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Characterization of skin lesions in mice following intradermal inoculation of *Haemophilus ducreyi*

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Summary. Twelve strains of *H. ducreyi*, which included two reference strains, were each inoculated intradermally into the flanks of CBA mice. All strains produced self-limited lesions which were macroscopically and microscopically typical of those seen in chancroid. Pustular nodules, about 5mm in diameter, developed at all inoculation sites by the second day when 10^7 organisms were inoculated. Approximately half of these lesions ulcerated and all had regressed by 2 weeks. Smaller nodules developed at about half the sites from the second to the fifth day when 10^6 or 10^5 organisms were given, but these did not ulcerate. No lesions were seen when 10^3 organisms were inoculated. Organisms were recovered from the lesions up to 11 days after inoculation. Specific *H. ducreyi* antigen, sought by a monoclonal antibody test, was detected in lesions up to 15 days following inoculation. Heat-killed organisms of *H. ducreyi* also produced nodules and ulcers although these were slightly smaller than those which developed after inoculation of viable bacteria. Similar lesions to those caused by *H. ducreyi* were produced after intradermal inoculation of about 10^8 viable or killed *Neisseria gonorrhoeae* organisms. Treatment of mice with ceftriaxone had little or no effect on the subsequent development of *H. ducreyi*-induced lesions. These findings indicated that the lesions were not produced specifically by viable *H. ducreyi* organisms. Ulcers were also produced following intradermal inoculation of purified lipopolysaccharide (LPS) from *H. ducreyi* or *N. gonorrhoeae*, but not by cell-free filtrates prepared from *H. ducreyi* cultures indicating a possible role for LPS in the pathogenesis of ulcerative skin lesions.

Keywords: *Haemophilus ducreyi*, lesions, antibiotic/heat treatment, LPS

Chancroid, a sexually transmitted genital ulcer disease caused by *Haemophilus ducreyi*, is endemic in many developing countries, particularly in Africa (Duncan *et al.* 1981; Nsanze *et al.* 1981) and South-East Asia (Rajan *et al.* 1983; Taylor *et al.* 1984) and has recently re-emerged as a significant component of genital ulcer disease in the United States (Schmid *et al.* 1987). The disease, which is characterized by painful genital ulcerations and frequently by regional lymphadenopathy, has received renewed attention following suggestions that it may be a significant cofactor in the heterosexual transmission of human immunodeficiency virus (Kreiss *et al.* 1986). Although the causative bacterium was first described 100 years ago, little is known of the pathogenesis

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of chancroid, largely because of the failure to develop a suitable animal model of the disease. Intradermal inoculation of rabbits with *H. ducreyi* results in the production of localized skin lesions, which do not, however, resemble typical chancroidal ulcers. Rabbit skin lesions, therefore, have not been considered a good model of the human disease (Hammond *et al.* 1978). Recently we described preliminary observations on a mouse model of *H. ducreyi* infection in which CBA mice were inoculated intradermally on each flank with one of three strains of *H. ducreyi* (Tuffrey *et al.* 1988). The lesions were similar to those seen in human cases of chancroid and all three strains of *H. ducreyi* were re-isolated from the mice during the first week.

In this communication we describe the results of inoculating different doses of *H. ducreyi* intradermally and the histology of the murine lesions. We have also attempted to determine how closely this model mimics the human infection and to identify the specific factors responsible for development of the lesions by inoculating mice with live and killed organisms, organism-free culture filtrates, and purified lipopolysaccharide extracted from cultures of *H. ducreyi* and *Neisseria gonorrhoeae*.

Materials and methods

Bacteria

Twelve strains of *H. ducreyi* were used. Ten, designated S3, S4, S15, S22, S23, S25, S26, S30, S31 and S35, isolated originally from men with classical chancroid in Carletonville, South Africa, were used after two passages. A further two were reference strains (IP 54.2 and 35000) obtained from Professor P. Piot, Institute of Tropical Medicine, Antwerp, Belgium. The bacteria were grown on solid medium comprising Columbia agar base (BBL), 10% sheep blood (Wellcome), 2.5% heat-inactivated foetal calf serum (Flow Laboratories), 1.5% IsoVitaleX (BBL) and vancomycin (3 mg/l). Plates were incubated in a candle extinction jar at 33°C for a minimum of 48 h, and the growth of bacteria was subcultured twice before colonies were harvested and emulsified in phosphate-buffered saline (PBS) to provide organisms for inoculation into mice. In certain experiments they were killed by boiling for 10 min prior to inoculation. Non-viability was confirmed by failure to culture. In experiments in which broth-grown *H. ducreyi* was used, the organisms were subcultured once on agar prior to inoculation into brain heart infusion broth (BBL) supplemented with 10% heat-inactivated foetal calf serum, 1% IsoVitaleX, 3 mg/l vancomycin and 10 ml/l hemin stock solution (BHI-Hemin medium). Hemin stock solution was prepared by adding 5 g hemin (Sigma, St. Louis, Mo, USA), 5 g L-histidine (Sigma) and 10 ml of triethanolamine (Sigma) to 250 ml of distilled water. The solution was sterilized by heating at 60°C for 30 min and stored subsequently in appropriate volumes at −20°C.

Neisseria gonorrhoeae, strain 4780, isolated originally from a patient with acute urethritis in South Africa, was cultured on GC agar base (Gibco) supplemented with 1.5% IsoVitaleX. The bacteria were passaged three times before use.

Preparation of cell-free filtrates from broth culture

A two-day 10 ml broth culture, containing approximately 1.4×10^7 colony-forming units (cfu) of *H. ducreyi*, was centrifuged at 4000 *g* for 10 min. The pellet was resuspended in 2 ml of BHI-Hemin medium and the supernatant fluid filtered through a 2 μm filter before inoculation into mice.

Extraction and analysis of lipopolysaccharide

The lipopolysaccharide (LPS) of the outer membrane was extracted from both *H. ducreyi* (strains S26 and 35000) and *N. gonorrhoeae* by the method of Johnson and Perry (1976). Endotoxic activity of LPS was mea-

sured by the *Limulus* amoebocyte assay (Sigma Chemical Co., St Louis, Mo.) (Levin & Barry 1964). Approximately 15 μg of each LPS extract was boiled in sample buffer for 10 min and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a discontinuous gel system with 12% (w/v) polyacrylamide resolving gel. All gels were run at a constant current of 30 mA, and fixed and stained using the silver stain method of Hitchcock and Brown (1983).

Mice and inoculation

Syngeneic CBA mice, 6–12 weeks old, bred and maintained in a specific pathogen-free unit, were used. The hair on the flanks of the mice was clipped and inocula of 0.1 ml were introduced intradermally. Observations were made daily during the following 2 weeks for lesions at the injection site. If present, these were graded from ± (< 1 mm diameter) to + + + (> 5 mm) as described previously (Tuffrey *et al.* 1988).

Detection of bacteria

Sterile cotton-tipped nasopharyngeal swabs (Medical Wire and Equipment Co. Ltd) were used to sample the skin lesions for viable bacteria, as described previously (Tuffrey *et al.* 1988). In addition, material on swabs was occasionally smeared on glass microscope slides, fixed with methyl alcohol for 10 min and tested for *H. ducreyi* antigen by indirect immunofluorescence using a specific monoclonal antibody.

Treatment with antibiotics

Mice were injected intraperitoneally with 4 mg of ceftriaxone (Hoffman La Roche, Welwyn Garden City, Hertfordshire) given each day for 5 days, starting the day before inoculation with either 8×10^6 or 8×10^7 cfu of strain S26 of *H. ducreyi*. The results of other experiments showed that this high dose of antibiotic was not toxic for the mice and that strain S26 of *H. ducreyi* was fully susceptible *in vitro* to ceftriaxone (MIC of

Table 1. Response of CBA mice to intradermal inoculation of different doses of *H. ducreyi*

Dose (cfu)	Strain	Proportion of sites with nodules	Proportion of sites with ulcers	Lesions on indicated days after inoculation: 2	3	4	5	6	7	9	13
	S26	12/12	4/12	+ + †*	+ +	+ +	+ +	+*	+	+	±
10^7	S30	12/12	8/12	+ +	+ + + †	+ + +*	+ +*	+	+	+	−
	IP54.2	8/8	5/8	+ +	+ +	+ + + †	+ + +	+	+	+	±
	S26	3/6		±	±	±	−	−	−	−	−
10^5	S30	3/6		±	±	±	±	−	−	−	−
	IP54.2	1/4		±	±	±	±	−	−	−	−
	S26	0/6		−	−	−	−	−	−	−	−
10^3	S30	0/6		−	−	−	−	−	−	−	−
	IP54.2	0/4		−	−	−	−	−	−	−	−
	PBS	0/6		−	−	−	−	−	−	−	−

Appropriate diameter of lesion: ±, < 1mm; +, 1–2 mm; + +, 3–5 mm; + + +, > 5mm.
* *H. ducreyi* re-isolated.
† Ulceration first seen.

0.002 mg/l) (Abeck *et al.* 1988). Control groups of mice did not receive antibiotic treatment.

Histopathology

Excised skin bearing nodules and/or ulcers was fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μm) were cut through various levels of the block and stained with haematoxylin and eosin.

Results

Skin lesions produced by viable H. ducreyi: dose–response

All the strains of *H. ducreyi*, including the reference strains, produced lesions in the mice, as described previously (Tuffrey *et al.* 1988). In all cases, the lesions were self-limited. The results of a dose–response experiment, in which strains S26, S30 and IP54.2 were inoculated, are shown in Table 1. When 10^7 bacteria were inoculated, large pustular nodules developed at all injection sites by the second day and these regressed during the next 2 weeks. However, about half the lesions ulcerated during this time. When 10^6 or 10^5 bacteria were inoculated, small nodules developed at about half the sites by the second day and these had regressed by the fifth day without ulcerating. No lesions developed after inoculation of 10^3 organisms.

Histology of lesions

Histological examination of the inoculated skin sites (Figs 1–4) showed inflammatory changes which comprised polymorphonuclear leucocytes and, later, small round cells and macrophages. Subsequently ulcerations were produced which were similar to those seen in chancroid.

Table 2. Isolation of *H. ducreyi* from and detection of antigen in skin lesions of CBA mice after intradermal inoculation

Strain of *H. ducreyi*	Detection of viable organism in lesions		Last day‡ on which organisms recovered	Detection of antigen in lesions	
	Proportion positive	Percentage positive		Proportion positive	Percentage positive
S3	3*/26†	11.5	5	17*/19†	89
S4	1/9	11	11	11/11	100
S15	2/10	20	8	NT	
S22	3/10	30	8	NT	
S23	0/7	0	—	6/7	86
S25	3/10	30	8	NT	
S26	7/49	14	8	NT	
S30	9/68	13	10	10/10	100
S31	0/5	0	—	7/7	100
S35	4/26	17	7	18/19	95
Ref IP54.2	2/16	12.5	5	NT	
Ref 35000	0/2	0	—	NT	
Total	34/238	14.2		69/73	94.5

* Number of lesions from which organisms isolated or in which antigen detected.
† Number of lesions tested.
‡ After inoculation.

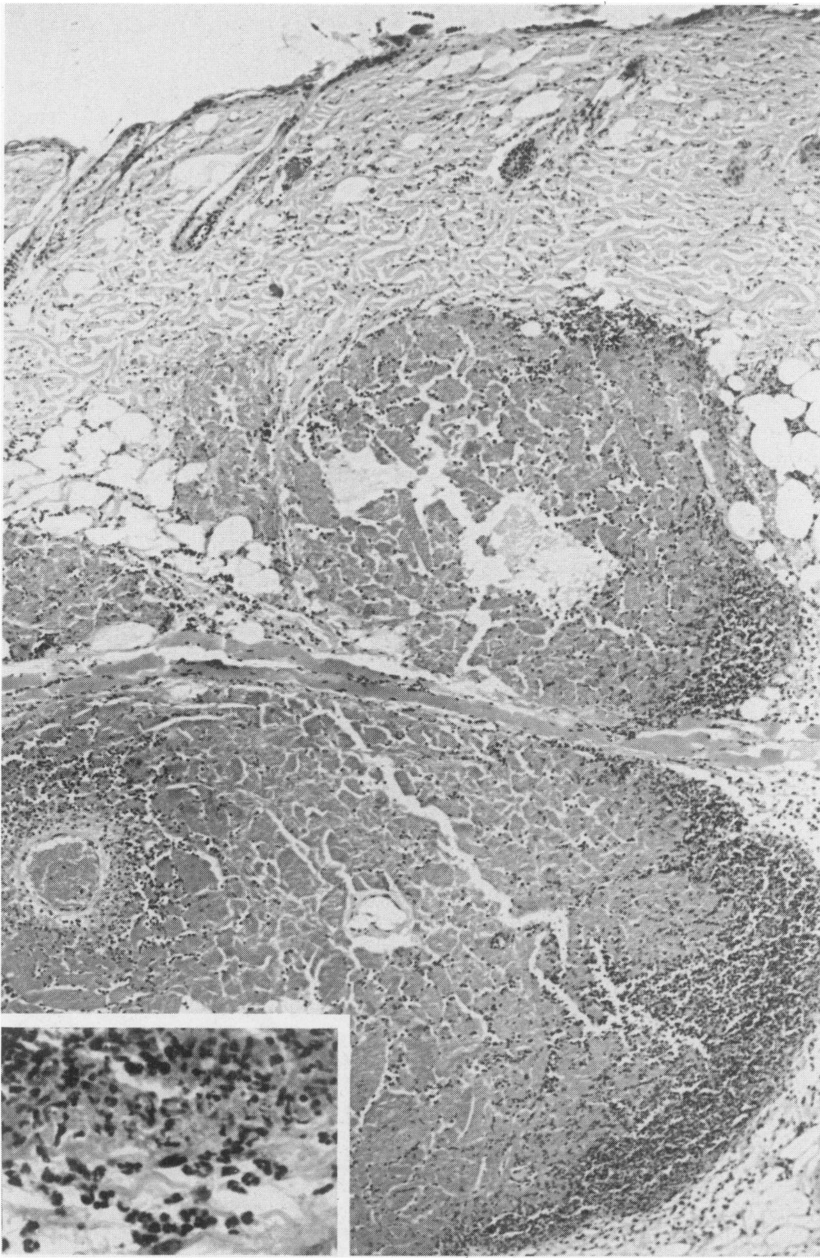

Fig. 1. Section through a nodule in mouse skin 2 days after inoculation of 10^7 cfu of strain S26 of *H. ducreyi* showing peripheral infiltration of the inoculated organisms with polymorphonuclear leucocytes (PMNL). H & E, × 102. Inset: Detail of edge of infiltrate showing PMNL. H & E, × 400.

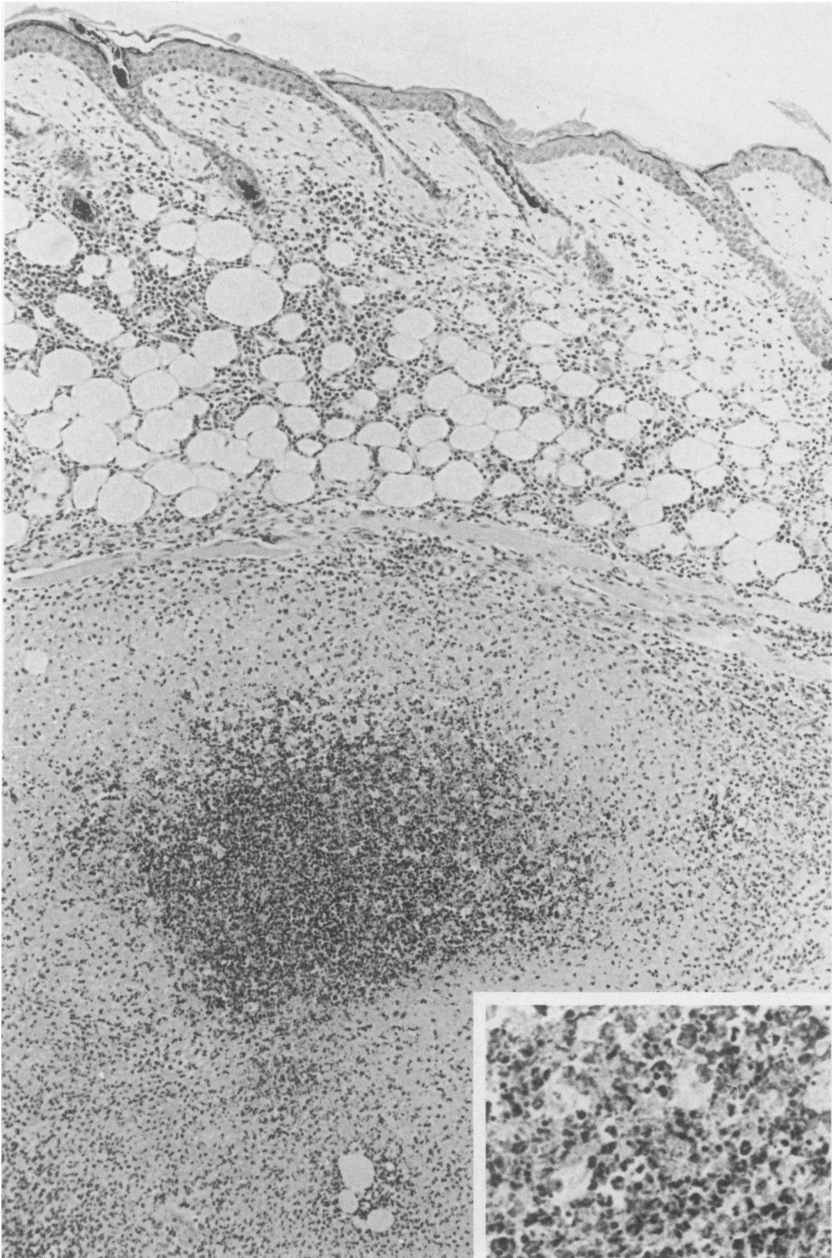

Fig. 2. Section through a pustule in mouse skin 7 days after inoculation of 10^7 cfu of strain S22 of *H. ducreyi* showing a chronic inflammatory focus at the inoculation site. H & E, × 102. Inset: Detail of centre of infiltrate showing PMNL, macrophages, lymphocytes and cellular debris. H & E, × 400.

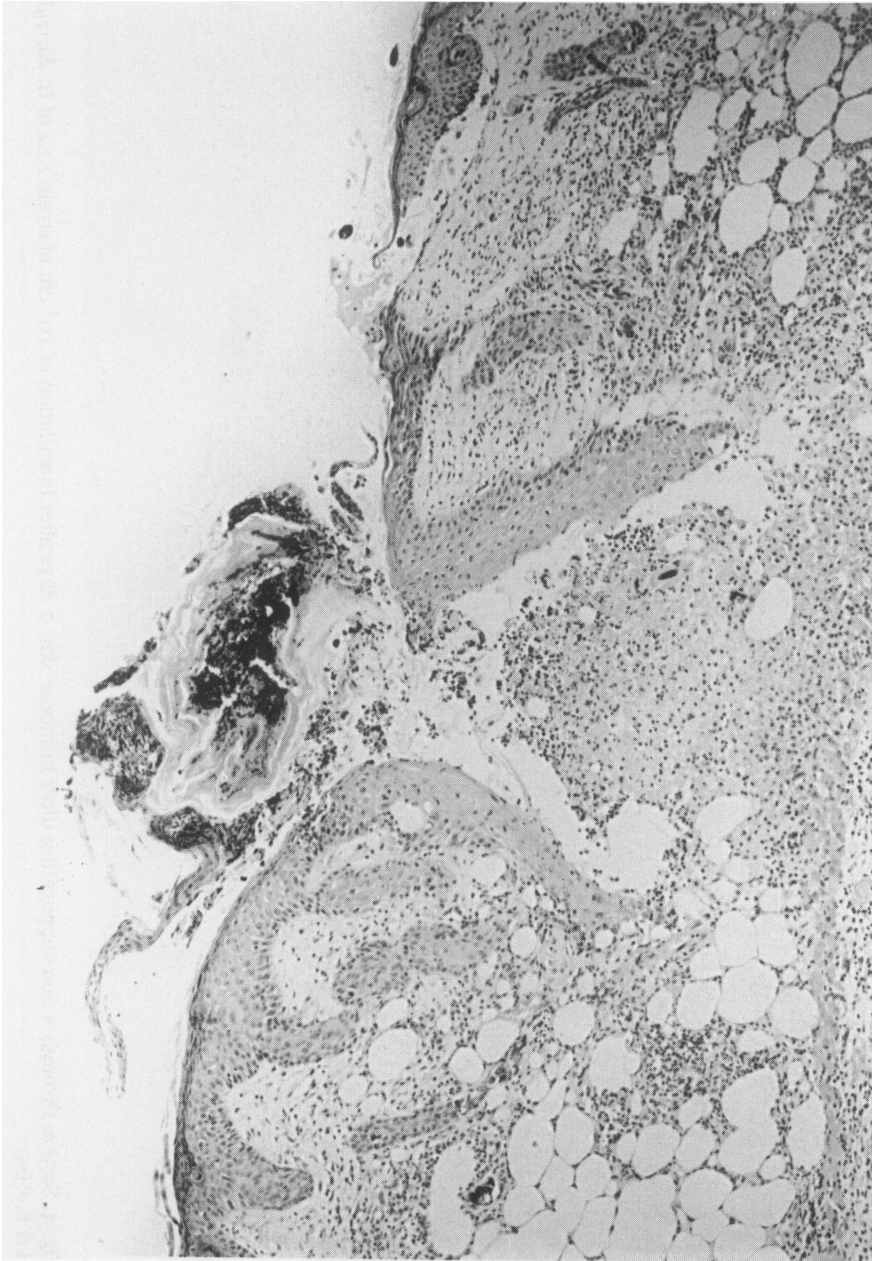

Fig. 3. Section through ulcerating lesion in mouse skin 7 days after inoculation of 10^7 cfu of strain S22 of *H. ducreyi*. H & E, × 102.

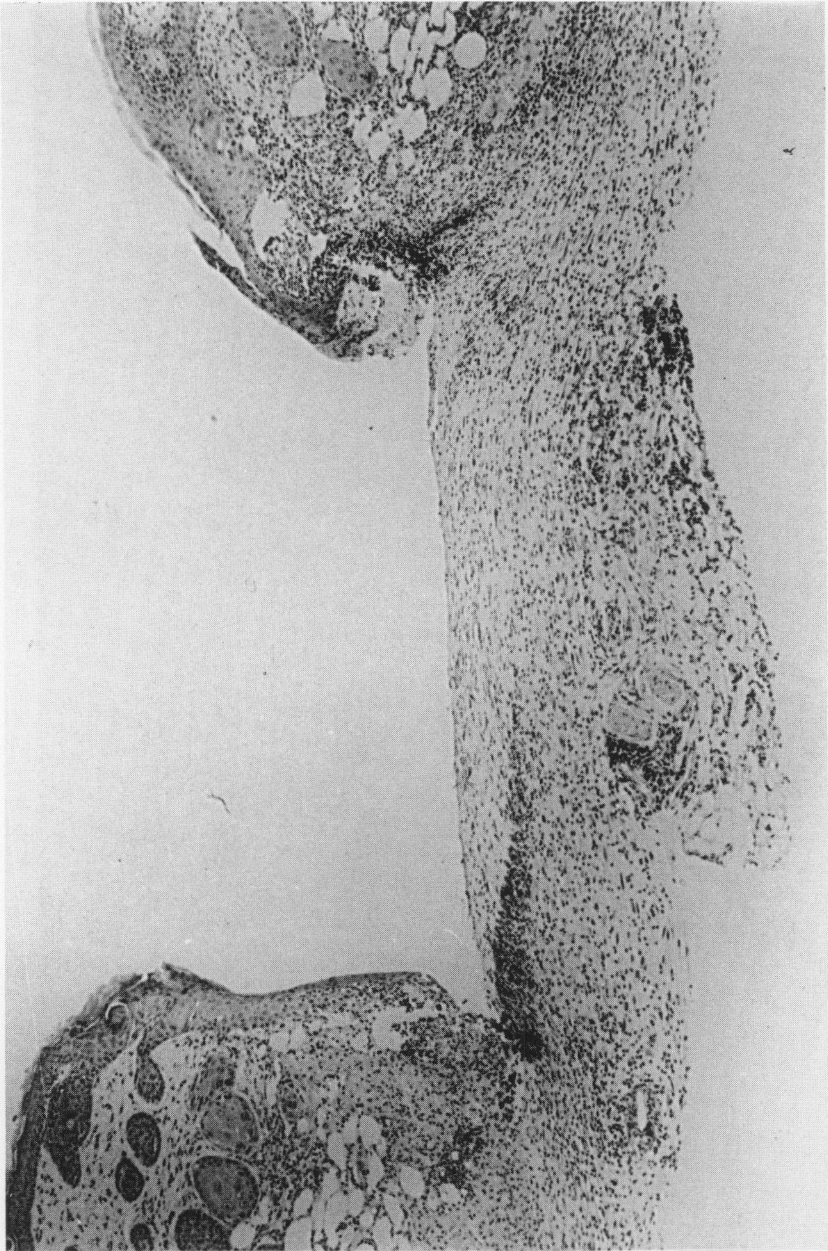

Fig. 4. Section through a non-suppurating ulcer in mouse skin 7 days after inoculation of 10^7 cfu of strain S22 of *H. ducreyi*. H & E, ×65.

Table. 3. Result of inoculating CBA mice with viable or heat-killed *H.ducreyi* or *N. gonorrhoeae*

Bacterium		Viable organisms	No. organisms in inoculum	Lesions* indicated on day after inoculation					
Species	Strain			2	3	4	6	10	14
H. ducreyi	S26	+	6.4×10^7	++	++†	++	++	+	−
		−		++	++	+	+	±	−
H. ducreyi	35000	+	1.5×10^7	++	++†	+	±	−	−
		−		±	++	+	±	−	−
N. gonorrhoeae	4780	+	6.3×10^8	++	++†	+	±	−	−
		−		++	++†	+	±	−	−

* See footnote to Table 1.
† Ulceration.

Detection of H. ducreyi

Organisms of eight of the ten low-passage strains of H. ducreyi were recovered from the lesions on at least one occasion by culture. Organisms were isolated from lesions up to 11 days after inoculation but, overall, only 14% of all isolation attempts were successful, a summary of such attempts being presented in Table 2. Specific *H. ducreyi* antigen was sought by the monoclonal antibody test in lesions produced by six of the strains and detected in all of them, in some up to 15 days after inoculation. Overall, antigen was detected on 94% of the occasions it was sought (Table 2).

Skin lesions produced by heat-killed bacteria

The effect of inoculating mice with killed organisms of strains S26, S30 and 35000 of *H. ducreyi* was compared with that of inoculating with viable organisms. The results for strains S26 and 35 000 are shown in Table 3; those for strain S30 were the same as for strain S26. Viable organisms of strain S26 produced lesions which persisted up to 10 days after inoculation, while those of strain 35 000, in a smaller dose, induced lesions up to day 6. Killed organisms of the three strains produced lesions which persisted for the same length of time as those produced by viable organisms, although killed organisms produced slightly smaller lesions.

Similar lesions to those caused by *H. ducreyi* were seen in mice following intradermal inoculation of 6.3×10^8 viable *N. gonorrhoeae*. Killed gonococci caused lesions of the same size which persisted for the same period of time. These results confirmed that ulcerative skin lesions were not produced specifically by viable *H. ducreyi* organisms.

Effect of antibiotic treatment

Treatment of mice with ceftriaxone had little or no effect on the subsequent development of skin lesions induced by intradermal inoculation of either 8×10^7 or 8×10^6 cfu of strain S26 of *H. ducreyi* (Table 4). Recovery of *H. ducreyi* from the antibiotic-treated mice was not achieved.

Effect of organism-free filtrates of H. ducreyi

The results of inoculating mice with agar-cultured and broth-cultured organisms of strain S26 of *H. ducreyi* as well as centrifuged organisms and organism-free filtrates from broth cultures of strain S26 are shown in Table 5. Lesions were produced only after inoculation of organism- associated material.

Table 4. Effect of treating CBA mice with ceftriaxone on lesions produced by strain S26 of *H. ducreyi*

No. organisms in inoculum (cfu)	Antibiotic treatment	Lesions* on indicated day after inoculation							
		1	2	3	4	5	7	9	15
8×10^7	None	++	++	+++†	++†	+++†	+	+	+
	Daily	+	++	++†	++†	++†	+	+	+
8×10^6	None	±	+	+	+†	+†	±	±	±
	Daily	±	+	+	+†	+†	±	±	±

* See footnote to Table 1.
† Ulceration.

Effect of lipopolysaccharide extracted from H. ducreyi and N. gonorrhoeae

The LPS preparations exhibited low molecular weight bands following SDS–PAGE, typical of those previously described for the rough LPS extracted from both *H. ducreyi* and *N. gonorrhoeae* (Abeck *et al.* 1987). The high endotoxic activity of the preparations was confirmed by gel-clot formation in *Limulus* assays.

LPS from strains S26 and 35 000 of *H. ducreyi* and strain 4780 of *N. gonorrhoeae* produced skin lesions in mice similar to those seen previously when viable or killed organisms were inoculated (Table 6). Undiluted LPS extracts caused ulcers the first day after inoculation and diluted extracts produced smaller and less severe lesions, the size and severity being dose related. All the lesions had regressed by 2 weeks, as seen after inoculation of whole organisms.

Discussion

The results of this study confirm that skin lesions are produced in mice following intradermal inoculation of viable *H. ducreyi* bacteria and that the macroscopic appearance of the lesions is similar to that seen in the human disease. The histological appearance of the murine lesions is also similar to that seen in the naturally occurring infection (Freinkel 1987; McCarley *et al.* 1988), with the sequential appearance of intradermal abscesses containing polymorphonuclear leucocytes, followed by ulceration, scab formation, and spontaneous healing.

The formation and nature of lesions in

Table 5. Result of inoculating CBA mice with cultures of *H. ducreyi* (strain S26) and organism-free filtrates

Inoculum	Lesions* on indicated day after inoculation					
	1	2	4	5	7	9
Agar grown organisms (2.5×10^7)	+	+++	+++†	+++	++	+
Broth grown organisms (1.4×10^5)	±	+	+	±	±	±
Centrifuged organisms (1.4×10^6)	±	+	+	+	+	±
Supernatant filtered fluid	–	–	–	–	–	–
Broth only	–	–	–	–	–	–

* See footnote to Table 1.
† Ulceration.

Table 6. Result of inoculating CBA mice with lipopolysaccharide from *H. ducreyi* and *N. gonorrhoeae*

LPS		Lesions* day on indicated after inoculation					
Origin	Dilution	1	2	3	4	5	7
H. ducreyi (strain S26)	neat	++†	++†	++†	++†	++	+
	1:2	++	++	+	++	++	+†
	1:8	+	+	+	+	+	±
	1:20	+	+	±	±	+	±
H. ducreyi (strain 35 000)	neat	++†	++†	++†	++	+++	++
	1:2	++†	++†	++†	++	++	+
	1:8	+	++	++	++	++	++
	1:20	±	+	+	+	+	+
N. gonorrhoeae (strain 4780)	neat	+++†	++†	++	++	++	++
	1:2	++†	++†	+†	++†	+	+
	1:8	+	++	+	+	++	++
	1:20	±	+	+	±	+	±

* See footnote to Table 1.
† Ulceration.

mouse skin was found to be dependent on the dose of organisms, only intradermal inoculation of inocula containing large numbers (> 10^7 cfu) resulting in ulcer formation. This is approximately the same inoculum size used by Hammond *et al.* (1978) in their rabbit intradermal model to differentiate between virulent and avirulent strains of *H. ducreyi*. Since we found that one of the reference strains (IP 54.2) induced lesions in mice, but had not done so in rabbits (Hammond *et al.* 1978), it would appear either that mouse skin is more sensitive than rabbit skin or that other factors, unrelated to virulence of the bacteria in humans or rabbits, are responsible for the production of lesions in mice. Resolution of this issue is important in understanding pathogenesis.

Although viable *H. ducreyi* bacteria were re-isolated from some mouse lesions several days after inoculation, their infrequent recovery compared with persistence of *H. ducreyi* antigen indicated that bacterial multiplication either did not occur or was minimal in murine skin, unlike the situation in the human disease. In fact, the results of subsequent experiments clearly indicated that viable bacteria were not required to produce lesions in mice. Thus, pretreatment of mice with an antibiotic known to be active against the inoculated strain did not inhibit the development of lesions and, furthermore, inoculation of heat-killed bacteria produced lesions of similar character to those resulting from the inoculation of viable bacteria. Such observations raised the question of which bacterial component was most likely to be responsible for producing the lesions. Since LPS had been suggested as a possible virulence factor (Odumeru *et al.* 1987) attention was given to this idea. The development of ulcers following inoculation of organism-associated, but not organism-free, fractions of *H. ducreyi* cultures was in favour of LPS being responsible, and the occurrence of characteristic lesions after inoculation of purified LPS and the demonstration that this was a dose-related response confirmed the important role for endotoxin in the pathogenesis of the lesions in mice. Since *N. gonorrhoeae*, like *H. ducreyi*, also produces rough LPS, it is perhaps not surprising that a

preparation of this bacterial species was also found capable of inducing lesions in these animals. It is feasible, therefore, that on occasion some lesions thought to be chancroid due to *H. ducreyi* are, in fact, due to gonococcal infection and in this context it is of interest that primary infection of the skin by *N. gonorrhoeae* has been shown to result, albeit rarely, in the formation of ulcers or other skin lesions in humans (Neubert *et al.* 1985).

In conclusion, while the mouse skin model of *H. ducreyi* infection described here mimics the human infection both macroscopically and microscopically, the failure of *H. ducreyi* to multiply within the murine lesions limits its use in antibiotic treatment studies and in those on disease progression. However, convincing evidence for the role of LPS in the pathogenesis of ulcers in the mouse suggests that LPS could be an important factor in the development of chancroid and that the murine model could be of value in vaccination studies using various organism-associated fractions and purified LPS as vaccine candidates.

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