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Electron Microscopic Study of Colonies of *Mycobacterium lepraemurium* [1]

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It has already been confirmed that *M. lepraemurium* can be cultivated on Ogawa's egg yolk medium by his method, and that the bacilli subcultured on the medium are surely *M. lepraemurium* ([10, 13, 14]). The colonies of murine leprosy bacilli grown on Ogawa's egg yolk medium were observed with scanning electron microscopy (SEM) by the authors. Thus far there has been little study of bacterial colonies by SEM ([1, 2, 6-8, 11, 16-22]). This paper deals with the relationships between the gross morphology and microstructure of colonies of murine leprosy bacilli by SEM, and the chemical nature of extracellular material in the colonies as clarified by transmission electron microscope (TEM) observations of ultrathin sections of the colonies stained with ruthenium red.

MATERIALS AND METHODS

The colonies of the Hawaiian strain of *M. lepraemurium* subcultured on Ogawa's egg yolk medium slants were observed with SEM. The rough type colonies of the 13th to the 14th subculture incubated for one to three months, and the smooth type colonies of the 14th to 15th subculture incubated for one to four months were used. Cold 2.5% to 3% glutaraldehyde in M/15 phosphate buffer (pH 7.2) was poured gently into the test tube containing the Ogawa's egg yolk medium, and the test tube was left in the refrigerator for 18 to 24 hours. After fixation, the fixative was removed and the test tube was cut transversely near the colonies. The site of medium having the colonies was cut off thinly and dehydrated by means of a graded ethanol series. Thereafter, the ethanol was replaced with iso-amyl acetate. The colonies were weakly adherent to the medium, and frequently dropped off. In such an event the slice of medium from which the colonies dropped off was removed, and the process described above was carried out without pinching the colonies with forceps. Iso-amyl acetate being replaced with liquid CO_2, the colonies were dried in a critical point dryer, HCP-1. Also when the colonies were put into the critical point dryer, the colonies which dropped off the medium were sucked into a pipette with iso-amyl acetate and poured on the filter paper. The filter paper having the colonies was put into a wire basket and placed in the chamber of the dryer. Colonies thus dried in the critical point dryer were cemented to aluminum stubs with adhesive. This was done with care not to distort the colonies by instrumental pinching. This was accomplished by lifting them by the forceps tip to which a small amount of adhesive was attached, and then transferring them to the adhesive which was on the aluminum stub. After the adhesive dried and hardened, the colonies were coated with gold in an ion-spattering apparatus (Giko IB-3). Some rough type colonies were dried by the freeze-drying method without use of organic solvent, and their features under SEM were compared to those of the other rough type colonies which were grown on the same slant and dried by the use of organic solvent, in order to examine whether the organic solvent used in the process of drying of the specimens affected the features of the colonies. Additionally, some rough colonies were disrupted gently with a Potter-Elvehjem type homogenizer, stained negatively with phosphotungustic acid solution in phosphate buffer (pH 7.4), and observed by TEM.

Ultrathin sections of the rough type colonies stained with ruthenium red were observed with TEM. Following Luft's method ([12]), the colonies were fixed in a refrigerator for two hours with 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.4) in which

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ruthenium red was dissolved at a concentration of 0.5 mg/ml. Then they were treated for four hours in a refrigerator with 1% solution of osmium tetroxide in 0.05M cacodylate buffer (pH 7.4) in which ruthenium red was dissolved in a concentration of 0.5 mg/ml. Subsequently, the specimens were refixed in a refrigerator for three to four hours with 1% solution of osmium tetroxide in 0.05M cacodylate buffer (pH 7.4). After refixation, they were dehydrated with the graded ethanol series, embedded in methacrylate resin, ultrathin-sectioned, and observed with TEM.

Some rough type colonies were treated with chloroform in the process of preparation. After dehydration with ethanol, they were first immersed in a 1:1 mixture of 100% ethanol and chloroform for 30 minutes, then in chloroform for one hour, next in a 1:1 mixture of chloroform and iso-amyl acetate for one hour, and finally transferred to iso-amyl acetate. After this they were dried in the critical point drying apparatus. Other colonies grown on the same slant were prepared with the ordinary method.

SEM used in this study was the Hitachi field-emission type SEM, HFS-2S, which was operated at 25 KV, and TEM is Akashi's AFM-80.

RESULTS AND DISCUSSION

In the typical rough type colonies of murine leprosy bacilli, filamentous strands which were 40-148 mμ (chiefly 70-120 mμ) in diameter, were observed between the bacilli; and granules which were not spherical, but disc-like, and 60-300 mμ (chiefly 100-200 mμ) in diameter, were occasionally found on the bacilli or filamentous strands (Fig. 1). These were not observed in the smooth type colonies, save that rarely short bridges were found between the bacilli (Fig. 2).

Among the colonies which were of rough type macroscopically, some had many interbacillary filaments and granules, but some colonies on the other slants had interbacillary filaments and granules only partially, and other colonies had only interbacillary filaments partially. Subcultivation of rough colonies repeated many times on Ogawa's egg yolk medium caused a change of type from rough to smooth in many colonies. The disappearance of interbacillary filaments and granules occurred prior to the macroscopic change of colony to the smooth type. The colony described above which had interbacillary filaments only partially is considered to have been in transition from rough to smooth type, and to be not a typical rough colony, though it showed a rough surface macroscopically. Therefore, in order to compare some aspects of the rough type with the smooth type, macroscopic discrimination between rough and smooth type is insufficient. Discriminative determination by means of SEM is needed.

The following studies were made to determine whether or not the interbacillary filaments described above are an artifact. First, the rough type colonies were dried by freeze-drying without the use of organic solvent and observed with SEM. The interbacillary filaments were observed also in these specimens (Fig. 3). Then rough type colonies, gently disrupted with a Potter-Elvehjem type homogenizer, were suspended in a small amount of physiological saline solution negative-stained with phosphotungustic acid solution in M/15 phosphate buffer (pH 7.4), and observed with TEM. Filaments which were 25-120 mμ in diameter were observed (Fig. 4). Therefore, it can be said that the interbacillary filaments are not an artifact formed by the use of organic solvent.

Next, the rough type colonies were stained with ruthenium red dissolved in the fixatives. Previously, leprosy bacilli and murine leprosy bacilli *in vivo* had been stained with ruthenium red by one of the authors, Okada ([15]). The bacilli isolated from human leproma or murine leproma were stained with ruthenium red during fixation, ultrathin-sectioned, and observed with TEM. A coat stained with ruthenium red which surrounded the outside of the cell wall of bacilli and having a jagged outer margin was observed on both *M. leprae* and *M. lepraemurium*. This finding indicates that both bacilli have a layer of complex carbohydrate outside the cell wall. Some leprosy bacilli and murine leprosy bacilli *in vivo* do not have this coating. The absence of the coating on these bacilli may be due to its removal during the isolation of bacilli from the lesion. Murine leprosy bacilli grown *in vitro* also have the same coating as that of bacilli *in vivo* (Fig. 5). When the bacilli adhered to each other, the coating was absent at the adhering site. The margin of

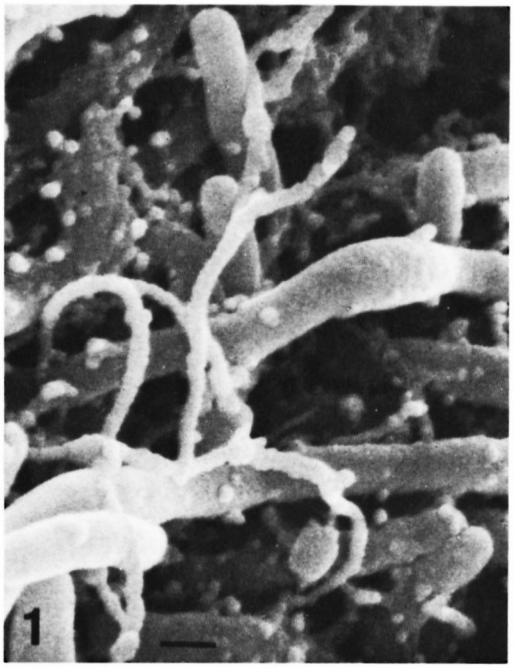


FIG. 1. Rough type colony of murine leprosy bacilli. The filamentous strands are present between bacilli, and many granules are observed on the bacilli or filamentous strands. × 35,000, scale bar: 0.2 μ.

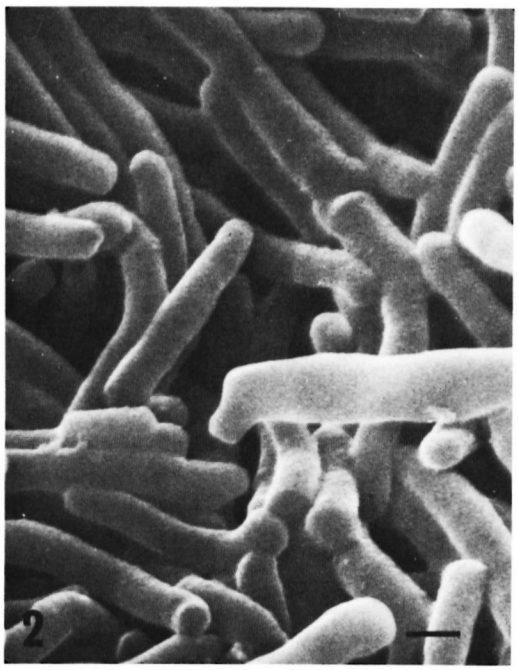


FIG. 2. Smooth type colony of murine leprosy bacilli. Neither interbacillary filament nor granule can be found. ×35,000, scale bar: 0.2 μ.

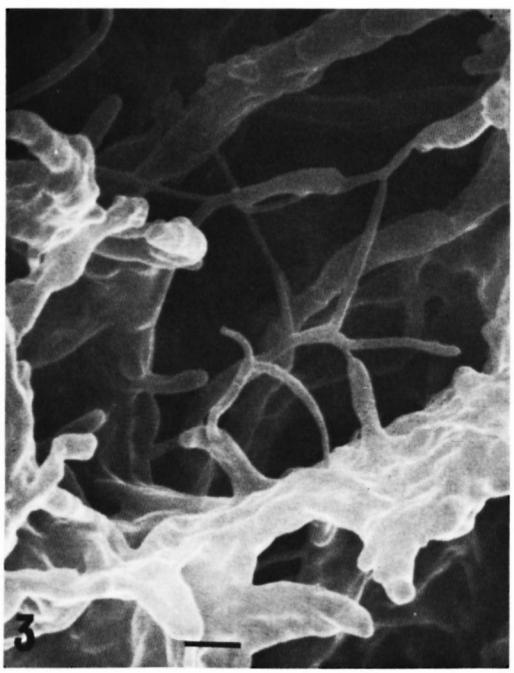


FIG. 3. Freeze-dried rough type colony of murine leprosy bacilli. The interbacillary filaments are present also in this specimen. × 35,000, scale bar: 0.2 μ.

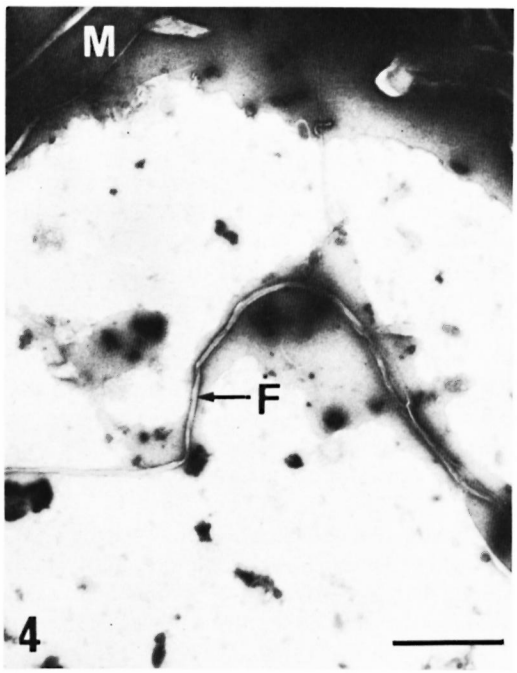


FIG. 4. Negative staining of rough type colony of murine leprosy bacilli which were destroyed gently and suspended in physiological saline solution. The filamentous strands are observed. M: murine leprosy bacilli; F: filamentous strand. ×14,000, scale bar: 1μ.

interbacillary filament was stained with ruthenium red and presented a tubule-like appearance (Fig. 6). Filaments sectioned transversely presented a ring-like appearance (Fig. 7). In the section of a colony not stained with ruthenium red, the interbacillary filament could not be seen clearly because of its low electron density.

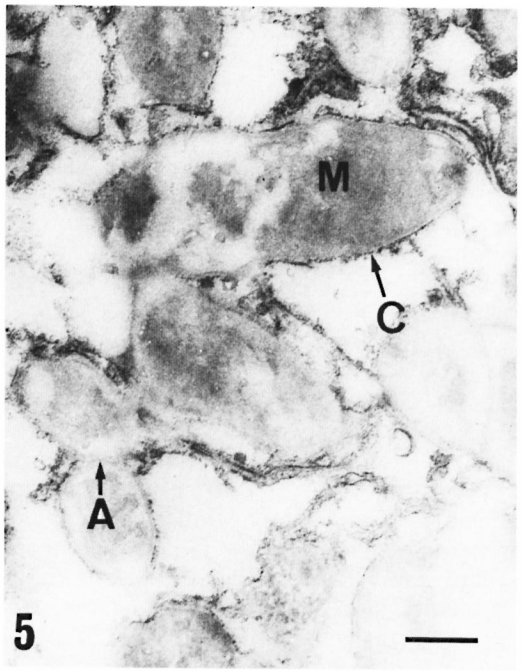
Judging from these facts, it can be concluded that the interbacillary filament is not an artifact produced in the process of preparation of the specimens.

Drucker and Whittaker reported that the well-separated, tangled filamentous bacilli growing vertically as well as laterally, are no doubt responsible for the rough appearance of colonies of *Nocardia graminis* NCTC 4728 ([7]). They also observed that the cells were haphazardly arranged within rough colonies of *Streptomyces scabies* and *Corynebacterium xerosis*, and that the smoother colonies of *Aerococcus sp.*, *Spirillium rubrum*, and *Vibrio metchnikovi* consisted of microorganisms which were regularly arranged ([6]). Takagi and Katsumoto studied colonies of S and R type of *Salmonella* and *Shigella* groups with SEM. They attributed the difference in gross morphology between S and R type to the difference of arrangement of bacilli in these colonies. They concluded that the smoothness or the roughness of colonies is related to whether the surfaces of cell bodies growing within colonies are hydrophilic or hydrophobic ([17, 18]). In the present study of murine leprosy bacilli, no difference of arrangement of bacilli in the R and S colonies was observed. Therefore, the cause of the difference in gross configuration of the colonies of microorganisms is not uniform among the different species of microorganisms.

Elmros and his colleagues ([8]) studied colonies of *Neisseria gonorrhoeae* with SEM. Virulent colonies of *Neisseria gonorrhoeae* have highly convex surfaces while colonies of avirulent strains exhibit a radial extension and flat upper surfaces, though the difference of gross form between both strains of this microorganism does not relate to the smoothness or roughness of the surface of the colony. An abundance of intercellular strands was found between cells in virulent colonies, but not in the avirulent colonies. They held that these strands seemed to anchor the cells to each other and to the agar surface and that the presence of such structures explained the highly convex surface of virulent colonies. Kraus and Glassman ([11]) also observed such strands in colonies of virulent types of gonococci.

In our ultrathin sections of colonies stained with ruthenium red, the homogeneous substance stained with ruthenium red filled up the space between clustered bacilli (Fig. 8). The homogeneous substance was connected with the coating of bacilli which stained with ruthenium red, having the same density as that of the coating. Accordingly, the homogeneous substance also consists of complex carbohydrate. In the colony observed with SEM, the space between bacilli was sometimes filled with a homogeneous substance. In some cases the marked deposit of homogeneous substance concealed the contour of individual bacilli (Fig. 9). The homogeneous substance observed with SEM seems to be identical to the substance which stained homogeneously with ruthenium red in ultrathin sections, and accordingly it also consists of complex carbohydrate. In papers dealing with the SEM studies of other bacteria, some authors have designated such homogeneous extracellular substance as "covering film" or "surface film" ([6, 7, 21]). However the term "film" is not appropriate, judging from the distribution of the substance shown in Figure 8. In the colony of some species of mycobacteria, the amount of this substance observed with SEM is considerable. It can then be held that the amount of complex carbohydrate included in the colony is quite considerable in such a case.

In the rough type colony treated with chloroform after dehydration with ethanol, interbacillary filaments were observed with SEM in the same manner as in control specimens not treated with chloroform (Fig. 10). Draper and Rees ([3-5]) observed filamentous or tape-like substances on the surface of murine leprosy bacilli or attached loosely to the bacilli which were isolated from livers or spleens of mice and thought that these composed the electron transparent zone surrounding murine leprosy bacilli when seen in host cells. They at first regarded this material as wax D_S and later as mycoside C. The interbacillary filaments found with SEM in the rough type colony in our study did not disappear on treatment with chloroform. Therefore, it can be concluded that it is not mycoside C, and is a newly found structure which is dif-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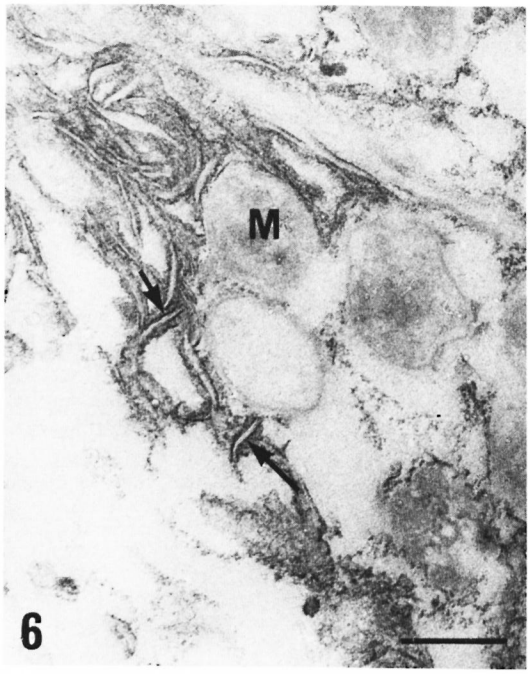
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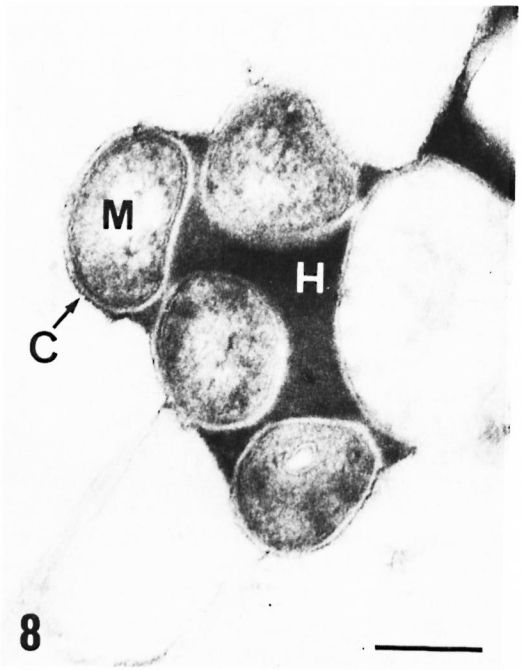
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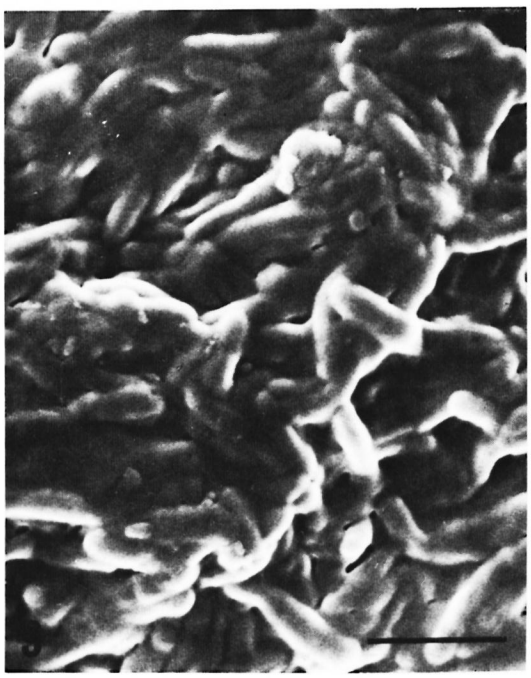

FIG. 9. Colony of murine leprosy bacilli. The extracellular substance fills up the space or valley between bacilli, and conceals the contour of each bacillus. × 17,500, scale bar: 1 μ.

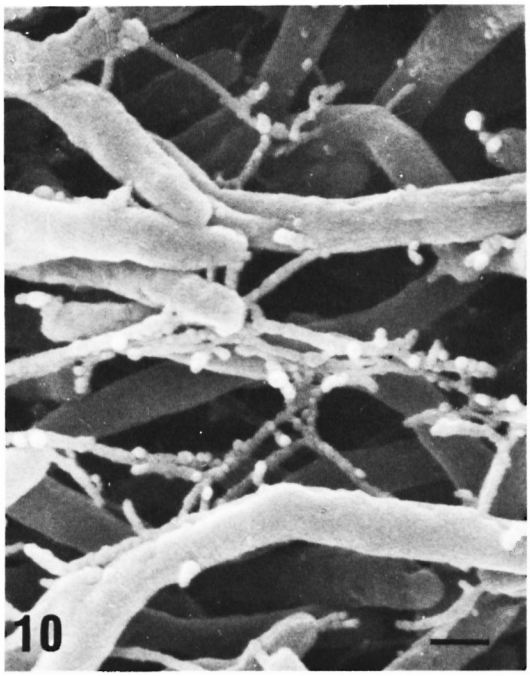

FIG. 10. Rough type colony treated with chloroform. The interbacillary filaments and granules are observed also in this specimen. × 35,000, scale bar: 0.2 μ.

ferent from the substance found by Draper and Rees and interpreted as composing the transparent zone surrounding bacilli within cells.

Rough type colonies incubated for one to three months and smooth type colonies incubated for one to four months were examined, but no differences were found in colonies incubated for different periods of time.

FIG. 5. Colony of murine leprosy bacilli stained with ruthenium red. The coat stained with ruthenium red surrounds the outside of bacilli, showing the jagged outer margin. The density of bacilli is low, because they were stained neither with uranium nor lead. When the bacilli adhere to each other, the coat is absent at the adhering site. M: murine leprosy bacillus; C: coat of bacillus; A: adhering site of two bacilli. ×48,000, scale bar: 0.2 μ.

FIG. 6. Rough type colony of murine leprosy bacilli stained with ruthenium red. The margin of interbacillary filaments are stained with ruthenium red, and presented the tubule-like appearance (arrow). M: murine leprosy bacillus. × 70,000, scale bar: 0.2 μ.

FIG. 7. Rough type colony of murine leprosy bacilli stained with ruthenium red. The interbacillary filament sectioned transversely presents the ring-like appearance, the margin being stained with ruthenium red (arrow). × 57,500, scale bar: 0.2 μ.

FIG. 8. Colony of murine leprosy bacilli stained with ruthenium red. The homogeneous substance stained with ruthenium red fills up the space or valley between bacilli, and is connected with the coat of complex carbohydrate surrounding the cell wall of bacilli. M: murine leprosy bacillus; C: coat of bacillus; H: homogeneous substance. ×70,000, scale bar: 0.2 μ.

SUMMARY

Colonies of the Hawaiian strain of murine leprosy bacilli, grown on Ogawa's egg yolk medium, were observed with a scanning electron microscope. In the rough type colony, filamentous strands which were 40-148 mμ (mostly 70-120 mμ) in diameter were found between the bacilli and designated as "interbacillary filament." Additionally, disc-like granules which were 60-300 mμ (mostly 100-200 mμ) in diameter were occasionally observed on the surfaces of bacilli and interbacillary filaments. Interbacillary filament and granules such as those found in the rough type colony could not be found in the smooth type colony. The interbacillary filament is not an artifact produced in the process of preparation of a specimen but is a newly found structure which is different from the filamentous or tape-like substance previously regarded by Draper and Rees as composing the transparent zone surrounding murine leprosy bacilli within host cells.

The cause of differences in gross configurations of R and S type colonies is not uniform among the different species of microorganisms.

The homogeneous extracellular substance which fills the space between bacilli in colonies of murine leprosy bacilli is a complex carbohydrate and is connected with a coating of complex carbohydrate surrounding the cell wall of bacilli.

RESUMEN

Se estudiaron las colonias del bacilo de la lepra murina, cepa Hawaii, crecidos en medio de Ogawa con yema de huevo, usando la microscopía electrónica de barrido (scanning). En la colonia del tipo rugoso, se observaron estructuras filamentosas de 48 a 148 mμ de diámetro (en su mayoría de 70 a 120 mμ) las cuales se denominaron como "filamentos interbacilares." Además, ocasionalmente se observaron gránulos discoides de 60 a 300 mμ de diámetro (en su mayoría de 100 a 200 mμ) sobre la superficie de los bacilos y de los filamentos interbacilares. En las colonias del tipo liso no se observaron ni filamentos interbacilares ni estructuras discoides. El filamento interbacilar no es un artefacto producido durante la preparación de la muestra sino que es una estructura recientemente encontrada que es diferente de la substancia filamentosa o acintada considerada previamente por Draper y Rees como componente de la zona transparente que rodea a los bacilos de la lepra murina dentro de las células huésped.

La causa de las diferencias en las configuraciones gruesas de las colonias de los tipos R y S, no es uniforme entre todas las diferentes especies de microorganismos.

La substancia extracelular homogénea que llena el espacio entre los bacilos en las colonias del *M. lepraemurium*, es un carbohidrato complejo que está conectado con una capa de carbohidrato complejo alrededor de la paréd celular de los bacilos.

RÉSUMÉ

On a étudié, au moyen du microscope électronique à balayage, des colonies de la souche hawaïenne de bacilles de la lèpre murine, cultivées sur le milieu au jaune d'oeuf d'Ogawa. Dans la colonie de type "rough", des extensions filamenteuses d'un diamètre variant de 40 à 148 mμ (le plus souvent entre 70-120 mμ) ont été observées entre les bacilles; on les a nommées "filaments interbacillaires". De plus des granules en forme de disque, d'un diamètre de 60-300 mμ (principalement 100-200 mμ), ont été observés, à l'occasion, sur la surface des bacilles et des filaments interbacillares. Filaments interbacillaires et granules tels que ceux qui ont été observés dans les colonies de type "rough" n'ont pas pu être retrouvés dans les colonies de type "smooth". Le filament interbacillaire n'est pas un artéfact qui serait produit au cours de la préparation de l'échantillon, mais bien une structure entièrement nouvelle, différente de la substance filamenteuse ou en ruban que Draper et Rees on considéré jadis comme faisant partie de la zone transparente qui entoure les bacilles de la lèpre murine à l'intérieur des cellules qui les hébergent.

La cause des différences observées dans les configurations des colonies de type R et S n'est pas uniforme pour les différentes espèces de microorganismes.

La substance extra-cellulaire homogène qui remplit l'espace entre les bacilles dans les colonies de bacilles de la lèpre murine, est un hydrate de carbone complexe; elle est en contact avec un revêtement d'hydrates de carbone complexes qui entoure la paroi cellulaire des bacilles.

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