KOR. JOUR. MICROBIOL. Vol. 17, No. 1, 1~15, 1979

Ultrastructure and Morphological Features of *Mycoplasma pneumoniae* during Culture Development

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Mycoplasma pneumoniae 細胞의 發達過程 중 微細構造 및 形態學的 特徵

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ABSTRACT

Mycoplasma pneumoniae strain CL-8 attached to broth-covered surfaces was examined sequentially during growth from single cells for morphologic and ultrastructural changes using several different electron microscopic techniques. Changes in morphology revealed both round and spindle shapes and observation of cell transitions suggested some type of morphological cycle. The round-to-ovoid cells observed in the early stages of growth appeared to be viable, and morphologically and ultrastructurally different from the spherical forms which were produced during the latter stage of growth. The spindle segments were detected until the late stationary phase. The knob-like structure of the segments appeared to be structurally the same as the terminal cored structure seen in thin sections and may be a growing point or an attachment site of the cell. A tubular structure was observed in the core of the terminal structure and a microtubule-like element appeared to bridge between some spindle segments. A matrix substance was observed around single cells as well in the intercellular space of the colonies prepared by critical-point drying and may be involved in adhesion of the organisms to solid surfaces. Asymmetrical triple-layered cytoplasmic membranes, surfaces, of which appeared to be structurally different each other, were observed in young cells, whereas symmetrical and thicker membranes were seen in older cells. Small bodies were found in 4d or older cultures and did not appear to contain any internal structures or an easily detectable unit membrane.

INTRODUCITON

During the past decade, light and electron microscopic studies have shown morphological and ultrastructural heterogeneity among mycoplasma species(Boatman, 1973; Domermuth *et al.*, 1964; Freundt, 1970; Zucker-Franklin *et al.*, 1966). *Mycoplasma pneumoniae* appears to be pleomorphic, with round(Furness *et al.*, 1968) and/or filamentous shapes

(Boatman, 1971; Collier, 1972; Kammer *et al.*, 1970; Knudson *et al.*, 1970) as its major morphologic forms.

The morphological and ultrastructural aspects of *M. pneumoniae* adherent to glass or plastic surfaces (Biberfeld *et al.*, 1970; Bredt 1968; Kim *et al.*, 1977) or to mammalian cells (Collier *et al.*, 1971; Powell *et al.*, 1976; Wilson *et al.*, 1976; Zucker-Flanklin *et al.*, 1966) have also been investigated. Kim *et al.*, (1977) reported that *M. pneumoniae* attahced to solid surfaces revealed segmentous cells consisting of a spindle body with one pointed and one knob-like end. Different strains of the organism attached to glass revealed similar morphology (Bredt, 1968, 1974; Hubbard *et al.*, 1971; Radestock *et al.*, 1977), Phase-contrast microscopy (Bredt, 1968; Hubbard *et al.*, 1971) and scanning electron microscopy (Biberfeld *et al.*, 1970; Kammer *et al.*, 1970; Klainer *et al.*, 1973) have been used to observe the sequential changes in the organism as it proliferated. It was difficult to observe the detailed morphology due to the limited resolving power of the instruments and successive observations have not been recorded throughout any growth cycle.

M. pneumoniae is considered as possessing a triple-layered "unit membrane", ribosomes, and the nuclear materials seen in other mycoplasma species (Domermuth *et al.*, 1964; Freundt, 1970; Maniloff *et al.*, 1972; Zucker-Flanklin, 1966). In addition, Biberfeld *et al.* (1970) and Collier *et al.* (1972) reported that the filamentous cells of *M. pneumoniae* had terminal cored structures which were proposed to be involved in multiplication, attachment, and pathogenicity.

The present work makes use of several electron microscopic techniques for successively examining the morphology and development of *M. pneumoniae* cells on the broth-covered solid surfaces and reveals some new morphological and ultrastructural features of this microbe.

MATERIALS AND METHODS

Organism and Culture Medium

Mycoplasma pneumoniae strain CL-8 (Somerson *et al.*, 1973) was isolated at Children's Hospital, Columbus, Ohio. Organisms were passed in artificial media 18 to 38 times. After storage at −70°C, *M. pneumoniae* was subjected to two passages for full recovery of the metabolic activities in SSR-2 broth medium (Sommerson *et al.*, 1973) in prescription bottles. In the early stationary phase, culture fluids were drained and glass adherent mycoplasmas were harvested and concentrated by scraping into fresh broth medium at one-tenth their original fluid volume. Cell clumps in the culture were dispersed for 5 min. by use of a Vortex mixer (Furness *et al.*, 1968). The culture was centrifuged at 2,000 × g to remove remaining aggregates and the supernatant fluid was passed through a 0.4μm Nuclepore membrane filter. This filtrate containing single cells was used as an inoculum.

The SSR-2 borth medium consisted of mycoplasma broth base buffered with HEPES (n-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid; Calbiochem, La Jolla, Ca.) and supplemented with Eagle's Minimal Essential Medium, fresh yeast extract, bovine serum fraction (Difco), and dextrose. In some instances, horse serum (Lot #27060, KC Biological, Inc.,

Lenexa, KS)was substited for the bovine serum fraction.

Counting of Colony Forming Unit(CFU)

The single cells were inoculated into 30 ml of SSR-2 broth medium in 8 oz. prescription bottles and incubated in horizontal position at 37° C in normal environment. Each day, the cultures were harvested by scraping with rubber policemen, dispersed for 5 min using a Vortex mixer, and followed by a serial ten-fold dilution in fresh broth medium. 0.02 ml of each dilution was put on the SSR-2 agar plate (Sommerson *et al.*, 1973). The plates were incubated at 37°C in a humid 5% CO_2. After 2 wk incubation, the plates were overlaid with 2 & sheep blood in 0.6% Ironagar No. 2 (Colab Lab. Inc., Chicago Height, Il.) with 0.85% NaCl. After an additional 2 d incubation, hemolytic plaques were counted.

Light and Electron Microscopy

The single cells were cultivated on the surfaces of formvar-and/or carbon-coated electron microscope grids and Epon 812 plats (Kim *et al.*, 1977) in Petri dishes containing SSR-2 broth medium at 37°C in a normal environment. At specified intervals, the materials containing adherent cells were removed from the culture fluids and prepared for further microscopic techniques.

For light microscopy, the cover slips which the organisms attached to were fixed with 3.25% glutaraldehyde in 0.1 M cacodylate buffer, *p*H 7.2 for 1 h, washed with distilled water, and air dried. The samples were shadowed with germanium by a shadow-casting technique which is used for electron microscopy and then were examined with a Zeiss photomicroscope(Kim *et al.*, 1977).

The organisms grown on the surface of formvar-carbon coated 200 mesh copper grids (E. F. Fullam, Inc., Schenectady, N. Y.) were fixed with 3.25% glutaraldehyde for 1 h, washed in distilled water, dehydrated through a graded ethanol series, and placed in amyl acetate. The grids were transferred into a wire container and critical-point dried using CO_2 in a Samdri PVT-3 (Biodynamics Res. Corp., Rockville, Md.)

For carbon replication of whole cells, the organisms attached to the formvar-coated 300 mesh copper grids were air dried after fixation with 3.25% glutaraldehyde and 1 h washing in distilled water. The samples were shadowed with palladium-gold and rotary coated with carbon (Kim *et al.*, 1977). Carbon replicas were made using the method of Bradley (1957). For replication of freeze-etched membrane, the cells harvested from the prsecription bottles were suspended in the cacodylate buffer containing 10% glycerol for 30 min. The cells were spun down at 8,000 × g for 30 min and small droplets of these preparations were placed on 3mm copper discs and were immediately frozen in liquid Freon-12 cooled in liquid nitrogen. The specimens were freeze-cleaved, etched, and shadowed with carbon in a Balzers freeze-etching apparatus(Balzers High Vacuum Corp., Santa Anna, Ca.) The carbon replicas were floated off on distilled water, cleaned through a graded sulfuric acid series, washed in distilled water, and then picked up on electron microscope grids.

Organisms were permitted to adhere to and grow on pieces of Epon 812 plate which had been submersed in fluid culture medium (Kim *et al.*, 1977).The Epon

pieces were removed from the culture fluids, fixed using Luft's ruthenium red technique(Luft, 1971) dehydrated through a graded ethanol series, and embedded in fresh Epon 812. Thin sections perpendicular to the old Epon surface were made and double post-stained with uranyl acetate and lead citrate (Echlin, 1964). All preparations for transmission electron microscopy were examined with a Phillips EM-300 electron microscope at an accelerating voltage of 80 to 100 kv.

The critical-point dried samples were sputter coated with gold in a Hummer Ⅲ coater(Technis, Alexandria, Va.) and examined with an Hitachi S-500 scanning electron microsope at voltage of 20 kv. Images of the specimens were recroded on Polaroid P/N films.

RESULTS

Sequential development *of M. pneumoniae* cells were examined by the modified light microscopy(shadowed). Cultures were started from single cells of 2×10^5 colony forming units per milliliter. By using this dilute inoculum, the growth pattern was extended over 11 days as seen in Fig. 1 and the pH of the culture was slowly declined during the growth curve. The lengthy period gave us an opportunity to observe the successive morphologic changes in the cells. Organisms attached to and grown on the cover slips revealed a changing morphology as shown in the modified light micrographs (Fig. 2). The single cells were primarily round forms with an occasional short filament. The samples from 1 d through 7 d short filaments produced from round cells or other filaments (Fig. 2A-2D).

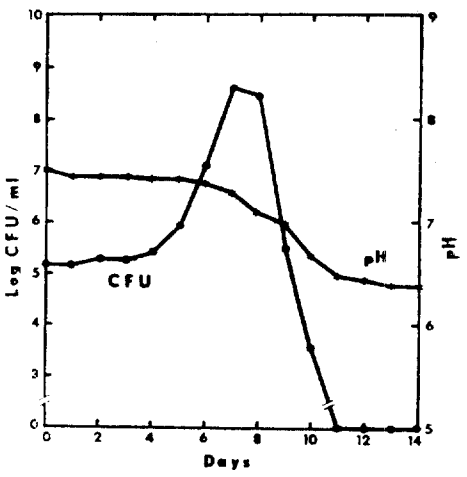

Fig. 1. Growth and *p*H curves of *Mycoplasma pneumoniae* in SSR-2 broth medium.

Those filaments became branched and overlapped each other to from a net work of filamentous figures and microcolonies. Later, through 11 d (Fig. 2E, 2F), the filaments had changed to beaded shapes and were further transformed to spherical froms.

To observe more detailed morphology of the cells during the growth cycle, we utilized critical-point drying, freeze-etching, carbon replication, thin sectioning, and scanning electron microscopy. The round single cells in 12 h samples (Fig. 3A) prepared by critical-point drying were 0.3 to 0.5 μm in diameter and revealed an extracellular matrix(M). A thin section of these single cells (Fig. 3B) attached to the Epon plate(OE) showed a triple-layered cytoplasmic membrane and ribosomes distributed throughout the cytoplasm. Electron dense substances were observed outside the cells and on the interface (I) between the old and new Epon (NE). A dark zone (Z) where the old and new Epon joined was clearly seen beneath the interface.

Carbon replicas of 3 d samples(Fig. 4

A, 4B) showed aggregates of round cells associated with some spindle-shaped segments, a few of which were linearly connected. The segment had knob-like ends(arrows) oriented outward from the microcolonies. One thin section (Fig. 5) from a series of serial sections reveals the inside of such a microcolony. Round-ovoidcells (R) were entangled in the colony and segmentous cells (S) stretched out at the periphery. In these developing clones, we frequently could find evidence of a lysing cell (LC) shown in the thin section in Fig. 5. Its triple-layered membrane (12 to 20 nm) was thicker than those of other cells in the colony. The inner dense layer was as thick as the outer layer. Electron dense substances appeared to deposit on both sides of the membrane. Very sparse cytoplasm was observed in this cell, whereas the cytoplasm was evenly-distributed in other presumably viable cells of the colony.

The critical-point dried 6 d sample(Fig. 6) showed that colonies(CL) were associated with many segments, forming a network. Some of the sements were connected with a thin thread-like structure (arrows). Round-to-ovoid cells were also observed in these colonies. A thin section (Fig. 7) showed many cross or sagittally sectioned terminal cored structures (arrows). These terminal structures were oriented randomly in the colony and their central cores had a tubular structure, about 8 to 36 nm in diameter(Fig. 7, 9C). The outer layer of the unit membrane in these cells was appatrently thicker or at least more heavily stained than the inner one. The carbon replicas of the freeze-etched membranes showed ultrastructure of their surfaces which were distinctively different between the inner(IM) and outer membranes(OM) (Fig. 8). The outer membrane contained globular particles, while the inner one showed many smooth -surfaced humps. The serial setions of a segmented filament reveal a structure which resembles a microtubule (MT) (Fig. 9A, 9B). This element (with a dimension of 15 to 30 nm in diameter)was observed to bridge two segmentous cells.

Rounding of the spindle-shaped cells is seen clearly in the scanning electron micrographs of colonies at two different ages. The 7 d colony(Fig. 10A) consisted of smooth, round-to-ovoid cells surrounded by many spindle rod structures, while the cells of an 11 d colony (Fig. 10B) were of spherical forms with a rougher surface. The thin section of a 10 d old colony (Fig. 11A) revealed mainly round forms and occasional filaments. The cytoplasm of these cells was sparse, but their membranes looked distinct and thickened. Some cell debris was also seen through out the colony. Small spherical bodies (SB) were found in the thin sections of 4 d or older samples. These small bodies seen in Fig. 11 were about 20 to 55 nm in diameter and increased in number with the age of the culture. Neither a unit membrane or internal structures were observed in these small elements(Fig. 11B).

DISCUSSION

The changing morpholgy observed in *M. pneumoniae* cells grown on solid surfaces suggested the existence of a growth (morphological) cycle or at least identifiable stages. Round forms were observed in early and very late stages of the gro-

with, while the spindle forms occurred until the late stationary phase. The round-to-ovoid cells in the early stages appeared to be morphologiclly and ultrastracturally different from the spherical forms of the later stage. The former was viable, while the latter non-viable and apparently products of involution. Changes in the morphology of this organism have been reported by others(Biberfeldetal, 1970; Hubbard *et al.*, 1971; Kammer *et al.*,1970). Our observations are supported by the facts (Bredt, 1968) that involutional deformation of the organism was faciliated with specific antiserum and delayed by fresh medium, together with observations obtained by Bredt *et al.* (1974) and Hubbard *et al.* (1971) with the phase-contrast microscope. The morphological changes of the cells during this growth cycle appeared to be directly correlated to the *p*H changes of the culture and the results are in agreement with those of Low *et al.* (1965).

Our observations suggest that the spindle filaments were produced from other filaments or round cells, and that round cells were produced in the same way. Serial sections of many young microcolonies revealed a cell with sparse cytoplasm which may be one of the older originating cells of the clone. Under our culture conditions, we never observed the long filaments reported to be uniform in diameter in *M. pneumoniae* (Boatman and Kenny, 1971;Collier 1972;Klainer and Pollack 1973; Willson and Collier, 1976). Strain variations have been reported in this organism (Lipman *et al.*, 1969) and this point along with different experimental conditions could explain this discrepancy.

A matrix-like substances was observed in th critical-point dried sampler and seen around single cells as in the intercellular space of the collonies. The matrix is probably a carbohydrate polymer, since thin sections revealed some dense substnces out side the cells, stained with ruthenium red. The matrix may be a normal component of the organism, because singe cells of *M. pneumoniae* appeared to adher to the surface as early as 3 h post ionoculation in this study. The matrix may be involved in adhesion of the cells to the solid surfaces or other cells.

The total thickness of the triple-layered membrane in young cells was very similar to those of other reports(Demermuth *et al.*, 1964; Freundt, 1970; Zucker-Flanklin *et al.*, 1976) and the membrane showed an asymmetrical configuration with a thicker outer layer and thiner inner layer. The ultrastructure of membranes has been studied by freeze-etching technique in several other mycoplasma species(Tillack *et al.*, 1970; Whites-caver and Furness, 1975). The surface structure of *M. pneumoniae* membrane examined by freeze-etching technique in his study showed that the inner layer was distinctively different from those of other species. It has been long recognized that are variations among species, strains, or types of the organism(Dormermuth *et al.*, 1964; Freundt, 1970). In addition, the use of different fixatives and staining substances also could be causing diverse results. The spherical old cells in this study revealed thicker symmetrical membrance. This may suggest that in the old lysing cells, proteinaceous substances released may be denatured and precipitatad on the membrane layers.

Apparently, the knob-like structures of the spindle segments were structurally the same as the terminal cored structures seen in thin sections. Biberfeld *et al.* (1970) and Bredt *et al.* (1974) seggested that the structure was involved in multiplication and in gliding movement(Radestock and Bredt, 1977)of *M. pneumoniae* cells. Actin-like protin providedby Neimark(Nk 1977) may suggest a relationship to the gliding movement, but it has not been localized in the organism. Collier *et al.* (1971, 1972) and Powell *et al.* (1976) proposed that the terminal structure was associated with attachment and pathogenicity of the organism. The randomly oriented structures throughout the colonies indicate that in addition to replication, attachment, and gliding movement, they may show a type of polarity as suggested previously (Kim *et al.*, 1977) and play a role as the growing point of the segment.

Wilson *et al.*, (1976) reported an electron dense central filament at the center of the core and a terminal button at the end of the terminal structure. Thin sections of the terminal structure showed a tubular structure in the core rather than a filament. The function of this tubular structure in the core was not determined. but appeared unconnected to the microtubulelike structure seen two segments. The microtubular structure may be a kind of membraneous tubule (Maniloff and Morowitz, 1972) produced during division of two segments, after which the segmenets separate.

The small bodies observed during the study were the same as those seen previously by scanning electron microscopy (Klainer and Pollack, 1973). Eng *et al.* (1971) showed small globular elements by immune election microscopy in *M. pneumoniae*. They provided evidence by specific immune precipitation that the elements were "non cell-bound" antigen on the organism. The observation of the small bodies in this study indicates that they are products of this organism and may be the same as the antigenic globular elements.

摘 要

*Mycoplasma Pneumoniae*의 細胞發達過程에 따라 形態의 變化 및 微細構造上의 特徵를 여러가지 電子顯微鏡的 技術을 利用하여 試驗하였다. 紡錘狀의 細胞들이 卵球形 또는 따른 紡錘形의 細胞로 부터 發生하여 連鎖狀 또는 分技狀으로 連結되는 網狀의 群體群을 形成하였다가 生長의 후반에는 다시 球形으로 變形되어 死滅되었다. 이러한 形態上의 變化를 수반하는 生活史는 培地의 *p*H 變化와 거의 一致하였으며, 生長초기에 나타나는 卵球形의 細胞들은 末期에 나타나는 球形의 老朽細胞들과 代謝指能이나 微細構造 및 形態的으로 區分되었다. 單一細胞나 群體內의 細胞들은 炭水化物性 體外媒質로 싸이어 있으며 이 媒質은 critical-point drying이나 ruthenium red에 依한 特異性 染色法에 依하여 잘 檢察되었으며 이는 細胞들의 粘着性과 gliding movement와 連關性이 있는 것으로 해석되었다. 이 微生物의 細胞內構造로서 terminal와 核物質은 細胞膜이 없는 三層으로 된 單位原形質膜에 依하여 포위되어 있었다. 特殊構造로 알려진 cored structure는 carbon replication이나 thin sectioning에 依하여 잘 觀察되었으며 이 기관은 細胞의 生長點 또는 附着部位로서의 作用을 하는 것으로 評價되었다. 紡錘形細胞의 teminal core 內에는 microtubalef이 觀察되었으며, 이와 類似한 微細管狀 構造는 紡錘形 細胞들의 連鎖狀連結部位에서도 觀察되었다. 原形質膜의 單位 構造는 幼細胞에서 非對稱이었으며 對稱形인 老細胞의 膜보다 얇았다. freeze-etching에 依한 幼細胞의 原形質膜의 外內層의 構造는 서로 相異했으며 다른

mycoplasma species와 區分되었다. 生長증기부터 이 微生物의 群位에는 抗原作用을 하는 것으로 해석되는 代體産物로서 small bodies가 發見되었는데 이 小體에는 어떤 內部構內 單位膜도 觀察되지 않았다.

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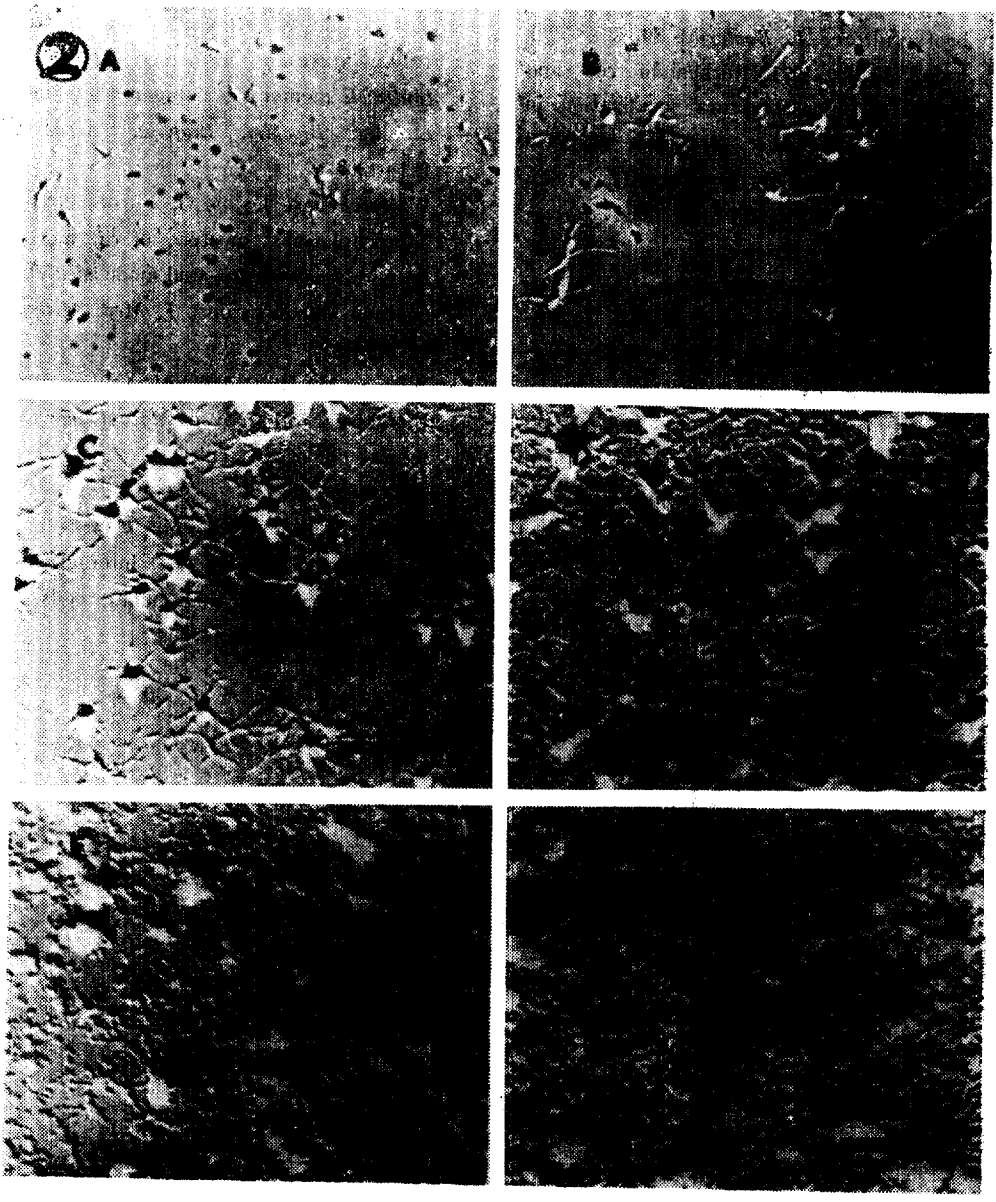

Fig. 2. Light micrographs of *Mycoplasma pneumoniae* showing sequential development single. A; 1 d, B; 3 d, C; 5 d, D; 7 d, E; 9 d, and F; 11 d after incubation. Bars represent 10 μm.

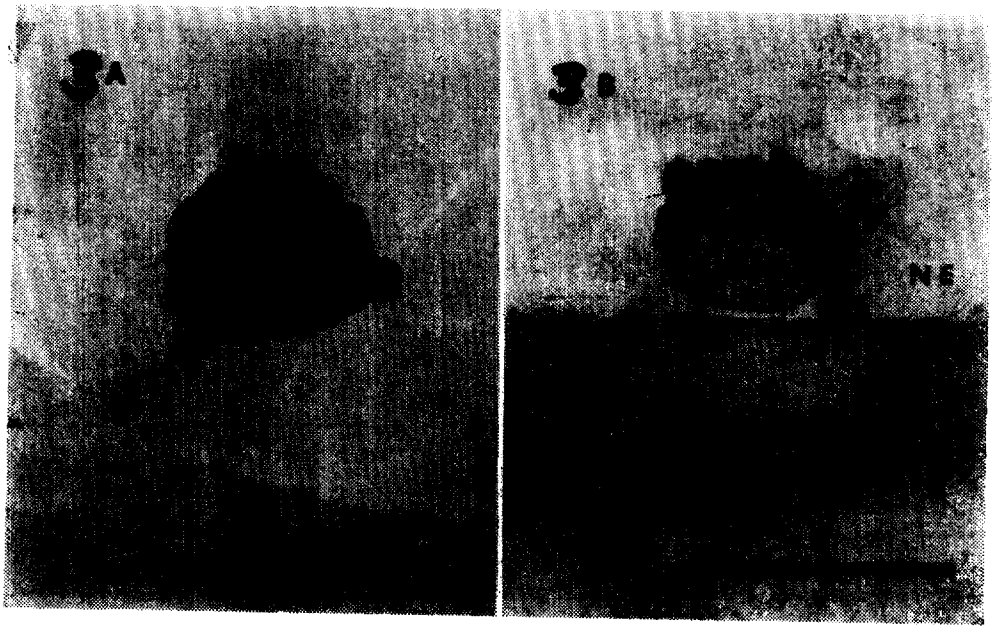

Fig. 3. Round single cells of *M. pneumoniae* attached to the surfaces which were prepared by (A) critical-point drying and (B) thin sectioning. The lower part (OE) of the picture B is the Epon piece which the organism attached to in the upper part (NE) is the new Epon which the sample was embedded in. A zone (Z) beneath the interface was formed by penetration of monomers into the already polymerized Epon. Bars represent 0.5μm.

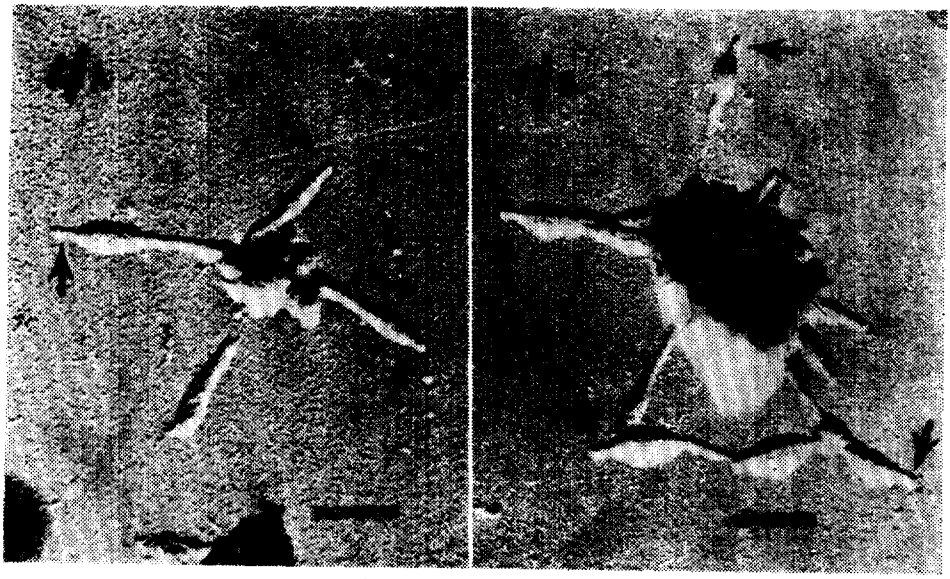

Fig. 4 Carbon replicas of 3 d *M. pneumoniae*. Aggregates of round cells were associated wth spindle cells which appeared to be produced from the round cells. The spindle segments possess knob-like structures (arrows) which oriented outward from the microcolonies. Bars represent 1 μm.

Fig. 5. Electron micrograph of 3 d *M. pneumoniae* microcolony thin sectioned. Round-to-ovoid cells (R) were packed in the colony and spindle cell (S) stretched out at the periphery. A lysing cell (LC) was frequently observed. Bar represents 1 μm.

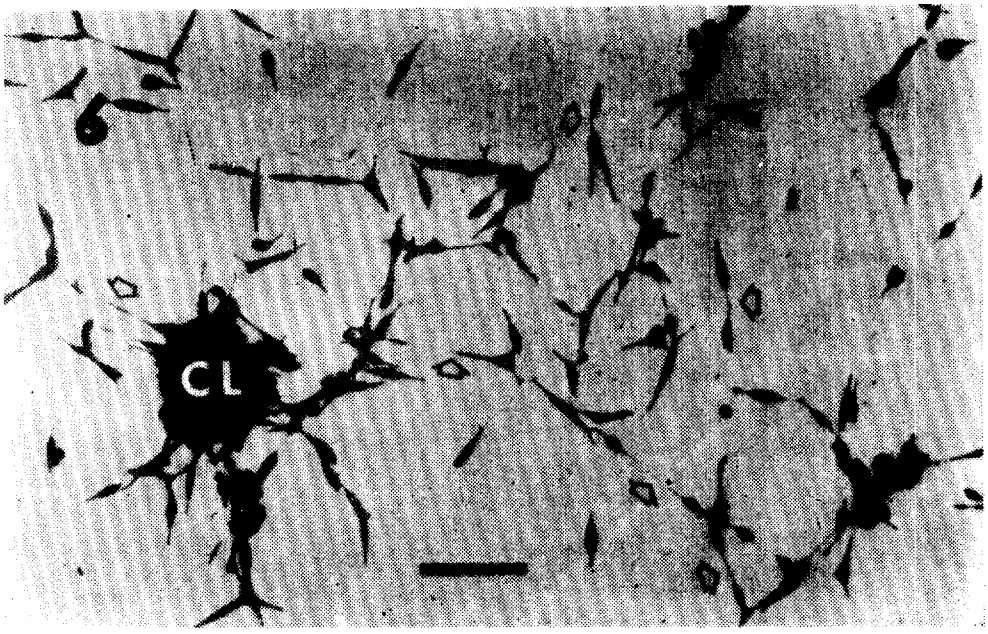

Fig. 6. Electron micrograph of 6 d *M. pneumoniae* prepared by critical-point drying. Colonies of round-to-ovoid cells were associated with many segments, forming a network. Spindle represents 2 μm.

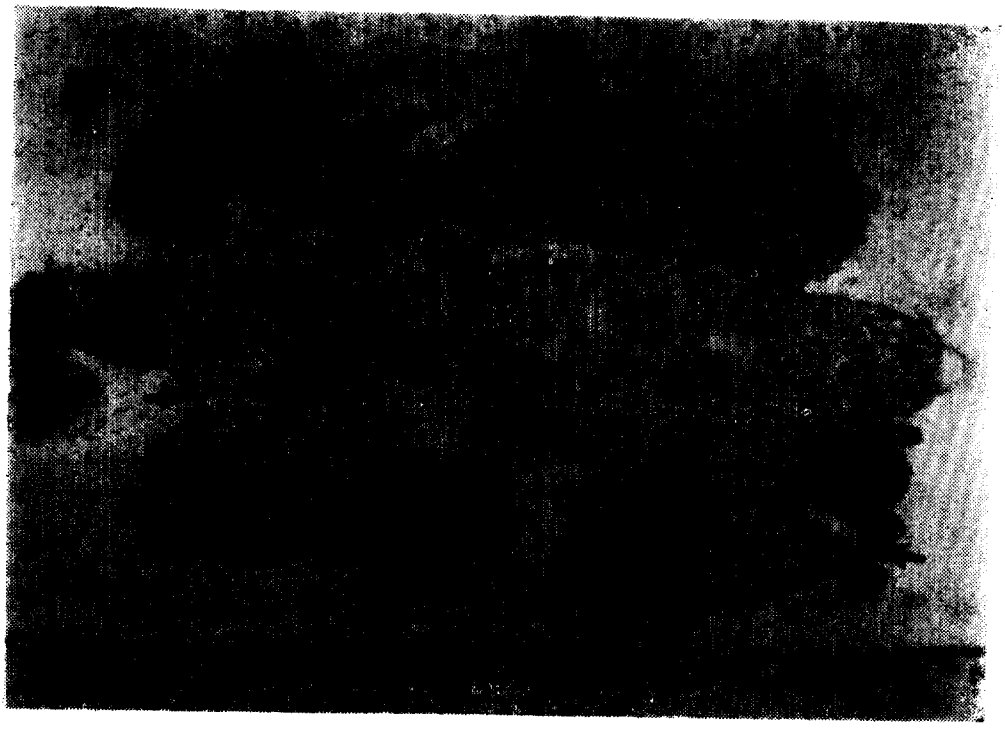

Fig. 7. Thin section of 6 d *M. pneumoniae* microcolony. Ribososomes were evenly distributed throughout the cytoplasm which was enclosed by asymmetrical triple-layered membrane. Many cross or sagittally sectioned terminal cored structures (arrows) were randomly oriented. Bar represents 1 μm.

Fig. 8. Carbon replicas of freeze-etched *M. pneumoniae*. The outer membrane (OM) contained spherical particles and the inner membrane(IM) showed many smooth-surfaced humps. Bars represent 0.1 μm.

Fig. 9. Serial sections of segmented *M. pneumoniae* cells. Two spindle cells were connected with a microtubular structure (MT). A segment in picture C possesses a cross sectioned terminal cored structure which showed a tubular structures (arrow) in the core. Bar represents 0.2 μm.

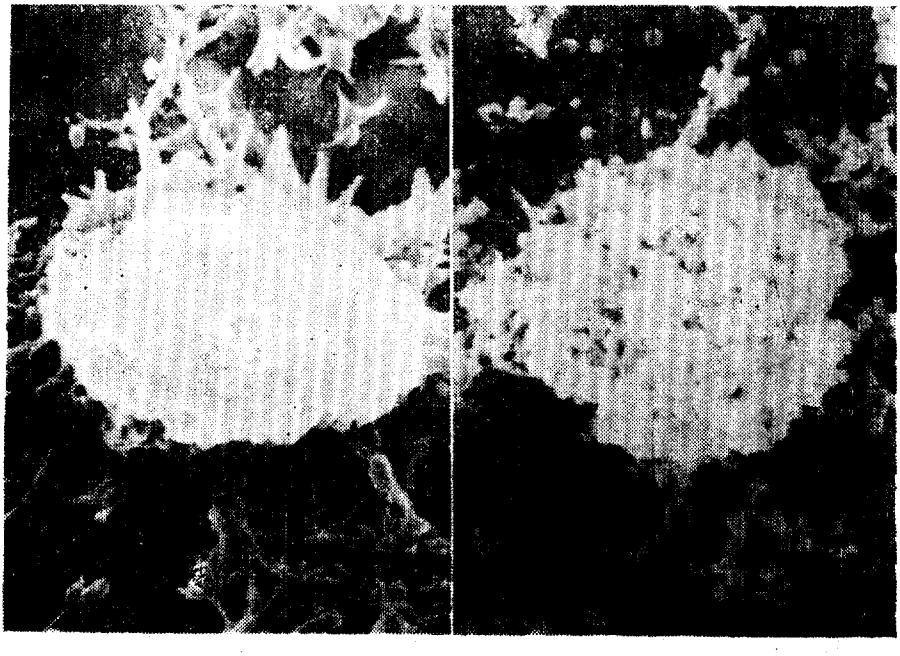

Fig. 10. Scanning electron micrographs of *M. pneumoniae* colonies at two different ages. The 7 d colony (A) consisted of smooth, round-to-ovoid cells surrounded by manyspindle cells of an 11 d colony (B) were completely spherical with rougher surfaces. Bars represent 1 μm

Fig. 11. Thin section of a 10 d *M. pneumoniae* colony (A). The cells were mainly round and their cytoplasm was sparse and their membranes looked distinct and thickened. Many. spherical small bodies (SB) were observed in the intercellutar spaces of the colony. Neither a unit membrane or any internal structure was observed inan enlarged picture B of the small bodies. Note that a triple-layered membrane of a mycoplasma cell (MC) is seen. Bars represent 0.5 μm in A and 0.1 μm in B.