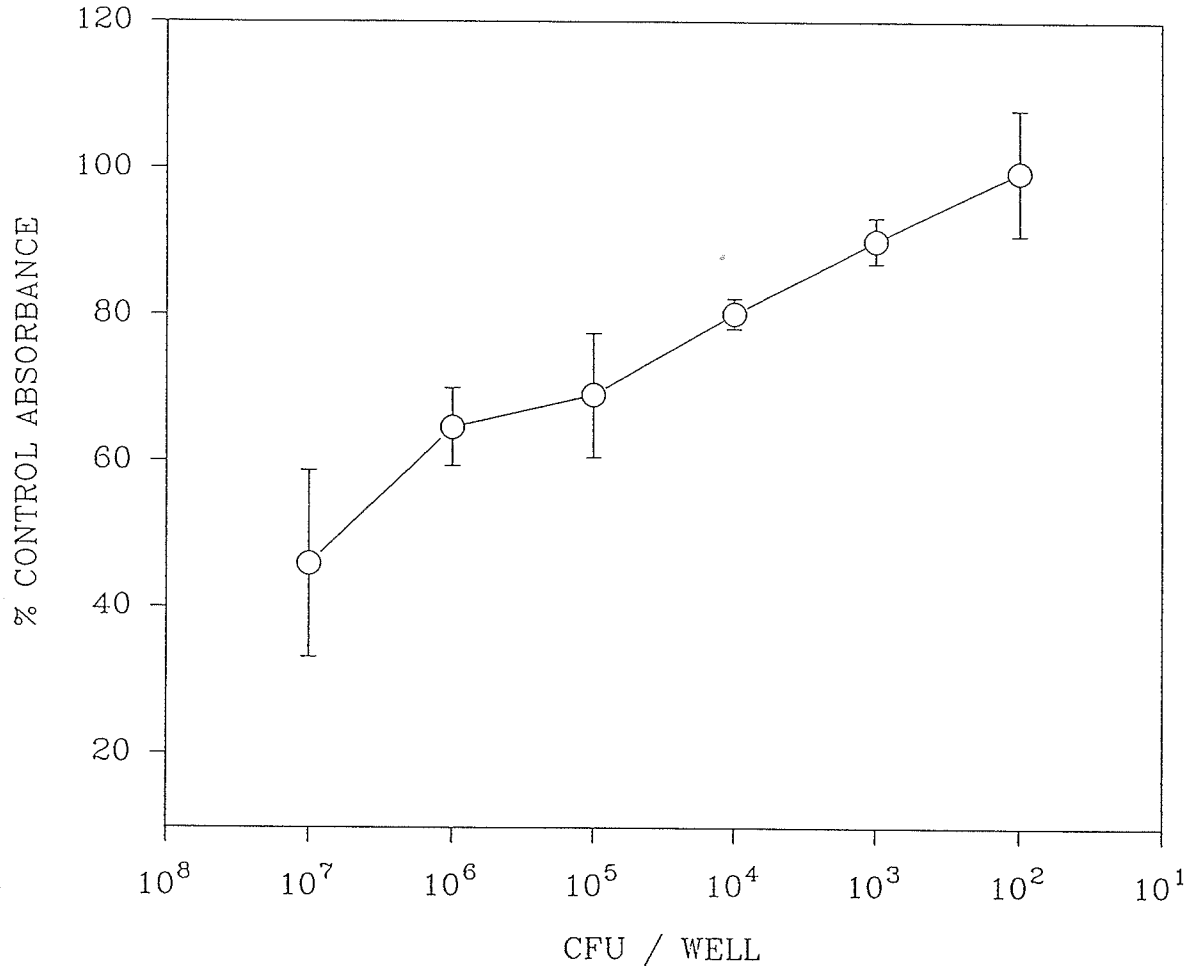
A) HFF cells were seeded at an initial concentration of 10^4 cells/well and diluted serially 1:10, whereupon they were either incubated overnight in SCM or SCM with antibiotics. The XTT assay was performed as described in section 6.1 of Materials and Methods.

B) H.ducreyi were seeded at an initial concentration of 10^7 cells/well and diluted serially 1:10, whereupon they were either incubated overnight in SCM or SCM with antibiotics. The XTT assay was performed as described in section 6.1 of Materials and Methods.

Table 1: The Ability of HFF Cells and H.ducreyi 35000 to reduce XTT to its Formazan Product

A) HFF cells				
Cell number	10^4	10^3	10^2	10^1
No antibiotics	1.305	0.858	0.206	0.193
24h antibiotic pulse	1.213	1.071	0.791	0.089
B) H.ducreyi				
Cell number	10^7	10^6	10^5	10^4
No antibiotics	1.552	1.109	0.890	0.442
24h antibiotic pulse	0.083	0.060	0.051	0.003

All data are expressed as absorbance values of 450/650 nm.


120
100
80
60
40
20
% CONTROL ABSORBANCE
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
CFU / WELL

2.1 - Live Bacterial Assays

The extent of monolayer damage was expressed as a percent of the absorbance of uninfected control monolayers. All H. ducreyi strains tested caused monolayer damage in an inoculum dependent manner, with cell damage occurring in amounts as low as 10^3 cfu/well (Fig 5), whereas H. influenzae (ATCC 11201) did not damage the monolayers (Fig 5b). Thus the CPE appeared to be specific to H. ducreyi and was not simply a result of bacterial growth.

2.2 - Cell-Free Culture Supernatants and Whole Cell Lystaes

Often the ability of bacteria to cause cell damage is due to a cytotoxin that is secreted. In broth culture, the toxin may be secreted and therefore is detectable in the growth medium. In our assay system, cell culture filtrates from both virulent and avirulent H. ducreyi strains grown in tissue culture medium did not appear to damage the monolayers (Fig 6a). To determine if an inducible toxin was the cause of the CPE, culture supernatants were obtained from HFF cell monolayers that had been damaged by H. ducreyi. Filtrates of this fluid were then inoculated onto confluent HFF cell monolayers, but no damage was apparent after 24 h (Fig 6b).

Whole cell lysates (Fig 7a) and insoluble components of H. ducreyi (Fig 7b) were tested for their ability to cause CPE. All fractions tested were at a protein concentration of 0.5 mg/ml in the first well and were serially diluted 1/2. No CPE was observed for any of the fractions tested (Fig 7), indicating a lack of cytotoxic activity in these preparations.

Figure 5: The Effect of Antibiotics on the Detection of a HFF cell Specific XTT signal.

Monolayers of HFF cells were infected with H.ducreyi 35000 as described in section 6.1. The first well contained approximately 1 X 10^7 cfu which was serially diluted 1:10.

A) The infected wells were incubated until a visua CPE was apparent (24h). One set of wells was treated with antibiotics (▽) and the other was not (●).

B) The CPE of H.influenzae b (▽) was compared to the CPE of H.ducreyi srtrains 35000 (▼), RO18 (●), A77 (■), and CIP542 (□).

The amount of HFF cell monolayer damage was assessed by the XTT assay described in section 6.1 of Materials and Methods.

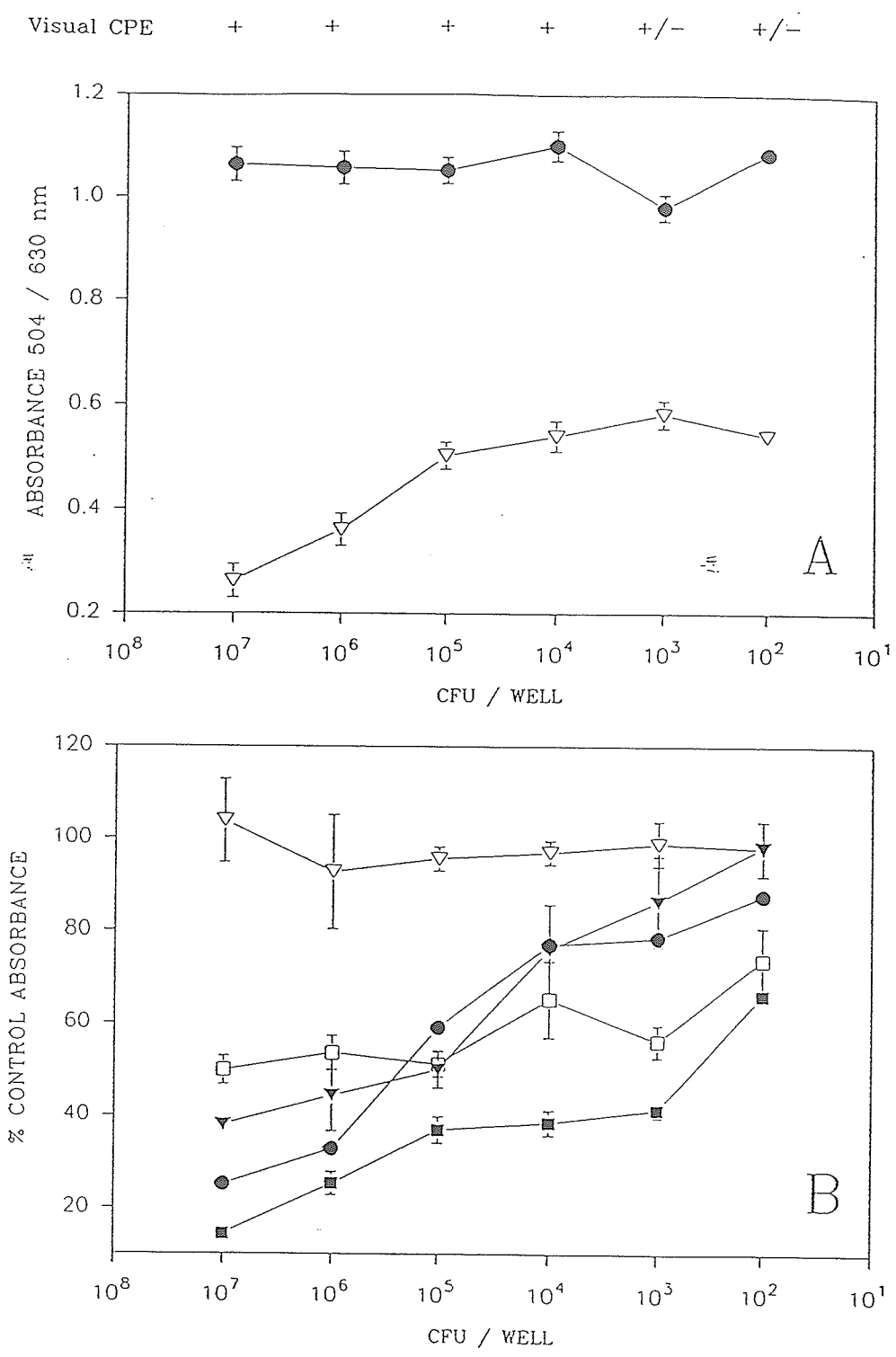
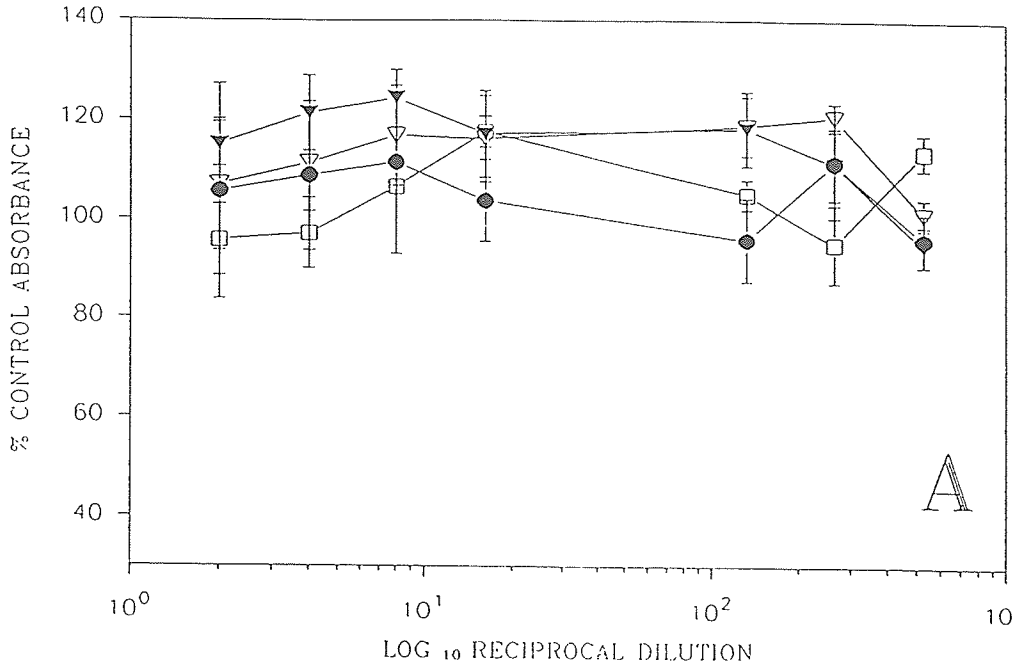

Visual CPE + + + + +/− +/−
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE 504 / 630 nm
A
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL
120
100
80
60
40
20
% CONTROL ABSORBANCE
B
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

Figure 6: Effect of Cell Free Culture Supernatants From *H.ducreyi* on HFF Cell Monolayer Viability.

Monolayers were exposed to filter sterilized culture supernatant from *H.ducreyi* 35000 (●), RO18 (▼), A77 (▽), and CIP542 (□) grown in SCM alone (A) or in association with HFF cells (B). Supernatant (100 µl) was added to the first well of each series and serialy diluted 1:2. Monolayer damage was assessed using the XTT assay described in section 6.2 of Materials and Methods.


140
120
100
80
60
40
% CONTROL ABSORBANCE
A
10^0
10^1
10^2
10
LOG 10 RECIPROCAL DILUTION

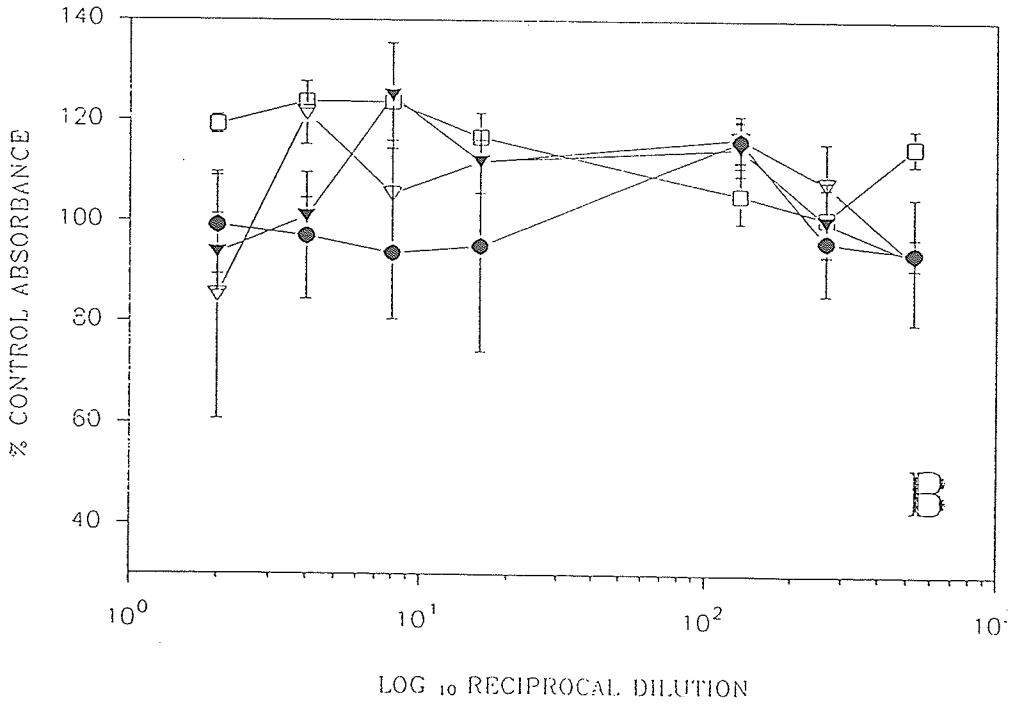
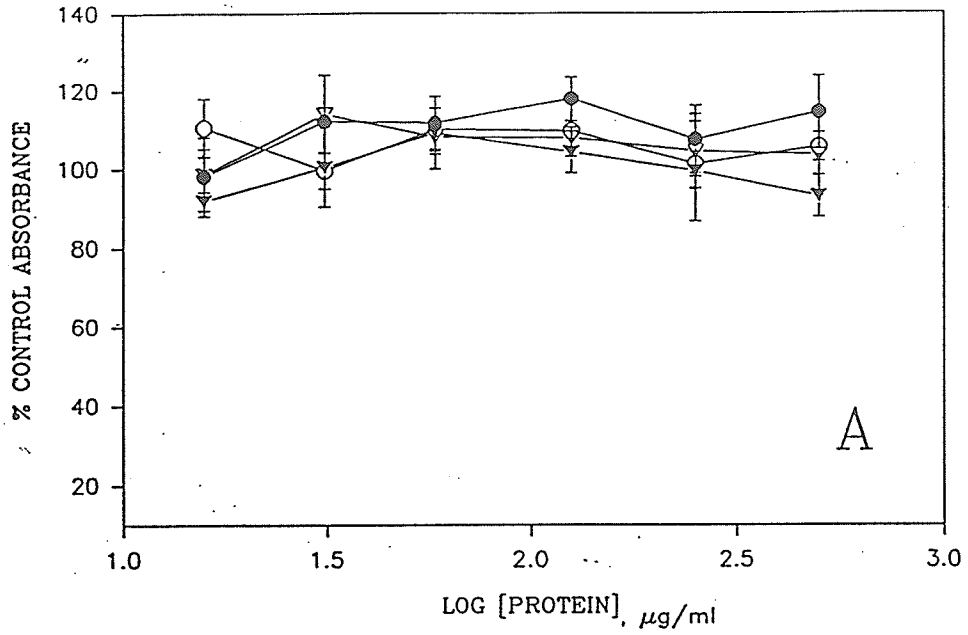
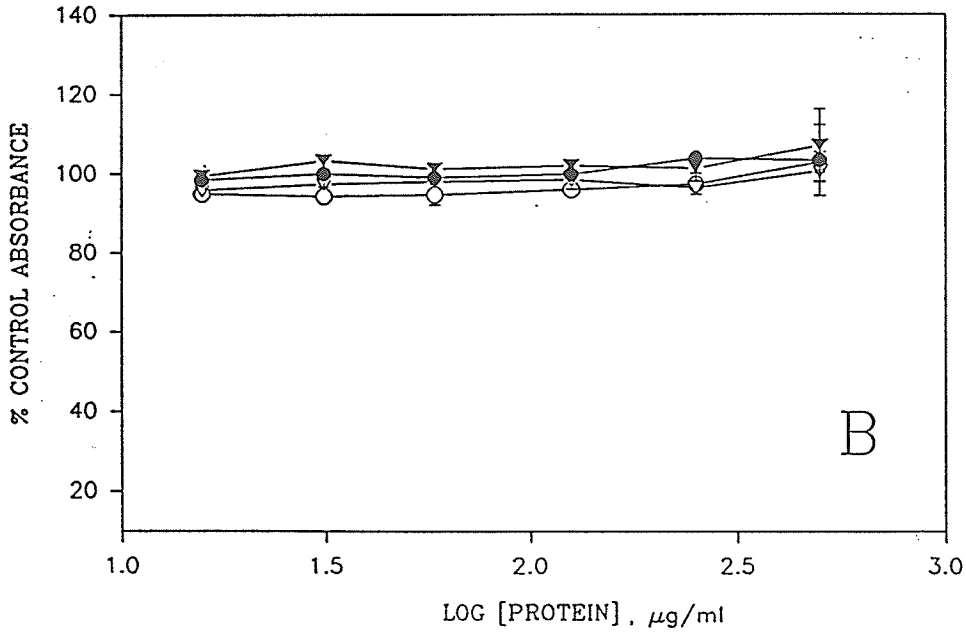

140
120
100
80
60
40
% CONTROL ABSORBANCE
B
10^0
10^1
10^2
10
LOG 10 RECIPROCAL DILUTION

Figure 7: Effect of Whole Cell Lysate and Insoluble Pellet Material on HFF cell monolayer viability.

HFF cell monolayers were incubated with (A) whole cell lysates of H.ducreyi 35000 (○), RO18 (◆), A77 (▼), CIP542 (▽) or (B) insoluble pellets of H.ducreyi 35000 (○), RO18 (◆), A77 (▼), and CIP542 (▽). All cell fractions were added at an initial protein concentration of 0.5 mg/ml. HFF cell damage was determined by the XTT assay described in section 6.2 of Materials and Methods.


140
120
100
80
60
40
20
% CONTROL ABSORBANCE
A
1.0
1.5
2.0
2.5
3.0
LOG [PROTEIN], µg/ml


140
120
100
80
60
40
20
% CONTROL ABSORBANCE
B
1.0
1.5
2.0
2.5
3.0
LOG [PROTEIN], µg/ml

2.3 - Gentamicin Killed Bacteria

In order to determine if live bacteria were required to exert a CPE, gentamicin killed H. ducreyi were inoculated onto HFF cell monolayers.

Gentamicin killed H. ducreyi were not able to exert a CPE at any of the concentrations tested, indicating that live, replicating bacteria are needed to damage HFF cells (Fig 8).

2.4 - Attachment and Microcolony Formation

The role of attachment and microcolony formation in HFF cell damage was also examined using the XTT assay. Previous investigations demonstrated that the avirulent H. ducreyi strain A77 attaches to HFF cells in significantly lower numbers than do virulent strains 35000 and RO18 (11). Washing HFF cell monolayers at 4h after infection with H. ducreyi served to remove any non- or loosely adherent bacteria. Thus, the resultant CPE would be exerted solely by adherent bacteria remaining on the HFF cell monolayer. The CPE exerted by the virulent H. ducreyi strains 35000 (Fig 9a) and RO18 (Fig 10a) was not significantly altered by the washing step. However the CPE caused by the avirulent strains A77 (Fig 9b) and CIP542 (Fig 10b) was decreased as a result of the washing step. Thus, these data suggest that the ability of H. ducreyi to attach to host cells may play a role in the ability of H. ducreyi to exert a CPE on HFF cells when selective pressures such as wash steps are used.

2.5 - Reversability of CPE

Further experiments were done to determine whether or not

Figure 8: The Ability of Gentamicin Killed H.ducreyi to Exert a CPE on HFF Cell Monolayers.

Monolayers of HFF cells were inoculated with gentamicin killed H.ducreyi as described in section 6.1. The first well contained approximately 1 X 10^7 cfu which was serially diluted 1:10. The XTT assay was performed as described in section 6.1 of Materials and Methods to assess HFF cell damage by gentamicin killed H.ducreyi strains 35000 (○), RO18 (●), A77 (Ψ), CIP542 (▼).

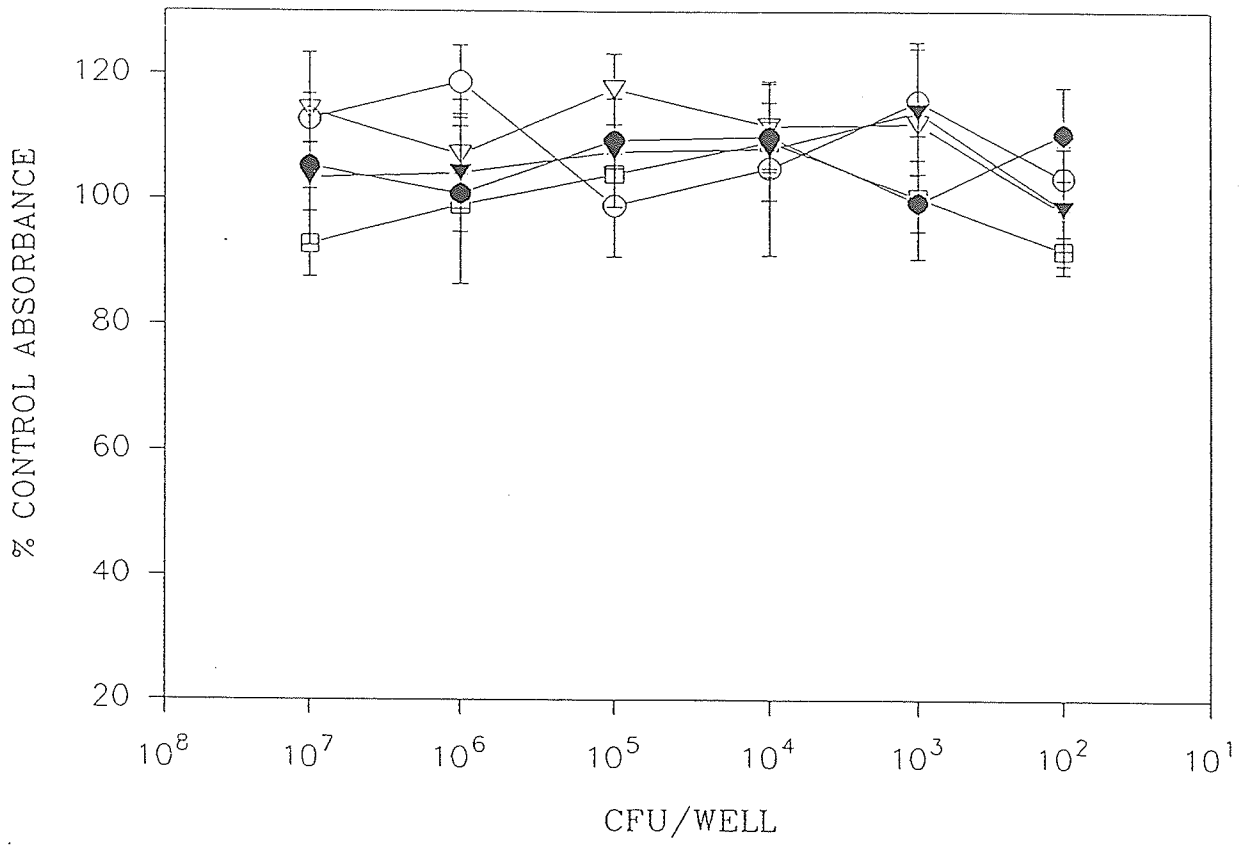
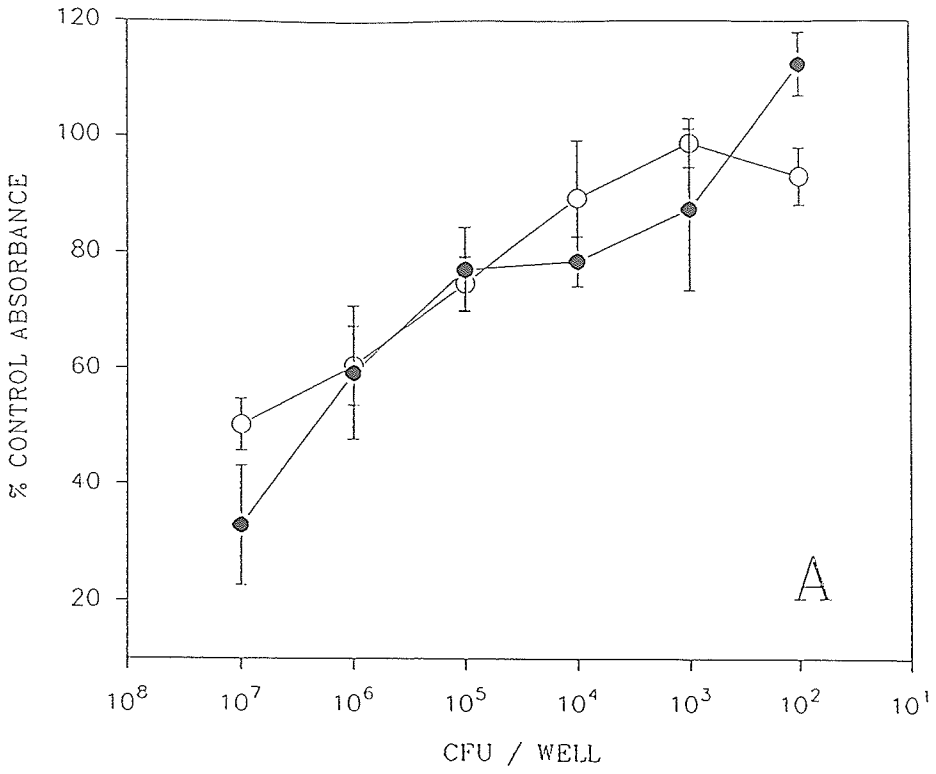

% CONTROL ABSORBANCE
120
100
80
60
40
20
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
CFU/WELL

Figure 9: Role of Attachment of H.ducreyi in CPE (I).

HFF cell monolayers were infected with H.ducreyi 35000 (A) or A77 (B) at an initial concentration of 1 X 10^7 cfu/well.

A) HFF cell monolayer damage by H.ducreyi 35000 was compared between infected monolayers in which HFF cell monolayers were washed at 4h (○), or unwashed(●). Both sets of monolayers were further incubated for a total of 24h before being assayed for damage using the XTT assay procedure described in section 6.4 of Materials and Methods.

B) HFF cell monolayer damage by H.ducreyi A77 was compared between infected monolayers in which HFF cell monolayers were washed at 4h (○) or unwashed(●). Both sets of monolayers were further incubated for a total of 24h before being assayed for damage using the XTT assay procedure described in section 6.4 of Materials and Methods.


120
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20
% CONTROL ABSORBANCE
A
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

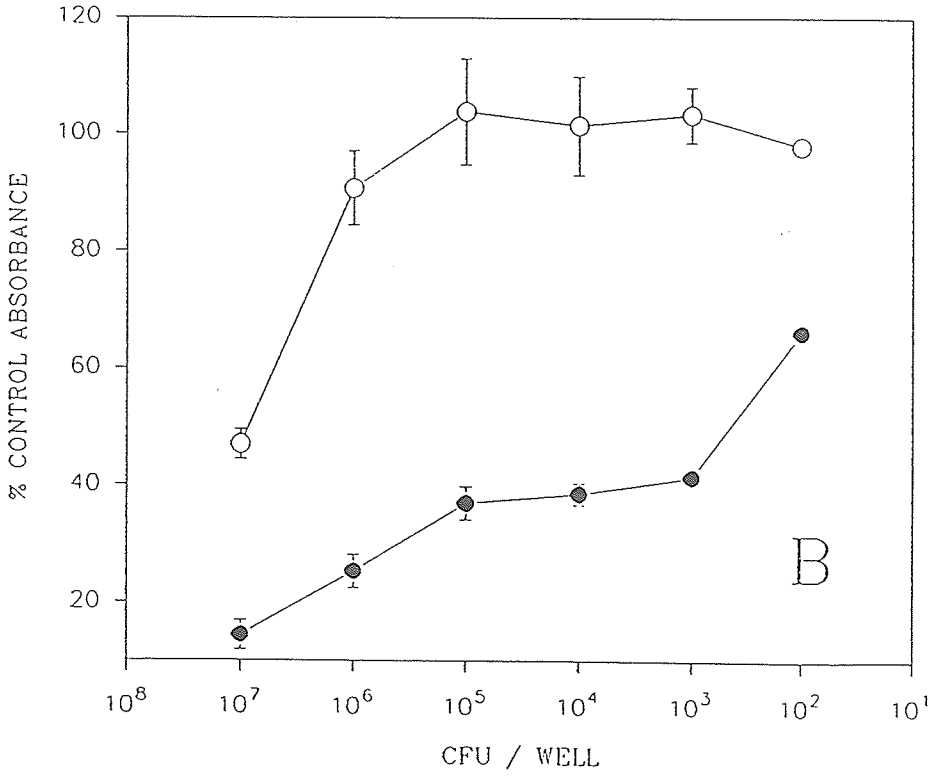
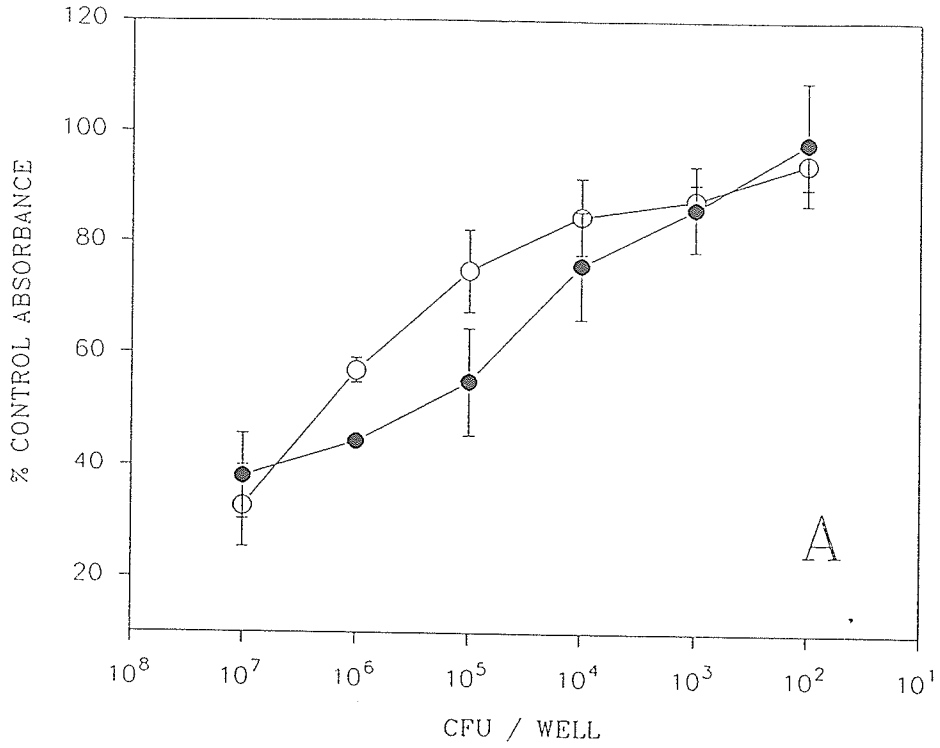

120
100
80
60
40
20
% CONTROL ABSORBANCE
B
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

Figure 10: Role of Attachment of H.ducreyi in CPE (II).

HFF cell monolayers were infected with H.ducreyi RO18 (A) or CIP542 (B) at an initial concentration of 1 X 10^7 cfu/well.

A) HFF cell monolayer damage by H.ducreyi RO18 was compared between infected monolayers in which HFF cell monolayers were washed at 4h (○), or unwashed(●). Both sets of monolayers were further incubated for a total of 24h before being assayed for damage using the XTT assay procedure described in section 6.4 of Materials and Methods.

B) HFF cell monolayer damage by H.ducreyi CIP542 was compared between infected monolayers in which HFF cell monolayers were washed at 4h (○) or unwashed (●). Both sets of monolayers were further incubated for a total of 24h before being assayed for damage using the XTT assay procedure described in section 6.4 of Materials and Methods.


120
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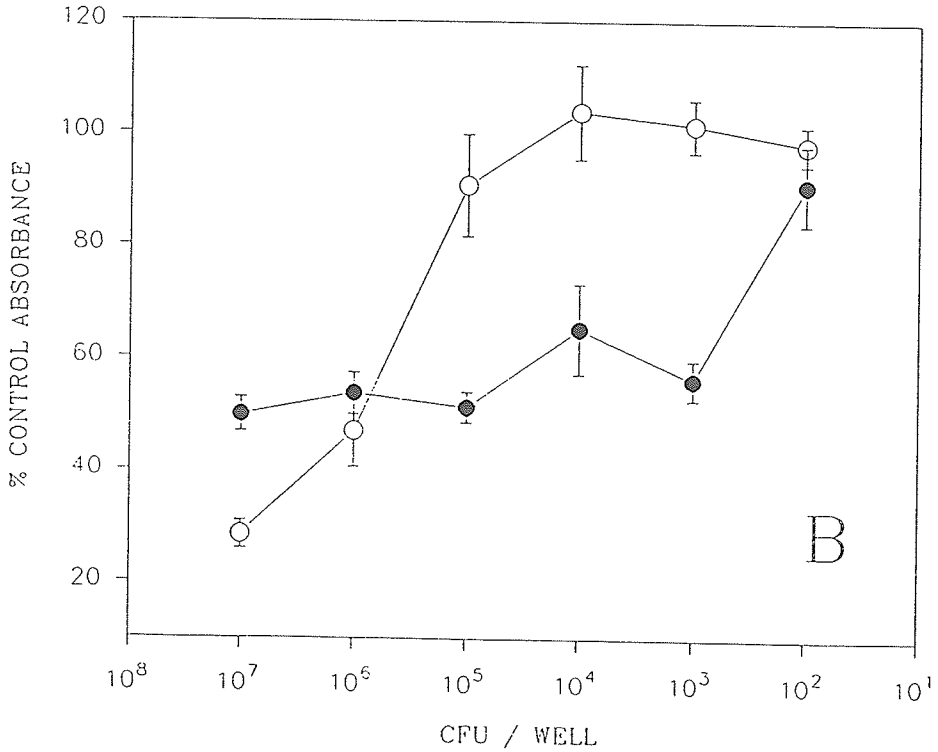
% CONTROL ABSORBANCE
A
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL


120
100
80
60
40
20
% CONTROL ABSORBANCE
B
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

the CPE produced by H. ducreyi was reversible. All of the H. ducreyi strains tested exerted a CPE within 24 h of inoculation (Fig 11,12). For all strains tested, no HFF cell damage was apparent when bacteria were killed 4 h after infecting the HFF cell monolayers and allowed to incubate for a further 20 h before being assayed for cytotoxicity (Fig 11 A,B, 12 A,B). Monolayers that were infected with H. ducreyi for 24 h, washed and then incubated for a further 72 h in media containing antibiotics did not show any improvement in monolayer condition (Fig 11 A,B 12 A,B). These results indicate that H. ducreyi damage to the HFF cell happens relatively late in the infection, and once the HFF cell is damaged, the CPE is not rapidly reversible.

2.6 - LOS-Mediated Cell Damage

The LOS of H. ducreyi and N. gonnorhoaeae have been shown to share a significant level of chemical and structural similarity (22,78). It has also been shown that the LOS of N. gonorrhoaeae is able to exhibit direct toxicity on fallopian tube mucosa in vitro (41). The ability of H. ducreyi LOS to exert direct cytotoxicity on HFF cell monolayers was therefore assessed.

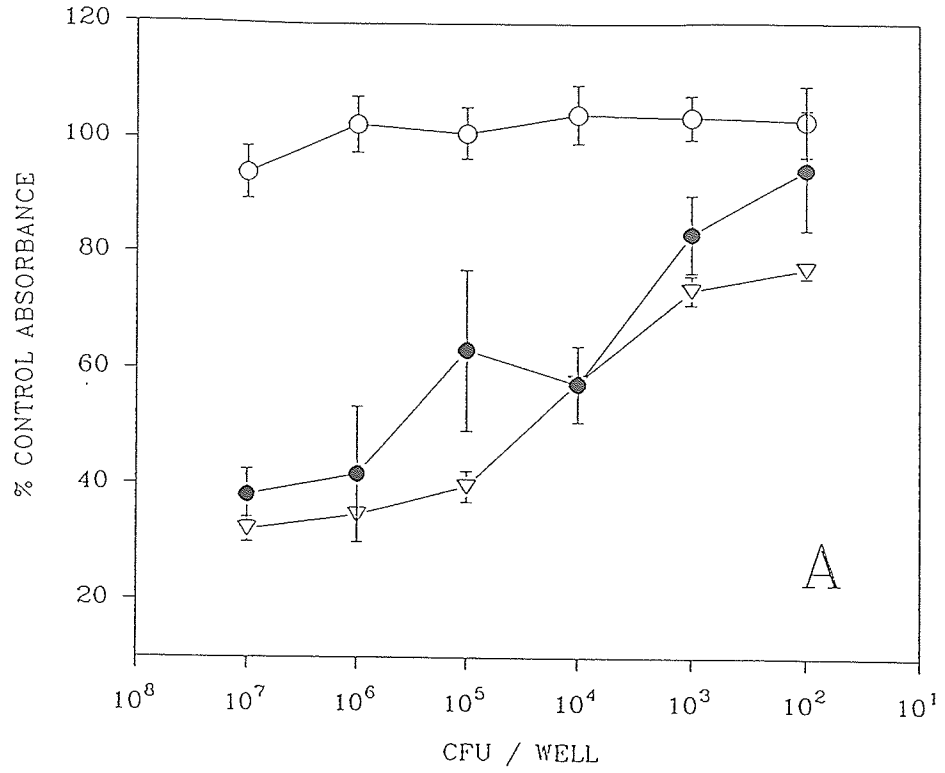
Bacterial LOS was obtained from H. ducreyi strains 35000, RO18, A77 and CIP542 using the phenol-water extraction method described in section 4.3. The purity of LOS preparations was assessed by visualizing Coomassie blue and silver-stained LOS

Figure 11: Reversability of CPE (I)

HFF cell monolayers were infected with H.ducreyi at an initial concentration of 1 X 10^7 cfu/well and treated as described below. In order to determine the capacity of HFF cells to regenerate after a CPE occurred, monolayers were assayed for damage 4h (○), 24h (●) and 72h after H.ducreyi were killed by an antibiotic pulse (▽).

A) HFF cell monolayer damage by H.ducreyi 35000 was compared between monolayers in which bacteria were killed with antibiotics at 4h post infection (○), at 24h (●), or incubated for 24h to allow sufficient time for a CPE to occurr, pulsed with antibiotics and allowed to regenerate for a further 72h (▽) before being assayed for damage using the XTT assay procedure described in section 6.3 of Materials and Methods.

B) HFF cell monolayer damage by H.ducreyi A77 was compared between monolayers in which bacteria were killed with antibiotics at 4h post infection (○), at 24h (●), or incubated for 24h to allow sufficient time for a CPE to occurr, pulsed with antibiotics and allowed to regenerate for a further 72h (▽) before being assayed for damage using the XTT assay procedure described in section 6.3 of Materials and Methods.


120
100
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60
40
20
% CONTROL ABSORBANCE
A
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

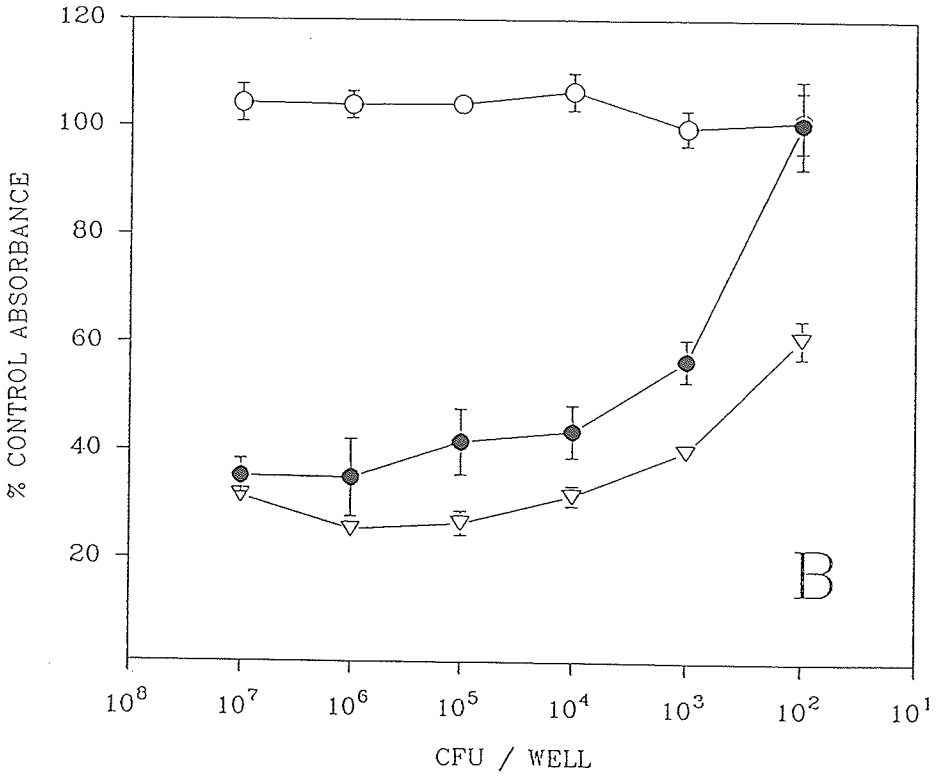
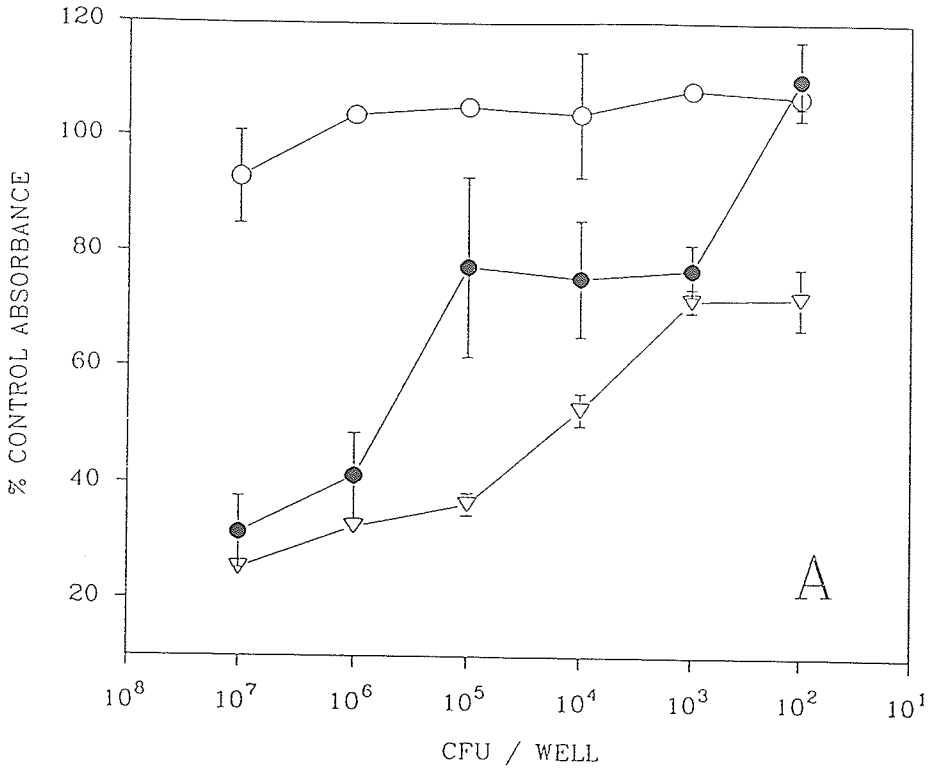

120
100
80
60
40
20
% CONTROL ABSORBANCE
B
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

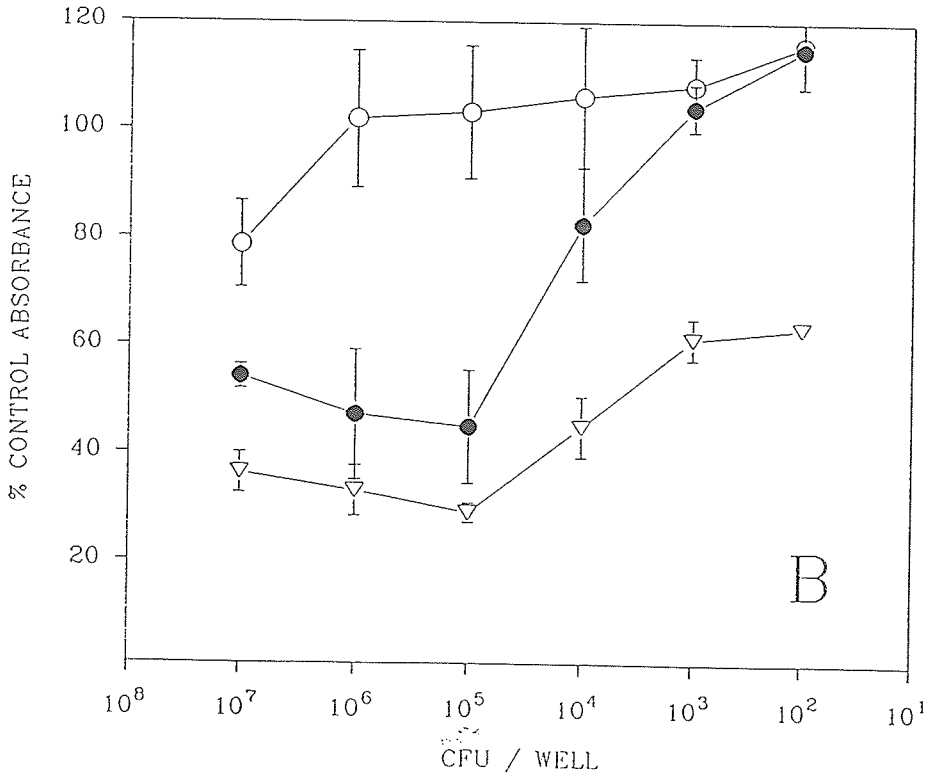
Figure 12: Reversability of CPE (II)

HFF cell monolayers were infected with H.ducreyi at an initial concentration of 1 X 10^7 cfu/well and treated as described below. In order to determine the capacity of HFF cells to regenerate after a CPE occurred, monolayers were assayed for damage 4h (○), 24h (●) and 72h after H.ducreyi were killed by an antibiotic pulse (▽).

A) HFF cell monolayer damage by H.ducreyi RO18 was compared between monolayers in which bacteria were killed with antibiotics at 4h post infection (○), at 24h (●), or incubated for 24h to allow sufficient time for a CPE to occurr, pulsed with antibiotics and allowed to regenerate for a further 72h (▽) before being assayed for damage using the XTT assay procedure described in section 6.3 of Materials and Methods.

B) HFF cell monolayer damage by H.ducreyi CIP542 was compared between monolayers in which bacteria were killed with antibiotics at 4h post infection (○), at 24h (●), or incubated for 24h to allow sufficient time for a CPE to occurr, pulsed with antibiotics and allowed to regenerate for a further 72h (▽) before being assayed for damage using the XTT assay procedure described in section 6.3 of Materials


120
100
80
60
40
20
% CONTROL ABSORBANCE
A
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL


120
100
80
60
40
20
% CONTROL ABSORBANCE
B
10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
CFU / WELL

preparations that were subjected to SDS-PAGE electrophoresis as described in section 3.0. In all preparations tested, no Coomassie blue staining was evident, confirming that there were no detectable contaminating proteins in the extracted LOS preparations (Fig 13A). Silver-stained gels revealed a series of low molecular weight bands indicative of H. ducreyi LOS (85) (Fig 13B). The LOS of H. ducreyi was quantified using the phenol-H_2SO_4 assay described in section 4.3.1 of Materials and Methods. The standard curve was constructed using a commercial preparation of E. coli 0157:B8 LPS (Sigma, St. Louis MO) of known concentration. The use of this technique provided a useful means for determining the relative concentration of LOS in a given sample, as the linearity of the standard curves was reliable (Fig 14). The LOS of all H. ducreyi strains tested were able to exert damage after 24 hours of incubation (Fig 15A). For the avirulent strains A77 and CIP542, visual and XTT quantifiable damage was observed up to a concentration of approximately 25 μg / well of LOS (Fig 15A). For the virulent strains 35000 and RO18, visual but not quantifiable damage was also evident at a 25 μg / well concentration, however the amount of damage observed was below the sensitivity of the XTT assay, and thus it was not readily detected (Fig 15A).

Figure 13: Purity of H.ducreyi LOS Preparations.

H.ducreyi LOS was phenol-water extracted, electrophoresed, and visualized as described in section 4.3 of Materials and Mehods.

A) **Coomassie blue stained LOS gels.** Aliquots of purified LOS from H.ducreyi 35000 (Lane 3), RO18 (Lane 4), A77 (Lane 5) and CIP542 (Lane 6) were extracted, subjected to electrocphoresis and visualized by Coomassie blue staining as described in section 4.3 of materials and Methods.

B) **Silver Stained LOS gels.** Aliquots of purified LOS from H.ducreyi 35000 (Lane 3), RO18 (Lane 4), A77 (Lane 5) and CIP542 (Lane 6) were extracted, subjected to electrphoresis and visualized by silver staining as described in section 4.3 of materials and Methods.

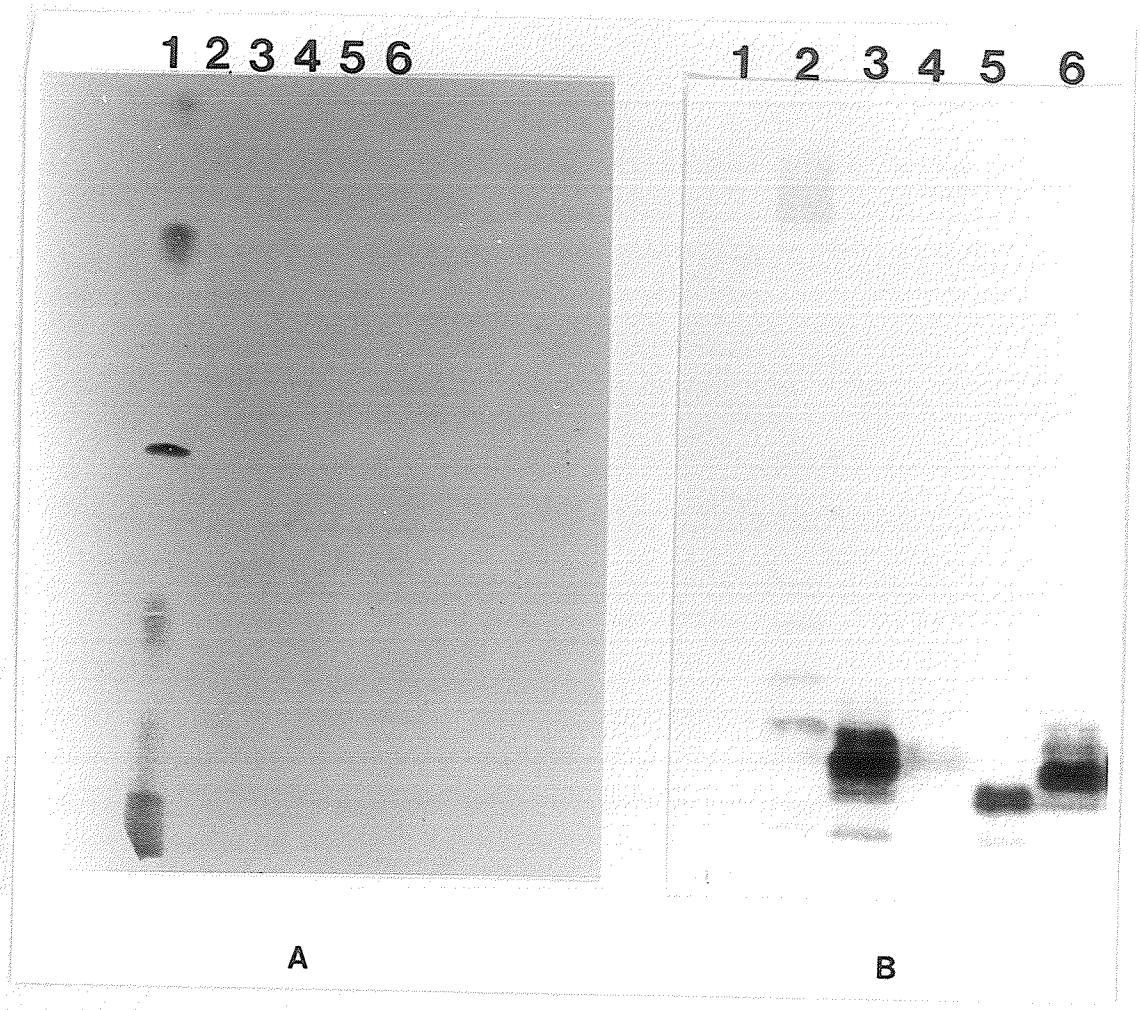

1 2 3 4 5 6
1 2 3 4 5 6
A
B

Figure 14: Linearity of Standard Curves for Phenol H_2SO_4 Assay for Detection of LOS.

H.ducreyi LOS was quantified using a phenol-H_2SO_4 assay described in section 4.3.1 of Materials and Methods. A standard curve was constructed using known amounts E.coli 0158:B7 LPS (O).

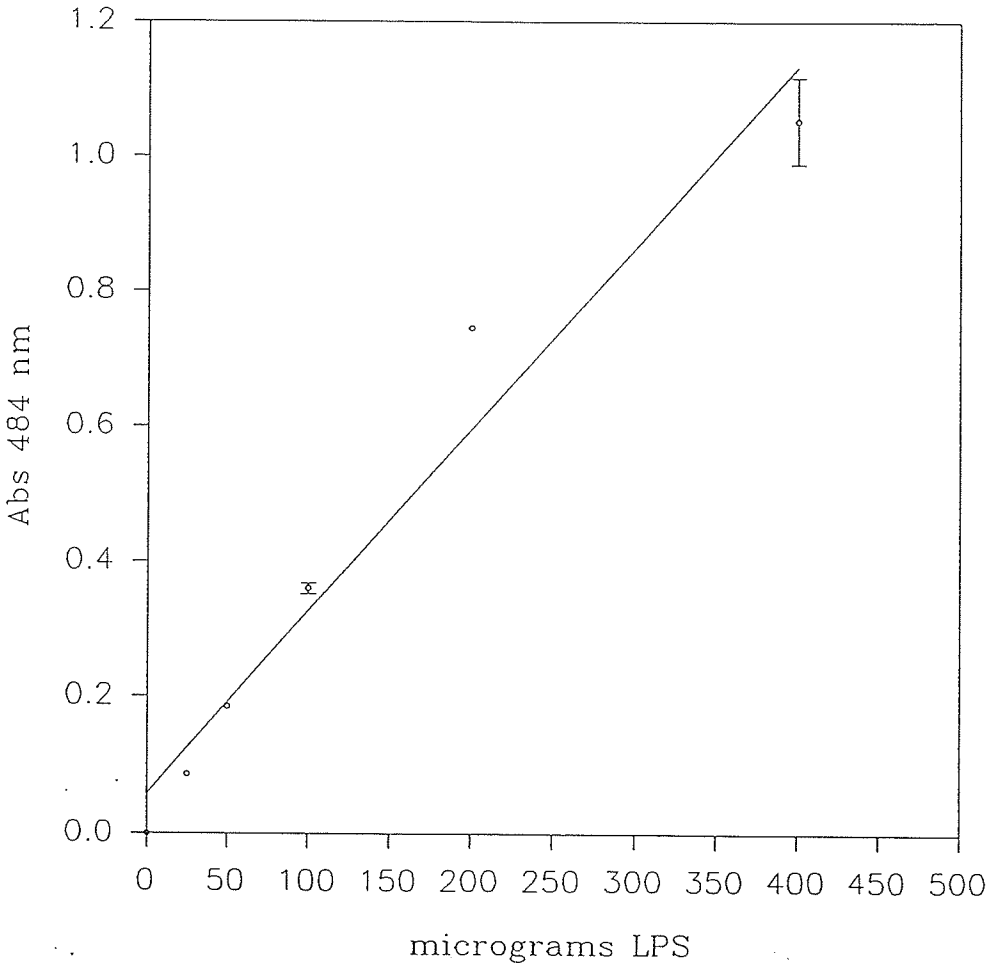
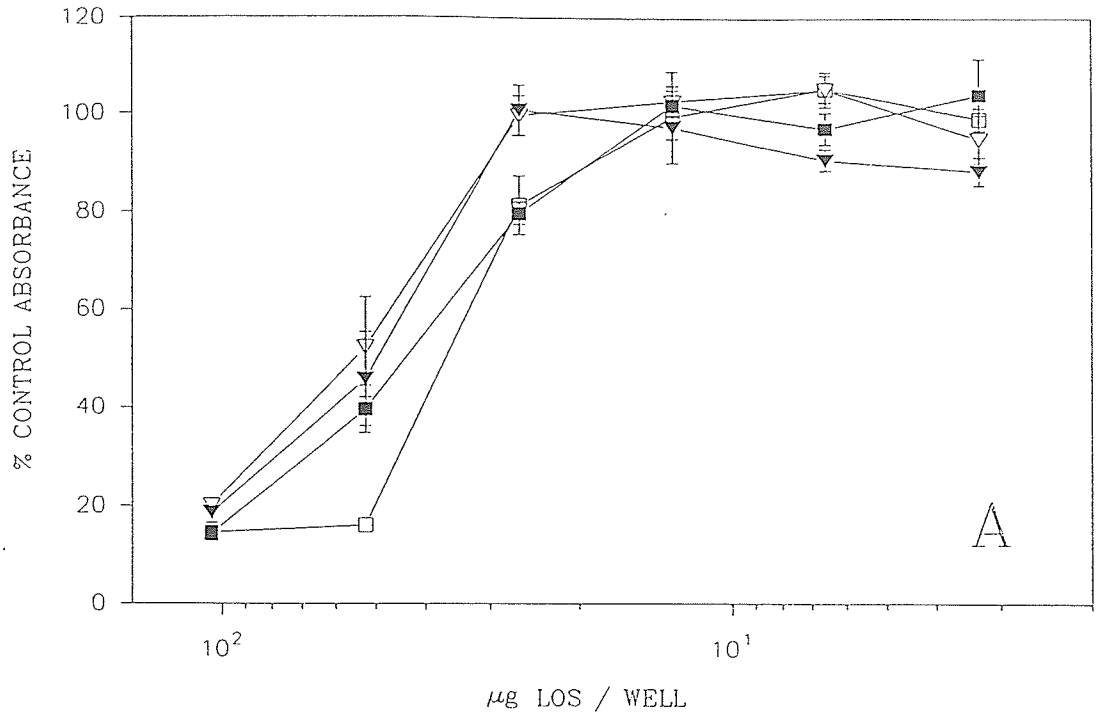
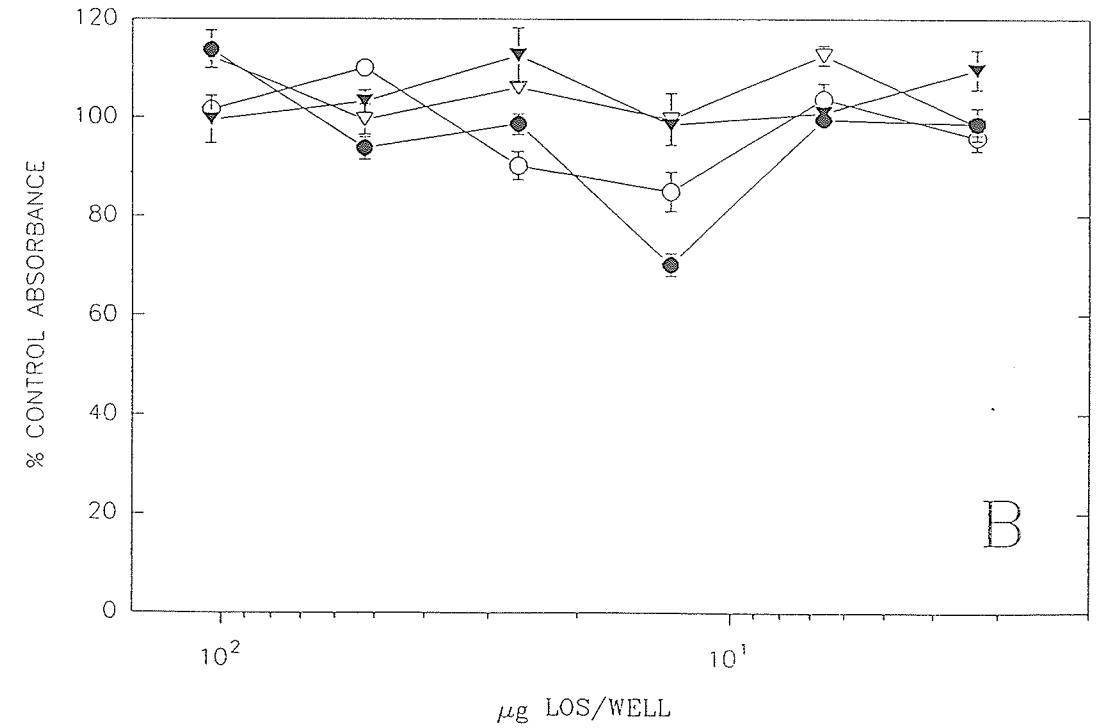

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs 484 nm
0
50
100
150
200
250
300
350
400
450
500
micrograms LPS

Figure 15: Effect of H.ducreyi LOS on HFF Cell Viability

A) Monolayers were exposed to purified LOS suspended in SCM from H.ducreyi 35000 (▽), RO18 (▼), A77 (□), and CIP542 (■). The LOS was added to an initial concentration of 100 μg/well in the first well and serially diluted 1:10. Monolayer damage was assessed using the XTT assay described in section 6.6 of Materials and Methods.

B) Monolayers were exposed to purified LOS incorporated into liposomal SUV's from H.ducreyi 35000 (▼), RO18 (▽), A77 (●), and CIP542 (○). The SUV incorporated LOS was added to an initial concentration of 100 μg/well in the first well and serially diluted 1:10. Monolayer damage was assessed using the XTT assay described in section 6.6 of Materials and Methods.


120
100
80
60
40
20
0
% CONTROL ABSORBANCE
10^2
10^1
μg LOS / WELL
A


120
100
80
60
40
20
0
% CONTROL ABSORBANCE
10^2
10^1
μg LOS/WELL
B

No damage was detected when H. ducreyi LOS was incorporated into liposomal SUV's (Fig 15B). Thus, the data suggest that LOS from both the virulent and avirulent H. ducreyi strains tested were equally able to damage HFF cells, and that free H. ducreyi LOS is required for HFF cell damage to occur.

3.0 - Shedding of LOS by H. ducreyi

In order to determine if H. ducreyi were able to shed LOS into the environment, the ability of H. ducreyi strains to shed LOS over time was investigated (Fig 16). At all time frames tested, H. ducreyi strains were able to consistently shed LOS in amounts comparable to the positive control, N. meningitidis (Fig 16).

Analysis of gels stained with the Silver stain method of Tsai and Frasch (118) revealed that both protein and carbohydrate components were being shed by the bacteria into the supernatants (Fig 17A). A series of low molecular weight bands corresponding to the LOS bands of H. ducreyi and N. meningitidis were seen on the silver stained gels, indicating that bacterial LOS was possibly present in the supernatant (Fig 17A). The presence of LOS in the supernatants was confirmed by H. ducreyi LOS-specific monoclonal antibody 2D2 (110) using Dot Blot Immunodetection (Fig 17B).

These data suggest that H. ducreyi are able to actively shed LOS, potentially releasing the bacterial LOS into the environment where they can exert damage on HFF cells once a sufficient concentration is achieved.

Figure 16: Shedding of LOS by H.ducreyi

H.ducreyi were incubated for 0, 1, 2, 6, and 24h in 0.01M HEPES buffer (pH=7.4) and relative amounts of carbohydrate (LOS) in the supernatants were determined using the colourimetric assay described in section 7.0 of Materials and Methods.

The relative amounts of carbohydrate (LOS) in the supernatants over time was compared between N.meningitidis ( ), H.ducreyi RO18 ( ) and A77 ( ).

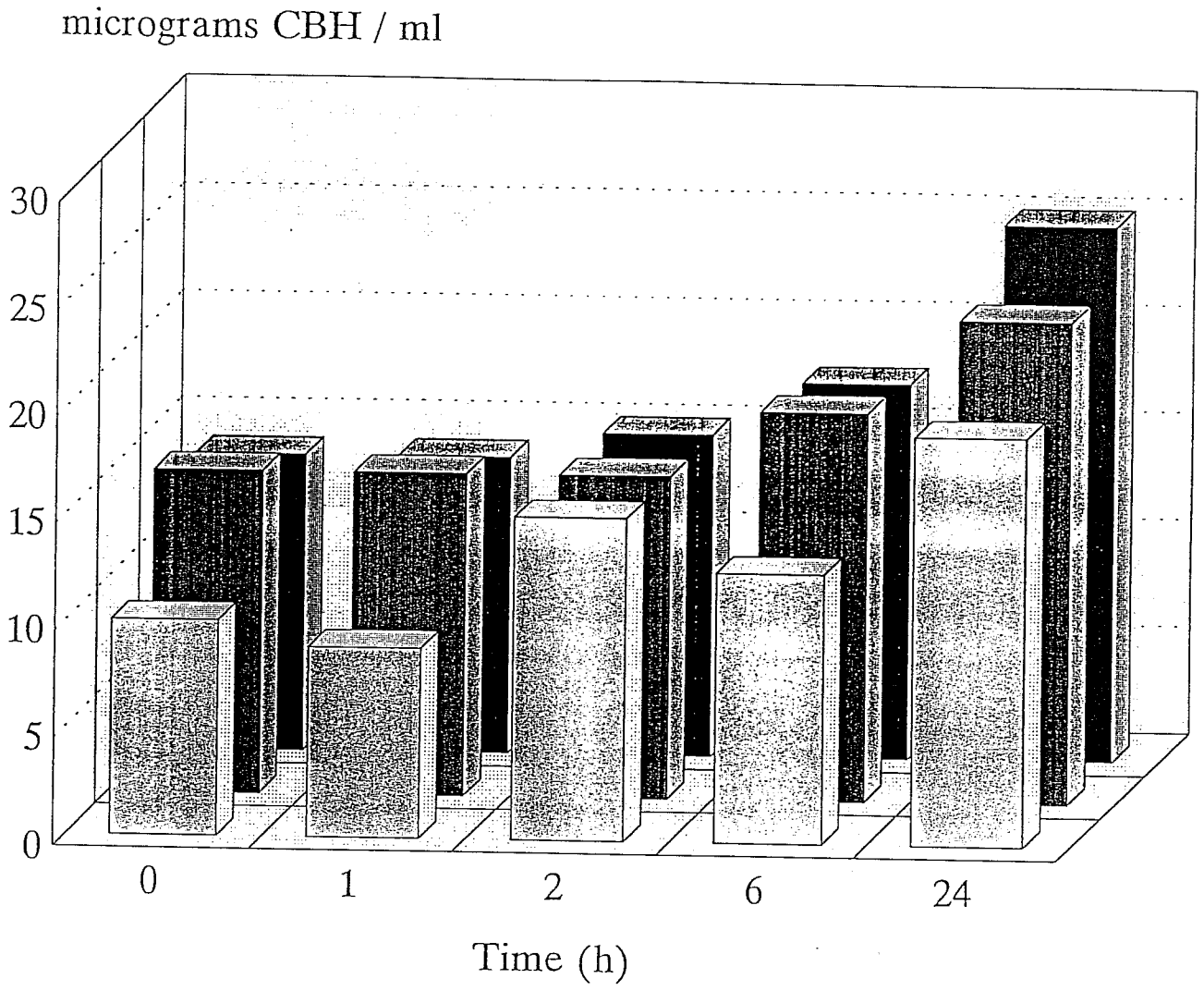
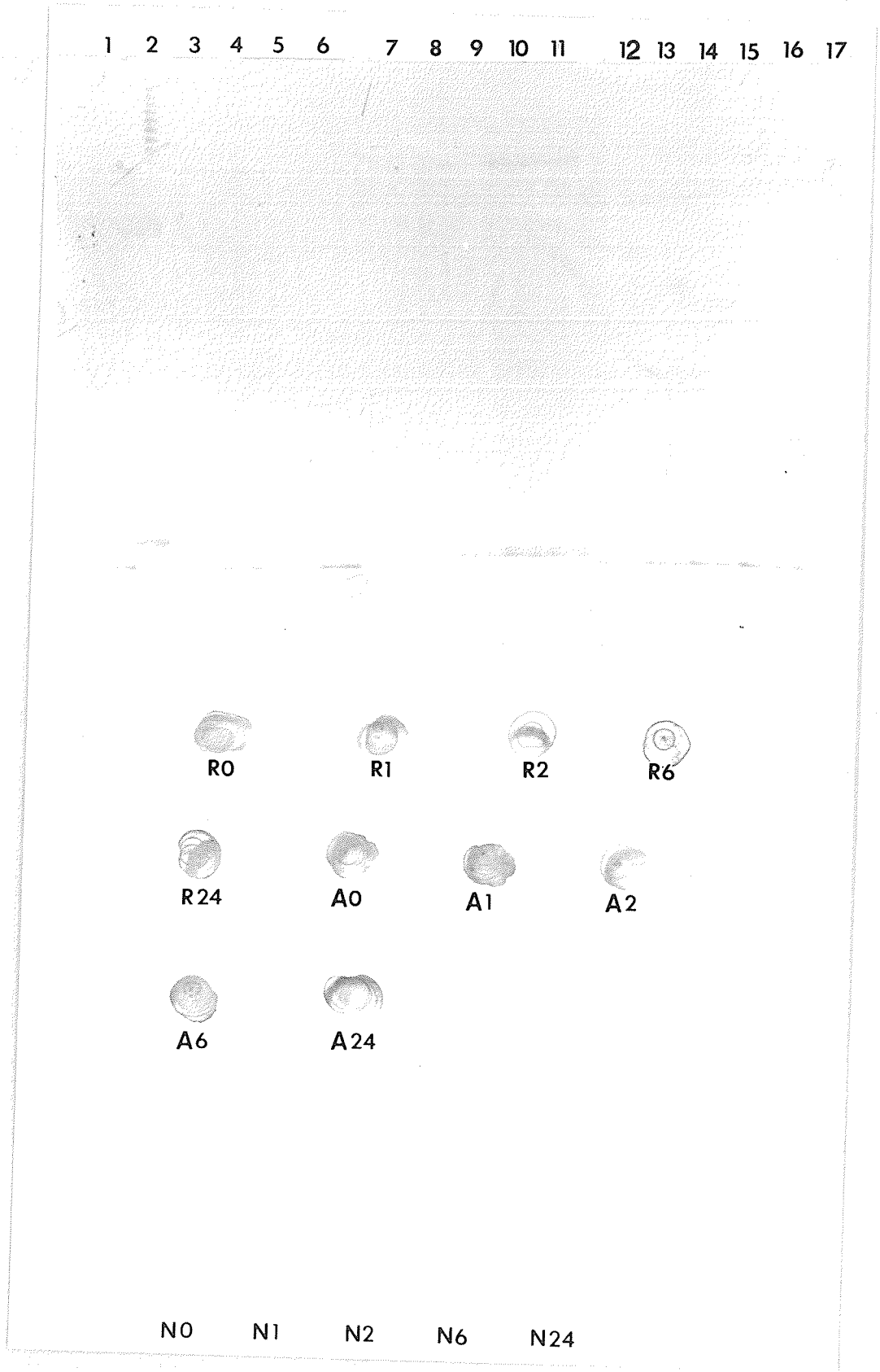

micrograms CBH / ml
30
25
20
15
10
5
0
0
1
2
6
24
Time (h)

Figure 17: Shedding of LOS by H.ducreyi: SDS-PAGE and Dot Blot Analysis of Supernatant Contents.

A) **Electrophoretic analysis of supernatants.** N.meningitidis supernatants at 0 (Lane 1), 1 (Lane 3), 2 (Lane 4), 6 (Lane 5), and 24 hours (Lane 6) and H.ducreyi RO18 supernatants at 0 (Lane 7), 1 (Lane 8), 2 (Lane 9), 6 (Lane 10) and 24 hours (Lane 11) as well as H.ducreyi A77 supernatants at 0 (Lane 12), 1 (Lane 13), 2 (Lane 14), 6 (Lane 15) and 24 hours (Lane 16) were concentrated 50-fold, electrophoresed and silver stained as described in section 7.0 of Materials and Methods.

B) **Dot blot immunodetection analysis of supernatants.** N.meningitidis supernatants at 0 (NO), 1 (N1), 2 (N2), 6 (N6), and 24 hours (N24) and H.ducreyi RO18 supernatants at 0 (R0), 1 (R1), 2 (R2), 6 (R6) and 24 hours (R24) as well as H.ducreyi A77 supernatants at 0 (A0), 1 (A1), 2 (A2), 6 (A6) and 24 hours (A24) were concentrated 50-fold, spotted onto nitrocellulose, probed using the H.ducreyi LOS specific monoclonal antibody 2D2 as described in section 7.1 of Materials and Methods.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
R0
R1
R2
R6
R24
A0
A1
A2
A6
A24
N0
N1
N2
N6
N24

IV.) DISCUSSION

The overall objective of this study was to use the *In vitro* HFF cell culture model (10) to study the ability of *H. ducreyi* to damage HFF cells. A more detailed understanding of the mechanism by which *H. ducreyi* damages HFF cells should further our understanding of the pathogenesis of *H. ducreyi*.

The ability of a microbial pathogen to establish a productive infection in its human or animal host can depend on a number of different factors. Generally, it is accepted that in order to establish itself in the host, a microbial pathogen must first gain entry into the host, localize itself to a certain cell type or ecological niche, and ensure its survival by multiplying in sufficient numbers (34).

A variety of reports have demonstrated that *H. ducreyi* are able to invade and adhere to keratinocytes (18,118) and epithelial cells (60,107). Our scanning electron microscopy (SEM) data demonstrate that the avirulent *H. ducreyi* strains A77 and CIP542 were not able to attach and form adherent microcolonies on the HFF cells to the same extent as the virulent strains 35000 and RO18. These data confirm Alfa *et al*'s (11) attachment data which demonstrated that the avirulent *H. ducreyi* strain A77 did not attach to HFF cells as efficiently as the virulent *H. ducreyi* strain 35000. Although the ability of *H. ducreyi* to enter the HFF cells could not be conclusively assessed by our SEM data, previous reports have shown that HFF cells are not able to internalize *H. ducreyi* to

a significant extent (11). Furthermore, the avirulent H. ducreyi strains A77 and CIP542 were not able to produce ulceration in the temperature dependant rabbit model of Purcell et al (91), whereas the virulent H. ducreyi strain 35000 was able to do so (91). These data taken in conjunction with our SEM data suggest a possible role for attachment in the ability of H. ducreyi to cause tissue damage in the host. Although HFF cell damage was observed at 24h post infection, an accurate assessment of CPE cannot be reliably achieved by observing SEM micrographs.

As a result, an adaptation of the XTT assay originally described by Scudiero et al (99,104) was employed to quantitate HFF cell damage. Although HFF cells reduced XTT to its coloured formazan product in an amount proportional to HFF cell number, the presence of H. ducreyi in the infected monolayers provided a problem as H. ducreyi were also able to reduce XTT. Our initial studies demonstrated that an overnight pulse of antibiotics rendered the XTT signal to be HFF cell specific, as H. ducreyi were killed by the antibiotic pulse, preventing them from reducing XTT. The modified XTT assay was successfully employed as a quantitative, reproducible and less subjective means of determining HFF cell damage. Using the conditions established in our initial studies, the limit of detection of the XTT assay was found to be 10^2 HFF cells.

Having adapted the XTT assay for CPE measurement of HFF cell damage, we examined the ability of a variety of H.

ducreyi strains to cause a CPE on HFF cells. For all H. ducreyi strains tested, the CPE was found to require live bacteria, as gentamicin killed H. ducreyi did not exert a CPE. In contrast, HFF cell monolayers infected with live bacteria exhibited a concentration dependant CPE for all H. ducreyi strains tested, whereas the related pathogen H. influenzae type b was found not to damage the HFF cell monolayer. The CPE was found to occur late in the infection, as no HFF cell CPE was detected at 4h post infection. When HFF cell damage was evident, it was not found to be readily reversible, indicating that the CPE was not due to a fast acting toxin, such as is seen with diphtheria toxin (24,25).

The possibility that in vitro damage caused by a microbial pathogen is due to a non-specific phenomenon such as pH has been raised in other cell culture models (36). Even though the pH of the culture medium was acidic for H. influenzae as evidenced by its bright yellow colour, no visual damage was observed for H. influenzae type b. Although H. ducreyi damaged the HFF cells, the pH of the culture medium did not become acidic. Therefore a specific H. ducreyi derived cytotoxic factor seems more likely to be the cause of CPE rather than a non-specific factor such as a shift in pH.

Our SEM data and observations in other reports point to the possibility that the CPE observed in our model may be affected by the ability of H. ducreyi to attach to and localize onto the HFF cell surface. Recent studies have demonstrated that H. ducreyi adheres to certain cultured cell

lines forming tightly adherent microcolonies on the eukaryotic cell surface. Although the ability of H. ducreyi to attach to various cell lines has been described, the role that attachment plays in the pathogenesis of H. ducreyi is not fully understood. Our data indicate that avirulent H. ducreyi strains A77 and CIP542 were able to damage HFF cell monolayers as effectively as virulent H. ducreyi strains 35000 and RO18, but this ability was significantly decreased if, after 4h post infection, unbound bacteria were washed off.

These results support previous studies that have demonstrated that H. ducreyi strains that do not bind well to to eukaryotic cells (11) are not able to produce ulcers in the temperature dependant rabbit model (91), whereas H. ducreyi strains that attach well to eukaryotic cells are able to produce ulcers in vivo. These data are further supported by TEM and light microscopy demonstrating different capacities for attachment and localization on HFF cells between virulent and avirulent H. ducreyi (10,11,12). These data taken together suggest that CPE may be modulated by the ability of the organism to remain localized by attaching to eukaryotic cells.

The inability of gentamicin killed bacteria to damage HFF cell monolayers suggests that viable H. ducreyi are needed for HFF cell damage to occur. The requirement of live bacteria for HFF cell CPE could be indicative of an exotoxin. Exotoxin producing bacteria such as E. coli accumulate toxin intracellularly before excreting it into the environment (94).

Thus, if H. ducreyi produced an exotoxin, certain subcellular fractions or culture supernatants should be able to cause a CPE on HFF cells, as the cytotoxin would be present in some of these preparations.

Our data do not support the presence of an exotoxin that is active against HFF cells, as incubation of HFF cell monolayers with cell free culture supernatants of bacteria grown either in tissue culture medium alone or in association with HFF cells suggested that H. ducreyi did not elaborate a diffusible exotoxin. Furthermore, no HFF cell damage was observed, even when culture supernatant was obtained from visibly damaged HFF cell monolayers. Neither whole cell lysates nor insoluble bacterial fractions of H. ducreyi caused HFF cell damage. Our results support by earlier reports that H. ducreyi seems to be relatively inert with respect to extracellular enzyme activity (1,2) and that cell-free filtrates were unable to cause ulcer formation in mice (121). Furthermore, it has been reported that inoculation of live H. ducreyi into permeable membrane inserts that kept the bacteria from HFF cell monolayers did not result in HFF cell damage (11).

In contrast, Purven and Lagergard (92) suggest that H. ducreyi elaborates a cytotoxin into its environment. The differences in results between our study and that of Purven and Lagergard (92) could be due to the fact that different clinical isolates were used in the two studies. The apparent differences could also be due to the use of transformed

epithelial cell lines by Purven and Lagergard (92), whereas primary human fibroblasts were used in our study. It is possible that H. ducreyi could elaborate an exotoxin into its environment that is specific only for cells of epithelial or transformed origin, indicating different mechanisms of cytotoxicity at work in the different studies.

Although it is quite possible that H. ducreyi is capable of excreting a cytotoxin, the exact role of a cytotoxin in the pathogenesis of H. ducreyi is unclear, as it has been recently reported that H. ducreyi strains described as non-toxin secreting were able to cause ulceration in animals (61,62) and humans (80). Recently, a haemolysin has been described for H. ducreyi (87,117). The effect that a H. ducreyi derived haemolysin could have on HFF cells has not been addressed, however the observation that E. coli haemolysin can damage macrophages in vitro (125) suggests the need to further study whether or not H. ducreyi haemolysin could damage HFF cells.

It is conceivable that attachment of H. ducreyi to the HFF cells could lead to a response by the eukaryotic cell that ultimately results in cell death. One possible mechanism could be that the H. ducreyi adhesin is directly responsible for eukaryotic cell damage, as has been described for Bordetella pertussiss (50,121). Alternately, a CPE could be the result of a cytotoxic component that is resident on the bacterial surface. However our observation that gentamicin killed H. ducreyi do not damage HFF cells suggests if the cytotoxic component were indeed resident on the bacterial surface, it

does not appear to be released from dead H. ducreyi in a form that can cause eukaryotic cell damage.

The observation that a large inoculum of either live or heat killed H. ducreyi can cause host tissue damage in animals (21,44,61,120,121) lends credence to the possibility that a heat stable component such as H. ducreyi LOS plays a central role in host cell damage. In fact, purified preparations of H. ducreyi LOS have been shown to cause ulceration in animals (21,44,61,120,121). However, data generated using the temperature dependant rabbit model of Purcell et al (91) suggests that purified LOS alone is not sufficient to cause ulceration, indicating that other host factors, such as complement or cytokines could also be involved in ulcer formation in the host.

Recent in vitro studies have demonstrated that host cell damage can result as a consequence of direct interaction with LPS (41,47). It has been demonstrated that H. ducreyi LOS is similar to the LOS of Neisseria gonorrhoaeae (21,22,37,78). The LOS of N. gonorrhoaeae has been shown to directly damage human fallopian tube mucosa in vitro (41). Given the structural similarities between the two species of LOS, it is possible that H. ducreyi LOS could directly cause HFF cell damage. To address this issue, purified H. ducreyi LOS was evaluated as a potential mediator of HFF cell CPE.

Incubation of H. ducreyi LOS from virulent and avirulent H. ducreyi strains with HFF cell monolayers resulted in a concentration dependant CPE. LOS from all H. ducreyi strains

tested was able to exhibit damage at amounts ranging from 25-200 μg LOS/well, or the equivalent of 10^8-10^9 cfu of bacteria (21). Subtle HFF cell changes were observed by microscopy in wells containing 13.5 μg/well LOS that was not detected by the XTT assay, indicating that HFF cell damage was occurring in these wells at a level that was beneath the sensitivity of the XTT assay. The ability of H. ducreyi LOS to damage HFF cells was prevented by incorporation of the LOS into liposomal single unilammellar vesicles (SUV's), indicating that free LOS was needed to exert cell damage. These results are consistent with our earlier observation that gentamicin killed H. ducreyi do not damage HFF cells, as H. ducreyi may need to release LOS into their environment in order for CPE to develop.

The ability of H. ducreyi to shed LOS was assessed by an indirect carbohydrate assay (32) as well as SDS-PAGE and immunoblot analysis using an H. ducreyi LOS specific monoclonal antibody. All H. ducreyi strains tested were able to shed LOS into their environment in amounts similar to the pathogen N. meningitidis. Gel electrophoresis of these supernatants demonstrated that low molecular weight bands corresponding to the LOS of H. ducreyi existed in the supernatants, an observation that was confirmed by dot blot immunodetection using an anti H. ducreyi LOS monoclonal antibody. Taken together, these data strongly suggest that all H. ducreyi strains tested are able to shed LOS, and this may be how HFF cell damage is elicited. Therefore, the ability of LOS to cause a CPE appears to be concentration dependant.

Culture supernatants derived from H. ducreyi grown in SCM tissue culture medium were shown not to be able to damage HFF cell monolayers, even when these supernatants were derived from H. ducreyi in association with damaged HFF cell monolayers. As estimated in our shedding assay, H. ducreyi RO18 and A77 were able to release LOS in amounts between 15-22 μg/ml of supernatant. Keeping in mind that the phenol-H_2SO_4 assay is used to detect carbohydrate and is therefore an indirect measure of LOS quantity, these data still indicate that the amount of LOS shed is about 10-fold less than that required to cause a CPE in HFF cells. The lowest LOS concentration needed to achieve HFF cell damage in our direct LOS experiments (250 μg/ml) was significantly higher than the LOS concentrations obtained from our shedding experiment (20 μg/ml) in 1 ml of supernatant. Furthermore, the observation that a slight visual CPE was observed that was not reflected in the XTT assay data for wells containing 13.5 μg/well concentrations of LOS points to the possibility that cytopathic changes could be taking place at lower concentrations of LOS that are below the sensitivity of the XTT assay. It appears that the LOS concentrations at the HFF cell surface where large numbers of replicating bacteria are in close association with the eukaryotic membrane is sufficient to cause HFF cell damage.

Taken together with observations published in other reports, our data suggest a possible mechanism for LOS in host tissue damage. To exert HFF cell damage, H. ducreyi must

first come in close proximity to the HFF cells, where LOS mediated damage can occur either by an attachment independent mechanism or an attachment dependant mechanism. In the attachment independent mechanism, live H. ducreyi that are in close proximity of HFF cells shed LOS directly onto the HFF cell surface, and HFF cell damage occurs when a sufficient concentration of LOS is present on the HFF cell surface. The requirement for H. ducreyi to be in close proximity to HFF cells for HFF cell damage to occur has been previously demonstrated in vitro (12).

In the attachment dependant mechanism, H. ducreyi attach to the HFF cell surface and grow, forming adherent microcolonies. During this process of growth, LOS is shed by the growing H. ducreyi onto the HFF cell surface in sufficient concentrations to cause HFF cell damage.

As the capacity of a bacterial pathogen to establish a productive infection most often depends on its ability to attach to a target cell or organ (34,50), the most dominant mechanism of tissue damage in vivo is most likely to involve the attachment dependant mechanism described in this study, as non adherent H. ducreyi would likely be removed from the area by host defenses. Our demonstration that virulent H. ducreyi strains are able to form HFF cell adherent microcolonies to a greater extent than avirulent H. ducreyi strains suggests that microcolony formation by H. ducreyi can serve as a protective measure against the phagocytic and killing capacities of neutrophils, a phenomenon that has been demonstrated for H.

ducreyi in vitro (84). Furthermore, the ability of H. ducreyi to release a cytotoxic compound in concentrations sufficient to cause cell damage would be greatly enhanced by its ability to form adherent microcolonies, as large numbers of bacteria would be present in a small area.

Although both in vivo (35,110) and in vitro (92,93) reports suggest that other cell types resident in the epidermis are also damaged as a consequence of H. ducreyi infection, the ability of H. ducreyi to cause in vivo damage to fibroblasts in the lower dermis could be explained by the attachment dependant mechanism of LOS mediated damage proposed in this study.

V.) SUMMARY AND CONCLUSIONS

In summary, we have characterized the HFF cell damage caused by H. ducreyi by using a quantitative, reproducible XTT assay. Our results demonstrated that the CPE caused by H. ducreyi did not appear to be due to a secreted exotoxin.

The CPE observed with HFF cells was demonstrated to be due to H. ducreyi LOS and was of two distinct types. The first type of CPE was attachment independent in nature, and involved the damage of HFF cells by H. ducreyi which were in close proximity to the HFF cells. In this type of CPE, all H. ducreyi strains tested were able to exert a CPE on HFF cells. A second mechanism that was attachment dependant in nature was also demonstrated. In this second type of CPE only virulent H. ducreyi which attach more efficiently to HFF cells than avirulent H. ducreyi were able to produce a CPE after the monolayers were washed. We suggest that the attachment dependant mechanism demonstrated in this study could be the more likely mechanism of fibroblast cell damage observed in vivo. Therefore, a three step mechanism of attachment, growth, and HFF cell damage by LOS is proposed.

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