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MÉMOIRES ORIGINAUX

The ultrastructure of *Rickettsia slovaca* in naturally infected females of the tick *Dermacentor marginatus*

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Ultrastructure de *Rickettsia slovaca* dans des tiques femelles de *Dermacentor marginatus* naturellement infectées.

RESUME. La structure fine de *Rickettsia slovaca* dans des femelles naturellement infectées de la tique *Dermacentor marginatus* est similaire à celle d'autres rickettsies du groupe « spotted fever ». *R. slovaca* se multiplie dans tous les organes et tissus examinés. Elle se trouve généralement dans le cytoplasme ; occasionnellement elle peut aussi envahir le noyau.

Les rickettsies des glandes salivaires ont été soigneusement étudiées. Elles se multiplient dans toutes les cellules des trois types d'acini (I-III), ainsi que dans les cellules des conduits évacuateurs. Le nombre de rickettsies éliminées par la salive atteint la valeur approximative de $10^4 EID_{50}$/ml.

SUMMARY. The ultrastructure of Rickettsia slovaca *in naturally infected females of the tick* Dermacentor marginatus *is similar to that of other members of the spotted fever group rickettsiae.* R. slovaca *was found to multiply in all organs, tissues and cells examined. It is primarily found free in the cytoplasm ; occasionally it may also invade the cell nucleus.*

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Special attention was paid to the rickettsiae in the salivary gland. They multiply in all cells of the three acini types I-III and also in the duct cells. The amount of rickettsiae eliminated via the saliva reached values of about $10^4 EID_{50}/ml$.

Introduction

Rickettsia slovaca, a member of the spotted fever group of rickettsiae, was discovered in Slovakia in 1968 (Brezina et al., 1969) but received its name only recently (Úrvölgyi and Brezina, 1978). The rickettsia was found to be pathogenic for man (Brezina, personal communication). Its main vector and reservoir is the tick *Dermacentor marginatus* (Řeháček et al., 1972 ; Župančičova-Majerská et al., 1972).

The fine structure of *R. slovaca,* cultivated in experimentally infected *Hyalomma dromedarii* and *Dermacentor marginatus* ticks and in chick embryo yolk sacs, has been described (Čiampor et al., 1978 ; Sixl-Voigt et al., 1973). No studies have been made on the ultrastructure of this rickettsia within naturally infected ticks.

The present study was undertaken to investigate the fine structure of *R. slovaca* in various cell types from different organs, and especially from salivary glands of infectious *D. marginatus* females collected in nature. Special attention was paid to the question whether salivary glands serve only for the collection and storage of rickettsiae prior to their release via saliva during feeding or if they also play a role in the propagation of the agent.

Material and methods

Infected ticks.

Adult *D. marginatus* infected with *R. slovaca* were collected in natural foci of this rickettsia in Central Slovakia ; the infected ticks were selected by the hemocyte test (HT) (Řeháček et al., 1971). All the ticks were fed for 3-6 days on guinea pigs and then a portion of them were reared for 3 days at about 24 °C to support further propagation of rickettsiae. Only specimens manifesting very high infection as judged by the HT were chosen for further examination.

Electron microscopy (EM) of the rickettsiae.

The infected females were dissected in physiological saline (pH 7,2). Salivary glands were withdrawn either as a single organ or en bloc together with adjacent organs like muscles, tracheae, nephrocytes, fat body, hemocytes, etc. One of the pair of glands was used for EM ; from the other gland a smear was prepared on a slide for the control of the rickettsial infection by the immunofluorescence method.

Fixation was carried out for 2 hours at 4 °C with 2,5 % glutaraldehyde (TAAB) in sodium cacodylate buffer (0,05 M, pH 7,2) containing 75 mM sucrose. After fixation tissues were washed overnight at 4 °C with the same buffer containing 150 mM sucrose. Postfixation occurred in 2 % O_8O_4 in the same buffer with 120 mM sucrose for 2 hours at 4 °C. All solutions contained 2 mM Ca^{2+} and 2 mM Mg^{2+}.

After a brief rinse in the wash solution, the specimens were dehydrated in cold ethanol and embedded in Spurr's resin (TAAB). Thin sections were cut with diamond knives and

stained according to standard procedures. They were examined in a Philips EM 201 fitted with a goniometer stage at 60 or 80 kV.

Demonstration of rickettsiae in tick saliva.

The semiengorged infectious *D. marginatus* females, treated for a few minutes with infra-red light (Barker et al., 1973), eliminated droplets of saliva contaminated with rickettsiae. These samples were collected from the tip of the hypostome into microtubes. The agent from this material was then titrated in chick embryo yolk sacs.

Results

Experimental results repeatedly showed that *R. slovaca* produces a generalized infection in all tissues and cell types of naturally infected *D. marginatus* females.

Rickettsiae were found not only to be stored but also growing and dividing as demonstrated by binary fission of the agent (*fig. 2, 5 and 6*). Cells infected either lightly or heavily with *R. slovaca* apparently did not exhibit cytopathic changes.

The majority of rickettsiae was found free in the host cell cytoplasm amongst the various cell organelles (*fig. 1-3*). In one case, a single rickettsia was observed to be enclosed in a secretory vesicle of a salivary gland cell (*fig. 4*) ; this however seems to be exceptional. Occasionally, rickettsiae also invaded cell nuclei (*fig. 1, 6 and 11*). Binary fission was observed in several cases (*fig. 6*). Rickettsiae were found singly or in groups ; in the latter case they were sometimes so densely packed that the electron-lucent « halos » surrounding the agents appeared confluent (*fig. 2*).

In general, *R. slovaca* from naturally infected *D. marginatus* exhibits ultrastructural features characteristic for rickettsiae from the spotted fever group ; it displays almost the same pattern as the agent cultivated in *H. dromedarii* and in chick embryo yolk sacs (Čiampor et al., 1978). *R. slovaca* is a typically rodshaped rickettsia with dimensions of 0.37-0.45 × 0.8-1.2 µm in our experimental conditions. The cell membrane is about 5-5.5 nm thick (*fig. 7-9*). It is separated from the cell wall by a periplasmic space of about 8-11 nm. The cell wall appears very similar to that of *R. prowazeki* or *R. rickettsii*, but differs from that of *R. tsutsugamushi* (Silverman and Wiseman, 1978 ; see also this paper for the terminology of the different cell wall components). The cell wall is composed of an inner leaflet of 5.2-5.5 nm which may show a very small clear space in the center, of an outer leaflet measuring 2-2.5 nm and of a clear space between both leaflets measuring 2-2.6 nm. On the outside of the outer leaflet appears a fuzzy microcapsular layer of about 16 nm thickness. External to this layer follows a clear zone (« halo ») of variable thickness up to 90-100 nm ; this corresponds to the slime layer described recently in *R. prowazeki* and *R. rickettsii* (Silverman et al., 1978).

The cytoplasm of *R. slovaca* contains numerous irregularly dispersed ribosomes (*fig. 8*) ; in some rickettsiae very fine fibrillar strands are visible which may represent DNA (*fig. 7*). On one occasion a network of dense DNA-like fibrils (*fig. 12*) was observed, which resembled the centrally located DNA-strands in *Coxiella bur-*

neti (Anacker et al., 1964 ; Gulevskaja and Kudelina, 1968) and which was previously postulated to be a nuclear equivalent (Stoker et al., 1956). A contamination of this tick with a few single rods of *C. burneti* cannot entirely be excluded, although this possibility appears very unlikely, especially in view of the absence of an intravacuolar localization which is typicial for *C. burneti.* Therefore, one must be cautious about this single observation of thick DNA-like strands. Similar centrally located filaments have not been observed in *R. canada* (Brinton and Burgdorfer, 1971).

Special attention was paid to the rickettsiae in the salivary glands. *R. slovaca* was present in all cells of the three types of acini I-III and also in the salivary duct cells (*fig. 1-7, 10 and 11*). Binary fission could be observed in all cell types. This clearly demonstrates that the salivary glands play an active role in rickettsiae propagation ; they are not simply organs in which rickettsiae are collected before their release into the host via the saliva.

In general, rickettsiae in salivary gland duct cells looked « healthy » and did divide ; on rare occasions however, some rickettsiae within the cytoplasm or the nucleus of such cells appeared to manifest malformations or degenerative changes. The cytoplasm and cell membrane were retracted from the cell wall. Sometimes, the cytoplasm was empty-looking ; ribosomes were rare or absent (*fig. 10 and 11*). In the nucleus, the cytoplasm of some rickettsiae also appeared condensed (*fig. 11*). At the moment these rare observations are unexplained.

The amount of rickettsiae eliminated via the saliva reached values of about 10^4 EID_{50}/ml. As the percentage of *D. marginatus* ticks infected with rickettsiae in Central Slovakia is very high (Řeháček et al., 1972) and most individuals of mainly goats and sheep are attacked by several hundred ticks, it follows that these animals are inoculated yearly with tremendous quantities of rickettsiae.

Discussion

The results obtained in the present study confirm again that *R. slovaca* belongs to the spotted fever group of rickettsiae. Typically the agent is located free in the cytoplasm ; it is not found in vacuoles surrounded by a limiting host membrane as observed with *C. burneti* (Handley et al., 1967) or with *R. sennetsu* (Anderson et al., 1965 ; Tanaka and Hanaoka, 1961). Occasionally, cell nuclei are infected by « healthy » looking rickettsiae ; images of dividing agents indicate that the environment of the nucleus is sufficiently favorable for propagation. Nuclear localization of *R. slovaca* was also reported in experimentally infected *H. dromedarii* (Čiampor et al., 1978). *R. slovaca* invaded cell nuclei in *D. marginatus* only occasionally, in the same manner as *R. rickettsii* rarely infected nuclei of *D. andersoni* cells (Burgdorfer et al., 1968).

With conventional cytological techniques the structure of the cell wall of *R. slovaca* appears very similar to that of typhus and spotted fever group rickettsiae, but is unlike that of *R. tsutsugamushi* (scrub typhus group) (Silverman and Wisseman, 1978).

To demonstrate the microcapsular layer and the slime layer on the outside of the outer cell wall leaflet, additional staining techniques are necessary : e.g. ruthenium red staining of *R. prowazeki* revealed the microcapsular layer, with a thickness of 12.5-16.5 nm, to be composed of subunits with a diameter of 8.5-10 nm and a periodicity of 10-12 nm (Popov and Ignatovic, 1976). Staining, with either 2 % phosphotungstate at pH 7 or with 0.5 % aqueous uranyl acetate at pH 4.5, demonstrated tetragonally arranged subunits in the outer layer of the envelope of *R. prowazeki* and *R. akari* which project about 7 nm above the cell wall with a periodicity of 13 nm (Palmer et al., 1974). The use of a specific antibody stabilization technique, and staining with methenamine silver and ruthenium red revealed a slime layer on the outside of the outer cell wall leaflet of *R. rickettsii* and *R. prowazeki,* which is largely polysaccharide in nature. This very labile structure is likely to correspond to the large, electron-lucent, halo-like zone, which is seen by conventional electron microscopy to surround rickettsiae of the typhus and spotted fever groups. It is speculated that this slime layer may contain major group — specific antigens ; it could be important in the attachment to host cells and/or it could function with an antiphagocytotic mechanism (Silverman et al., 1978). Use of the above mentioned special staining techniques will presumably reveal similar surface structures in *R. slovaca.*

The numerous ribosomes of *R. slovaca* are irregularly dispersed in the cytoplasm. Ribosomes were not observed to follow a membraneous pattern with a dense aggregate to one side of the vacuoles, as has been found for example in *R. prowazeki* in hemocytes of experimentally infected *H. dromedarii* (Bird et al., 1967) ; nor were ribosomes concentrated at the cell periphery, often in close association with the cell membrane, as described for *C. burneti* (Burton et al., 1975).

In *R. slovaca,* the electron-translucent spherical structures which are commonly present in *R. typhi* (Ito et al., 1978) and in *R. prowazeki* from ticks (Bird et al., 1967), from lice (Silverman et al., 1974) or from monkey kidney cell cultures (Anderson et al., 1965) were not observed. Such structures were also absent in *R. rickettsii* (Anderson et al., 1965). It seems therefore that these structures may be typical for typhus group rickettsiae. However, such spherical structures were observed in *R. slovaca* within tick salivary glands by Sixl-Voigt et al. (1973). Their significance is not really understood ; they may represent inclusions of lipids or other substances which are easily removed during specimen preparation (Ito et al., 1978).

In *R. slovaca* from *D. marginatus* the crystalloid structures described in *R. canada* within hypodermal cells of *D. andersoni* (Brinton and Burgdorfer, 1971) and within chick embryo cells (Avakyan et al., 1972) were not observed.

Several species of rickettsiae are supposed to display various morphological forms ; e.g. *R. canada* can be differentiated into three morphologically distinct forms according to the staining intensity of the cytoplasmic matrix, the dispersion of ribosomes and the presence of crystalloid bodies that have a « striated beaded lattice structure » in the cytoplasm (Brinton and Burgdorfer, 1971). Based on slight differences in the ultrastructure of *R. canada, R. prowazeki, R. sibirica* and *C. burneti,* Russian rickettsiologists distinguished « vegetative and resting forms » of rickettsiae (e.g.

Avakyan et al., 1972). The existence of the above described forms in *R. slovaca* have not yet been observed here.

In *R. slovaca, R. sibirica, R. conorii* and *R. akari,* which were cultivated in *H. dromedarii,* Čiampor et al. (1978) described the appearance of an electron-transparent space at both poles of rickettsiae. This space forms « pole caps » or sometimes, after folding, « pole tails ». To date similar structures in *R. slovaca* from naturally infected *D. marginatus* have not been observed. Whether this difference is due to the different host system or to the different preparation methods, needs further investigation.

Despite a careful study of many tissue sections, neither the actual process of release of rickettsiae into the salivary gland lumen nor the presence of free rickettsiae in the salivary ducts was observed. This may perhaps be explained by the fact that the ticks were no longer salivating at the moment of dissection and fixation.

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LEGENDS

Fig. 1. Salivary gland acinus (type II). Rickettsiae are visible within the nucleus (N) and the cytoplasm (→) of a « water cell ». CC : « cap cell ». S : secretory cell (terminology of the cell types according to Meredith and Kaufman (1973). X 9'780.

Fig. 2. Secretory cell of a salivary gland acinus (type III). The cytoplasm contains many rickettsiae surrounded by an electron-lucent « halo ». → : dividing rickettsia. ER : rough endoplasmic reticulum. X 22'900.

Fig. 3. Salivary gland acinus (type I). The cytoplasm contains a rickettsia (R) surrounded by a « halo » (*). G : Golgi region. N : nucleus. X 37'400.

Fig. 4. Secretory cell from a salivary gland acinus (type II). A rickettsia (R) enclosed in a secretory vacuole (S) ; this has been observed only once. ER : rough endoplasmic reticulum. G : Golgi region. X 41'600.

Fig. 5. Salivary gland acinus (type II). Dividing rickettsia in a secretory cell. → ← : plane of division. Electron-lucent « halo » : *. X 98'000.

Fig. 6. Salivary gland acinus (type II). Many rickettsiae are located within the nucleoplasm (N) of a secretory cell. → ← : dividing rickettsia. X 42'400.

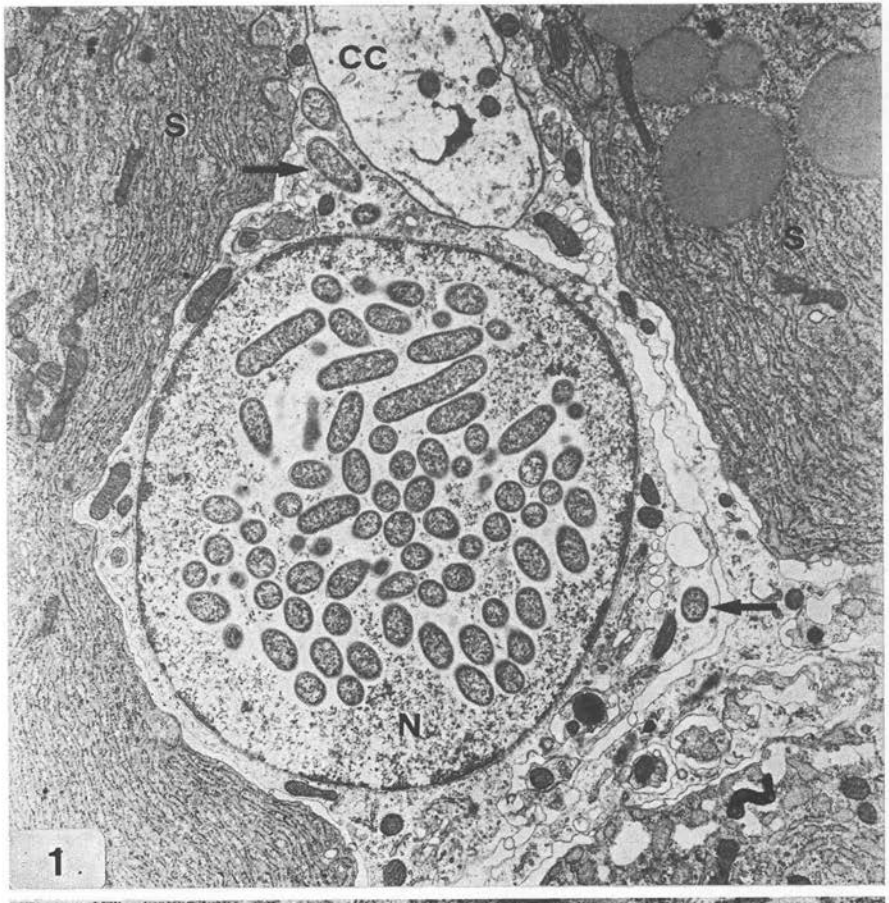
Fig. 7. Secretory cell of a salivary gland acinus (type II). Fine fibrillar material (→), possibly representing DNA, is visible within the rickettsia. → : cell membrane. ⟶ : cell wall. * : « halo ». X 183'000.

Fig. 8. Rickettsia in a midgut cell. R : ribosome-like particles. 1 : cell membrane. 2 : periplasmic space. 3 : electron-dense inner leaflet of the cell wall, sometimes showing a fine clear space in the center. 4 : electron-transparent space between the outer and the inner leaflets. 5 : electron-dense outer leaflet of the cell wall. 6 : microcapsular layer. * : electron-lucent clear space (« halo ») corresponding to the slime layer. Terminology according to Silverman and Wisseman (1978). X 186'800.

Fig. 9. Rickettsia in the cytoplasm of a hemocyte. For symbols refer to Fig. 10. X 201'000.

Fig. 10-11. Some rickettsiae with an altered ultrastructure have sometimes been observed amongst « healthy » looking rickettsiae (*) in the cytoplasm (fig. 10) or in the nucleus (fig. 11) of salivary gland duct cells. The cytoplasm appears to be retracted from the cell wall ; it may be empty-looking or condensed. M : mitochondria. N : nucleus. Fig. 10 : X 48'700. Fig. 11 : X 26'900.

Fig 12. Midgut cell. This single observation shows a rickettsia containing a network of thick strands (→). X 91'200.



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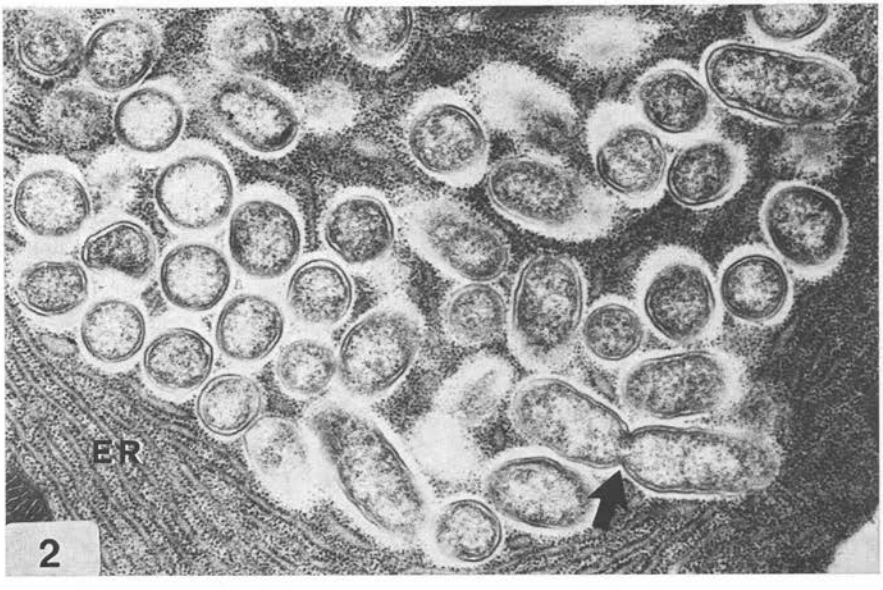

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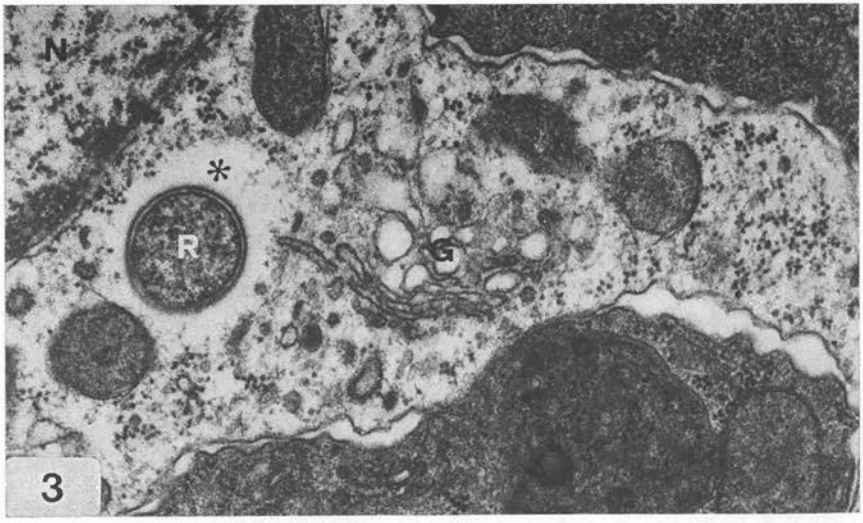
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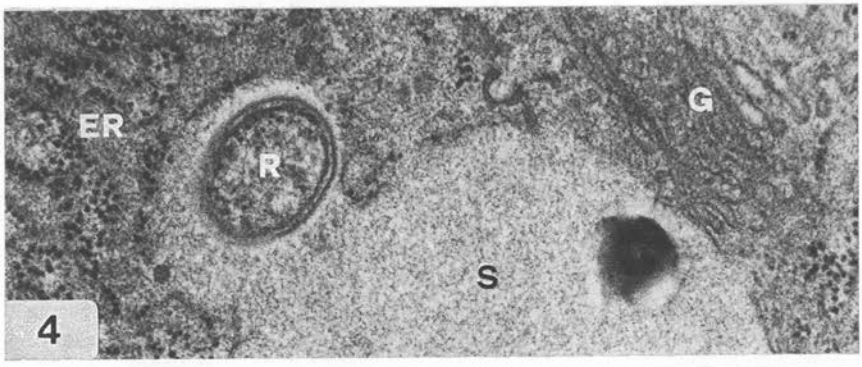
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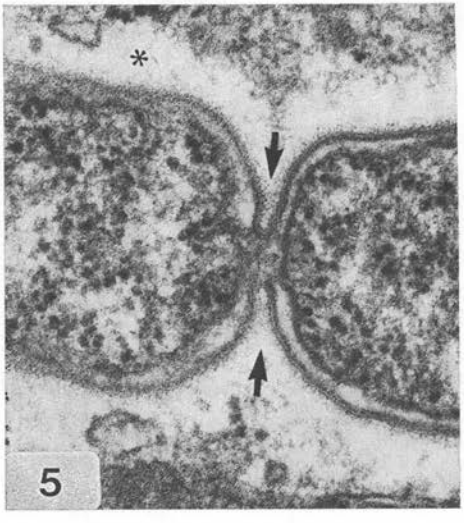
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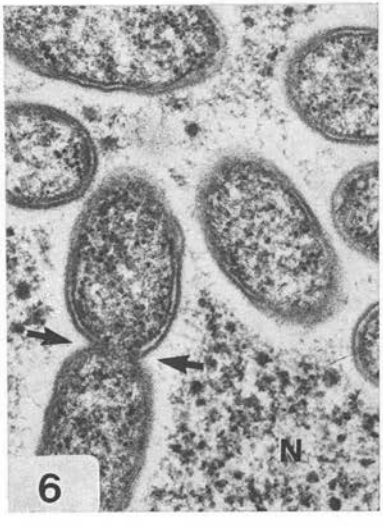
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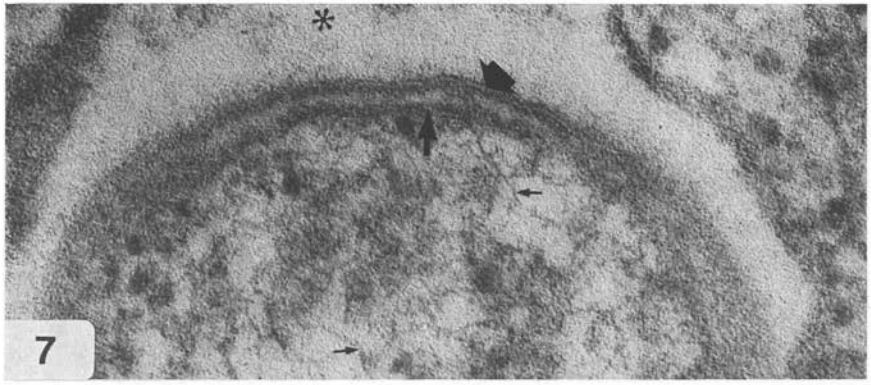
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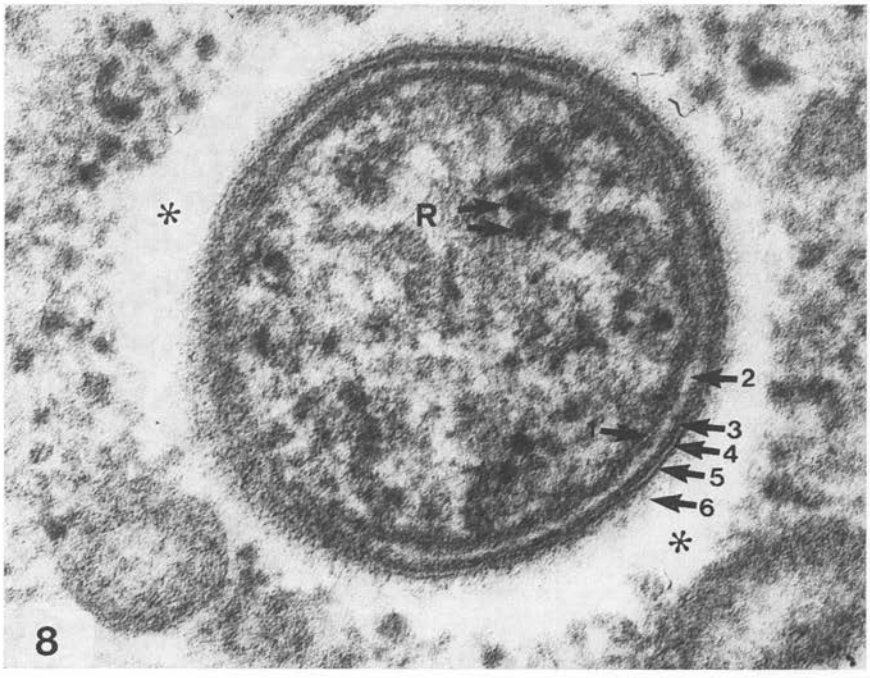
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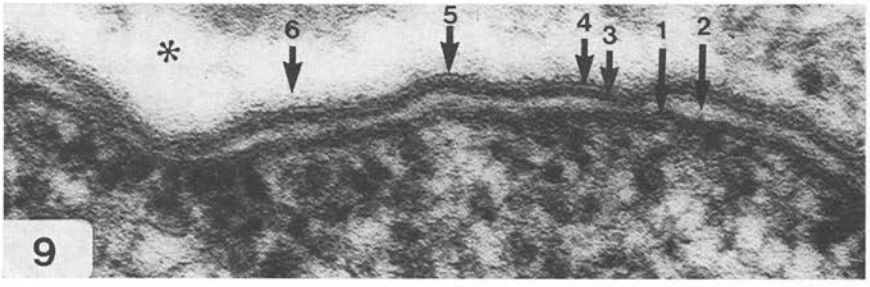
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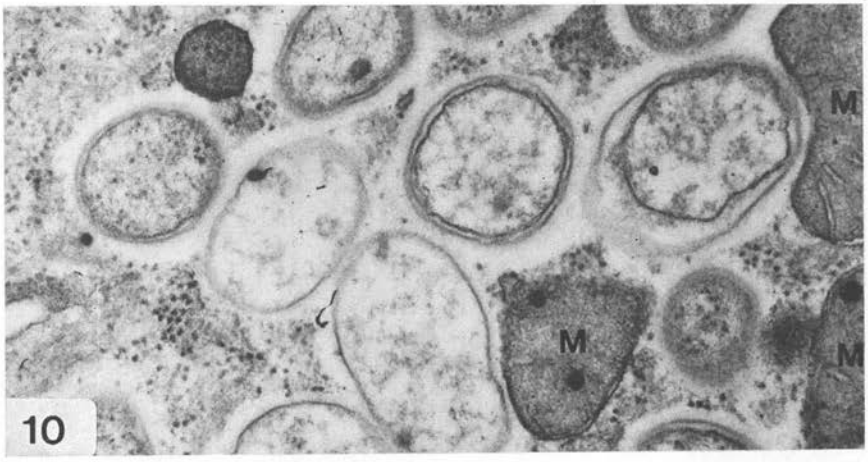

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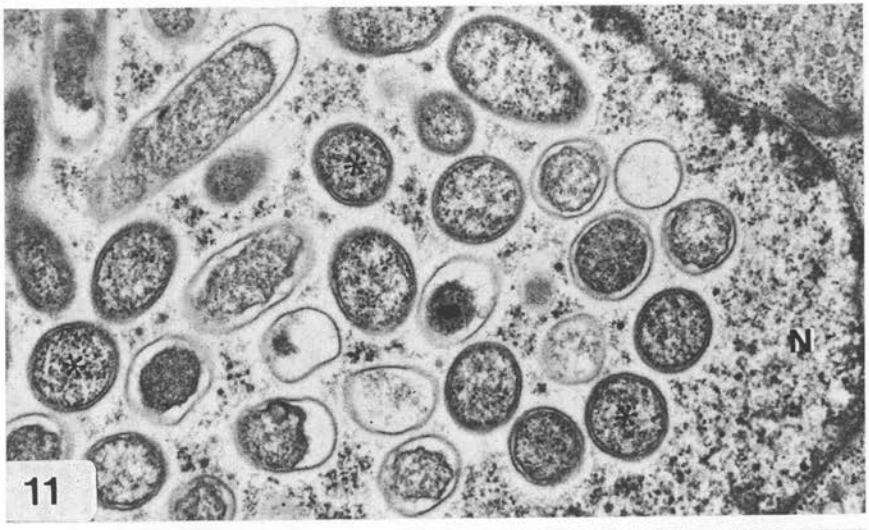

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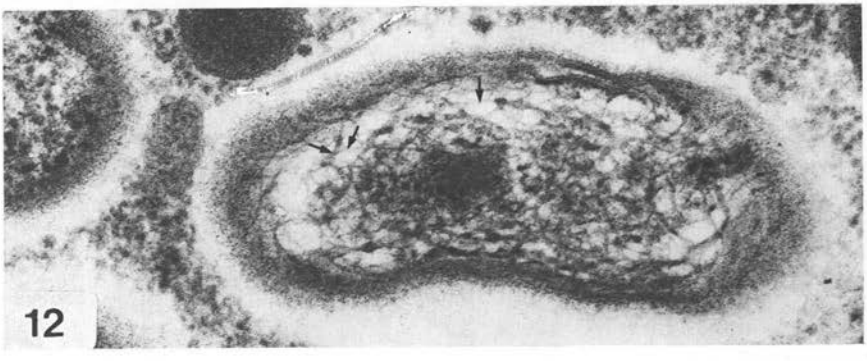

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