Cell-wall defective variants of *Treponema pallidum**

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Microbial variants possessing defective cell walls have attracted considerable interest in recent years, because of the possibility of their having a role in the pathogenesis of disease. There is evidence that, in the infected host, a bacterium may undergo damage to its cell wall and assume a temporarily altered form with altered properties, yet remain capable of reversion to the parent bacterium. The characteristics of such microbial variants (consistent with damage to or deficiency of cell wall structure) are alterations in morphology, physiology, and cultural characteristics, including a Gram-negative non-rigid outer covering, resistance to penicillin, and colonial down-growth below the agar surface (McGee and Wittler, 1969). Most bacteria with damaged cell walls can exist in at least four phases, the protoplast phase, spheroplast phase, transitional phase, and L-phase, each differing from the others and from the classical bacterial phase (McGee, Wittler, Gooder, and Charache, 1971).

The nature of the disease pattern in syphilis, with its periodic course, frequent negative or equivocal results of microscopic examination, and resistance to treatment, suggested the possible existence of altered microbial forms to early investigators. Levaditi (1941), using silver-impregnation methods, observed granule-like forms which he assumed represented involutinal changes in *Treponema pallidum* in material from syphilitic lesions of men, rabbits, and mice. He postulated that a granular

stage in human infection existed in which treponemes were not demonstrable and that this was responsible for latent and late syphilis. Similar observations were reported by DeLamater (1952), DeLamater, Haanes, and Wiggall (1951a), and DeLamater, Haanes, Wiggall, and Pillsbury (1951b), who described various stages of granules, buds, and cysts, apart from the mature spirochaetes.

In a review of possible filterable forms of treponemes, Klieneberger-Nobel (1951) postulated an alternation between the treponemal phase and a granular phase, which, she assumed, might represent the regenerative or L-phase, the latter being resistant to therapy and responsible for periods of latency.

Rose and Morton (1952) concluded that, although morphological alterations occur in the growth of cultivable treponemes, they could find no evidence supporting the hypothesis of a life cycle. Hardy and Nell (1961) produced similar alterations in dead material by variations in osmotic pressure and believed these represented degenerative forms rather than an intermediate stage of a life cycle. Ustimenko (1965) reported the production of 'L-forms' of *T. pallidum* *in vitro* with the aid of penicillin or immune serum. The present study was undertaken to determine whether the production of cell-wall defective morphological variants of *T. pallidum* could be confirmed, to identify the particular variant phases produced, and to determine whether such variant forms were viable.

Material and methods

ORGANISMS

The organisms employed were *T. pallidum*, the Nichols (non-cultivable strain), pathogenic for rabbits and carried by passage in rabbit testes, and the non-pathogenic *T. pallidum* strains, Kazan 2, Kazan 5, Kazan 8, Reiter, and Nichols (cultivable).

STANDARD GROWTH MEDIA

The standard fluid medium contained Bacto Heart Infusion Broth (2.5 per cent. w/v), magnesium sulphate

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($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (0.01 per cent. w/v), Bacto yeast extract (0.5 per cent. w/v), sodium thioglycollate (0.076 per cent. w/v) (Nutritional Biochemical Corporation, Cleveland, Ohio), Bacto agar (0.1 per cent. w/v), sucrose (10 per cent. w/v), and filter-sterilized inactivated horse serum (20 per cent. v/v). The pH was adjusted to 7.4 before the addition of sucrose or serum. Incubation was at 37°C. in polycarbonate anaerobic jars containing BBL Gaspak, hydrogen-carbon dioxide generators.

The standard solid medium contained the same constituents, plus agar in a final concentration of 1.2 per cent. (w/v).

PENICILLIN GRADIENT PLATES

Penicillin-free plates (100 × 15 mm.) contained standard solid growth medium with 20 per cent. (v/v) horse or rabbit serum. In these a 5 × 50 mm. trough was made at one end of the plate as described by Roberts and Wittler (1966). The plates were then inoculated with 0.3 ml. of a 24-hr broth culture of the treponeme. After distributing the inoculum over the surface of the agar, 0.25 ml. penicillin in varying concentrations from 0.01 to 100,000 u./ml. was placed in the trough. The plates were incubated anaerobically at 37°C. and examined on alternate days for 1 week and thereafter every 3 or 4 days up to 1 month. Agar to agar transfers from penicillin-containing gradient plates to penicillin-free solid medium were done by cutting agar blocks with a surface of 1 cm². from an area of growth. The block was inverted onto a fresh plate and streaked with a small metal spatula one-half of the distance across the plate.

Agar to fluid medium passages were also done by transfer of agar blocks containing growth to penicillin-free fluid medium.

PENICILLIN-CONTAINING PLATES

These were prepared by adding from 0.01 to 10,000 u./ml. final concentration of penicillin and 20 per cent. (v/v) final concentration of sterile horse or rabbit serum to the solid medium immediately before pouring the agar into 60 × 15 mm. and 100 × 15 mm. sterile plastic Petri dishes.

METHICILLIN

This was prepared as a stock aqueous solution containing 100,000 u./ml. for testing in standard fluid medium.

PENICILLINASE

The potency of penicillinase was checked by determining the concentration that would inhibit the effect of 1 u./ml. of penicillin G on *Staphylococcus aureus* (Oxford strain) kindly supplied by Miss Sylvia Cary, Walter Reed Army Institute of Research.

LYSOZYME

Lysozyme (Sigma Chemical Company, St. Louis, Mo.) in a final concentration of 100 to 200 µg./ml. in serum-saline (one part horse serum to nine parts saline) was freshly prepared for each experiment. The solution was filter-sterilized through a 0.22 µ Millipore filter.

24-hr-old cultures of the treponeme were harvested by centrifugation, resuspended in 5 ml. serum-saline (1 : 10 dilution of horse serum in saline) and centrifuged again for 5 min. The pellet was suspended in 2 ml. standard fluid medium without agar containing 20 per cent. (v/v) horse serum. Half of this suspension (1 ml.) was treated with lysozyme (1 ml.) solution containing 200 µg./ml.; the other half of the suspension was used as a control for viable counts. The lysozyme-treated suspension was incubated at 37°C. From this suspension after 30 min. and after 1 hr serial 10-fold dilutions were made. From each dilution tube, 1 ml. was transferred into each of four fresh tubes of standard fluid medium, incubated at 37°C. for 48 hrs and then passed through at least five serial subcultures in fresh fluid medium without lysozyme. The fifth subculture was then grown in fluid medium, harvested, and re-suspended, and 1 ml. of the suspension was re-exposed to 1 ml. lysozyme solution (100 µg./ml.). The whole procedure was repeated and the culture exposed a third time to lysozyme (100 µg./ml.). Plates were inoculated from each transfer.

VIABLE CELL COUNTS

At least eight tubes of 24-hr cultures of the treponeme in fluid medium were harvested by centrifugation at 8,000 r.p.m. for 5 min. The pellet was washed once in 5 ml. serum-saline (1 : 10 dilution of horse serum in saline) and centrifuged again for 5 min. The pellet was suspended in 2 ml. of standard fluid medium without agar, to which 20 per cent. (v/v) horse serum had been added. From this suspension serial 10-fold dilutions were made in the initial three tubes and 2-fold dilutions in subsequent tubes. From each dilution tube, 1 ml. was transferred into each of four fresh tubes of standard fluid medium. Tubes were examined for the presence of motile organisms in wet mount preparations, and an end-point was calculated according to the method described by Cochran (1950). From each dilution tube, 0.1 ml. samples were plated on each of four plates on the standard solid medium containing 20 per cent. (v/v) horse serum. Plates were read for presence or absence of colonial growth after 6 to 7 days of incubation. Determination of viable units in cultures before and after treatment with lysozyme and penicillin were done in a similar manner.

LIGHT MICROSCOPY

Cultures in fluid medium were examined by various microscopic techniques.

(1) *Wet mounts*

These preparations were examined by phase contrast and by dark-field microscopy for evaluation of the motility of treponemes.

(2) *Giemsa staining*

Air-dried methanol-fixed smears were stained with 2 per cent. (v/v) Giemsa stain in phosphate buffer at a pH of 6.8, followed by dehydration as described by Klieneberger-Nobel (1962).

(3) *Acridine orange staining of live cultures* (Vital staining)
Acridine orange (Fisher Scientific Company, N.Y.) was

prepared as a stock 1 : 5,000 solution in distilled water. Based on the technique described by Rothstein (1958), 0.9 ml. of an actively growing culture in fluid medium was mixed with 0.1 ml. of the 1 : 5,000 solution of acridine orange. A small drop of the mixture was placed on a clean glass slide and covered with a 22 × 40 mm. cover slip. The slides were examined with a Leitz Fluorescence Orthoplan microscope equipped with an HBO 200 mercury bulb as the source of the blue-violet fluorescence excitation light. A 12 volt, 60 watt tungsten bulb was used for the standard dark-field illumination. The wave-lengths for the barrier filters were 490 or 570 mμ. The exciter filter was a 2 mm. thick BG filter.

(4) *Acridine orange staining for fixed smear*

Smears of actively growing cultures were fixed in equal parts of an ether-95 per cent. ethanol mixture for 15 min. The methods of staining described by Von Bertalanffy and Bickis (1956) and by Dart and Turner (1959) were used. The acridine orange solution and the buffers used were previously filter-sterilized through a 0.22 μ Millipore filter. Slides were examined by the Leitz fluorescence microscope.

(5) *Gram staining*

This was done on methanol-fixed smears.

EXAMINATION OF COLONIES

(1) *Unstained*

The colonies were examined *in situ* with a colony microscope (magnification up to 90 ×).

(2) *Stained*

Dienes' method (Madoff, 1960), employing methylene blue and azure II, was used to stain colonies *in situ* on agar.

ELECTRON MICROSCOPY

(1) *Negative stain*

Actively growing 1 to 4 day-old cultures in fluid medium or a suspension of the pathogenic treponeme obtained from infected rabbit testicles were centrifuged at 8,000 r.p.m. for 10 min. and the pellet suspended in 1 ml. normal saline. A large drop of suspension was placed on a piece of dental wax. Grids previously coated with 1 per cent. (v/v) collodion were placed on the surface of the drop of culture suspension for 30 sec. The grids were then stained with 1 per cent. (w/v) phosphotungstic acid at pH 7.0 for 30 sec. The excess acid was removed with filter paper. The grids were examined with a Siemens IA Elmiskop at 60 Kv.

(2) *Ultrathin sections*

A suspension of organisms was prepared by the method used for negative stains. A modification of the standard method of fixation reported by Kellenberger, Ryter, and Séchaud (1958) was used. Instead of suspension of the pellet in melted agar as described by these authors, the pellet was placed directly in 0.5 per cent. (w/v) uranyl acetate for 2 hrs, followed by standard procedures for dehydration and embedding (Luft, 1961). Thin sections were cut on a Reichert OM U₂ ultramicrotome and stained with uranyl acetate and lead citrate.

Results

The morphological and cultural characteristics of the parent *T. pallidum* strains were studied and compared with those of variant forms of the organism induced by penicillin and lysozyme.

Ultrastructure of T. pallidum

Although pathogenic and nonpathogenic treponemes are indistinguishable by light microscopy, certain ultrastructural differences between these organisms have been described (Ovčinnikov and Delektorskij, 1966, 1969). These observations on the ultrastructure of the parent organisms had to be verified before investigating morphological changes in the variant forms under various experimental conditions. Therefore the nonpathogenic treponemes, Kazan 2, Kazan 5, Kazan 8, and Reiter strains of *T. pallidum* and the pathogenic Nichols (non-cultivable) strain obtained from infected rabbit testes were examined. The ultrastructural observations made were in accord with the descriptions of Ovčinnikov and Delektorskij (1966, 1969), Jepsen, Hougen, and Birch-Andersen (1968), Pillot and Ryter (1965), and Pillot, Ryter, and Ginger (1966). In the pathogenic (Nichols strain) and nonpathogenic treponemes, the axial fibrils were twisted around the cytoplasmic helix (Fig. 1). Usually three fibrils were present at the region of insertion, where the fibrils appeared to bend, forming small hook-like structures that were inserted into basal granules or pits (Figs 1 and 2). In cross-sections the fibrils lay between the cell wall and the cytoplasmic membrane. There were usually six fibrils centrally in the pathogenic organisms and four to eight in the nonpathogenic strains studied. The cell wall and cytoplasmic membrane were each composed of trilaminar zones with outer and inner electron dense layers and an intermediate lighter area. There appeared to be some distinct morphological differences between the pathogenic Nichols and the nonpathogenic Kazan and Reiter strains. The pathogenic Nichols treponemes had tapering acuminate extremities (Fig. 1) and fibrils which inserted into basal granules that were at some distance from the extremities of these organisms. The nonpathogenic treponemes (Fig. 2) displayed blunt, somewhat rounded extremities and fibrils that inserted fairly close to the extremities of these organisms.

Growth of treponemes

This was observed using the standard fluid and solid media that were modified by varying the concentration and type of serum used, the concentration of agar used, and by the omission of osmotic stabilizers.

FIG. 1 *Electron micrograph of pathogenic T. pallidum (rabbit Nichols strain). The extremity is tapering and slightly elongated. Axial fibrils are twisted around the cytoplasmic helix and appear to insert some distance from the extremity. Negative contrast. $\times 60,000$*

(1) IN FLUID MEDIUM

The Kazan 8 strain showed moderate growth in 24 to 48 hrs in fluid medium containing either horse or rabbit serum in a concentration of 10 per cent. (v/v). When the concentration was increased to 20 per cent. (v/v), growth was more satisfactory with horse serum than with rabbit serum. Growth of classical treponemal forms was unaltered when osmotic stabilizers magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and/or sucrose were omitted from the medium.

(2) ON SOLID MEDIUM

When preincubated plates were inoculated with 24 to 48-hr actively growing cultures, a few colonies could be detected as early as 48 hrs, but maximum

FIG. 2 *Electron micrograph of a Reiter treponeme. The extremity is rounded. Axial fibrils insert fairly close to the extremity. Negative contrast. $\times 60,000$*

growth occurred at 6 to 7 days. The colonies were raised, convex, and discrete, measuring 0.5 to 0.8 mm. in diameter. Agar concentrations varying from 1.2 to 2 per cent. (w/v) were tested for ability to support treponemal growth. A final agar concentration of 1.2 per cent. (w/v) was found to be optimal for colony formation.

Penicillin effect on treponemes in fluid medium

The morphology of treponemes and variant forms at varying time intervals and in different concentrations of penicillin was studied by means of wet mounts, Giemsa stains, acridine orange, and by electron microscopy. The effect of penicillin in concentrations of 0.01 to 10,000 u./ml. was tested on treponemes of the Kazan 8 strain grown for 24 hrs in standard growth medium containing 20 per cent. (v/v) horse serum. Use of the standard fluid medium containing osmotic stabilizers ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, sucrose) resulted in better growth of variant forms of treponemes than the same medium without these stabilizers.

WET MOUNTS, DIENES, and GIEMSA STAINS

These showed that, at a penicillin concentration of 0.01 u./ml. and incubation for 12 hrs, there were 80 per cent. treponemes in a culture containing both treponemes and variant forms. About 20 per cent. of the culture consisted of round phase dense bodies measuring 0.2 to 0.4 μ in diameter. At 24 hrs, moderate numbers of treponemes were still noted.

FIG. 3 Culture of Kazan 8 strain in fluid medium exposed to penicillin (0.1 u./ml.) for 16 hrs. Multiple round bodies and fewer treponemes are seen. Giemsa stain. $\times 320$ (AFIP Neg 70-2564)

After 48 hrs, no treponemes could be identified and only occasional round bodies were present.

Addition of penicillin to produce a concentration of 0.1 u./ml. had a more severe effect. After exposure for 16 hrs there were 70 per cent. round bodies, which in Giemsa stains (Fig. 3) were usually purple and varied in size from 0.2 to 0.4 μ in diameter, whereas treponemes were usually deep pink. Within 48 hrs no treponemes or round bodies could be identified.

With a final concentration of 10 u./ml. penicillin, there were only 20 per cent. nonmotile treponemes and 75 per cent. round bodies after 24 hrs; no treponemes or round bodies were detected after 48 hrs. At penicillin concentrations of 100, 1,000 and 10,000 u./ml., no organisms (treponemes or round bodies) were found by wet mounts at 24 and 48 hrs.

ACRIDINE ORANGE vital-stained preparations from the cultures treated with 0.1 u./ml. penicillin revealed numerous variant forms and only occasional treponemes. Most of the former displayed a greenish fluorescence after 28 hrs exposure, suggesting the presence of DNA-containing material; the reddish fluorescence typical of RNA was found in fewer variant forms.

ELECTRON MICROSCOPY of cultures of the Kazan 8 strain treated with 0.01 or 0.1 u./ml. penicillin revealed the presence of round, oval, or irregular variant forms and occasional treponemes. Some of the variant forms displayed disrupted axial fibrils (Fig. 4).

FIG. 4 Ultrathin section of Kazan 8 culture in fluid medium treated with penicillin (0.01 u./ml.) for 24 hrs. Remnants of fibrils in longitudinal section (upper arrow) and transverse section (lower arrow) are visible in the variant form. $\times 60,000$

The variants were usually surrounded by a double unit membrane (Figs 5 and 6) and their cytoplasm displayed a delicate fibrillar pattern. Sometimes delicate cytoplasmic fibrils were interspersed between

denser ribosomal aggregates (Figs 6, 7, and 8). Remnants of damaged treponemes were associated with occasional variants (Fig. 5). More variant forms and fewer treponemes were observed in cultures

FIG. 5 *Ultrathin section of Kazan 8 culture in fluid medium treated with penicillin (0.01 u./ml.) for 48 hrs. Variant forms (A), (B), surrounded by double unit membrane. A portion of a damaged treponeme is seen (C). $\times 30,000$*

FIG. 6 *Ultrathin section of Kazan 8 culture in fluid medium treated with penicillin (0.1 u./ml.) for 24 hrs. Variant forms (A), (B), are surrounded by double unit membranes. Delicate cytoplasmic fibrils are seen (B). $\times 30,000$*

containing higher concentrations of penicillin.

NEGATIVE STAINS FOR ELECTRON MICROSCOPY were done on a culture to which 0.1 u./ml. penicillin had been added. Preparations were stained hourly for

8 hrs after the addition of penicillin. Similar preparations were made of a control culture without penicillin. At 4 hrs, the control appeared unaltered, whereas the penicillin-treated culture showed that the cell walls of some of the organisms appeared hazy

FIG. 7 *Ultrathin section of Kazan 8 culture in fluid medium treated with penicillin (0.1 u./ml.) for 24 hrs. Variant forms of varying electron densities are seen surrounded by double unit membranes. $\times 90,000$*

FIG. 8 *Ultrathin section of Kazan 8 culture in fluid medium treated with penicillin (0.1 u./ml.) for 24 hrs. Variant forms exhibit delicate fibrillar cytoplasm between denser areas of ribosomal material. $\times 90,000$*

and there was disruption of some fibrils. At 6 hrs after treatment, some organisms appeared fragmented. At 8 hrs after treatment, a few round bodies were detected, whereas treponemes were well preserved in the control cultures.

Subcultivation of penicillin treated fluid cultures

'Blind' passages were made from cultures treated with penicillin concentrations of 0.01 to 0.1 u./ml. to determine the viability of penicillin-treated treponemes. A total of fifteen serial transfers were made at 24-hr intervals from penicillin-treated cultures to fresh penicillin-containing and penicillin-free fluid medium. There was no evidence of growth in the serial transfers.

Penicillinase effect

Penicillinase was tested for its ability to inhibit or reverse the effect of penicillin. It was found that penicillinase added in final concentrations of 0.1 to 10 u./ml. to a 24-hr culture of Kazan 8 in fluid medium 30 min. after the addition of penicillin (0.01 u./ml.) resulted in partial inhibition of the effect of penicillin. Penicillinase was ineffective if added to this system 1 hr after the addition of penicillin.

Methicillin effect

The addition of methicillin to the fluid medium in final concentrations of 0.01 to 10,000 u./ml. resulted in the induction of variant forms similar to those produced by penicillin within 24 to 48 hrs in a culture of the Kazan 8 strain. A total of twelve serial transfers were made from methicillin-treated cultures to fresh methicillin-containing and methicillin-free medium. Growth was not detected in the serial transfers.

Penicillin-gradient plates

Plates with troughs that contained various concentrations of penicillin were employed as a means for producing variants of the Kazan 8 strain on solid medium. Plates without penicillin were used as controls and were incubated under the same conditions. Colonies were noted in the control plates after 48 hrs. The penicillin gradient plates showed a few scattered colonies in the area furthest from the trough in plates with a penicillin concentration of 0.01 to 0.1 u./ml. and no growth in plates containing higher penicillin concentrations. The colonies were very small, rounded or irregular, cream-coloured, and glistening, measuring 0.1 to 0.2 mm. in diameter. Studied by Dienes-stained preparations, the colonies appeared to grow centrally into the agar for a depth

of 6 to 8 μ . They contained 70 per cent. round bodies and 30 per cent. treponemes, the latter usually at the periphery. The round bodies in the centre of the colonies were smaller and denser with a granule-like appearance and measured 0.1 to 0.2 μ in diameter. The colonies were kept under observation for possible L-form transformation, and studied at weekly intervals with Dienes-stained preparations. Blocks excised from portions of agar containing growth were transferred to fresh penicillin-free and penicillin-containing plates. No growth was detected in any of the subcultures.

When a specified concentration of penicillin was incorporated in the serum agar medium before pouring plates, much the same results were obtained. In plates containing penicillin in final concentrations of 0.01 to 10 u./ml. a moderate number of very small colonies appeared after 5 to 7 days of incubation. The colonies were similar to those found in the penicillin-gradient plates and were most abundant in plates containing 0.01 u./ml. penicillin. They appeared to grow into the agar forming central cores 6 to 12 μ in depth. Colonies were not found in plates containing concentrations of penicillin higher than 10 u./ml.

Filtration procedures

Filtration experiments were undertaken to determine whether filter membranes would serve to separate variant forms from treponemes by permitting cell-wall defective variants to grow through the filter pores onto the surface of agar plates as described by Wyrick and Gooder (1971).

24 hour cultures treated with penicillin in concentrations of 0.01 to 100 u./ml. for 24 hrs were subsequently inoculated onto previously sterilized Millipore filters (0.22 to 0.6 μ pore diameter) which were placed on the surface of plates containing the same concentrations of penicillin and also inoculated onto similar filters placed on penicillin-free plates. After 1 to 2 weeks incubation the filters were transferred to fresh plates with and without penicillin. There was no evidence of any growth through the membranes on the original plates or in subsequent transfers.

Lysozyme effect

Experiments were conducted to determine whether lysozyme could induce the formation of cell-wall defective variants of the Kazan 8 strain of treponemes.

After the first treatment with lysozyme for 1 hr at 37°C., before dilutions were prepared, the suspension was examined by wet mounts. The suspension contained 80 per cent. treponemes, of which 70 per

cent. were motile. At the same time electron microscopy of the lysozyme-treated suspension before dilution and transfer, showed that some treponemes displayed mild to moderate damage of the cell wall with disruption of the fibrils. Other treponemes appeared intact. The few variant forms appeared similar in structure to those in penicillin-treated cultures. Some forms were composed of both a large body and portions of damaged treponeme (Fig. 9). Serial transfers in fluid medium (lysozyme-free) showed almost the same type of growth as the untreated control cultures.

The second treatment of the culture with lysozyme (100 µg./ml.) for 30 min. at 37°C. resulted in more severe damage; wet mounts and Dienes-stained preparations of the organisms showed a greater number of round bodies (60 per cent.) and a lesser number of treponemes (40 per cent.).

After the third treatment with lysozyme (100 µg./ml.) for 30 min. at 37°C. there was even greater damage to the treponemes. Wet mounts of transfers to fluid medium made from 10-fold dilutions in lysozyme-free fluid medium and incubated for 24 hrs showed that 20 per cent. of the culture consisted of treponemes (nonmotile or rarely motile) and 80 per cent. round bodies. The latter varied in size from 0.2 to 0.8 µ in diameter and were phase dense and phase pale. Viability estimates of suspensions transferred into fluid medium following the

third lysozyme treatment showed a significant decrease in growth, 2.72×10^3 organisms/ml. in the test culture in comparison to 2.17×10^4 organisms/ml. in the control. When the fluid medium test cultures were plated onto solid medium, colonies grew down into the agar to a depth of 3 to 6 µ. Dienes-stained preparations of 1-week-old colonies (Fig. 10) revealed numerous round to oval phase-dense and phase-pale bodies and scattered peripheral treponemes. Electron microscopy of cultures in fluid medium showed more numerous variant forms and fewer treponemes. The former showed peripheral cytoplasmic aggregates and scattered disrupted fibrils (Fig. 11) or more uniformly dispersed ribosomal material. The plates were incubated for 6 weeks and colonies were examined by the Dienes stain technique at frequent intervals. Typical L-form colonies were not found. Serial passages of 1-week-old colonies in lysozyme-free fluid medium did not result in growth.

In conclusion, initial treatment with lysozyme was not lethal to the treponemes, but subsequent exposure of the recovered culture to lysozyme resulted in cell wall damage to most of the organisms which were then unable to propagate.

Discussion

The addition of low concentrations of penicillin (0.01 to 0.1 u./ml.) to solid medium inoculated with the

FIG. 9 *Ultrathin section of Kazan 8 culture in fluid medium after initial lysozyme treatment. Remnants of a damaged treponeme (arrow) are visible as part of a large variant of irregular shape.*
× 60,000

FIG. 10 Colony from culture of Kazan 8 treated thrice with lysozyme. There are multiple phase-dense and phase-pale round bodies with occasional peripheral treponemes. Dienes-stained preparation. $\times 1,200$

FIG. 11 Ultrathin section of Kazan 8 culture in fluid medium treated thrice with lysozyme. The variant form displays peripheral cytoplasmic aggregates and scattered disrupted fibrils (arrows). $\times 90,000$

Kazan 8 strain of *T. pallidum* resulted in the production of small colonies which at low magnification did not exhibit the 'fried-egg' appearance that is typical of L-form colonies or of mycoplasmas (McGee and Wittler, 1969; Dienes, 1968; Hayflick, 1969).

Nevertheless, examination of Dienes-stained preparations of the colonies at high magnification with phase microscopy revealed the presence of central cores growing down into the agar and containing small granule-like bodies similar to those of previously

described L-form colonies of other bacteria (Roberts, 1966; Roberts and Wittler, 1966; Hijmans, Van Boven, and Clasener, 1969; Kagan, 1968). These colonies also contained a few treponemes, and therefore appeared similar to unstable L-form colonies. Although colonial morphology seemed to indicate that the variant forms could have been unstable L-phase variants, it is more likely that they were transitional-phase variants, since they were unable to propagate in serial subculture on agar as L-form colonies (McGee and others, 1971). The variant forms contained some cell-wall constituents, thus excluding the possibility of their being protoplasts. Some of the variant forms that developed in fluid medium cultures were probably spheroplasts, since some remnants of cell wall with double unit membranes could be detected, and since their ability to propagate in subcultures as spheroplasts was negligible. The ultrastructural features of the variant forms, together with their ability to grow as small colonies upon initial transfer to agar, suggest viability, although the possible association of some nonviable degenerative forms cannot be excluded.

The deleterious effect of penicillin on treponemes was similar to that reported by earlier workers (Eagle and Musselman, 1944; Mutermilch, Gérard, and Delaville, 1959). Penicillin-induced damage to the cell wall of treponemes in fluid medium was observed as early as 8 hrs after exposure and in higher concentrations was irreversible with penicillinase. In the Kazan 8 strain, typical self-propagating L-form colonies could not be produced using concentrations of penicillin and conditions for growth similar to those employed by Ustimenko (1965) who reported the production of 'L-form' colonies using the Stavropol 7 and 9 strains. A closer examination of Ustimenko's work suggests that she too may have produced unstable L-phase variants or transitional-phase variants, similar to the variant forms observed in the studies from this laboratory (Rodrigues, 1971). These views are based on Ustimenko's description of penicillin-induced L-transformation in semi-solid media without explicit evidence of serial propagation of these variants as colonies on solid media, in fulfillment of currently accepted criteria for L-form colonies (McGee and others, 1971).

Kagan (1968) included Ustimenko's work in her review of L-forms of bacteria and described the variants of the *T. pallidum* strains induced by Ustimenko as unstable L-phase variants. It is not surprising if the variants induced by Ustimenko and by the present author did indeed differ somewhat in structure or properties, since both the culture medium employed and the particular strains tested were different. Ovčinnikov and Delektorskij (1971) des-

cribed unstable 'L-forms' of *T. pallidum* (cultivated strain No. 8) as seen by electron microscopy. It is uncertain from the text whether these structures were true unstable L-phase variants that could be serially propagated on appropriate agar.

The effect of lysozyme as an inducing agent for the production of cell-wall defective variants was studied, since this enzyme had been reported to induce such variants from some bacteria (Ryter and Landman, 1968; King and Gooder, 1970a, 1970b; Wyrick and Gooder, 1971). Lysozyme was one of the enzymes used to study lytic effect on the pathogenic Nichols strain of *T. pallidum* by Jones, Nevin, Guest, and Logan (1968), who described damage to the protoplasmic cylinder, with no apparent damage to the cell wall. In the Kazan 8 strain, however, definite cell-wall damage by lysozyme was observed in the present study. The damage was less severe than that induced by penicillin, but was otherwise similar.

The use of acridine orange as a vital stain for treponemes has not been previously described. It was useful for studying treponemes and variant forms, permitting visualization of the relative content of RNA or DNA in the organisms.

Summary

Ultrastructural study of selected nonpathogenic strains of *T. pallidum* (Kazan 2, Kazan 5, Kazan 8 and Reiter) and the pathogenic Nichols strain permitted visualization of the cell wall, cytoplasm, and fibrils of these organisms.

Induction of morphological variants of the Kazan 8 strain was attempted, using penicillin and lysozyme. Penicillin appeared to have a deleterious effect on treponemes in fluid and solid media. With higher concentrations of penicillin (100 to 10,000 u./ml.) the organisms were completely destroyed. With lower concentrations (0.01 to 0.1 u./ml.) variant forms were noted in the fluid and solid medium. By electron microscopy, variants frequently showed double unit membranes, disrupted axial fibrils, and occasional remnants of the treponemes from which the variant was derived. Morphologically, these variants represented spheroplasts, unstable L-phase variants, or transitional-phase variants. On the basis of cultural criteria, most of these variants were probably transitional-phase variants. Self-propagating L-phase variants that produced L-form colonies were not encountered in these experiments.

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Variants de *Treponema pallidum* présentant des défauts de la membrane cellulaire

SOMMAIRE

L'étude de l'ultrastructure de souches choisies de *T. pallidum* non pathogènes (Kazan 2, Kazan 5, Kazan 8 et Reiter) et de la souche Nichols pathogène a permis une visualisation de la paroi cellulaire, du cytoplasme, et des fibrilles de ces organismes.

On a tenté d'obtenir des variants morphologiques de la souche Kazan 8 en se servant de la pénicilline et du lysozyme. La pénicilline semble avoir un effet nuisible sur les tréponèmes en milieux liquides et solides. A des plus hautes concentrations de pénicilline (100 à 10 000 u/ml) les organismes ont été complètement détruits. A de plus faibles concentrations (0,01 à 0,1 u/ml) on observa des formes variantes dans le milieu liquide aussi bien que dans le milieu solide. Au microscope électronique, les variants montrent fréquemment une portion à double membrane, des fibrilles axiales brisées et, de temps en temps, des restes des tréponèmes d'où provient le variant. Morphologiquement, ces variants représentent des sphéroplastes, des variants instables en phase L où des variants en phase de transition. Sur la base des critères de culture, la plupart de ces variants furent très probablement des variants en phase de transition. Au cours de ces expériences, on n'a pas rencontré de variants en phase L se développant par eux-mêmes pour produire des colonies de formes L.