CHARACTERISATION OF AN UNUSUAL BACTERIUM ISOLATED FROM GENITAL ULCERS

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SUMMARY. The preliminary characterisation of an unusual gram-negative bacillus isolated from genital ulcers in Swaziland is reported. Like *Haemophilus ducreyi*, it is an oxidase positive, nitrate-reductase-positive gram-negative rod that forms streptobacillary chains in some circumstances; it was therefore called the “ducreyi-like bacterium” (DLB). Distinguishing features of DLB are production of α -haemolysis on horse-blood agar, stimulation of growth by a microaerophilic atmosphere and by a factor produced by *Staphylococcus aureus*, a strongly positive porphyrin test, and a remarkable ability to undergo autolysis. DLB has a guanine+cytosine value of *c.* 50 mole% but it cannot be classified, even at the genus level, until more taxonomic data are obtained.

INTRODUCTION

Haemophilus ducreyi is an important cause of genital ulcers in many developing countries. A reliable serological diagnostic test is not available and microscopic examination of gram-stained smears from lesions is useful in only a limited number of cases (Kampmeier, 1977). A definitive diagnosis of chancroid can be made only by isolation and identification of *H. ducreyi* from the lesions. However, this may be complicated by the presence of bacteria that can be confused with *H. ducreyi* (Aoki *et al.*, 1952; Leibovitz, 1954; Deacon *et al.*, 1956; Kilian, 1976) and other micro-organisms of unknown pathological significance (Chapel *et al.*, 1978). During a recent study of genital ulcers in Swaziland, an unusual gram-negative bacillus was isolated from several lesions that clinically resembled chancroid on culture media used for isolation of *H. ducreyi*. Morphologically these isolates resembled *H. ducreyi* and they have, therefore, been called ducreyi-like bacteria (DLB). In this paper the preliminary characterisation of these isolates is reported.

MATERIALS AND METHODS

Patients. One hundred and fifty-five consecutive patients with genital ulcers attending the

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clinic for sexually transmitted diseases at the Outpatients' Department of Mbabane General Hospital, Mbabane, Swaziland, were included in the study.

Primary isolation of DLB. After thorough cleaning of the genital ulcer with sterile physiological saline, material was collected from its base with a calcium alginate swab (Inolex, code 60-50-15) and seeded directly on to two solid culture media: (i) Diagnostic Sensitivity Test (DST) Agar (Oxoid) with horse blood 5%, IsoVitaleX (Baltimore Biochemical Laboratories) 1% and vancomycin 3 µg/ml; and (ii) Hammond's medium which consists of Gonococcus (GC) Agar (Difco), with haemoglobin (Difco) 1%, IsoVitaleX 1% and vancomycin 3 µg/ml (Hammond *et al.*, 1978). All plates were incubated at 35°C in candle jars and examined daily for 10 days. Isolates were subcultured every 72 h on to the medium originally used for primary isolation and they were lyophilised for storage and transport after less than 15 passages.

Haemophilus ducreyi. Eight strains of *H. ducreyi*, for comparative studies, were kindly provided by the Pasteur Institute, Paris, Professor A. Ronald, Winnipeg and Dr W. Sturm, Amsterdam.

Growth and morphological characteristics. Anaerobic and microaerophilic conditions were obtained with hydrogen and carbon dioxide generators (GasPak, BBL) in GasPak jars (BBL) with and without catalyst. Requirement for X and V factors were determined with impregnated filter-paper strips (BBL) on Columbia Agar (BBL). Enhancement of growth by staphylococcus filtrate (SF) was studied on DST Agar containing horse blood 5% with and without SF 10%. SF was prepared by incubating *Staphylococcus aureus* strain ATCC 25923 in Trypticase Soy Broth (Bio-Mérieux, Marcy-l'Etoile, France) at 37°C for 48 h with vigorous stirring; cultures were centrifuged at 1500 *g* for 1 h and the supernate was sterilised by membrane filtration (pore size 0.22 µm). The presence of streptobacillary chains was determined by examination of gram-stained smears of fluid that had condensed on DST-Agar slopes after incubation for 24 and 48 h at 35°C in a microaerophilic atmosphere.

Biochemical tests. The ability of strains to produce hydrogen sulphide was tested with lead acetate paper strips (Merck Chemicals), and National Collection of Type Cultures (NCTC) micromethods (Clarke and Cowan, 1952; Shaw and Clarke, 1955) were used in tests for reduction of nitrate and production of indole, urease, phenylalanine deaminase, lysine and ornithine decarboxylase and arginine dihydrolase. Carbohydrate fermentation reactions were tested by the method of Brown (1974), the oxidase reaction by Kovács's method (Kovács, 1956) and porphyrin production by the technique of Kilian (1974). The ability of strains to produce β-galactosidase was tested with paper disks containing o-nitrophenyl-β-D-glycopyranoside (ONPG discs, Bio-Mérieux; Le Minor and Ben Hamida, 1962) and alkaline phosphatase by the method of White and Pickett (1953).

Preparation of bacterial DNA and measurement of base composition. Large quantities of DLB were grown in petri dishes on DST agar with horse blood 5% and SF 10%. Cultures were incubated at 35°C in a microaerophilic atmosphere for 24 h before removal of bacterial colonies. Bacterial DNA was prepared by the method of Marmur (1961). The base composition of the total genome DNA was measured by the thermal denaturation method (De Ley and Van Muylem, 1963) and calculated by the equation of Marmur and Doty (1962) as modified by De Ley (1970).

Electron microscopy. DLB were grown on Columbia Agar with horse blood 5% and Biosate Peptone (BBL) 3%. After incubation at 35°C in a microaerophilic atmosphere for 18 h intact bacterial colonies with small blocks of underlying agar were cut. These were fixed by the method of Ryter and Kellenberger (1958) and embedded by the method of Spurr (1969). Thin sections (50 nm) were stained with uranyl acetate and lead acetate and examined at 80 kV accelerating voltage in a Jeol electron microscope.

RESULTS

DLB were isolated from the genital ulcers of 25 of 155 patients (16.1%). Nineteen isolates survived transport to Belgium.

DLB were isolated on both media used for primary isolation; 15 of the surviving strains were initially isolated on the supplemented DST agar, while

another four strains were isolated on Hammond's medium. On both media DLB appeared as small translucent, punctiform colonies after incubation for 2-7 days.

At the beginning of these studies, two factors were found that enhanced the growth of these organisms: (i) a microaerophilic atmosphere which gave better results than either air plus 5% CO₂ or a strictly anaerobic atmosphere, and (ii) the presence of contaminating colonies of *S. aureus*. This second factor led to the preparation of SF, which was added to the agar medium in various amounts. An SF concentration of 10% produced optimal growth for all strains.

The growth characteristics of DLB and *H. ducreyi* are shown in table I. Although they differ in many respects, confusion may occur because of the common site of primary isolation and the frequent finding of gram-negative bacilli in smears from genital ulcers. After incubation for 48 h, the colonies of DLB on Columbia Agar with horse blood 5% were smooth and translucent, produced a yellow pigment and exhibited a discrete α -haemolysis. In contrast, colonies of *H. ducreyi* were opaque, grey, rough and β -haemolytic. Unlike *H. ducreyi*, DLB did not require V factor for growth, indicating that the growth factor in SF is not nicotinamide dinucleotide. The growth factor in SF was heat stable (121°C for 1 h), unaffected by trypsin (0.5% for 30 min at 37°C) and may be present in Columbia Agar (BBL) with horse blood 5%, because growth was almost maximal on this medium and the addition of SF 10% resulted in only slight enhancement of growth. Biosate peptone, a major constituent of BBL Columbia Agar, at a final concentration of 4%, enhanced growth significantly. Moreover, DLB did not require X factor for growth, a finding that was confirmed by a strongly positive porphyrin test. *H. ducreyi* was consistently negative in the porphyrin test.

Both *H. ducreyi* and DLB formed streptobacillary chains, but those of DLB were much shorter than those of *H. ducreyi*. However, the most striking feature of DLB was its ability to undergo autolysis. Gram-stained smears of 48-h cultures showed almost exclusively lysed bacterial cells (fig. 1b) and even

TABLE I
Growth characteristics of DLB (8 strains) and *Haemophilus ducreyi* (19 strains)

Character	Reaction or property of	
	<i>Haemophilus ducreyi</i>	DLB
Morphology of colonies	Grey, opalescent, pleomorphic, rough	Translucent, homogeneous, smooth.
Chain formation	Long chains	Short chains
Motility	—	—
Maximal growth after	72 h	48 h
Lysis after 48 h	—	+
Favourable atmosphere	Increased CO ₂ (5%)	Microaerophilic
Temperature range for growth	32-36°C	25-42°C
V-factor requirement	—	—
X-factor requirement	+	—
SF requirement	—	+

DLB = ducreyi-like bacteria; SF = staphylococcus filtrate; — = all strains negative; + = all strains positive.

Fig. 1.—Gram-stained smears of DLB after growth on: a. Columbia blood agar after 24 h; b. Columbia blood agar after 48 h; c. Columbia blood agar with Biosate Peptone 4% after 24 h; d. Columbia blood agar with 4% Biosate peptone after 48 h.

after 24 h, many bacteria appeared swollen, with indistinct borders (fig. 1a). Between 24 and 48 h, the number of viable organisms consistently dropped by a factor of $c. 10^3$. This autolysis could be prevented in part by raising the concentration of Biosate Peptone in the medium to 4%. After incubation for 24 h the bacteria appeared healthier, although still rather pleomorphic (fig. 1c), and this appearance was maintained at 48 h although some bacteria had lysed (fig. 1d).

Attempts to grow DLB in liquid media were disappointing. In Columbia Broth with horse blood 5%, saponin 1% and Biosate Peptone 3%, the viable

Fig. 2.—Ultra-thin section through colony of DLB showing bacteria that contain several membrane-enclosed cytoplasmic 'packages' within one cell-wall structure. Inset: detail of cell-wall structure.

count increased from 10^4 cfu/ml to 5×10^7 cfu/ml after 24 h but declined rapidly on further incubation.

The spontaneous lysis of DLB made electronmicroscopic studies difficult and early attempts yielded pictures of empty shells. However, when the bacteria were grown on media supplemented with Biosate Peptone 4%, better results were obtained (fig. 2). The cell wall is characteristic of most gram-negative organisms; an "outer membrane" is separated from the cytoplasmic membrane by a more electron-translucent zone. There was no evidence of cell division. However, many cells showed expansions of the cytoplasmic membrane, delineating vast compartments within the cell. Some cells contained two of these structures, but there was no associated pinching of

TABLE II
Biochemical characteristics of 19 strains of DLB

Test	Result	Test	Result
Production of:		Production of:	
indole	—	arginine dihydrolase	+/-
urease	—	ornithine decarboxylase	+/-
porphyrin	++	Fermentation of:	
hydrogen sulphide	+	glucose	—
catalase	—	xylose	+
oxidase	+	lactose	—
β galactosidase	—	sucrose	—
β lactamase	—	mannitol	—
phenylalanine deaminase	—	deoxyribose	—
alkaline phosphatase	—	Reduction of nitrate	+
lysine decarboxylase	—		

— = All strains negative; + = all strains positive; ++ = all strains strongly positive; +/- = variable (66.6% of 39 tests for ornithine decarboxylase and 77.9% of 77 tests for ornithine decarboxylase were positive; all strains gave at least one positive result in each test).

the cell wall. The significance of this compartmentation is not evident. In view of the spontaneous lysis of these bacteria, many lysed cultures were examined for evidence of infection with bacteriophages. No such evidence was obtained.

The biochemical characteristics of DLB shown in table II. The oxidase reaction was slowly positive, and production of hydrogen sulphide could usually be demonstrated after 2–3 days. The porphyrin test was strongly positive after 4 h and nitrate reduction was weakly positive. Tests for arginine dihydrolase and ornithine decarboxylase were usually positive, but the results were not uniformly reproducible.

The extraction of DNA from DLB proved difficult, probably as a result of the tendency of the bacteria to undergo spontaneous lysis. The DNA-base composition of two strains was determined; they had guanine + cytosine values of 49.6 and 50.6 mol%.

DISCUSSION

Although on morphological grounds DLB appear to belong to the genus *Haemophilus*, they do not meet the classical criteria established for this genus (requirement of X and V factors), nor the criteria proposed by Kilian (1976), i.e., reduction of nitrate and production of alkaline phosphatase. Moreover, the DNA-base composition of DLB (guanine + cytosine *c.* 50 mol%) differs from that for *Haemophilus* spp. (38–42 mol%; Zinnemann and Biberstein, 1974). Classification of DLB at the genus level remains speculative, and more comparative data are required. However, the uniformity of growth, morphological and biochemical characteristics of the DLB isolates tested indicates that they are a homogenous group of bacteria that could belong to a single species.

All the strains described in this study were isolated from genital ulcers of presumed venereal origin and more investigation of the pathogenicity of these organisms and their possible sexual transmission is required before an aetiological role can be assigned. However, because DLB have been isolated from a large number of genital ulcers on a selective medium previously used for

isolation of *H. ducreyi*, it is important that they be differentiated from *H. ducreyi*.

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