Studies in venereal disease

I. Isolation of L-phase organisms of N. gonorrhoeae from patients with gonorrhoea*

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In some cases of acute urethritis no microbial cause for the inflammation can be found.

L-phase organisms have been isolated from various clinical infections (Mattman, 1968). They may be the aetiological agents in some cases of urethritis in which no causative organism can be isolated. This was suggested, by Holmes, Johnson, and Floyd (1967a) and Holmes, Johnson, Floyd, and Kvale (1967b), in an earlier report in which they proposed that penicillin treatment might induce the gonococcal L-form and that these L-forms might cause post-gonococcal urethritis. No clinical evidence was presented, but Holmes, Gutman, Belding, and Turck (1971) have reported a case in which growth of gonococcal L-phase was obtained from 'sterile' synovial fluid.

No reports have so far been published of the isolation of the gonococcal L-phase from the genital area, and this paper describes methods and media for isolating the gonococcal L-phase.

L-phase organisms reverting to *N. gonorrhoeae* were grown from patients who attended with suspected gonorrhoea. One patient who gave a pure growth of L-phase organisms reverting to *N. gonorrhoeae* is described in detail.

Material and methods

During the spring of 1972 a total of 1,540 cultures were taken from the urethras of 609 men and the cervixes of 408 women attending the Venereal Disease Unit in Uppsala with suspected gonorrhoea. Prostatic massage was performed on 113 men recently treated for gonorrhoea with penicillin or tetracycline. Prostatic massage was also performed on forty men strongly suspected of having gonorrhoea although earlier urethral cultures had been negative. In all these cases urethral specimens were also taken before the prostatic massage, which was carried

out after the patients had passed urine. The clinical material is to be presented in detail elsewhere (Wallin, to be published).

MEDIA

The fluid *TA-medium* used for transporting the specimens to the laboratory was trypticase soy broth with sucrose 5 per cent. (w/v). For conventional cultures of the specimens the medium used was Gc agar base (Difco) 36 g., haemoglobin 10 g., Isovitalex 1 per cent. (v/v), aqua dest. to 1,000. For the isolation of L-phase organisms the following G-medium was developed: brain heart infusion (Difco) 37 g., sucrose 100 g., 25 per cent. yeast extract 20 ml., horse serum 100 ml., Isovitalex 1 per cent. (v/v), 2 per cent. haemin solution 1.6 per cent. (v/v), Noble Agar 12 g., and aqua dest. to 1,000.

This medium is translucent and permits inspection of the plates under the high magnification which is necessary for early detection of the minute L-phase colonies of *N. gonorrhoeae*.

CLINICAL AND MICROBIOLOGICAL PROCEDURE

Specimens were obtained with charcoal-coated cotton swabs. The swabs were then put into a protective medium (TA-medium) and transported to the laboratory where they were inoculated on arrival on to conventional media and on to osmotically stabilized media (G-plates). They were then incubated at 37°C. in 10 per cent. CO₂ and 95 per cent. humidity and read after 48 hrs. The G-plates were read in a stereo microscope (Zeiss, Model Stereo 4) at magnifications between × 40 and × 100; they were inspected again for suspected L-colonies after 3, 4, and 5 days. Suspected L-colonies were cut out and subcultured twice a week on G-plates with the push-agar-block technique until reversion took place. The reverting bacteria were then identified according to current bacteriological standard techniques. The final identification of *N. gonorrhoeae* was made by a fluorescent antibody technique by the method of Danielsson (1965).

Results

197 isolations of *N. gonorrhoeae* were made, 140 from males and 57 from females. In 24 (12 per cent.) of these specimens L-phase organisms reverting to

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*L-phase organisms are defined as 'independent growth variants of bacteria that lack a rigid cell wall and have a potential reversibility to the parent strain' (Hijmans, van Boven, and Glasener, 1969).

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TABLE I Numbers of cultures with growth of *N. gonorrhoeae* in bacterial and L-phase from patients with gonorrhoea

Figures within brackets denote total number of specimens in which L-phase cultures were possible

Medium used and type of growth	Source			Total
	Females		Males	
	Cervix	Urethra	Prostatic secretion	
Growth of gonococcal L-phase	5 (50)	19 (126)	— (3)	24
Growth of gonococcal L-phase in pure culture	—	1	—	1
L-phase cultures destroyed by heavy bacterial overgrowth	7	11	—	18
Total <i>N. gonorrhoeae</i> L-phase	5	20	0	25
Growth of <i>N. gonorrhoeae</i>				
On osmotically stabilized media only	2	6	1	9
On conventional media	55	131	2	188
Total <i>N. gonorrhoeae</i> in bacterial phase	57	137	3	197

N. gonorrhoeae were also isolated (Table I); nineteen were isolated from males and five from females. In nine cases (4.5 per cent.) *N. gonorrhoeae* was not isolated on conventional media but grew as bacteria or in mixed L-phase and bacterial phase on osmotically stabilized plates only.

In three of 113 patients treated for gonorrhoea, *N. gonorrhoeae* was isolated from urethral material obtained by prostatic massage. One of the patients has been treated 4 days before with two doses of 1 g. ampicillin, and one with doxycycline, 200 mg. the first day and then 100 mg. daily for 8 days. In both *N. gonorrhoeae* was also isolated from the urethra before prostatic massage. The third patient was treated with epicillin (6-(D-2-amino-2-(1,4-cyclohexadiene-1-yl)acetamidol)-penicillinaic acid) 2.5 g. + probenecid 1g.; 4 days later *N. gonorrhoeae* was isolated from a urethral swab only after prostatic massage and only on the osmotically stabilized medium.

None of the three patients had strains resistant to penicillin or tetracycline. All of them emphatically denied the possibility of re-infection.

The forty men strongly suspected of having gonorrhoea in spite of earlier negative urethral cultures, all had negative prostatic fluid cultures.

In one patient L-phase organisms in pure culture reverting to *N. gonorrhoeae* were isolated. The L-phase organisms had typical colonial morphology (Figs 1 and 2, overleaf), did not grow on conventional media, and did not stain by Gram's method.

A description of this case is given below.

Case report

A 23-year-old man with no previous history of urethritis attended the Venereal Diseases Clinic complaining of

urethral discharge for 7 days and burning on micturition for 3 days. He had had sexual intercourse with one partner without a condom 14 and 3 days before he attended. He had taken no antibiotics for the past month.

Examination

There was moderate purulent urethral discharge. Smears stained with methylene blue showed many leucocytes but no intra- or extracellular diplococci. A specimen was taken with a charcoal-coated swab and sent for conventional and L-phase cultures. No treatment was given.

Course (Table II)

When he returned 1 week later his symptoms were unchanged. Smears still showed a moderate amount of leucocytes but no diplococci. Conventional culture on the first occasion was negative, but the L-phase culture yielded a growth of L-phase organisms in pure culture which reverted to *N. gonorrhoeae*. The result of the L-phase culture was not known to the clinician at the second visit, and the patient was considered to have non-specific

TABLE II Course of symptoms and cultures

Date	13 Jan.	20 Jan.	2 Feb.	8 Feb.
Clinical symptoms
Conventional culture	negative	negative*	negative	negative
L-phase culture	positive	not done	contaminated	negative
Treatment (Doxycycline)

*This specimen was sent in Stuart's medium

FIG. 1 Two L-phase colonies of *N. gonorrhoeae*, 3 days old, in the vicinity of a colony of *Staphylococcus epidermidis*. Original magnification: $75 \times$ (Zeiss Stereo 4)

FIG. 2 Phase-contrast micrograph of a young (48 hrs) L-phase colony of *N. gonorrhoeae* (Zeiss phase-contrast microscope). Original magnification: $320 \times$

urethritis. This was treated with doxycycline (200 mg. the first day and then 100 mg. daily for 8 days).

The patient was examined twice more, 20 and 26 days after his first visit. There was a slight urethral discharge, and smears showed only a few leucocytes and no diplococci; on each occasion cultures on both conventional and osmotically stabilized media were negative.

Consort

The patient's sexual partner, a 24-year-old woman, was examined twice after the recovery of the gonococcal L-phase organisms. She had no history of gonorrhoea or other genital or urinary-tract infection. She denied having had any other recent partner and had not been taking drugs of any kind. Examination before and just after

menstruation gave normal results. Smears and cultures on conventional media of material from the cervix, urethra, and rectum were negative, as was a cervical specimen on the osmotically stabilized medium.

Discussion

In a recent electron microscope study, Ovčinnikov and Delektorskij (1971) demonstrated the presence of 'L-forms' of gonococci in urethral scrapings from patients with untreated gonorrhoea. As 'L-forms' may be induced by antibiotics interfering with the cell wall synthesis and also by leucocyte lysozyme action (Amano, Seki, Fujikawa, Kashiba, Morioka, and Ichikawa, 1956), one would expect L-phase organisms of *N. gonorrhoeae* to be induced in some cases of gonorrhoea whether treated or not. The L-phase organisms might then survive *in vivo* because of the acidity (Gnarpe and Edebo, 1970) and the increased osmolality of the purulent environment, although they would undergo lysis when cultured on conventional hypotonic media. The data presented in this report confirm the findings of Ovčinnikov and Delektorskij that L-phase organisms of *N. gonorrhoeae* do exist *in vivo*. Our patient with a pure growth of gonococcal L-phase organisms indicates that L-phase organisms of *N. gonorrhoeae* may be the cause of some symptomatic cases of acute urethritis although they give negative cultures on conventional media. L-phase organisms reverting to *N. gonorrhoeae* were isolated from 12 per cent. of the specimens from our patients with gonorrhoea, mostly in mixed bacterial and L-phase growth. In 4.5 per cent. of the specimens *N. gonorrhoeae* was isolated on osmotically stabilized media only. These isolates might represent L-phase organisms *in vivo* which reverted to the bacterial phase of growth on osmotically stabilized media within 48 hours. It therefore seems to be advisable to use osmotically stabilized media as well as the conventional media for culture in order to reveal the true aetiology of some cases of acute urethritis.

Further investigations are now being undertaken in an attempt to establish the actual incidence of gonococcal L-phase organisms in acute and relapsing genital infections.

Summary

Methods and media for isolating L-phase organisms of *N. gonorrhoeae* are described. In about 12 per cent. of patients with gonorrhoea, L-phase organisms of *N. gonorrhoeae* were isolated from the urethra or the

cervix. Some of the specimens of *N. gonorrhoeae* were isolated only on osmotically stabilized media, which therefore seem to be of importance in detecting gonorrhoea. The case history of one patient who gave a pure growth of gonococcal L-phase organisms is presented.

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Etude sur les maladies vénériennes

I. Isolement de *N. gonorrhoeae* en phase L* chez des malades atteints de gonococcie.

SOMMAIRE

On décrit les méthodes et les milieux permettant d'isoler des formes L de *N. gonorrhoeae*. Des formes L de *N. gonorrhoeae* furent isolées de l'urètre ou du col chez environ 12 pour cent des gonococciques. Quelques uns des spécimens de *N. gonorrhoeae* furent isolés uniquement sur les milieux osmotiquement stabilisés, qui paraissent ainsi avoir de l'importance pour la détection de la gonococcie. On présente l'observation d'un malade chez lequel on obtint une culture pure d'organismes gonococciques en phase L.

*Les organismes en phase L sont définis comme des 'variants indépendants de culture de bactéries dépourvus de membrane cellulaire rigide et qui peuvent redonner la souche originelle'. (Hijmans, Van Boven et Cleansener, 1969).