FORMATION OF GRANULAR STRUCTURES BY LEPTOSPIRAE AS REVEALED BY THE ELECTRON MICROSCOPE

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The formation of granules in leptospirae was noticed many years ago, but there is still no agreement as to their biological significance. Some authors are inclined to interpret them as products of disintegration of dead or degenerated organisms (Marchoux and Couvy, 1913; Gonder and Gross, 1919; Uhlenhuth and Fromme, 1930) while others consider that they play a definite role in the life-cycle of the organisms (Sergent and Foley, 1908; Balfour, 1911, 1912; Fantham, 1911; Meirowsky, 1914; Nicolle and Blanc, 1914; Leishman, 1918, 1920; Leipold, 1926; Bessemans et al., 1942; Gastinel and Mollinedo, 1942; Jakob, 1949). Granular structures develop spontaneously in aging cultures (Babudieri, 1949), but some workers (Timmerman, 1927; Herreweghe, 1943; Babudieri, 1949) have produced granulation artificially in young cultures by means of physical and chemical methods (cooling, heating, homologous antiserum, sodium taurocholate). The present paper is concerned with those granules which are formed naturally.

MATERIALS AND METHODS

Organisms. Observations were carried out on pure Wijnberg and Lisboa strains of Leptospira icterohaemorrhagiae and on Leptospira canicola, strain Utrecht—all kindly supplied by Dr. J. C. Broom of the Wellcome Laboratories of Tropical Medicine, London—and also on a strain of a Leptospira isolated from a case of "abacterial" cystitis (Czekalowski and Horne, 1951; Czekalowski and McLeod, 1953) and for brevity called here L.Cz.2

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2 This strain was designated as Leptospira Czekalowski by Professor J. W. McLeod, University of Leeds, England, and is at present in the

Medium and cultivation. The organisms were grown in a medium consisting of 12.5 per cent meat extract, 0.1 per cent Witte peptone, and 10 per cent suitable rabbit serum inactivated at 56 C for 30 minutes. The completed medium adjusted to pH 7.4, was sterilized by passing through a Maassen filter and distributed into tubes (6 by 3/4 in) in 10 ml volume. A few subcultures of each strain were made at the time and incubated at 29 C for varying periods up to 12 months.

Preparation of leptospirae for examination. The cultures were centrifuged at +4 C in an MSE refrigerated centrifuge and the sediment resuspended in all glass-distilled water. Washing was repeated 5 to 7 times and centrifugation carried out at 5,000 rpm for 30 minutes each time. From the final deposit suspensions of suitable densities were made for both electron and dark ground microscopy.

The preliminary experiments were carried out on organisms fixed with a few drops of two per cent osmic acid prior to washing, but this method produced preparations covered with slime and debris (figure 1) which obscured the structural details that could be observed in the washed specimen (figure 2). During the process of repeated washings, however, the unfixed leptospirae were not found to undergo any profound changes in morphology, apart from various degrees of straightening of the bent (hooked) end parts and occasional slight but uniform shallowing of coils (figure 3). In view of these findings, the following procedure was finally adopted:

Leptospirae were washed 3 to 5 times, fixed with a few drops of two per cent osmic acid for 5 minutes, and then washed twice more. This procedure was never observed to cause the formation

Leptospira Type Collection of Dr. J. C. Broom, Wellcome Laboratories of Tropical Medicine, London.
Figure 1. *Leptospira icterohaemorrhagiae*, Wijnberg strain, fixed with osmic acid prior to washing. Morphology of the organism is obscured by slime and debris.

Figure 2. Normal appearance of *Leptospira Czekalowski* after two washings and fixation afterwards by osmic acid.

Figure 3. Straightening of the bent (hooked) ends and slight shallowing of coils as the result of repeated washing (*Leptospira Czekalowski*).
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or appearance of any structures resembling granules in the cultures which lacked them before the treatment.

Electron microscopy. Specimens were mounted by allowing drops of the prepared suspension to dry on the usual collodion covered grids and shadowing with chromium (1 mg at 10 cm and 5°). Examination was carried out in a RCA EMU 2C electron microscope at an instrumental magnification of 8,800 with subsequent photographic enlargement.

Dark ground microscopy. This was carried out with a Leitz 2 mm oil immersion objective (N.A. 1.3) and a Leitz oil immersion, dark ground condenser (D 1.20 a).

RESULTS

Periodical examinations of the four strains of Leptospira were carried out using both electron and dark ground microscopy. It was noticed that toward the end of the second week of cultivation individual organisms began to show apparent

Figure 4. Two Leptospira Czekalowski with terminal granules; one granule apparently enveloped in a well defined membrane and shows internal structure which is consistent with its being a part of the leptospiral body.

Figure 5. Leptospira Czekalowski unshadowed; fixed by osmic acid. Small, dense, internal granules.
changes in morphologic structure when compared with normal leptospirae. A gradual but obvious transition occurred from forms displaying regular, symmetrically arranged, and closely knit coils (figures 2 and 3) to forms with shallower, irregular spirals, interspaced by straightened parts of organisms (figure 4). In a few days within these "deformed" organisms, some dense areas appeared which are difficult to interpret as little is known about the significance of granulations of this type in spirochetes in general. Figure 5 represents one of these leptospirae, unshadowed and fixed with osmic acid, in which several internal granules are visible. These granules are spaced quite regularly and may represent equally well accumulated nuclear material or local concentrations of cytoplasm. During the fourth and fifth week of cultivation another type of granules appeared. These granules were very much broader than the cross-section of leptospirae and were situated in any part of the leptospiral body (figures 4, 6, 7, and 8). They are either single (figures 4, 6, and 7) or multiple (figures 8 and 9). Later on they are shed "free". The number of these granular structures increased with the progressive aging of the cultures (figures 10 and 11) pari passu with the number of motile vegetative forms, until the fifth or seventh month of cultivation when no vegetative organisms could be seen by dark ground microscopy. The electron microscope reveals these granules in the early stage of their development as spheroidal bodies (figures 4, 6, 7, 8, 10, and 11) surrounded by a delicate cell membrane within which is contained a dense mass of leptospiral body in the form of twisted ropes (figures 6 and 7). A more advanced granule, and especially a "free" one, when viewed by the dark ground microscope appears to be enveloped by a refractile membrane with the interior consisting of an even more refractile central mass. The electron microscopy of the parallel specimen confirms these observations (figure 12) and makes it clear that the "free" granule is al-

**Figure 6.** *Leptospira icterohaemorrhagiae*, Lisboa strain. Formation of a terminal granule.

**Figure 7.** *Leptospira icterohaemorrhagiae*, Wijnberg strain. Formation of a terminal granule.
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Fig. 8. *Leptospira icterohaemorrhagiae*, Lisboa strain. Multiple formation of granules in different positions of the leptospiral body.

Fig. 9. *Leptospira icterohaemorrhagiae*, Wijnberg strain (light shadowing). Formation of numerous cyst-like granules in different parts of the leptospiral body. The numerous narrow filaments seen in the photograph are leptospiral axistales and will be described elsewhere.

Fig. 10. *Leptospira Czekalowski*. A small clump of granules with the associated leptospiral bodies.

**DISCUSSION**

The significance of granules in the life-cycle of spirochetes in general is far from clear, and while some investigators observed the presence of granules even by direct methods, others denied those findings. Occasional observations, however, made on *Treponema* and *Borrelia* strongly suggest...
that their spiral form is not the only one which these organisms may assume (Herxheimer, 1905, 1906; Schaudinn and Hoffmann, 1905; Novy and Knapp, 1906; Dutton and Todd, 1907; Leishman, 1910; Noguchi, 1911, 1912, 1917; Fantham, 1916; Inada et al., 1916; Levaditi et al., 1927, 1930; Levaditi, 1930; Levaditi and Li Yuan Po, 1930; Manouélián, 1930; Wartin and Olson, 1930; Ingraham, 1932; Morton and Anderson, 1942; Bessemans et al., 1943; Mudd et al., 1943; Hampp, 1946; Gelperin, 1949; Bisset, 1952). Certain authors suggest that at least some spirochetal granules may represent "germinative units" (Hampp et al., 1948), and DeLamater and his colleagues, by means of phase-contrast microscopy, showed in a most convincing way that Treponema pallidum possesses a very complex life-cycle with formation of single and multiple "reproductive cysts" from which new organisms emerge. It seems also that the main vegetative method of reproduction in Treponema pallidum—at least during the most active stages of development—is by means of a transverse division, but formation of the cysts does take place even in young cultures, though in small numbers only (DeLamater et al., 1950a,b,c, 1951a,b). It is unlikely that any of the granules formed in lepto-spiral cultures are "spore-like" structures because their resistance to heat and to noxious substances is not greater than that of normal leptospirae (Van Thiel, 1948). It should not be overlooked, however, that they may represent a resting stage in the life history of this spirochete. This hypothesis gains support from the experiments carried out by Bessemans et al. (1942) who by means of a micromanipulator isolated some granules from the culture of a water leptospirae (strain Gand) and grew from them "single-cell

Figure 11. Leptospira canicola, strain Utrecht, resembles figure 10 but shows a heavier texture of leptospirae.

Figure 12. A single, "free" granule completely formed, with central mass consisting of leptospiral fragments; surrounded by a well defined membrane (Leptospira Czekalowski).
cultures” of the identical organism. We are satisfied that the spheroidal bodies described and reproduced in this paper are not artifacts or products of degeneration or disintegration of leptospiroa. They represent definite and characteristic bodies originating in leptospiroa and each surrounded by a distinct, well defined membrane (figures 9 and 12). The formation of granules represents a rhythmic and constant process and hence these granules must play a role in the life-cycle of leptospiroa. We are inclined to interpret them either as a resting phase or as reproductive bodies of this organism.

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SUMMARY

Two types of granules have been described in the four strains of Leptospira examined. Small granules with diameter not wider than that of the cell itself may represent either nuclear or cytoplasmic material locally accumulated. The large granules may be situated in any part of leptospiral body but were found most frequently in the terminal position. When “free” the large granules are circular and well delineated by a cell membrane, with the central part consisting of leptospiral fragments embedded in a homogeneous substance. We are satisfied that these granules are not artifacts or products of degeneration or disintegration of leptospiroae, and that their constant and rhythmic appearance points to their playing some role in the life cycle of the leptospiroae. It is suggested that these granules represent either a resting stage or are reproductive bodies of leptospiroae.

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