Mechanisms of Skin Adherence, Penetration and Tissue Necrosis Production by Haemophilus Ducreyi, the Causative Agent of Chancroid

Paul Gerson Unna-Preis
Cutan-mikrobielle Wechselbeziehungen

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Mechanisms of skin adherence, penetration and tissue necrosis production by Haemophilus ducreyi, the causative agent of chancroid

Paul Gerson Unna-Preis: Cutan-mikrobielle Wechselbeziehungen

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1. ABSTRACT

***Haemophilus ducreyi* (*H. ducreyi*) strains, representing both reference strains and low-passage isolates, were investigated in terms of surface structures and enzymatic equipment. The interaction of these factors with host tissue was analysed using new in vitro- and in vivo-models.**

By electron microscopy studies there was no evidence of an extracellular capsule or surface appendages such as pili or flagella. Interaction of all isolates tested with the lectin *Phaseolus vulgaris* suggests N-acetyl-D-glucosamine units as common structural features of *H. ducreyi* cell envelope polysaccharide. In attachment to epithelial cells more than one hemagglutinin might be implicated as different haemagglutination patterns could be observed whereby the activity was not heat -labile, but was abolished by formaldehyde. Hydrophobic interactions might be of importance as well as strains showed a wide range of reactions from hydrophobic to hydrophilic, low hydrophobicity being more marked with the older strains. No elaboration of degradative enzymes based on the measurement of enzymatic activity using insoluble dye-protein complexes could be detected in case of *H. ducreyi*, using Azocoll and Remazol Brilliantblue hide powder for detection of proteolytic activity and elastin-orcein for detection of elastase activity.

In vitro studies using human keratinocytes and Vero cells did not show any morphological changes when incubated with *H. ducreyi* culture filtrates.

In vivo studies with a new mouse model for *H. ducreyi* infection could confirm the results of the in vitro studies. Mere contact to undamaged skin both of whole cell organisms, live or heat-killed, and of culture filtrates did not lead to any reaction or even damage of mouse skin. However, when the outer epidermal layer was overcome by intradermal injection of shaved mice ulcers developed. Tissue necrosis production was not bound to live organisms as dead ones showed the same effect. There is great evidence that this tissue necrosis is associated with *H. ducreyi* lipopolysaccharide (LPS) because intradermal injection of purified *H. ducreyi* LPS lead to the same reaction pattern.

For the first time a cell mediated immune response could be demonstrated in case of *H. ducreyi* infection as different antigen preparations of *H. ducreyi* isolates induced proliferation of lymphocytes isolated from healthy unexposed individuals and from a chancroid-sensitized male. In the latter case measured cell responses were much stronger. The dose-dependent phenomenon was associated with interleukin-2 production.

In summary, *H. ducreyi* isolates do not exhibit cytotoxic effects on the epithelial cells of the skin. Where the actual interaction of *H. ducreyi* and host-defence mechanisms takes place are the dermal and subcutaneous layers into which invasion of *H. ducreyi* is probably managed through pre-existing superficial abrasions. Damage of host tissue seems likely to be a result of interaction of *H. ducreyi* LPS and host inflammatory mediators. Within the infection process a cell mediated immune response develops without being protective.

The new mouse model for *H. ducreyi* infection should facilitate future studies on the immunology of *H. ducreyi infection*, together with the possibility of assessing diagnostic, therapeutic and, in particular prophylactic approaches. *Key words: Haemophilus ducreyi; chancroid; adherence; electron microscopy; lectins; haemagglutination; hydrophobicity; proteolytic activity; elastase activity; cell activation; interleukin-2; cell lines; epidermal keratinocytes; mouse model; lipopolysaccharide.*

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2. INTRODUCTION

2.1 Historical aspects of chancroid with special consideration of Unna's involvement

Chancroid, or soft chancre (ulcus molle or chancre mou) was first differentiated from syphilis, or hard chancre, by Bassereau in France in 1852 (Bassereau, 1852). It was not until 40 or 50 years later, in 1889, however, that the presumptive etiological agent was seen in pure form by Auguste Ducrey at the University of Naples. It was then that he published his well-known report on the identification of the "virus" of the soft chancre (Ducrey, 1889). Although he did not succeed in culturing the organism on laboratory media, he was able to produce skin lesions on the forearm of his patients by intradermal auto-inoculation of material from their lesions. It was actually due to an initiative of Unna that Ducrey published his observations in the renowned German journal "Monatshefte für praktische Dermatologie" and again due to Unna's study entitled "Der Streptobacillus des weichen Schankers" (Figure 1) that Ducrey's experiments gained international acceptance (Unna, 1892). Unna himself found similar organisms in tissue sections from chancres and the associated inguinal bubo. Instead of staining by Gram's method Unna introduced a new staining procedure based on methylene blue. Its original formula is seen in Figure 2. Like Ducrey Unna was not able to grow the organism in vitro. 8 years later, in 1900, Bezançon, Griffon and Le Sourd were the first who isolated the organism on a solid medium using nutrient agar plus 20% whole fresh rabbit blood (Bezançon et al, 1900).

At the time of establishment of the genus *Haemophilus* with the report of the Society of American Bacteriologists and classification of bacterial types in 1920, the bacillus of Ducrey was included in this genus, although not given a specific name

Monatshefte für Praktische Dermatologie.

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Aus Dr. Unnas dermatologischem Laboratorium zu Hamburg.

Der Streptobacillus des weichen Schankers.

Von

P. G. Unna.

Die Ätiologie des weichen Schankers ist noch völlig dunkel. Die Zeiten sind allerdings unwiderruflich dahin, wo man über seine Identität mit der syphilitischen Initialsklerose debattieren konnte. Die ganze Welt ist überzeugt, dafs für ihn ein spezifischer Organismus existiert, der gelegentlich schon einmal gefunden und rein kultiviert werden wird. Bisher aber hat die Auffindung eines anerkannten Schankerbazillus noch auf sich warten lassen. Die einzige Arbeit, welche von den meisten Forschern als ein positiver Fortschritt in dieser Richtung betrachtet wurde, war die Beschreibung eines Bazillus durch Ducrey[1] im Jahre 1889. Dieser Forscher konnte wenigstens natürliche Reinkulturen desselben aufzeigen in Sekreten solcher Schanker, welche künstlich extragenital fortgeimpft waren. Aber auch Ducrey ist es bisher nicht gelungen, diesen Bazillus seiner Sekretpräparate zu kultivieren und durch Rückimpfung die ätiologische Bedeutung desselben zu erhärten. Ebenfalls ist es demselben bisher nicht möglich gewesen, dieselbe Bazillusart konstant in dem Gewebe des weichen Schankers und in einer Verteilung aufzufinden, welche die klinische und histologische Beschaffenheit des weichen Geschwüres erklärt.

Von der letzteren Seite her ist es mir nun möglich gewesen, wie ich glaube, die Ätiologie des Ulcus molle um einen wesentlichen Schritt zu fördern.

In 5 hintereinander untersuchten reinen Fällen, in denen die klinische Diagnose von mir über allen Zweifel sichergestellt war und welche eben deshalb zur histologischen Untersuchung der Affektion ausgeschnitten und in absolutem Alkohol eingelegt waren, fand ich gelegentlich dieser Untersuchung zu meiner Überraschung einen und denselben

[1] Experimentelle Untersuchungen über den Ansteckungsstoff des weichen Schankers und über die Bubonen. *Monatsh. f. prakt. Derm.* 1889. pag. 387.

Fig. 1. Unna's report in "Monatshefte für praktische Dermatologie".

(Winslow et al, 1920). *Haemophilus ducreyi* (*H. ducreyi*) was the species designation in the first edition of "Bergey's Manual of Determinative Bacteriology" (Bergey et al, 1923).

2.2 Chancroid – the present situation

Chancroid is endemic in South and East Asia, Africa and India. In these regions chancroid accounts as one of the most frequent sexually transmitted diseases. Although traditionally regarded as a disease of tropical climates, recently outbreaks in Greenland (Lykke-Olsen et al, 1979), Canada (Hammond et al, 1980) and some European countries, including West Germany (Munich (Braun-Falco & Neubert, 1977), West-Berlin (G. Stüttgen, 1981)) have been described. Reports on the frequent isolation of *H. ducreyi* from patients in Sheffield (McEntegart et al, 1982) give further evidence of the potential for the disease in temperate climates.

Genital ulcers in patients with chancroid develop usually after an incubation period of 4 to 7 days, with rarely less than 3 days or more than 10 days. Within 2 to 3 days a pustule forms that soon ruptures, resulting in a painful, sharply circumscribed ulcer with ragged, undermined edges and without induration (Figure 3).

The base of the ulcer, in contrast to the smooth base of a syphilitic chancre, is irregular with many projections and depressions. There is little inflammation of surrounding skin. Several varieties of chancroidal ulcers have been described (Ballard et al, 1989), examples of a so-called "dwarf chancroid" and a "papular chancroid" (ulcus molle elevatum) are shown in Figure 4 and 5, resp.

Painful, tender lymphadenopathy may be present in up to 50% of patients with chancroid (Figure 6).

H. ducreyi ulcers develop both on skin and mucosal surfaces in about the same frequency. For example, "...in males most common sites of involvement are the distal prepuce, the mucosal surface of the prepuce on the frenulum..." (Morse, 1989). Furthermore, extragenital location of chancroid can involve any part of the skin or mucous membranes. Recently, Marckman et al. (1989) reported about a case of pedal chancroid without history or clinical signs of primary genital infection.

Diagnosis has to be based on culturing the organism. The clinical differentiation of chancroid from two other common causes of genital ulcers, syphilis and herpes simplex, is not reliable (Salzman et al, 1984).

Microsopic characteristics in case of *H. ducreyi* infection by direct examination of clinical material on smears that are Gram-stained or stained following the method of Unna, are a so-called "school of fish" arrangement, in which the bacilli are grouped in long parallel columns between cells or shreds of mucus, or long chains of cells (Figure 7). However, these features tend to be infrequently seen judging smears prepared from clinical material (Nsanze et al, 1981). Sturm et al. reported (1987) that only 1 (5.3%) of 19 patients with the clinical diagnosis of chancroid had a positive smear.

frischen vorzuziehen. Sehr empfehlen kann ich zu dieser wie zu vielen anderen Zwecken folgende Lösung:

Methylenblau		
Kalii carbonici	aa	1,0
Aquae destillatae		100,0
Spiritus		20,0
M. Coque ad remanent.		100,0
Adde:		
Methylenblau		
Boracis	aa	1,0
in Aqua destillata		100,0
Soluta misce. S.		

Zusammengesetzte Methylenblau-Lösung.

Fig. 2. Unnas formula for staining of *H. ducreyi* organisms.



Fig. 3. Chancroid. Sulcus coronarius. Two sharply circumscribed ulcers filled with yellow exudate in a Munich case proven by culture.

Today *H. ducreyi* is generally considered resistant against penicillin (mainly plasmid-mediated resistance) and tetracycline (both chromosomal- and plasmid-mediated) (Abeck et al. 1988). According to the "1989 Sexually Transmitted Diseases Treatment Guidelines" released by the Centers for Disease Control (1989) recommended regimens comprise erythromycin base 500 mg orally 4 times a day for 7 days or ceftriaxone 250 mg intramuscularly in a single dose. Ciprofloxacin 500 mg orally 2 times a day for 3 days is recommended as an alternative regime. The same applies to trimethoprim/sulfamethoxazole 160/800 mg orally 2 times a day for 7 days although it is noted that the susceptibility of *H. ducreyi* to this combination of antimicrobial agents varies throughout the world. Isolates resistant against this drug have been reported in a high percentage from South East Asia (Taylor et al, 1985).

Recently, we could report about a patient with chancroid presenting in our department who did not respond to therapy with trimethoprim/sulfamethoxazole. The *H. ducreyi* strain being isolated from the ulcer had a minimum inhibitory concentration of 32 ug/ml to this drug when tested by an agar dilution method, in that way being resistant. Changing therapy to oral ciprofloxacin healing was rapidly achieved (Abeck et al, 1990).

The importance of chancroid has risen dramatically worldwide since it could clearly be demonstrated by several investigators that chancroid is a major risk factor for aquisition of HIV-1 (Greenblatt et al, 1988; Simonsen et al, 1988). One of our patients suffered from chancroid for the first time two years ago and was seronegative for HIV-1 at this time. One and a half years later the HIV-test gave a positive result (Korting et al, 1989). A connection between the genital ulcer disease in this patient and acquisition of HIV-1 seems likely.



Fig. 4. Chancroid. "Dwarf chancroid" (from Ballard et al., 1989).

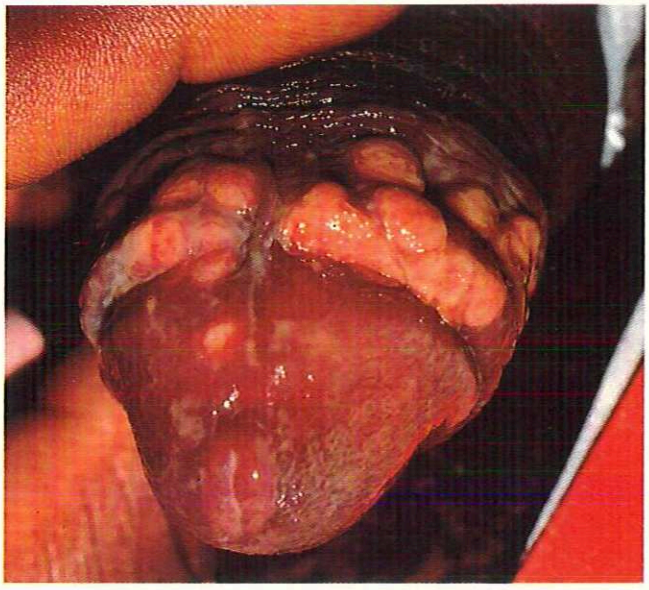


Fig. 5. Chancroid. "Papular chancroid" (from Ballard et al., 1989).



Fig. 6. Chancroid. Penile ulceration and unilateral lymphadenopathy (from Ballard et al., 1989).

3. AIMS OF THE STUDY

The pathogenesis of infection due to *H. ducreyi* is poorly understood. However, a better understanding of the interaction between the organism and its target, i.e. the skin and mucosal surfaces primarily of the genital organs, is necessary in order to provide a rational basis for interfering in the infection process on an early stage or to prevent infection at all. Our knowledge of the way of penetration of *H. ducreyi* through normal skin is so far still based on speculations. Essential factors for adherence and the mechanisms by which the organism produces tissue necrosis are completely unknown. The rapidity, however, ulcers arise due to the break-down of the host's tissue suggests rapid destruction due to a toxin or a substance or a group of substances toxic to the connective tissue underlying the epithelial surface.

The aims of the present study are to

- investigate and characterize cell-surface structures of *Haemophilus ducreyi* that might be essential for the adherence of the organism
- look for substances that might be able to prevent adherence of *Haemophilus ducreyi* to eukaryotic cells, including human keratinocytes
- study the metabolism of *Haemophilus ducreyi* in terms of proteolytic or other enzyme activities and their role in tissue necrosis production
- evaluate the role of *Haemophilus ducreyi* lipopolysaccharide in the infection process induced in living skin
- analyse the in vitro effect of *Haemophilus ducreyi* antigens on activation of mononuclear cells and interleukin-2 production of the host
- introduce readily available and cheap in vitro– and in vivo-assays for the assessment of *Haemophilus ducreyi*-related

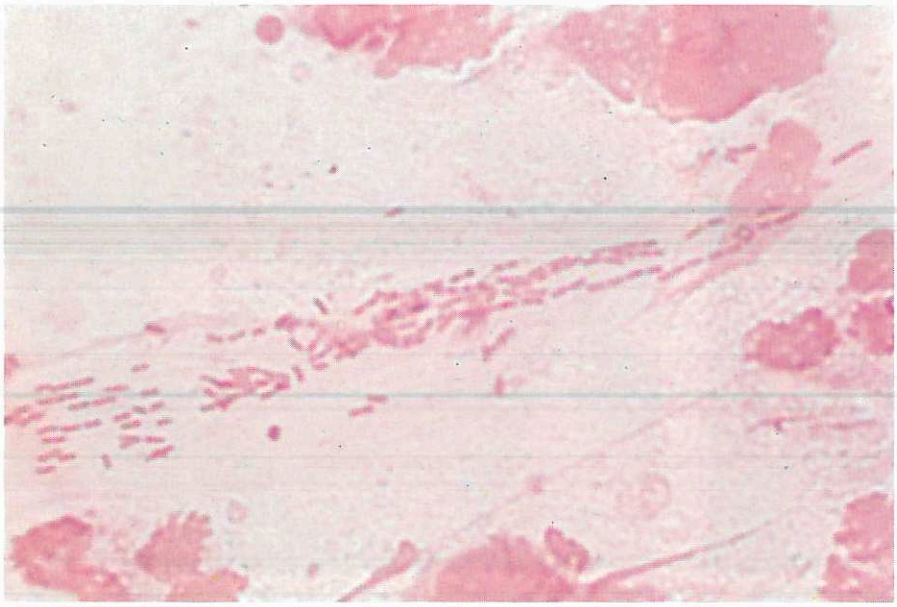


Fig. 7. Chancroid. Direct smear, Gram-stained; characteristic "schools of fish".



Fig. 8. H. ducreyi colonies on a solid medium containing sheep blood: characteristic colonial morphology.

virulence factors making the definitive evaluation of chemotherapeutic agents and vaccines possible

4. MATERIAL AND METHODS

4.1 In vitro studies with H. ducreyi and various cell lines

4.1.1 Bacterial isolates and growth conditions

H. ducreyi isolates used in the following studies comprised both reference strains and low-passage isolates. Among the reference strains *H. ducreyi* isolate designated 35 000 is an isolate from Winnipeg, Canada, the isolate designated CIP-542 has been obtained from the Institut Pasteur, Paris, and isolates designated ANA and D-14 are from Sheffield, U.K.. The isolates have been described previously (Abeck & Johnson, 1987). They have been subcultured several times. The remaining strains are clinical isolates from South Africa (kindly provided by Dr. R. C. Ballard) and from the Gambia (kindly provided by Dr. R. C. Wall). In addition, two strains of *H. ducreyi*, isolated in our own department were included in some of the studies.

H. ducreyi strains were identified by the criteria of Kilian (1976). Depending on the study culturing of the organisms was performed both on solid media and in a liquid medium in a candle jar with increased humidity at 35°C for 48 h as described previously (Abeck et al, 1987). The solid media consistéd either of Mueller-Hinton agar, chocolated horse blood (5%) and IsoVitaleX (1%) or Columbia agar, sheep blood (10%), foetal calf serum (2.5%) and IsoVitalex (1.5%). For the liquid medium brain heart infusion broth, foetal calf serum (10%), IsoVitalex (1%) and hemin stock solution (1%) were used.

On solid media the polymorphic colonial morphology characteristic for *H. ducreyi* giving the appearance of mixed flora from pure culture could be well observed (Figure 8).

4.1.2 Investigations on cell surface-structures and -composition as possible virulence factors

4.1.2.1 Electron microscopy studies of the cell wall

It is well established that attachment of microorganisms to their target surfaces is an initial step in the evaluation of infection and plays an important role in the development of the infectious process (Beachey et al, 1982; Ofek & Beachey, 1980). In case of *Neisseria gonorrhoeae* infection previous studies have indicated that pili are responsible for gonococcal attachment to epithelial cells and their presence is associated with virulence (Swanson, 1973).

The structure of *H. ducreyi* was investigated by electron microscopy in order to gain information on surface appendages as possible virulence factors.

Table I. *Lectins and their carbohydrate specifities.*

Lectin	Abbrevation	Specifity
Concanavalin A	Con A	a-D-Man > a-D-Glc < a-D-GlcNAc
Lens culinaris	LcH	a-D-Man > a-D-Glc
Tichosanthes kinlowii	TKA	D-Gal
Griff. simplicifolia I	GS-I	a-D-Gal > a-D-GalNAc
Arachis hypogeae	PNA	D-Gal-ß-(1– > 3) > ß-D-Gal NH_2 = a-D-Gal
Glycine max	SBA	a-D-GalNAc > ß-D-GalNAc > a-D-Gal
Dolichos bifloris	DBA	a-D-GalNAc > a-D-Gal
Griff. simplicifolia II	GSA-II	a-D-GalNAc = ß-D-GalNAc
Solanum tuberosum	STA	$(ß\text{-D-GalNAc})_{2-5}$ > ß-D-GalNAc
Triticum vulgaris	WGA	$(ß\text{-D-GalNAc})_3$ > $(ß\text{-D-GalNAc})_2$ > (ß-D-GalNAc)
Limax flavus	LFA	N-Acetylneuraminic acid
Phaseolus vulgaris	PHA-E	D-GalNAc
Ulex europaeus I	UEA-I	a-L-Fuc
Lotus tetragonolobus	Lotus A	a-L-Fuc = 2–0-Me-D-Fuc

a = alpha; Man = Mannose; Glc = Glucose; Fuc = fucose; Gal = Galactose; GlcNAC = N-acetylglucosamine; GalNAc = N-acetylgalactosamine; 2–0-Me-D-Fuc = 2–0-methylfucose

Bacteria and growth conditions

All reference strains and six low-passage isolates from South Africa were examined. Bacteria both grown on solid medium and in liquid medium (see 4.1.1) were analysed.

Electron microscopy

For ultrathin sections organisms growing on solid or in liquid medium were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, buffered in chromate-buffer (pH 7.2) and processed conventionally. In order to stain acid mucopolysaccharides ultrathin sections of *H. ducreyi* were post-fixed in the presence of ruthenium-red. For negative staining 1% potassium phosphotungstate was used after fixation in glutaraldehyde.

4.1.2.2 Lectin binding properties of cell envelopes

Lectins are plant or animal proteins or glycoproteins of non -immune origin with binding specifity for simple and complex carbohydrates. Interaction with lectins has been used to obtain structural information about the cell wall polymeres of several Gram-negative and -positive microorganisms (Doyle et al, 1972; Goldstein & Staub, 1970).

Interaction with lectins can be exploited to obtain independent chemical information about occurence of specific sugars, the configuration of anomeric linkages, and the location or position (terminal nonreducing or internal) of sugar residues in polysaccharide molecules.

In the present study a collection of *H. ducreyi* strains were examined for their ability to be agglutinated by a panel of 14 different lectins.

Bacteria and growth conditions

Fourty-three *H. ducreyi* isolates, including all reference strains and low-passage strains from South Africa, the Gambia and another isolate from Munich were included into the study. Culturing was performed on solid sheep blood medium (see 4.1.1).

Lectins

The 14 different lectins were obtained in ready-made panels from E.Y. Laboratories, San Mateo, Calif.. They were all of plant origin. The specifities of the lectins used are listed in Table 1.

Agglutination assay

H. ducreyi strains from 48 hours' cultures were transferred with a cotton swab to tubes containing Tris-methylamine-buffered saline (pH 7.4). After adjustment to a density of approximately 10^9 colony-forming units per ml, a portion (100 ul) of the cell suspension was added to each of the 14 wells in the panel containing the predried lectins. The panels were then rotated at room temperature on a bench top rotator for 15 minutes. Agglutination reactions were read at 5, 10 and 15 minutes without a change of the reaction pattern observed after the first reading. Tris-methylamine-buffered saline served as the negative control.

4.1.2.3 Studies on haemagglutination and cell surface hydrophobicity

Attachment of bacteria to membranes of the alimentary (Jones, 1977), urogenital (Svanborg-Eden & Hansson, 1978) and respiratory tract, is believed to be mediated by cell-surface adhesion of the respective pathogens and receptors on the host-cell surface (Beachey, 1980) resulting in the colonization of the infection site by the pathogen. Since similar mechanisms are involved in attachment and agglutination of erythrocytes in vitro, the haemagglutination (HA) test has proved useful for studying bacterium-host cell-surface interaction and mechanisms of attachment (Old, 1985). Although correlations between piliation and HA could be demonstrated for many bacteria, HA is not, however, always caused by fimbriae (Duguid et al, 1979). In addition, hydrophobic interactions have been implicated in a wide array of adhesion phenomena (Rosenberg & Kjelleberg, 1986).

We investigated some surface properties of *H. ducreyi* which may relate to adherence: direct haemagglutination as a model of attachment to host cells and hydrophobicity which could promote adherence of *H. ducreyi* organisms to target cells.

Bacteria and growth conditions

Reference strains 35 000, IP-542, D-14 and seventeen further low-passage isolates all from South Africa with the exception of one strain from the Gambia were included into the study and cultured on solid sheep blood medium (see 4.1.1). After 48 hours' incubation dense bacterial suspensions were made in PBS (pH 7.2) and adjusted photometrically to a density of approximately 10^9 colony forming units per ml.

Haemagglutination test

Blood from sheep, pig, calf and human (groups A, B, AB, and 0) was collected into Alsever's solution and used either fresh or within 24 h of collection after storage at 4 C. The red cells were separated by centrifugation, washed three times in PBS and resuspended in PBS at 1.5 per cent (v/v) which produced optimum haemagglutination. Reactions were performed in microtitre plates. After addition of the reactants (red cells last), the plates were agitated by side tapping, covered and incubated static in air at 37°C for 2 h followed by room temperature overnight. Each test was performed in triplicate. The results were read on a plate magnifier and HA was recorded as – (negative) or + (positive). Autoagglutination of the bacteria was excluded by mixing them with PBS instead of erythrocytes.

To check stability of the reaction patterns, the agglutination patterns of five low-passage isolates were determined again after they had been subcultured ten times.

Bacterial cell treatments

Bacterial suspensions were subjected to one of the following treatments: I. 2% (v/v) formaldehyde for 1 h at 37°C, II. 2% (v/v) formaldehyde for 1 h at 60°C, III. boiling at 100°C for 10 min, IV. incubation with trypsin (Sigma) (50 ug/ml, final concentration) at 37°C for 30 min in PBS containing 1.0 mM Ca^{2+}. Incubation was in a shaking water bath. The treated cell suspensions were washed four times in PBS and finally re-suspended in PBS to the original density.

A parallel test, in which 1.5% (wt/v) D-mannose in saline was substituted for the saline in the mixture, was used to determine whether HA was inhibited by mannose (Old, 1985).

Bacterial cell-surface hydrophobicity

The cell surface hydrophobicity of the isolates was measured using the method described by Rosenberg et al. (1980). The hydrocarbons used were n-hexadecane and p-xylene (both purchased from Sigma). To round-bottom test tubes (10 mm inner diameter) containing washed cells suspended in PBS and adjusted to an optical density (at 540 nm) of approximately 1.0, volumes of the hydrocarbons studied, ranging from 0 to 0.5 ml, were added. The mixtures were vigorously agitated for one min using a vortex mixer. After 20 min, the aqueous phase was removed with a Pasteur pipette and transferred to a cuvette, and the optical density was measured.

4.1.3 Studies on extracellular enzymes of Haemophilus ducreyi

Virulence of bacteria is associated very often with elaboration of degenerative enzymes such as mucinases, elastases, lecithinases or phospholipases. Of particular interest are the proteolytic enzymes. In case of *Pseudomonas aeruginosa* (*P. aeruginosa*) an important role of protease activity in the adherence of this organism to mammalian buccal epithelial cells could be noted (Woods et al, 1981). Further pathogenic activities of proteases from *P. aeruginosa* are inactivation of blood plasma, alpha 1-proteinase inhibitor and normal human immunoglobulin G (Holder, 1985). In addition, Fischer and Allen (1958) have produced corneal ulcers in rabbits through the use of either intact cells or proteolytic preparations obtained from the organism.

In the present study we investigated the presence of intracellular and extracellular excretion of protease and elastase by cultures of *H. ducreyi*.

Bacteria and growth conditions

H. ducreyi reference strains 35 000 and IP-542 and eight other low-passage isolates from South Africa were investigated and cultured as described above (see 4.1.1). As positive control a *P. aeruginosa* strain isolated from a venous leg ulcer was included.

General principle

Measurement of enzymatic activity was performed by using insoluble dye-protein complexes. The digestion of these complexes by proteolytic enzymes releases dye into solution which can be directly measured by photometric methods after removal of the remaining complex by filtration.

Sample preparations

Bacteria were grown in 18 ml of liquid medium for 48 h followed by centrifugation of the culture samples at 4 000 g for 30 min at 4°C. Protease and elastase contents of the supernatant were processed. The pellet was washed three times with sodium phospate-buffered saline (PBS, pH 7.4), resuspended in PBS and desintegrated in 2 ml portions by ultrasonic treatment for 30 sec four times at 4°C with the microtip of a Branson sonifier set at no. 2.

Protein aestimation

Protein concentration of the supernatant and the pellet samples was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

4.1.3.1 Proteolytic enzyme activity

Detection of proteolytic activity was performed by two different dye-release methods, using as substrates either Azocoll, an insoluble, ground collagen to which a bright-red azodye is attached (Chavira et al, 1984) or Remazol Brilliantblue hide powder, a substrate derived from hide powder labeled covalently with Remazol Brilliantblue (Rinderknecht et al, 1968).

Azocoll assay

Azocoll with a specification of 100 – 250 mesh was purchased from Calbiochem..

pH optimum for the reaction system was at 8.0, the incubation time was 60 min and dye absorbance was determined at 520 nm. For plotting the standard curve trypsin-solutions with known concentrations were used. Enzyme activity was found to be linear to enzyme concentration up to a trypsin concentration of 2 ug/ml. The proportional factor $P_{Azocoll}$ (c/E) characterizing direct proportion of extinction (E) and trypsin concentration (c) was 0.88 ug/ml.

Remazol Brilliantblue hide powder assay

Remazol Brilliantblue hide powder was purchased from Sigma. pH optimum for the reaction system was at 8.0, the incubation time was 60 min and dye-absorbance was determined at 595 nm. For plotting the standard curve trypsin-solutions with known concentrations were used. Enzyme activity was found to be linear to enzyme concentration up to a trypsin concentration of 20.0 ug/ml. The proportional factor $P_{RBB\ Hide}$ (c/E) characterizing direct proportion of extinction (E) and trypsin concentration (c) was 28.6 ug/ml.

1.3.2 Elastase activity

Detection of elastase acticity was performed with orcein-elastase (Johnson et al, 1967) which was purchased from Sigma.

pH optimum for the reaction system was 8.8, the incubation time was 150 min and dye-absorbance was determined at 590 nm. For plotting the standard curve elastase-solutions with known concentrations were used. Enzyme activity was found to be linear to enzyme concentration up to a elastase concentration of 20.0 ug/ml. The proportional factor $P_{Orceon\text{-}Elastin}$ characterizing direct proportion of extinction (E) and elastase concentration (c) was 61.5 ug/ml.

4.1.4 Cytotoxicity studies of H. ducreyi using human human keratinocytes and Vero cells

Tissue culture systems have proved useful for a wide array of bacterial pathogenicity studies. For example, measurement of cytotoxicity and viability have been successfully used for investigating the action of toxins in culture (Guerrant et al., 1974).

In the present study cytotoxicity of H. ducreyi was studied using both a primary, i.e. human keratinocyte, and an established, i.e. Vero cell culture.

Bacteria and growth conditions

The reference strains IP-542 and ANA as well as four Gambian isolates and another one from Munich (60/90) were used in the experiments. Isolates were grown in liquid medium consisting of Dulbecco's MEM enriched with 1.5% IsoVitaleX, 2.0% hemin (Sigma) and 10% new-born calf serum (Gibco) at 35°C for 48 h in a humidified atmosphere.

A *P. aeruginosa*-isolate was cultured using the identical medium at 37°C for 24 h.

Bacterial cultures were centrifuged (20 min; 4°C; 2000 g) and the supernatant filter-sterilized (Millipore^R; 22 um pore size) and further processed.

Cell culture

Studies were performed using both African green-monkey kidney (Vero) cells and human keratinocytes.

Vero cells were grown in medium consisting of Dulbecco's modified Eagle's medium (Gibco) containing 0.37% $NaHCO_3$, 200 ug/ml gentamycin and 10% new-born calf serum (Gibco).

Epidermal keratinocytes were isolated from foreskin, the epidermis was separated from the deeper layers of skin by cold trypsinization overnight, followed by disaggregation with proteolytic enzymes. Primary cell cultures seeded on metomycin C treated NIH 3T3 fibroblasts reached subconfluence after about 15 days. Culture medium consisted of Dulbecco's MEM supplemented with 20% HAM's F12 (Gibco), 10% foetal calf serum (Gibco), 2% adenine (Serva), 0.1% TIT-stock solution (Gibco), 0,1% T_3 (Sigma), 0.1% cholera toxin (Sigma), 0.1% hydrocortisone, 1% glutamine (Sigma) and 200 ul/ml gentamycin.

Cell culture infection

After trypsinization with trypsin (0.025%)– and EDTA (0.02%) -solution in PBS human keratinocytes and Vero cells were resuspended in 10 ml of culture medium and cells were counted using methylene blue stain. About 3×10^5 cells were centrifuged on acid-cleaned cover glasses and final volume adjusted to 1 ml per cover glass.

Monolayers were inoculated with 1 ml of the supernatant suspensions, and the cultures were incubated at 37°C for 48 h. All morphological changes were recorded at 24 h and 48 h. Experiments were performed in triplicate. Uninoculated medium served as a negative control.

Staining

Cells were fixed by air drying for 24 h. Staining was performed with a combined May-Gruenwald (4 min)- and Giemsa (20 min) -stain. After washing off excess stain from the monolayer preparations were air-dried for another 24 h. Fixation was done with Eukitt^R. The preparations were examined and photographed with an Olympus BH2 phase-contrast microscope.

Assessment of cytotoxic effects

Morphological changes observed in the cyto– and nucleoplasm of the cells were analysed by a pathologist.

4.1.5 Effect of different antigen preparations on activation of mononuclear cells and interleukin-2 production

A humoral immune response to infection with *H. ducreyi* could be demonstrated both in humans and in animals, but this response is apparently nonprotective since reinfection is common and repeated

autoinoculations were observed in earlier human studies of chancroid. A cellular immune response to infection with *H. ducreyi* has not been reported, seems nevertheless likely due to interaction with human lymphocytes during tissue necrosis production.

The following study was performed in order to investigate the cellular immune mechanisms in *H. ducreyi* infection.

Bacteria and growth conditions

Studies were performed with *H. ducreyi* strains S 22 and S 27, both low passage isolates from South Africa. Whereas S 22 was isolated from a chancroidal ulcer without associated lymphadenopathy, in case of S 27 associated lymphadenopathy was observed. Strains were cultured on solid sheep blood-containing medium (see 4.1.1).

Antigen preparations

3 antigen preparations of the strains were used: i whole antigen (formalin killed), ii sonicated whole antigen, iii outer membrane preparation (which was performed as previously described (Abeck et al, 1988a). Antigens were suspended in RPMI 1640 medium (Gibco).

Protein estimation

Protein content of the preparations was determined following the method of Lowry (1951).

Lymphocytes. Human peripheral blood mononuclear (PBM) cells both from healty adult volunteers without a history of chancroid and in addition from a man who had suffered from chancroid a year ago were isolated as previously described (Miller & Lewis, 1987). In brief, PBMs were isolated by Ficoll-Paque (Pharmacia) centrifugation of heparinized peripheral blood. After three times' washing with PBS without calcium and magnesium (Serva) PBMs were suspendended in culture medium.

Culture conditions. PBMs were cultured in RPMI 1640 (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine (2mM), 2-mercaptoethanol ($2x10^{-5}$M), Hepes (25 mM), and 10% v/v heat-inactivated pooled human AB serum (Gibco). AB serum was added to all cultures to avoid confusing nutritional deficiency of patient plasma with true immunosuppressive activity as recommended by Farmer and co-workers (1986).

Proliferation assay

Portions of PBM cultures were placed in 96 well flat-bottomed microtiter plates (Greiner) at a concentration of 5x10 mononuclear cells/ well in a volume of 200 ul in triplicate. Antigen preparations (10 ug/ml each) and mitogens PHA-P and Con A (both from Sigma) were added at the suboptimal doses at a final concentration of 10 ug/ml. The cells were cultured in a humidified atmosphere of 5% CO_2 for 72 h.

During the last 17 hrs of culture, the cells were pulsed with 1 uCi/well of [methyl-^{3}H] thymidine (specific activity 5 Ci/ mmol; Amersham). The cells were harvested onto glass fiber filter paper using a semiautomatic harvester (Skatron). The incorporation of (methyl-^{3}H) thymidine into DNA was measured in a liquid scintillation counter (Packard and Canberra).

Il-2 production

Preparations of PBMs were stimulated with 0.5 ug/ml purificd PHA in RPMI 1640 with 2% foetal calf serum, 1% glutamine, and antibiotics as suggested by Alvares et al. (1979).

Il-2 assay

Il-2 activity was measured by a the method described by Gramatzki et al. (1982). Cell cultures were performed in triplicate in 96 flat-bottom microtiter plates (Greiner). Each well was incubated with 100 ul volumes of the antigen preparations in a serial twofold dilution, ranging from 1:2 to 1: 4096 of each antigen-extract. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h. Thereafter, each supernatant was collected and after centrifugation (250g, 10min) stored at −80°C until tested for IL-2 activity. As controls phytohemagglutinins PHA-P and PHA-M, tested in different concentrations, were used. The Il-2 production was performed sterile to avoid the need for sterilization, which could reduce Il-2 activity.

The Il-2 activity was detected using the Il-2 dependent T cell line (CTL-L) as described previously (Gershon & Naot, 1984).

Statistics

The Wilcoxon rank sum test was employed. A value of $P \leq 0.05$ was considered significant.

4.2 In vivo studies in a mouse model

4.2.1 Effect of cell- and supernatant preparations

The classical test of virulence of *H. ducreyi* is the rabbit intradermal test, consisting of the development of a papule and ulcer following cutaneous inoculation. This model was introduced by Reenstierna in the early 1920s (Reenstirna, 1921). Disadvantages of this animal model are extensive costs for the animals needed and a lack of similarity between the ulcers produced in the rabbit and those seen in humans. For these reasons a mouse model of *H. ducreyi* infection is developed and the effects of *H. ducreyi* preparations gained from cell suspensions or culture supernatants are investigated.

Bacteria and growth conditions

Three strains of *H. ducreyi* were used for the experiments and represented one reference strain (IP-542) and two low-passage isolates from South Africa designated S26 and S30. Strains were either grown on sheep blood-containing medium or in liquid medium (see 4.1.1). Colonies on agar were emulsified in PBS.

Mice

Syngeneic 6–8-week-old CBA mice, bred and maintained in the specific pathogen-free unit at the Clinical Research Centre, Harrow, U.K. were used in all experiments.

Inoculation procedure

Mice were challenged as follows:

a. approximately 10^7 live organisms (per site) suspended in 0.1 ml portions of PBS were brought onto the flanks of undamaged skin and close skin contact was achieved by an adhesive tape for different periods of 12, 24, 48, and 72 hours.
b. approximately 10^7 live organisms (per site) suspended in 0.1 ml portions of PBS were inoculated intradermally and/ or subcutaneously on the flanks of shaved mice.
c. 0.1 ml portions of the supernatants of *H. ducreyi* strains grown in liquid medium for 48 hours were brought onto the flanks of undamaged skin and close skin contact was achieved by an adhesive tape for different periods of 12, 24, 48, and 72 hours.
d. approximately 10^7 heat-killed (boiling of the organisms for 10 minutes with control of complete death by culture on agar plates) organisms (per site) suspended in 0.1 ml portions of PBS were inoculated intradermally on the flanks of shaved mice.

In all experiments inocula of PBS were used as controls. For the experiments b and d observations were made daily during the following two weeks and swabs were taken from resulting lesions for culture.

4.2.2 Effect of lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria, and consists of three components, namely, lipid A, core oligosaccharide and 0-antigenic carbohydrate side chains (Hitchock et al, 1985). LPS molecules consisting of all three components are refered to as smooth LPS, while LPS which lacks 0-antigenic side chains and consists only of lipid A and core oligosaccharide is refered to as rough LPS. In a recent study we were able to show by using the techniques of SDS-polyacrylamide gel electrophoresis and silver staining that *H. ducreyi* produces rough LPS (Abeck et al, 1987). Rough LPS exhibits only low molecular weight

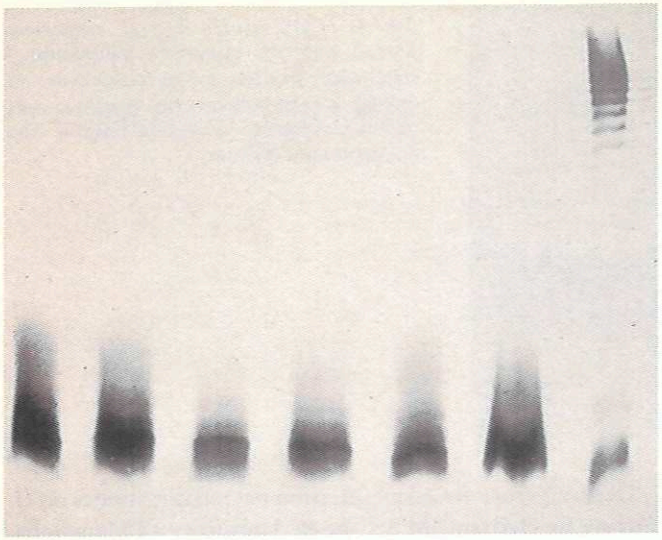


Fig. 9. LPS profiles of 5 strains of *H. ducreyi* (beginning from the left side), 1 isolate of *Neisseria gonorrhoeae* and *Escherichia coli* (on the right) analysed by SDS-PAGE.

bands on silver-stained gels in contrast to smooth LPS that produces a ladder-like appearance in silver-stained gels, each "rung" representing an increase in molecular weight due to the presence of additional 0-antigenic side chains. Figure 9 shows the "rough" LPS profiles of five *H. ducreyi* strains, of one *Neisseria* gonorrhoeae isolate and the "smooth" LPS profile of an *Escherichiacoli* isolate. The chemical composition of *H. ducreyi* LPS and the relationship to virulence has been investigated by Odumeru and co-workers (1987).

From studies of toxicity of *Neisseria gonorrhoeae* for human fallopian tube mucosa there is definite evidence that gonococcal LPS is responsible for cell damage (Melly et al, 1982). We investigated the effects of purified *H. ducreyi* LPS on tissue necrosis production in the newly developed mouse model for *H. ducreyi* infection.

Bacteria and growth conditions

The study was performed with *H. ducreyi* isolates already described under 4.2.1.

Mice

The same breed as described under 4.2.1 was used.

LPS extracts

LPS was extracted by the George Hunter Laboratory procedure for extraction and purification of gonococcal LPS which is a modification of the method described by Johnson and Perry (1976). LPS was extracted from the three strains, starting with about 100 plates of heavy growth each. After centrifugation *H. ducreyi* organisms were sonicated while kept on ice. The disrupted cells were heated to 69°C and equal volumes of hot phenol (95%. w/w, 69°C) were added. The extraction was carried out at 69°C for 15 minutes with constant stirring. The preparation was then cooled at 4°C and centrifuged at 1000 g for 10 min. The water phase was collected and kept, while the interface and phenol-phase were re-extracted with an equal volume of distilled water.

The aqueous layers of successive extractions were pooled and dialysed against running tap water until no phenol smell was detected. The crude phenol-water extract was freeze-dried, weighed and resuspended in distilled water. Insoluble material was removed by centrifugation at 2000 g for 10 min. Supernatant was treated with DNAse and RNAse (Sigma), final concentration 10 ug/ml for 1 h at 37°C and centrifuged at 100 000 g for 18 h. After freeze-drying the pellet the enzyme treatment and centrifugation were repeated twice. The final pellet was resuspended in distilled water, freeze-dried and weighed. The preparations were free of contamination by nucleic acids and proteins as determined by carbocyanine dye assay and measurements of A_{260} and A_{280}.

Inoculation of mice

Inoculation of 0.1 ml portions of LPS extracts from each isolate with concentrations of 1, 10, and 20 ug/ml were inoculated intradermally on the flanks of shaved mice. Observations were made daily during the following two weeks.

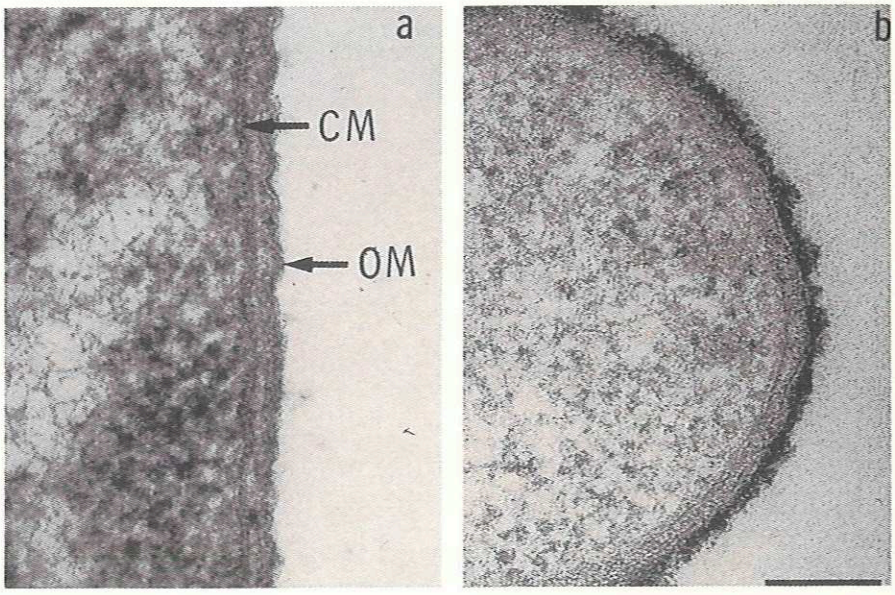


Fig. 10. (a) Ultrathin section of *H. ducreyi* processed conventionally and showing a typical Gram-negative cell wall with a cytoplasmic membrane (CM) and rugose outer membrane (OM). (b) Ultrathin section of *H. ducreyi* post-fixed in the presence of ruthenium red so as to stain acidic mucopolysaccharides. The only staining seen is a narrow zone closely apposed to the OM, without evidence of a thicker extra-cellular capsule.
The bar represents 100 nm.

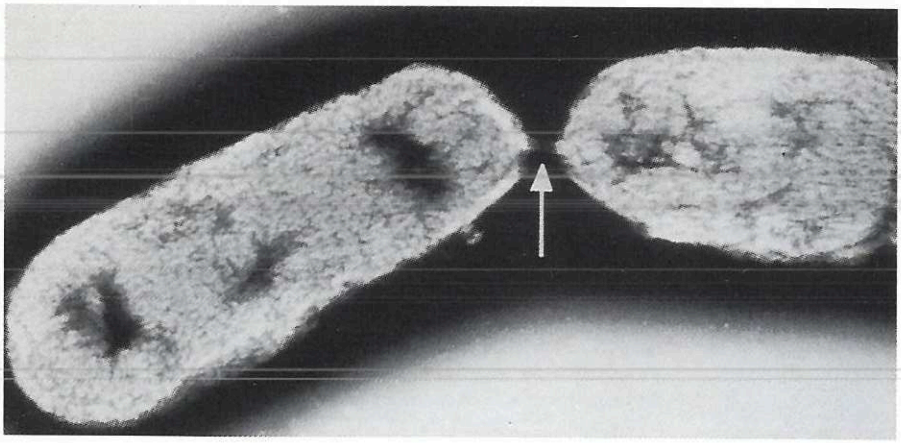


Fig. 11. Cells of *H. ducreyi*, negatively stained with 1% potassium phosphotungstate after fixation in glutaraldehyde. A linkage is seen between two daughter cells (arrow). Absence of pili and flagella. The bar represents 500 nm.

4. RESULTS AND DISCUSSION

5.1 In vitro studies of H. ducreyi-host interaction

5.1.1 Structural investigations

5.1.1.1 Electron microscopy studies

Ultrathin sections of *H. ducreyi* revealed a cell wall typical for Gram-negative bacteria with a cytoplasmatic membrane surrounded by a rugose outer membrane (Figure 10a). There is no evidence of an extracellular capsule (Figure 10b) and examination of cells by negative staining again failed to show surface appendages such as pili or flagella (Figure 11). An interesting feature seen by electron microscopy is the presence of linkages holding adjacent cells together end to end (Figure 11). These linkages appear to be responsible for the characteristic appearance of *H. ducreyi* in short chains when examined by Gram's stain.

Differing from our results electron microscopy studies on *H. ducreyi* by Bertram (M.Sc. thesis, University of Mannitoba, Winnipeg) using additional Alcian blue which like ruthenium red stains acidic polysaccharides could reveal the presence of discontinuous distribution of extracellular material which could be stabilized as a continuous layer by polyvalent rabbit antiserum, although lacking the fine structure detail of antibody -stabilized, ruthenium-red stained capsular material of *H. influenzae* type b. Anyhow, host surface invasion seems not to be linked to pilus-like structures in case of *H. ducreyi* infection.

5.1.1.2 Lectin characterization

The lectin agglutination patterns of the 43 *H. ducreyi*-isolates are shown in Table 3. Although the isolates showed a diversity of reaction patterns, it was possible to group strains from

Table II. *Lectin agglutination patterns of 43 Haemophilus ducreyi isolates.*

Origin of strain	Number of isolates	ConA	LcH	TKA	GS-I	PNA	SBA	DBA	GS-II	STA	WGA	LFA	PHA-L	UEA	Lotus
South Africa	3	+	+	+	+								+		
	2	+	+	+	+			+	+			+	+		
	4	+	+	+	+				+			+	+		
	2	+	+	+					+			+	+		
	2	+	+	+	+	+	+	+	+	+	+	+	+		
	1	+	+						+		+	+	+		
	4	+	+		+					+		+	+		
	2	+	+		+			+			+	+	+		
	2		+	+						+	+	+	+		
	2								+	+		+	+		
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gambia	3	+	+	+	+	+			+		+	+	+		
	1	+	+	+	+						+	+	+		
	2	+	+	+		+					+		+		
	3	+	+		+				+		+	+	+		
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thailand	2		+	+					+	+	+	+	+		
	1										+	+	+		
	1											+	+		
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
France (IP-542)	1		+						+			+	+		
Canada (35000)	1	+	+	+	+		+		+	+	+	+	+		
FRG (M)	1	+	+	+	+	+	+	+	+	+	+	+	+		

Table III. *Haemagglutination patterns of 20 strains of H. ducreyi.*

HA pattern	No. of strains	Agglutination of erythrocytes from			
		Human	Sheep	Pig	Calf
I	9	+	−	−	−
II	5	+	+	+	+
III	3	+	+	−	+
IV	3	+	+	+	−

different geographical areas by lectin agglutination patterns. All strains reacted with *Phaseolus vulgaris*, which has D-Galactose-N-acetylglucosamine-specificity suggesting the occurence of N-acetyl-D-glucosamine units as common structural features of *H. ducreyi* cell envelope polysaccharides. Furthermore, interactions of isolates with soy-bean agglutinin (Glycine max; SBA), Dolichos bifloris (DBA)– and Griffonia simplicifolia II (GSA-II)-lectins suggest that alpha-N-acetylD-galactosamine (alpha-D-GalNAc) units and beta-D-Gal linked to GalNAc and (or) GlcNAc may also occur as structural features of the polysaccharide component of the cell-envelope of some *H. ducreyi*-strains and could be in this way possible candidates for a lectin-specific anti-*H. ducreyi* vaccine.

5.1.1.3 Haemagglutinating reactions and hydrophobicity

H. ducreyi-strains reacted with the erythrocytes tested. On the basis of positive reactions with human erythrocytes and the three different animal species four different HA patterns were observed (Table 3).

Human erythrocytes of groups A and AB were agglutinated by all strains. One strain and three strains, resp. failed to agglutinate erythrocytes of groups B and O.

No change of the HA pattern was found after repeated subculture.

The different HA patterns among the strains suggest that more than one hemagglutinin may be implicated.

Further characterization of the agglutination process revealed that the activity was not heat-labile and was not inhibited by D-mannose. However, bacterial suspensions after treatment with trypsin, which may degrade protein adhesins, and formaldehyde failed to agglutinate red cells.

Measurement of the bacterial hydrophobicity with n-hexadecane or p-xylene as test hydrocarbons, revealed marked differences among the isolates tested. Affinity to p-xylene was in general stronger than to n-hexadecane. However, marked interstrain variations with each of the two substances was noted, ranging from no affinity (strain IP-542) to a maximum of 46% affinity (South African strain) for n-hexadecane and similarly from no affinity to a maximum of 74% affinity (both isolates from South Africa) for p-xylene. For the nine isolates belonging to HA pattern I, which reacted with human erythrocytes only, the affinity to the hydrocarbons tested was less than 15%. In contrast, isolates of HA pattern II, which reacted with all erythrocytes tested, revealed an high affinity to both n-hexadecane and p-xylene with over 40% and 60%, respectively.

5.1.2 Studies on the elaboration of degradative enzymes

5.1.2.1 Investigations on proteolytic activity

Table 4 shows the values of protease-activity in supernatant and pellet samples of *H. ducreyi* and *P. aeruginosa* measured using either Azocoll or Remazol Brilliantblue hide powder. Whereas for *P. aeruginosa* high proteolytic activity both intra- and extracellularly could be demonstrated, in case of *H. ducreyi* strains no detectable activity was seen.

5.1.2.2 Investigations on elastase activity

Table 5 shows elastase-activity in supernatant and pellet samples of *H. ducreyi* and *P. aeruginosa*. Although some activity could be demonstrated for *H. ducreyi* supernatant samples

Table IV. *Results of proteolytic activity determination by the assay using Azocoll or Remazol Brilliantblue hide powder.*

Strain	Azocoll-Assay						Remazol Brilliantblue Hide Powder					
	supernatant			pellet			supernatant			pellet		
	Er	Ee	Er-Ee	Er	Ee	Er-Ee	Er	Ee	Er-Ee	Er	Ee	Er-Ee
3500	+0.001	−0.006	+0.007	−0.012	−0.008	−0.004	−0.001	−0.005	+0.004	+0.008	−0.006	+0.014
IP542	−0.007	−0.001	−0.006	+0.013	+0.002	+0.011	−0.003	0.000	−0.003	+0.008	+0.001	+0.007
3138	−0.006	0.000	−0.006	+0.003	+0.002	+0.001	−0.001	−0.001	0.000	−0.007	+0.001	−0.008
2686	−0.018	−0.010	−0.008	+0.007	−0.005	+0.012	0.000	−0.006	+0.006	+0.003	−0.003	+0.006
F 6	+0.016	−0.007	+0.023	+0.011	+0.008	+0.003	−0.005	−0.001	−0.004	+0.002	−0.006	+0.008
F 35	−0.006	−0.004	+0.010	+0.005	−0.001	+0.006	+0.008	+0.005	+0.003	−0.006	−0.001	−0.005
S 5	−0.005	−0.002	−0.003	−0.002	−0.003	+0.001	+0.009	+0.001	+0.008	0.000	0.000	0.000
S 22	−0.001	0.000	−0.001	+0.011	+0.020	−0.009	−0.001	+0.003	−0.004	−0.014	−0.015	+0.001
S 25	−0.005	−0.008	+0.003	−0.002	+0.007	−0.009	−0.001	−0.002	+0.001	−0.004	+0.001	−0.005
YD 3	+0.001	−0.003	+0.004	−0.002	−0.002	0.000	+0.001	−0.002	+0.001	−0.001	0.000	−0.001
*	−0.003	−0.003	0.000	+0.003	+0.002	+0.001	0.000	−0.001	+0.001	−0.001	−0.003	+0.002
P. aer	+3.342	−0.019	+3.374	+3.361	−0.013	+3.374	+0.501	−0.013	+0.514	+0.586	−0.009	+0.595

Abbrevations: Er, extinction of tested bacterial solution
Ee, correction factor taking into account bleeding of the samples caused by the brown colour of the supernatent which is getting weaker during incubation through influence of heat and interaction with the buffer
* extinction of test sample without bacteria pre-incubation

Table V. *Results of elastase determination by the assay using orcein-elastase.*

Strain	supernatant			pellet		
	Er	Ee	Er-Ee	Er	Ee	Er-Ee
3500	+0.041	−0.010	+0.051	+0.039	−0.006	−0.045
IP542	+0.030	−0.012	−0.042	+0.083	−0.010	+0.093
3138	+0.029	−0.007	+0.036	+0.035	−0.001	+0.036
2686	+0.031	−0.017	+0.048	+0.063	−0.011	+0.074
F 6	+0.042	0.000	0.042	+0.058	−0.014	+0.072
F 35	+0.028	−0.004	+0.032	+0.054	−0.009	+0.063
S 5	+0.038	−0.005	−0.043	+0.066	−0.011	+0.077
S 22	+0.022	−0.009	+0.031	+0.069	0.000	+0.069
S 25	+0.045	−0.008	+0.053	+0.064	−0.016	+0.080
YD 3	+0.030	−0.005	+0.035	+0.037	−0.013	+0.050
*	+0.034	−0.008	+0.042	+0.057	−0.009	+0.066
P. aer	+0.123	−0.021	+0.144	+0.119	−0.008	+0.127

Abbrevations: Er, extinction of tested bacterial solution
Ee, correction factor taking into account bleeding of the samples caused by brown colour of the supernatant which is getting weaker during incubation through influence of heat and interaction with the buffer
* extinction of test sample without bacteria pre-incubation.

which was about one fifth of the one found in samples with *P. aeruginosa* and almost the same activity was found in pellet samples of *H. ducreyi* and *P. aeruginosa,* it could be shown by analysing the activity in sterile liquid medium that the determined elastase activity in the supernatant in case of *H.ducreyi* was due to proteins already present in the medium. Whether intracellular elastase activity is present in *H. ducreyi* has to be further investigated and cannot be answered by the method used.

In summary, our data suggest that elaboration of degradative enzymes is not involved in the interaction of *H. ducreyi* and host surfaces.

5.1.3 H. ducreyi – host interactions studied on primary and established cell cultures

No cytotoxicity of *H. ducreyi* filtrates to human keratinocytes as well to Vero cells was observed, both with reference strains and with low-passage isolates. Figures 12 and 13 show unaffected Vero cells and keratinocytes, resp. after 48 hours' incubation with *H. ducreyi* supernatants.

In contrast, filtrates of *P. aeruginosa* were cytotoxic for both cell types. Affected Vero cells showed long filamentous tendrils and an irregular cytoplasm (Figure 14). Interaction with human keratinocytes resulted in a shrinking of the cyto-toplasm (Figure 15).

From our data apparently showing an absence of cytotoxicity of *H. ducreyi* filtrates to human keratinocytes and Vero cells it can be concluded that *H. ducreyi* isolates do not have the potency for disruption of the outer covering of the skin consisting of multiple layers of keratinocytes.

5.1.4 Cell-mediated immune response

H. ducreyi antigen preparations were found to be able to induce proliferation of lymphocytes both from normal unexposed individuals and from a chancroid-sensitized subject, with stronger cell response seen for the strain isolated from a patient with associated lymphadenopathy.

The sonicated *H. ducreyi* preparation showed the strongest response of cells with both strains, followed by whole cell preparation. Outer-membrane preparations revealed the weakest cell response.

The phenomenon of lymphocyte stimulation was dose-dependent.

The antigen response of cells from the chancroid-sensitized subject was significantly higher than those of cells from unexposed individuals ($p < 0.01$).

Il–2 production

No Il-2 production was detected in any of the unstimulated cultures, but could be measured in the antigen-stimulated

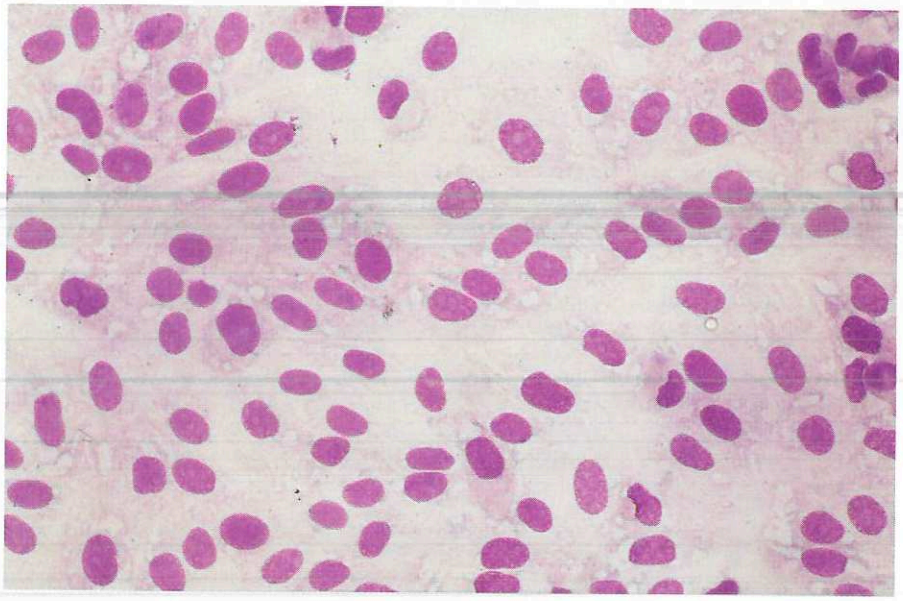


Fig. 12. Vero cells; incubation with *H. ducreyi* filtrates (S 26) for 48 h. Absence of morphological cell changes. (x40)

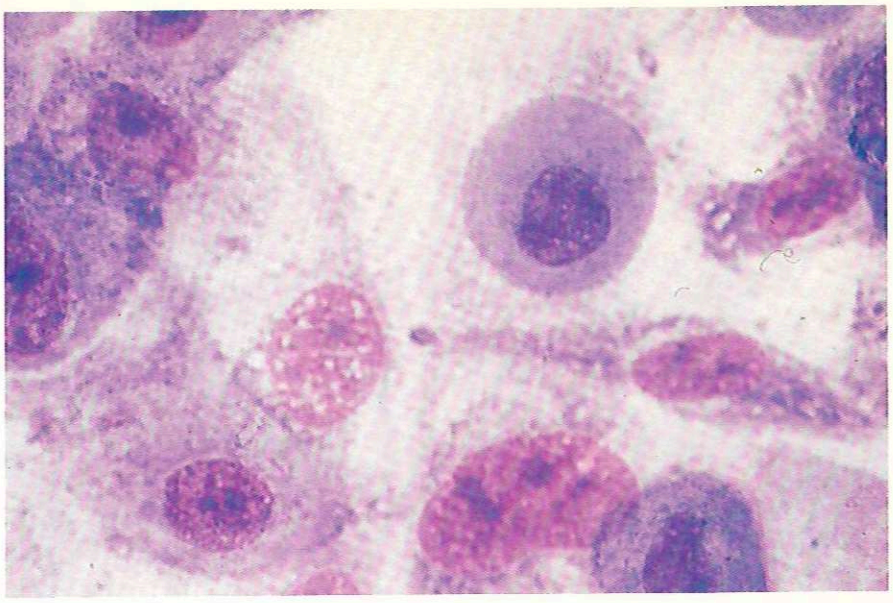


Fig. 13. Human keratinocytes; incubation with *H. ducreyi* filtrates (S 26) for 48 h. Absence of morphological cell changes. (x100)

cultures. Again, Il-2 production was significantly higher in *H. ducreyi*-antigen stimulated mononuclear cells of the subject with a history of chancroid.

From the results presented here it can be assumed that a cell-mediated immune response occurs during *H. ducreyi* infection which, however, does not lead to resistance to infection. A cell-mediated immune response can also be suggested from findings that increased levels of soluble interleukin-2 receptors in serum and urine samples of patients with chancroid can be detected which are reduced by therapy (unpublished data). The chancroid sensitized subject had a history of one year after infection. Up to now there are no data to tell for how long this cell-mediated immune response will differ significantly from the one seen in unexposed individuals.

The method presented here could be very helpful in assessing possible antigen preparations which are potential candidates for a chancroid vaccine. Presenting antigens by sonication of whole cells had the strongest effect on cell proliferation.

5.2 In vivo-interaction of H. ducreyi antigen preparations and skin

5.2.1 Effect of whole cells and culture supernatents

The mere contact both of live *H. ducreyi* organisms and *H. ducreyi* culture supernatant as in the experiments a and c did not lead to any reaction or even damage of mouse skin.

The results of experiment b are summarised in Table 6.

Nodules were seen at every site of injection from the first day after inoculation (Figure 16) and they became red and pustular (Figure 16). Some of the lesions ulcerated after 2 to 3 days in mice inoculated with strains S26 or S30, and a little later in those given IP-542 (Figure 16).

Approximately half of all the sites developed ragged, full thickness skin ulcers with rounded crater-like edges, typical of those occuring in chancroid.

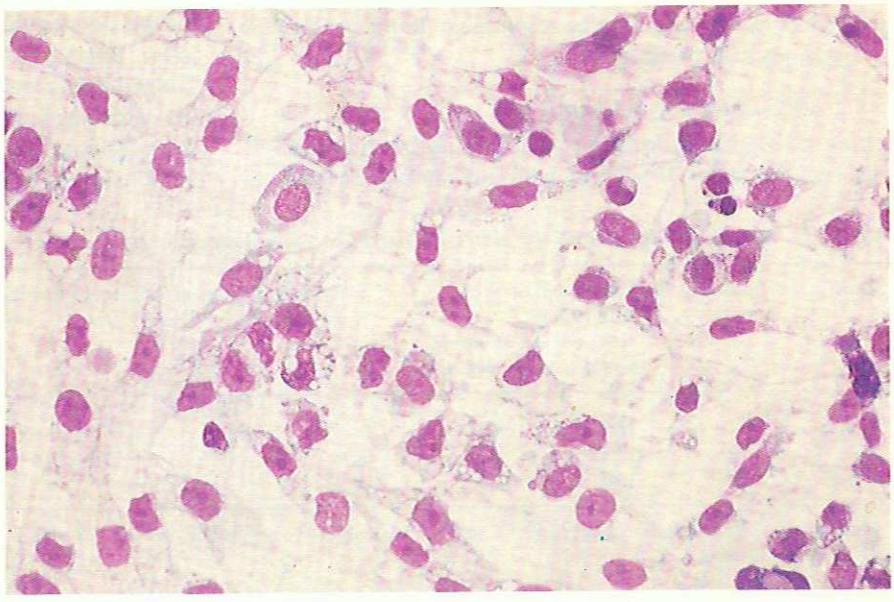


Fig. 14. Vero cells; incubation with *P. aeruginosa* filtrates for 48 h. Cells showing cytotoxicity from filtrates. (x40)

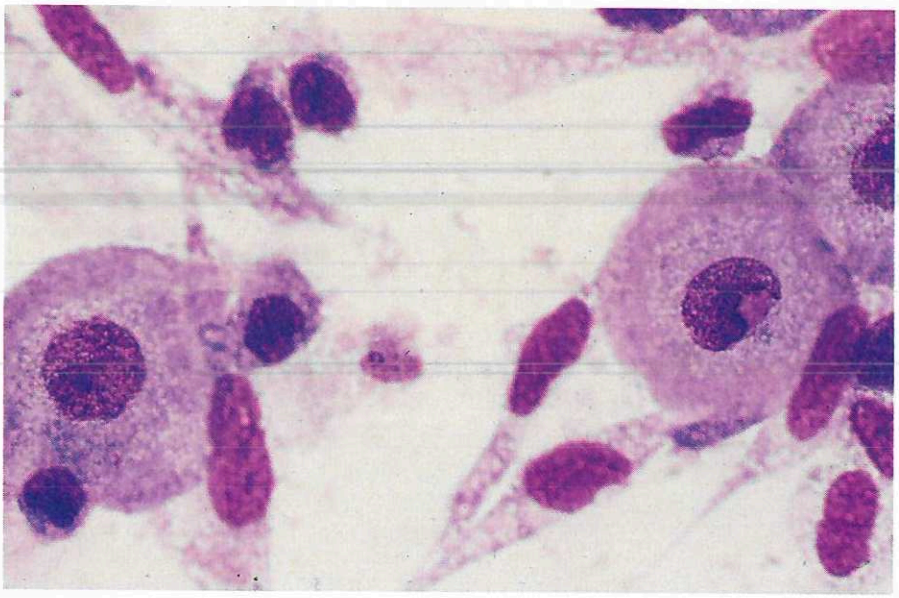


Fig. 15. Human keratinocytes; incubation with *P. aeruginosa* filtrates for 48 h. Cells showing cytotoxicity from filtrates (x100).

After one week the lesions were drying out, leaving scabs, and healing was complete after about 14 days.

H. ducreyi was recovered up to 6 days after inoculation from pus and ulcers produced by all 3 strains.

No lesions were seen at sites inoculated with PBS alone. The lesions seen in the mouse model closely resembled those seen in human disease resulting in sharply circumscribed ulcers with ragged undermined edges.

It is difficult to demonstrate conclusively that *H. ducreyi* organisms multiply in the skin of young adult mice, but this seems likely because the organisms were recovered nearly one week after inoculation.

Our results clearly prove that *H. ducreyi* organisms are not able to penetrate the host tissue actively as for example by elaboration of degradative enzymes. Penetration is only possible through abrasions. Furthermore, tissue necrosis production does not depend on live organisms because heat-killed ones had the same effect in the animal model.

In this way endotoxins being released by the organisms during the infection seem to be responsible for the production of tissue necrosis production.

5.2.2 Effect of lipopolysaccharide

After intradermal inoculation of LPS all three strains developed within about 12 and 24 hours of inoculation signs of inflammation at the injection site independently from the LPS concentration chosen. Skin damage could be seen for the first time after 24 hours with preparations of highest LPS concentration of isolate S30. Within the next 24 hours the same effects could be observed with the other samples tested resulting in severe ulceration as seen and described after intradermal injection of whole organisms (5.2.1).

The results gained from the experiments strongly sugggest that tissue necrosis production of *H. ducreyi* is a result of interaction of LPS and inflammatory mediators of the host.

6. PRINCIPLE FINDINGS

With *H. ducreyi* the initial contact to host tissue does not depend on pilus-like structures as it is frequently the case with other microorganisms causing sexually transmitted disease, such as *Neisseria gonorrhoeae* or *Trichomonas vaginalis*.

Attachment might be lectin-mediated as *H. ducreyi* cell envelops present a wide variety of different structural features of the polysaccharide components. In common to all *H. ducreyi* isolates is a N-acetyl-D-glucosamine unit as can be judged from reaction of all strains tested with *Phaseolus vulgaris*. Haemagglutination studies indicate that more than one hemagglutinin is involved in initial attachment and hydrophobic bonds being of importance. Studies looking for extra–

Table VI. *Results of inoculating the skin of CBA mice with Haemophilus ducreyi.*

Strain	Number of mice	Proportion of sites with lesions		Severity of lesions after 2 to 13 day due to inoculation of 10^7 organisms (day 0)							
		nodules	ulcers	2	3	4	5	6	7	9	13
S 26	6	12/12	4/12	++[a,b]	++	++	++	+[a]	+	+	+/−
S 30	6	12/12	8/12	++	+++[b]	+++[a]	++[a]	+	+	+	
IP-542	4	8/8	5/8	++	++	+++[b]	+++[a]	+	+	+	+/−

Approximate diameter of lesion: +/−, −1 mm; +, 1–2 mm; ++, 3–5 mm; +++, −5 mm
[a] *H. ducreyi* isolated
[b] Ulceration first seen

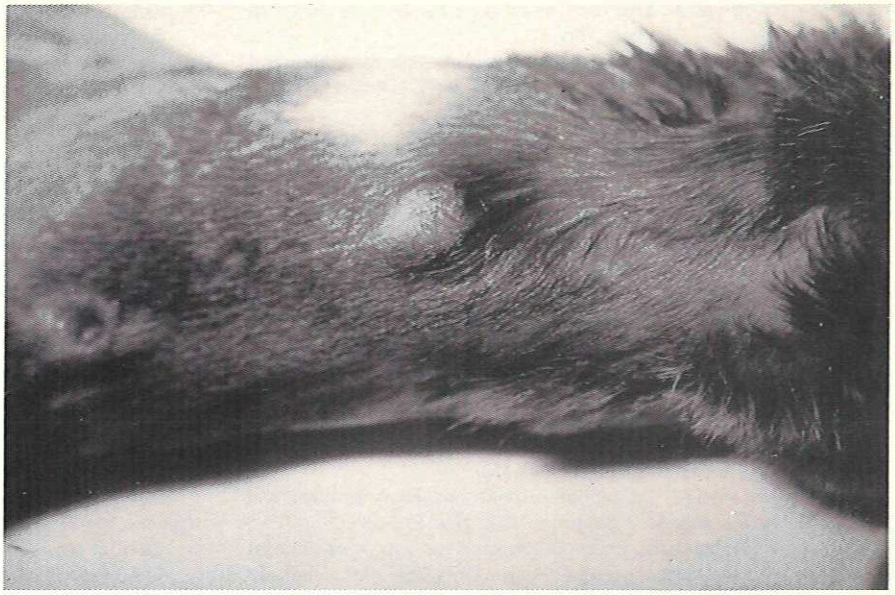


Fig. 16. Typical ulcer (left), nodule (centre) and initial inflammatory reaction (right) in skin of a CBA mouse inoculated with *H. ducreyi*.

and intracellular production of enzymes of proteolytic or elastase did not reveal any activity.

Incubation of culture filtrates with human keratinocytes and an established cell line did not show any cytotoxic effects so that it seems likely that *H. ducreyi* is not able to destroy epidermal surfaces as it can be demonstrated with *Pseudomonas aeruginosa*. This corresponds to findings in a new mouse model of *H. ducreyi* infection. Undamaged mouse skin was an impregnable barrier for both whole cells and culture filtrates. Pre-existing skin lesions seem to be necessary in order to initiate infection as intradermal or subcutaneous injection of *H. ducreyi* organisms resulted in ulcer production. Relevant interaction of *H. ducreyi* and host skin does not take place on the epidermal level but on the dermal level. Tissue damage in the sense of ulceration is not bound to live organisms as injection of dead ones produced identical tissue necrosis. It is likely that *H. ducreyi* lipopolysaccharide is the responsible toxic substance as in the mouse model ulcer production was observed after injection of purified LPS. Although this substance must be very toxic to the connective tissue as judged by the rapidity with which the ulcers break down, disease is self-limited both in animal and in men and apparently non-protective as it is well-known from clinical reinfection.

Cell mediated interleukin-2 immune response is a major feature of the inflammatory reaction as could be demonstrated for the first time. Although different in persons not yet or already exposed to *H. ducreyi* it does not protect against re-infection in men. However, in this way infection by *H. ducreyi* fulfills an important general requirement necessary for developing a vaccine, namely the activation of antigen-presenting cells to initiate antigen processing and production of interleukins. *H. ducreyi* LPS possibly coupled to protein is a major candidate for such a vaccine as are certain carbohydrates.

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