GRANULE FORMATION IN LEPTOSPIRES

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The results of an electron-microscopic study of changes in the ultrastructure of leptospires under the influence of specific and nonspecific factors are described. The study of many photomicrographs showed that the outer membrane of the leptospires is a delicate structure, easily broken by various precodures. As a result, granules described as spherical swellings, pools, cyst-like formations, and spores are formed. Penicillin not only causes changes in the outer membrane but also leads to the formation of granules within the cytoplasm. It is postulated that different forms of leptospires are degeneratively changed forms produced by the action of specific and nonspecific factors.

The problem of granule formation in spirochetes and its role in the biology of these microorganisms is still unsolved and a matter for dispute. On the basis of the discovery of atypical forms of spirochetes, including formations of granule type, some investigators consider that spirochetes have a certain life cycle of development in which granules are one of the stages. Others consider that these forms are found perfectly regularly during the investigation of material from patients and also after cultivation under unfavorable conditions and are degenerative forms of spirochetes. Two types of granular structures have been described morphologically in leptospires, where they may exist both inside and outside the cytoplasmic cylinder. A particularly debated question is that of the origin and role in the biology of leptospires of certain structures located outside the body of the leptospire and described as "swellings," "spheres," "granules," "cyst-like formations," and "spores" [1, 2, 4, 5]. Attempts have been made to interpret these forms as one stage in the life cycle of leptospires and the possible developmental cycle of leptospires from these spheres has actually been described and attempts made to obtain a viable culture of leptospires from them [6, 7-9]. However, such experiments have not yet yielded reliable results. By contrast it is stated that spherical structures may arise under unfavorable conditions of cultivation of leptospires, e.g., in a medium containing an excess of sodium chloride [3]. The nature of the granules located within the cytoplasmic cylinder likewise has not been fully explained although the chemical nature of some of them (a concentration of nucleoproteins or polysaccharides) has recently begun to be elucidated as the result of combined electron-microscopic and cytochemical investigations.

This paper describes the results of an electron-microscopic study of granule formation in leptospires depending on the action of various specific and nonspecific factors of them.

EXPERIMENTAL METHOD

A 10-12 day culture of Leptospira icterohaemorrhagicae, strain Sud'in, grown on distilled water with 10% rabbit serum at 28° C was used. The culture of leptospires was treated with various dilutions of specific antiserum (from 5 min to 2 h), to the action of formalin vapor for 18 h, 3% NaCl solution (1.5 h), heating to 56° C for 5 min, irradiation with ultrasound from 30 sec to 1.5 min at 100 mA and 1-2.5 MHz in the UZDV-2 apparatus, and with sodium salt of benzylpenicillin and nystatin. Specimens for electron microscopy were prepared with Caulfield's fixative at 4° C for 48 h. All manipulations connected with centrifugation of the leptospires (3000 rpm for not more than 10 min) were carried out only after their preliminary fixation. After

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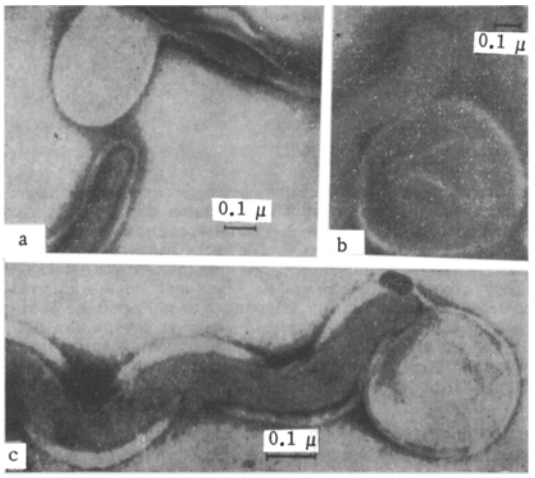

Fig. 1

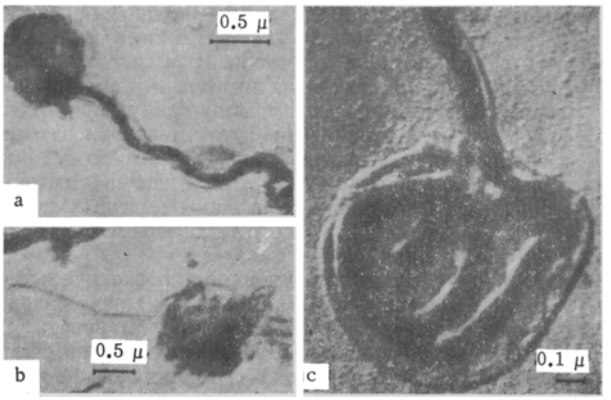

Fig. 2

Fig. 1. L. icterohaemorrhagicae, strain Sud'in. Spherical swellings on ends and body of leptospire: a) after treatment with formalin vapor (18 h); b) after treatment with hypertonic NaCl (3% solution, 1.5 h); c) after exposure to ultrasound (2.5 MHz, 30 sec). Caulfield's fixing solution, negatively stained with phosphotungstic acid.

Fig. 2. L. icterohaemorrhagicae, strain Sud'in. Combined action of heating to 56° C for 5 min and specific antibodies: a) initial stage of formation of "spherical swellings;" b) rupture of spherically swollen end of leptospire, remnants of outer membrane can be seen; c) "spherical" formations on end of leptospire with cytoplasmic cylinder visible inside. Fixation with Caulfield's solution, shadow-cast with chromium.

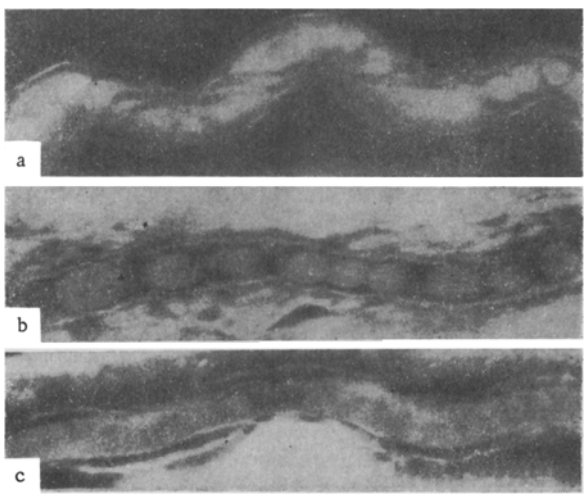

Fig. 3. L. Icterohaemorrhagicae, strain Sud'in. Formation of granules inside cytoplasmic cylinder: a) treatment with ultrasound (2.5 MHz, 30 sec), 60,000×; b) treatment with ultrasound (1 MHz, 30 sec): cytoplasmic cylinder containing granules can be seen, the outer membrane is broken up, 108,000×; c) single exposure to penicillin (0.2 unit/ml, after 72 h): granules can be seen inside the cytoplasm, 42,000×.

centrifugation the residue was washed with distilled water and applied to collodion and Formvar films. Some samples were dried and shadow-cast with chromium while others were stained with 2% phosphotungstic acid for 30 sec. Investigations were carried out in the ÉM-7 and UMV-100 electron microscopes.

EXPERIMENTAL RESULTS

Hollow spherical swellings on the ends and body of the leptospires were found reasonably constantly during the electron-microscopic study of the ultrastructural changes produced by the action of specific immune antibodies, especially in the midzone, during the first 5–10 min of their interaction with the culture of leptospires, by the action of formalin vapor, by treatment of the cultures of leptospires with ultrasound (Fig. 1), and also after a single exposure to penicillin. After spraying with chromium these structures appear as electron-translucent "pools" on the body and ends of the leptospire, sometimes clearly bounded by the outer membrane. On negative staining with phosphotungstic acid the outer membrane is clearly visible, and only occasionally is it absent. The interior of the pools consists of homogeneous osmiophilic material. Sometimes the walls of the cavity are collapsed and they appear wrinkled. The frequent discovery of spherical formations in response to the harmful action of specific and nonspecific factors suggests that they are forms arising through degenerative changes in the leptospires in connection with disturbance of the rigid properties of the outer membrane. As a result of these changes, the outer membrane begins to swell, and the resulting cavity becomes filled with electron-translucent hyaloplasm. The possibility of rupture of the outer membrane

in some places on the body with escape of the colloidal part of the cytoplasm outside cannot be ruled out. After fixation with osmium it can be seen as an electron-translucent drop on the surface of the leptospire. The absence of spherical "hollow" structures in living leptospires when examined under the dark-ground microscope and their regular discovery in almost all cells on electron microscopy [1], described in the literature, indicate that certain injurious factors arise during preparation of specimens for electron microscopy, causing the appearance of "spherical swellings" on the body of the leptospire. This applies in particular to the prolonged centrifugation of the leptospire culture if not previously treated with the fixative, at speeds exceeding 3000 rpm. It is also evident that not all fixatives are physiological, and some may give rise to changes in the vulnerable outer membrane. All these factors must be taken into account when specimens are prepared for electron microscopy and, in particular, in special investigations undertaken to study the morphological structure of leptospires.

The "cyst-like formations" or "spores" arising on the ends of leptospires were found particularly often after a short exposure of the culture of leptospires to hypertonic sodium chloride solution or after heating for a moderately short time. During exposure to these factors the process of formation of the "cyst-like" forms on the ends of the leptospires, their detachment from the body, and the appearance of granular forms resembling "cysts" and "spores" can be followed (Fig. 2). "Spores" are often found after the combined action of the above factors and specific immune serum. The "cyst-like" granules, whether connected to the body of the leptospire or lying separately from it, are bounded by the outer membrane; the cytoplasmic cylinder twisted into a spiral lies inside the granule. Sometimes the contents of the granule, especially after a short rise of temperature, consist of an amorphous mass. As a rule structures of this type arise only at one end of the leptospire.

In the study of ultrastructural changes taking place in leptospires under the influence of antibiotics (penicillin, nystatin) and also as a result of exposure to ultrasound, granular structures were found inside the cytoplasmic cylinder (Fig. 3). The granules arising during brief exposure to ultrasound fill the cytoplasmic cylinder sometimes for a considerable distance. They are large, circular in shape, and almost equal in size to the diameter of the cylinder. The intracytoplasmic granules formed after a single exposure to various doses of penicillin (0.1-0.6 unit/ml) are rather small and are situated in various parts of the cytoplasm. The ability to form intracytoplasmic granules persists in leptospires during successive passages on nutrient media not containing penicillin. Whereas the mechanism of granule formation during exposure to ultrasound can be exaplained by the appearance of cavities inside the cytoplasm as a result of which it becomes separated into granules, the mechanism of granule formation during exposure to penicillin and nystatin has not yet been exaplained. Further research is necessary to shed light on their chemical nature and to explain the ability of the organisms to form granules during passage on media without penicillin.

The electron-microscopic study of ultrastructural changes in leptospires under the influence of specific and nonspecific factors thus showed that the outer membrane is easily broken with the formation of various types of granules, both connected with the body of the leptospire and lying separately. Some antibiotics, notably penicillin, not only have an injurious action on the outer membrane, but also lead to granule formation within the cytoplasmic cylinder. Ability to form granules persists in some leptospires even after consecutive passages on nutrient media without penicillin.

The writers consider that the appearance of atypical forms of spirochetes, including structures of granule type, may be the result of their exposure while in the patient's body to both specific antibodies and antibiotics, especially penicillin, which is used particularly for the treatment of syphilis and leptospirosis. This explains the fact that atypical forms of spirochetes, including formations of the granular type, are regularly found in material taken from patients for testing. During cultivation of leptospires various degenerative forms may also arise on account of changes in the pH of the medium, an increased concentration of salts, and changes in the temperature of cultivation. Recent investigations aimed at obtaining cultures of leptospires from granular formations [7] are not convincing for the methods used to verify the formation of granular forms (microscopy) are not sufficiently sensitive. Microscopic examination of one or two drops of material does not provide reliable evidence of the absence of morphologically unchanged leptospires in the whole of the material.

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