Replication and Sequential Development of Adherent *Mycoplasma Pneumoniae* Studied by Light and Electron Microscopies

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光學 및 電子顯微鏡技術에 依한 *Mycoplasma pneumoniae*의 分裂과 連續分化에 關한 硏究

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국문초록

*Mycoplasma pneumoniae*의 形態的 分化過程을 光學 및 Carbon replication과 critical point drying 전자현미경 技術을 이용하여 觀察하였다. 單細胞로 부터 시작하는 全分化 과정에서 이 細菌은 球形과 紡錘形의 細胞形態로 特有한 分化를 하며 이 과정은 培養液의 pH變化와 一致하여 進行되었다. 本 硏究 結果로 부터 *M. pneumoniae*의 單細胞에서 시작되는 새로운 生活回路를 다음과 같이 提起하였다. 單細胞期를 지나 第1分裂期에는 球形細胞가 주로 對數增殖期의 초기에 二分法에 의하여 분열하고 그 후에 방추형 細胞가 分節法에 의하여 분열하는 第2分裂期가 뒤 따른다. 紡錘形의 긴 細胞는 極性을 갖고 세포 한쪽 끝으로 부터 成長하여 수 많은 방추형세포에 의한 網狀期가 된다. 이와 같은 生長回路는 좋은 生長 환경에서는 반복되지만 pH가 6.8 이하로 떨어지거나 다른 條件들이 나빠지면 細胞表面이 粗惡한 球形으로 變形되어 死滅期에 들어가 모든 細胞는 生命力을 상실하게 된다.

INTRODUCTION

Although the replication of mycoplasmas has been studied extensively[11,12,13,21,22,26], the roles of the round and filamentous forms in the growth cycle of *Mycoplasma pneumoniae* remains uncertain. Clyde[9] and Furness *et al.*[12] reported that *M. pneumoniae* was made up of round cells and that round elementary bodies[12] were the basic reproductive units of the organism. Other workers have seen filamentous shapes with the phase-contrast[5,6,7,14,25,27] and with electron microscope[1,2,3,10,15,17,19,23,24,31].

Furness *et al.* [12] observed "synchronized" growth curves of *M. pneumoniae* from filtered and UV-irradiated cultures and concluded that the round elementary bodies were exhibited by binary fission. Bredt[6] reported that single filaments were the basic units of the organisms and that the rounding of the filamentous cells was an involution step. He also claimed that binary fission was the mode of replication, but that a growth cycle was not evident.

Sequential development of *M. pneumoniae* cultures has been observed starting with single cells and employing phase-contrast microscopy[5,6,7,14,16]. Growth

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of this organism has also been examined with the scanning electron microscope[1,3,15,19]. It was noted that filamentous cells, which had been produced from round cells or short filaments,made a network of long and branched filaments and colonies, and that these cells eventually converted to spherical forms[28].

The present work makes use of several microscopic techniques in examining the sequential changes in *M. pneumoniae* units as they replicate and develop into microcolonies attached to a surface. We have correlated the various morphological forms seen on surfaces with phases in growth and replication of the organism.

MATERIALS AND METHODS

Organism and Culture Medium

M. pneunoniae strain CL-8[30] was isolated at Children's Hospital, Columbus, Ohio. In studies in the attachment and development of *M. pneumoniae* on solid surfaces, we used organisms that had been passaged in artificial media for 18~39 times. We used the SSR~2 broth medium formulation[30] to grow the organisms. It consists of mycoplasma broth base buffered with HEPES(N-2-hydroxyethylpiperazine -N'-2-enthanesulfinic 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 (KC Biological, Inc., Lenexa, KS, lot #27060) was substituted for the serum fraction.

After storage at −70°C, *M. pneumoniae* was subjected to two passages in SSR-2 broth medium in prescription bottles. At exponential phase, culture fluid were drained and glass adherent mycoplasmas were harvested and concentrated by scraping them into fresh broth medium at one-tenth their original fluid volume. Prior to use as an inoculum, cell clumps were deaggregated for 5 min by use of a Vortex mixer. Cultures were incubated at 37°C.

Light Microscopy

A concentrated mass of mycoplasmas was subjected to filtration through an 0.4μm Nuclepore membrane filter(VWR Scientific, Columbus, OH). The filtrate was added to SSR-2 broth medium and cover slips were placed in plastic cell culture dishes[18]. At intervals, cover slips were removed from the culture fluid, and organisms attached to cover slips were fixed for 1 h in 3.25% glutaraldehyde in 0.1M cacodylate buffer, pH7.2. They were washed with the buffer, air dried, shadowed with germanium, and examined with a Zeiss photomicroscope[17].

Critical-point Drying

A culture was centrifuged at 2,000Xg to remove aggregates and the supernatant fluid was passed through an 0.4μm Nuclepore membrane. Using this filtrate, organisms were grown on the surface of formvar and carbon coated 200 mesh copper grids(E.F. Fullam, Inc., Schenectady, N.Y.) in SSR-2 medium. They were fixed with 3.25% glutaraldehyde for 1h, washed 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).

Carbon Replication

The organisms attached to the fromvar coated 300 mesh copper grids in SSR-2 broth medium were air dried after fixation with 3.25% glutaraldehyde for 1h and washing in distilled water. The samples were shadowed and rotary coated with carbon[17]. Carbon replicas were made using the method of Bradley[14].

Determination of the Number of Colony Forming Units(CFU)

We used PPLO broth medium(Difco) supplemented with Bacto-Mycoplasma supplement-S, in which the organisms grew in suspension rather than attached to a surface. At specified intervals, samples were taken, deaggregated using a Vortex mixer for 5min, and subjected to serial ten-fold dilution in the broth medium. About 0.02ml of each dilution was placed on SSR-2 agar plates[30]. After 14d incubation in a humid environment, the plates were overlaid with 0.6% Ionagar(Colab Lab., Inc., Chicago Heights, IL) preparded with 2% sheep blood in saline. After 2d additional incubation, the number of CFU's was determined from a cunt of the number of the hemolytic plaques[24].

Correlation of Morphology and pH of the culture

Deaggregated cells of a normally harvested culture

were added to replicates of six petri dishes containing SSR-2 broth medium and formvar and carbon coated 200 mesh copper grids. At various intervals during incubation, culture fluids were examined for changes in pH, and the organisms attached to the grids were morphologically examined. The CFU was determined with the suspension culture cultivated in Difco's PPLO broth medium inoculated with the same organisms. Each day of the growth cycle, one of the six culture fluids was replaced with fresh SSR-2 broth medium, pH 7.5. The pH change of the replaced medium and the morphological change of the attached organisms were rocorded for an additional 3d incubation.

RESULTS

Sequence of Morphological Changes

We used both light and electron microscopy to follow changes in the form and in the replication of *M. pneumoniae* cells. Cultures were initiated from units which appeared to be single cells and the smallest structures capable of replication. As observed through light microscopy from 12h through 3d(Fig. 1B~1D), both round and short filamentous forms showed size changes and evidence of replication. Filaments became longer and branched to produce a network of filamentous figures and microcolonies within 3d. At 5d(Fig. 1E), the filaments had changed to beaded shapes and by 7d were further transformed to spherical forms(Fig. 1F).

Cultures were also started from single mycoplasma units with an inoculum which contained less organisms (2×10^5/m*l*). Under these circumstances, the growth pattern ostensibly was complete within 11 to 12days. To reveal more details of events occurring in the first five days, we produced carbon replicas of cells adherent to the grids(Fig. 2). Single mycoplasma units were adherent to the surface within 6h after inoculation; micrographs revealed round forms and occasional spindle-shaped short filaments(Fig. 2A, 2H).

At 12 to 24 h post incubation, we observed round forms occurring singly or in pairs(Fig. 2A, 2B). These round cells appeared to replicate by binary fission(Fig. 2B~2D), although the surfaces of some cells(Fig. 2E) possessed protrusions we have called growing points(GP). We observed some filamentous cells attached to the round ones, while others were elongated(Fig. 2I) or with short branches free of other forms. The filaments connected to round cells also increased in number and showed increased branching(Fig, 2F, 2G).

By 36 to 48 h, there were single and paired round cells, but many of them had formed small aggregates. In 2.5 to 3d samples, the round cells were dominant and had formed larger aggregates, sometimes in association with filamentous cells. The knob-like structures of the filaments connected to the aggregates were pointed away from the microcolonies(arrows on Fig. 2F, 2G, 2J, 2K). At 4d, samples revealed as many filamentous as round forms. Filaments were connected to the round cell aggregates and were more elongated. They also appeared in preparations free of round forms, and some filaments showed structures projecting from them(Fig. 2J). The number of CFU's appeared to increase slowly and the pH was still greater than 7.4.

By days 5 and 6, spindle forms were predominant, occurring singly or in chains, and despite some variation in length, were similar to the short filamentous forms(Fig. 2H) observed in the begining of the cycle. These forms had a large body with one pointed end and one knob-like structure at the opposite end. The pointed ends of some spindles attached to the knob-like structures of other spindle forms(Fig. 2G, 2K). Apparently, many segmented filaments(S) were formed in that way and thread-like structures(T) remained, linearly connecting spindle-shaped cells (Fig. 3).

in 7 to 8d samples, many spindle-filaments appeared to touch each other and form a network of cells and microcolonies. However, some ends of single or branched knob-like structures were unattached. By 7~8 days, the number of CFU's was about 5×10^8/ml and the pH at 7.3 or lower.

By 9 to 10d, the network of the filamentous units was disappearing. After the 9th day, cells lost their spindle shape, the tips of filaments were round, and filaments became misshapen(Fig. 4). Cell surfaces were rough and spherical forms reappeared. In the

Table 1. Correlation of morphology, pH, and CFU of the culture during one growth cycle(I) and following incubation after replacement with fresh medium(II)

Incubation in days	I characteristics of the culture during one growth cycle			II morphology[a]/pH following incubation after replacement with fresh medium[c]		
	morphology[a]	pH	CFU/ml[b]	day 0.5	day 1.5	day 3.0
0	R, F	7.5	4.8×10^5			
1	F, C	7.35	2.5×10^6	F/7.4	F/7.25	F/6.6
2	F, C, N	7.1	2.0×10^9	F/7.3	F/6.6	S/5.9
3	F, C, N, S	6.8	2.8×10^9	F/7.2	F/6.8	S/5.7
4	F, C, S	6.3	2.7×10^4	S/7.45	S/7.4	S/7.3
5	S	6.1	0	S/7.45	S/7.4	S/7.3
6	S	6.2	0	S/7.4	S/7.4	S/7.3

[a]R, round; F, filamentous; C, colony; N, network, S, spherical

[b]CFU's were obtained from suspension culture using Difco's PPLO broth medium

[c]Each day of the growth curve, the culture fluid was drained and replaced with fresh medium(pH 7.5) The morphology and pH of the culture in the new medium were examined for an additional 3d incubation.

period of 11 to 12d, the rough-surfaced spherical forms were predominant(Fig. 5). Degeneration of the filamentous cell is seen clearly in the critical point drying electron micrographs of the colonies of three different ages(Fig. 6). The 9d filamentous cells(Fig. 6A) lost their segmentous characteristics and rounded ends of the segments, 10d cells(Fig. 6B) became more rounded and somewhat curved, and the cells of 11d colony(Fig. 6C) were completely spherical and their surfaces were rough.

Correlation of Morphology and pH of the Culture

We determined whether rounding of the filamentous cells represented the appearance of degenerate forms or was part of a continuing developmental process. For this experiment, we used a heavy inoculum which shortened the growth curve to 4 days. Mycoplasma cultures were examined daily for 6 days post-inoculation(Table 1; I). As the pH of the culture fluids gradually decreased, the morphology of the attached cells changed from round or short filaments to long segmented filaments. At the end of the stationary phase(3d), there were both filamentous and spherical forms. The pH of the culture fluid was about 6.8. In the remainder of the cycle, the pH was below 6.8, the spherical forms remained, and the number of CFU's rapidly decreased to zero.

We also looked for changes in morphology in experiments where we replaced culture fluids with fresh medium(pH 7.5). The results obtained by replacement and by an additional 3d incubation are seen on the right(II) in Table 1. When cultures with fluids at a pH of 6.8 or above were replaced with fresh medium(samples from 1, 2 and 3d), organisms retained their filamentous morphology and the decline in the pH continued. The filamentous shapes were maintained until the pH descended to about 6.6. At that point(and at points lower), the filamentous shapes converted to spherical forms. When the culture fluid at a pH below 6.8 was replaced with fresh medium (samples from 4, 5, and 6d) only spherical forms were observed and the pH of the new medium remained the same. As determined from the number of CFU's in culture, with a pH below 6.6, the mycoplasmas were not capable of developing colonies.

DISCUSSION

Both round and spindle-shaped forms of adherent *M, pneumoniae* appeared to be able to replicate. The round forms have been previously observed[9,12] in anaerobic cultures. Spindle-shaped filaments have also been reported by others[5,6,7,25]. They appeared to be different from a variant "rho" form[29] which in some species was produced in a particular medium.

We did not see the striated fibers found in that form. We never observed the long filaments reported to be uniform in diameter in this species[1,3,10,19,24,31], but strain variations do occur in this species[20] and could explain this discrepancy. Our interest centered on single mycoplasmas as they attached to surface and changed in form and number of round cells. Perhaps, round cells did not separate after fission, but remained as aggregates. After aggregates of round cells appeared, microcolonies produced filaments by forming protrusions (growing points) randomly on the cell surface. Apparently, the knob-like structures seen on filamentous cells were structurally the same as the protrusions on the round cells.

Filamentous forms appeared to be produced from the knob-like ends of existing filaments. This mode of replication or "segmentation" was different from the "fragmentation" formulated by Freundt[11]. The segments appeared, one by one, from the knob-like ends of the segmented filaments. Bredt *el al.* [5,6,7] and Hubbard *el al.*[14] used phase-contrast microscopy to witness divisions of short filamentous cells and to conclude that mycoplasmas multiplied through binary fission. From the viewpoint that cytoplasmic separation must be preceded by nuclear division, segmentation does not differ from binary fission. The segmentation described here suggests a type of polarity. Biberfeld *et al.* [1] reported that in addition to involvement in replication of the organism, the knob-like structure was concerned with the locomotion. Bredt[6] and Radestock *et al.* [25] reported that the motility of the organism was led by the knob-like end. There probably are some relationships between the polarity of growth and movement of the filamentous cells.

The morphological changes during the replicative cycle of *M. pneumoniae* were coincident with the changes in pH and the number of viable cells in the culture. Results obtained from a correlation of morphology with pH and viability indicated that at pH 6.8 or lower the rounding of the filaments involves degeneration of cells and loss of reproductive capability. The threshold pH for the irreversible rounding of the cells is around 6.6. The morphological changes in the cells appear to be directly related to the pH changes in a culture, and our results are in agreement with those of Low *et al.*[21]. The facts[6,8] that involutional deformation of the organism is facilitated with specific antiserum and delayed by fresh broth medium, together with the observations obtained by Bredt[5,6] and Hubbard *et al.*[14] with the phase-contrast microscope, support our findings.

Our evidence is against the concept that "elementary bodies" are produced and released from filamentous cells. Binary fission appears to be followed by segmentation. Exponential growth occurred during the period of segmentation. This observation suggested that segmentation may be a major mode of replication and a spindle-shape as the major morphologic form when the organism was cultivated to attach to a solid surface. While length of the growth cycle was directly proportional to the size of inoculum, regardless of the size of inoculum or the length of the growth cycle, the cell morphology proceeded as we have described and the pH at which certain morphological forms

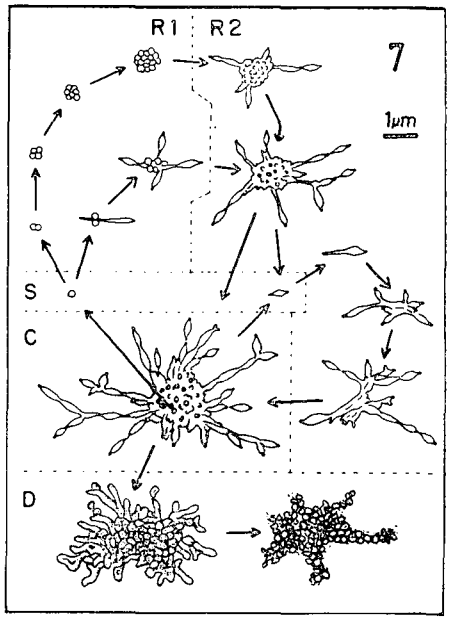

Fig. 7. Schematic diagram of a proposed development cycle of *M. pneumoniae*. The cycle could be started from single cells(S) into replication stage by binary fission(RI) confluency(C). The cells in the stage of confluency could go into another cycle or 'degeneration stage(D), depending on the conditions.

appeared was approximately the same.

Kammer *et al.* [15] proposed three phashs in the morphological changes of *M. pneumoniae*. From the data obtained in this and our previous studies[16,17,18], we have reconstructed a replication cycle started from single cells of *M. pneumoniae*(Fig. 7). Our divisions in association with replication and morphological development are shown as follows: stage of replication through binary fission and segmentation, stage of confluency, and a degeneration stage with rounding of cells. Round cells and spindle-shaped segments(Fig. 7; S) appearded to be two types of reproductive unit. Round cells produced from the center of colonies replicated by binary fission(Fig. 7; RI). New round cells resulting from fission may not separate, but remain as aggregates. By forming growing points on the cell surface, round cells produced filamentous cells. Spindle segments were produced, one segment at a time, from the knob-like projections of other segments(Fig. 7; R2). The segments were liberated from the ends of the segmented filaments. Under favorable growth conditions, the round cells and spindle segments were produced continuously by both modes of replication and by repetition of the cycle, grew to from a confluent mass of organisms attached to a surface(Fig. 7; C). If conditions became adverse(e.g. low pH), the mycoplasmas assume a spherical wrinkled shape which has no reproductive capability(Fig. 7; D).

Summary

The morphological development of *Mycoplasma pneumoniae* attached to solid surfaces was examined by light and electron microscopies. Critical point drying and carbon replication techniques revealed that during the growth cycle of developing microcolonies, the morphological form coincided with the pH of the culture. *M. pneumoniae* appeared to have a well defined morphology associated with age of the culture. The organisms were dimorphic, with round cells capable of reproduction and segments consisting of a spindle shaped body with one pointed and one knob-like end. Starting with single cells, there were the following stages in the development of a culture: replication stage through binary fission and segmentation, stage of confluency, and a degeneration stage into rough spherical forms. The round cells appeared to replicate by binary fission during the lag and early log phases of growth, while spindle segments replicated by segmentation during most of the logarithmic growth. The growth of the filaments and replication of the segments occured at the knob-like ends, showing a type of polarity, and formed a meshwork across the surface. This development could be cycled under favorable growth conditions, but the culture aged and when the conditions became adverse(e.g. pH 6.8 or lower), filamentous cells converted to spherical forms, losing their reproductive capability.

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Figure Legends

Fig. 1. Light micrographs showing sequential development of *M. pneumoniae* initiated from single cells. A; 3h after inoculation, B; 12h, C; 1d, D; 3d, E; 5d, and F; 7d. Bar represents 10μm.

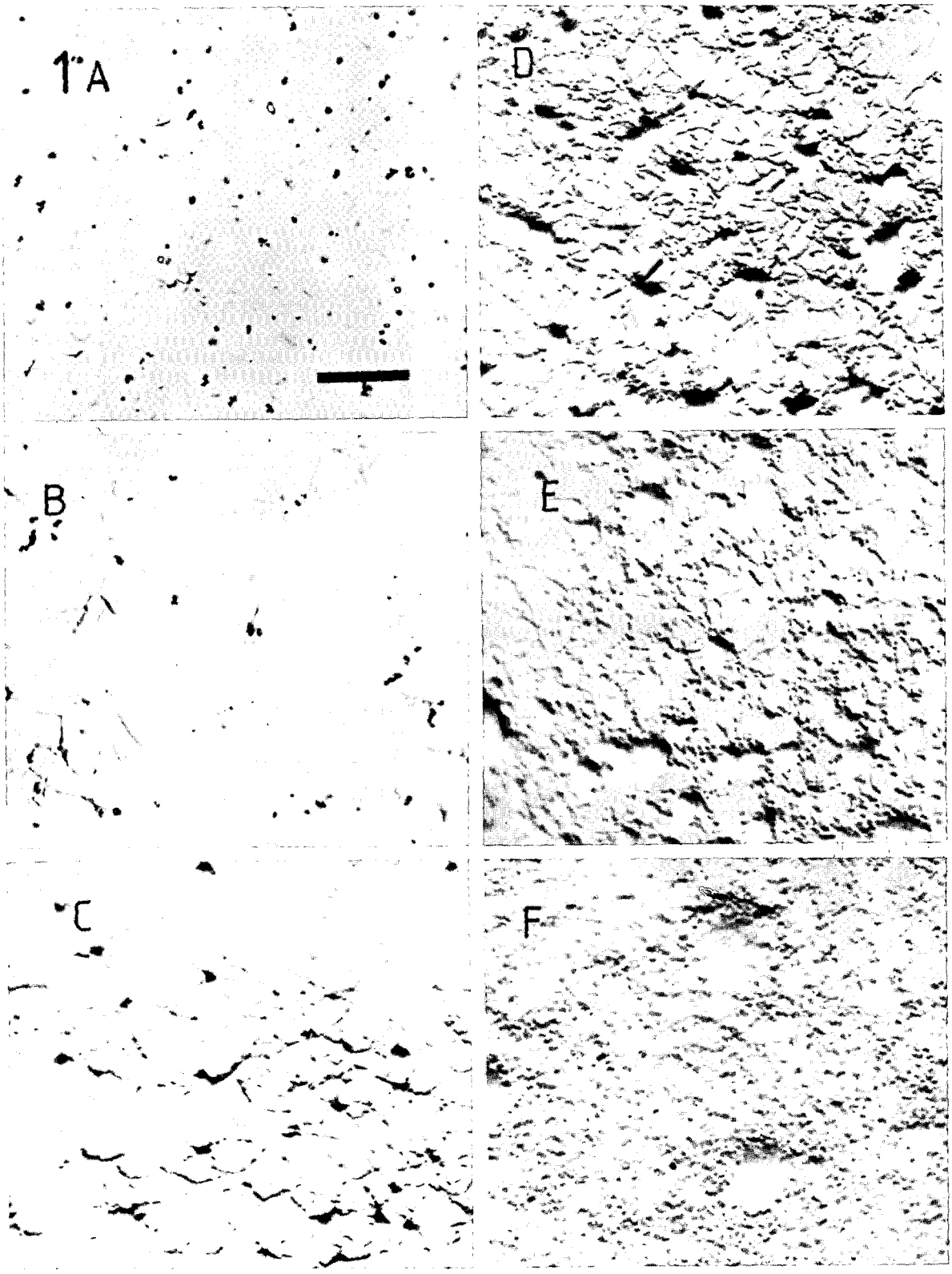
Fig. 2. Carbon replicas of 6h to 5d *M. pneumoniae*. A round cell(A) appeared to replicate by binary fission (B-D). Segmentous filaments were produced from round cells by forming growing points(GP) as shown in E. A segment(H) appeared to replicate by segmentation(G-K), pointing knob-like structures(arrows) always outward from the center of microcolonies. Bars represent 1μm.

Fig. 3. Carbon replica of a 7d old *M. pneumoniae* microcolony. Many segmented filaments were produced from the colony. Thread-like structures(T) are seen to be connected between two segments. A segment(S) was produced from a round cell. Bar represents 2μm.

Fig. 4. Carbon replica of 9d old *M. pneumnniae* cells. The cells lost their spindle shape and filaments became misshapen. Bar represents 1μm.

Fig. 5. Carbon replica of an 11d *M. pneumoniae* cells. The cell surfaces were rough and spherical forms reappeared. Bar represents 1μm.

Fig. 6. Electron micrographs of critical point dried *M. pneumoniae*, showing degeneration during the period of 9 to 11d. The 9d filamentous cells(A) lost their segmentous characteristics and rounded ends of the segments. 10d organisms(B) became more rounded and somewhat curved. 11d cells(C) were completely spherical. Bar represents 1μm.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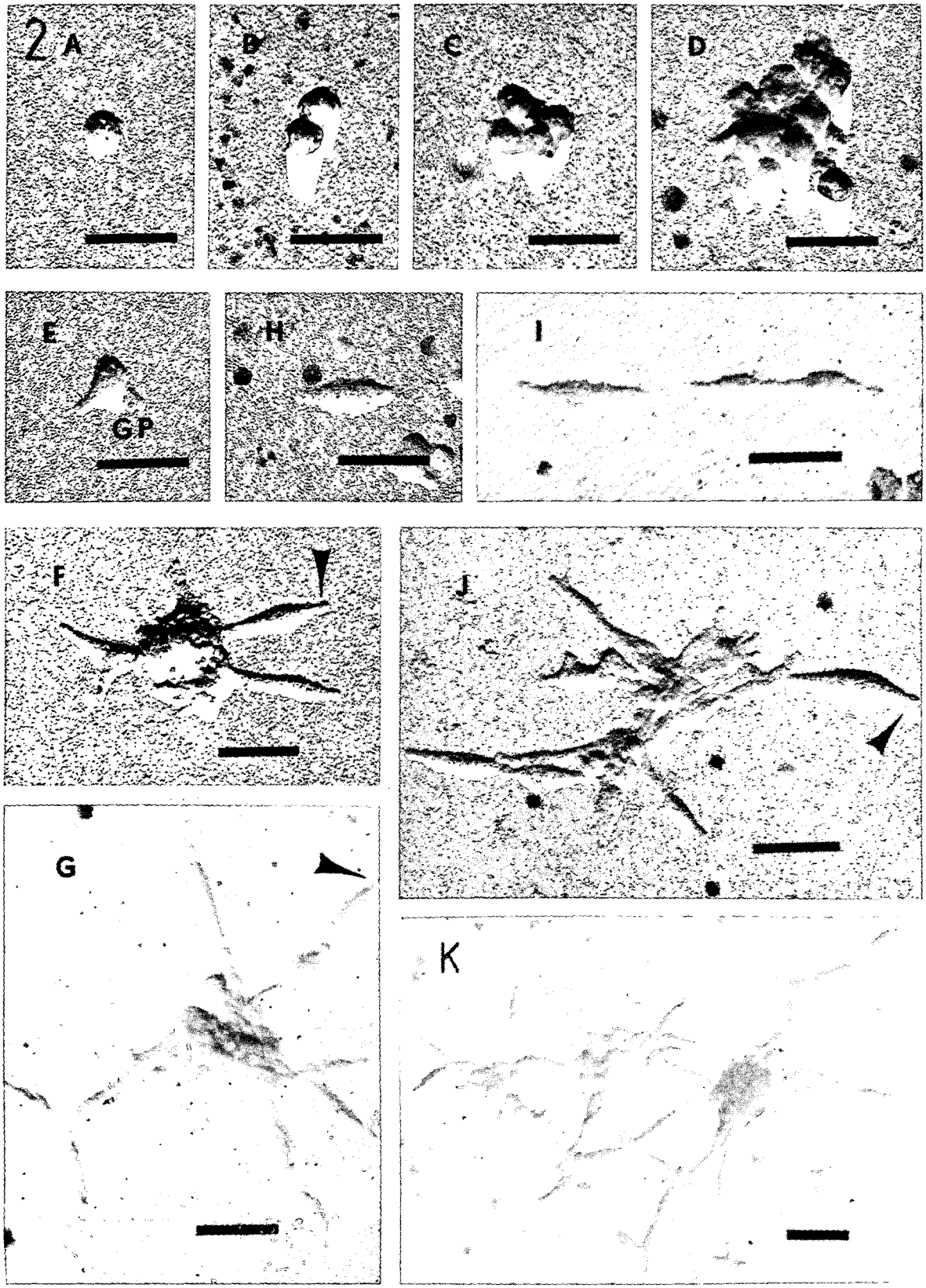
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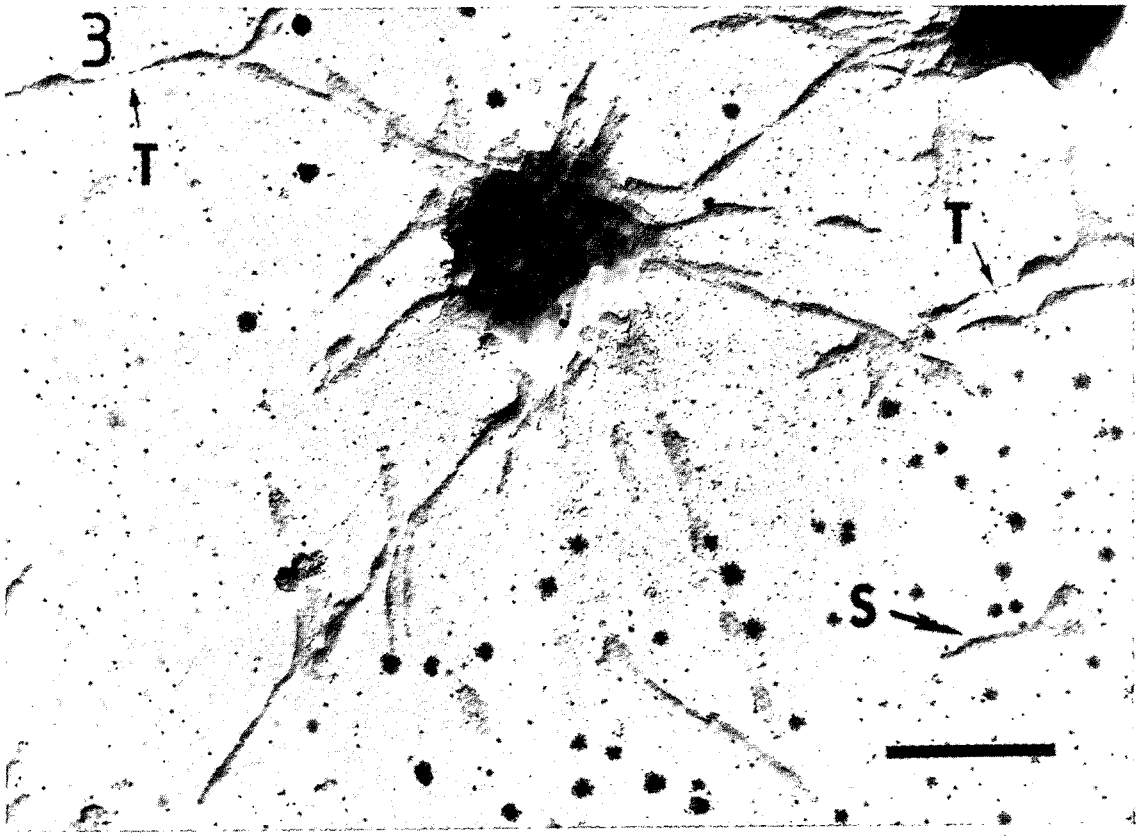
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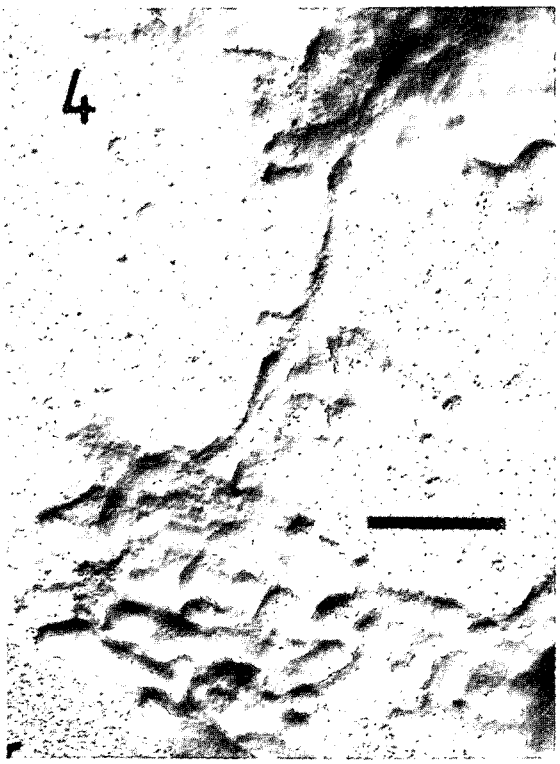
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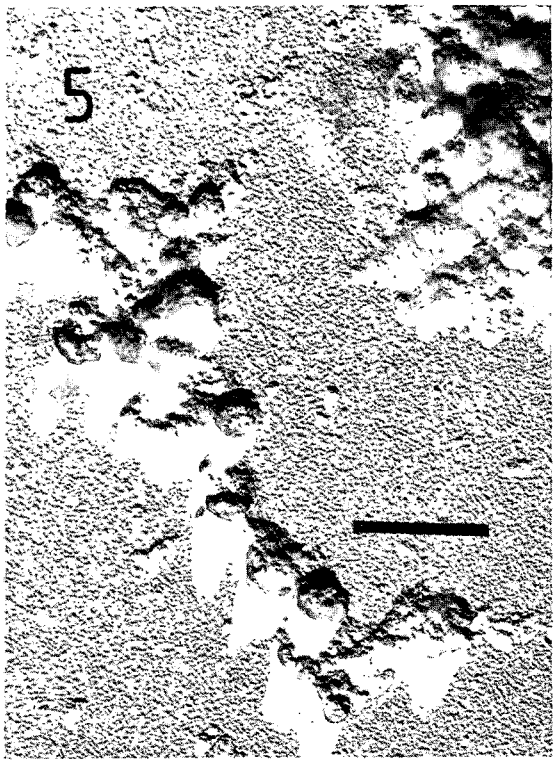
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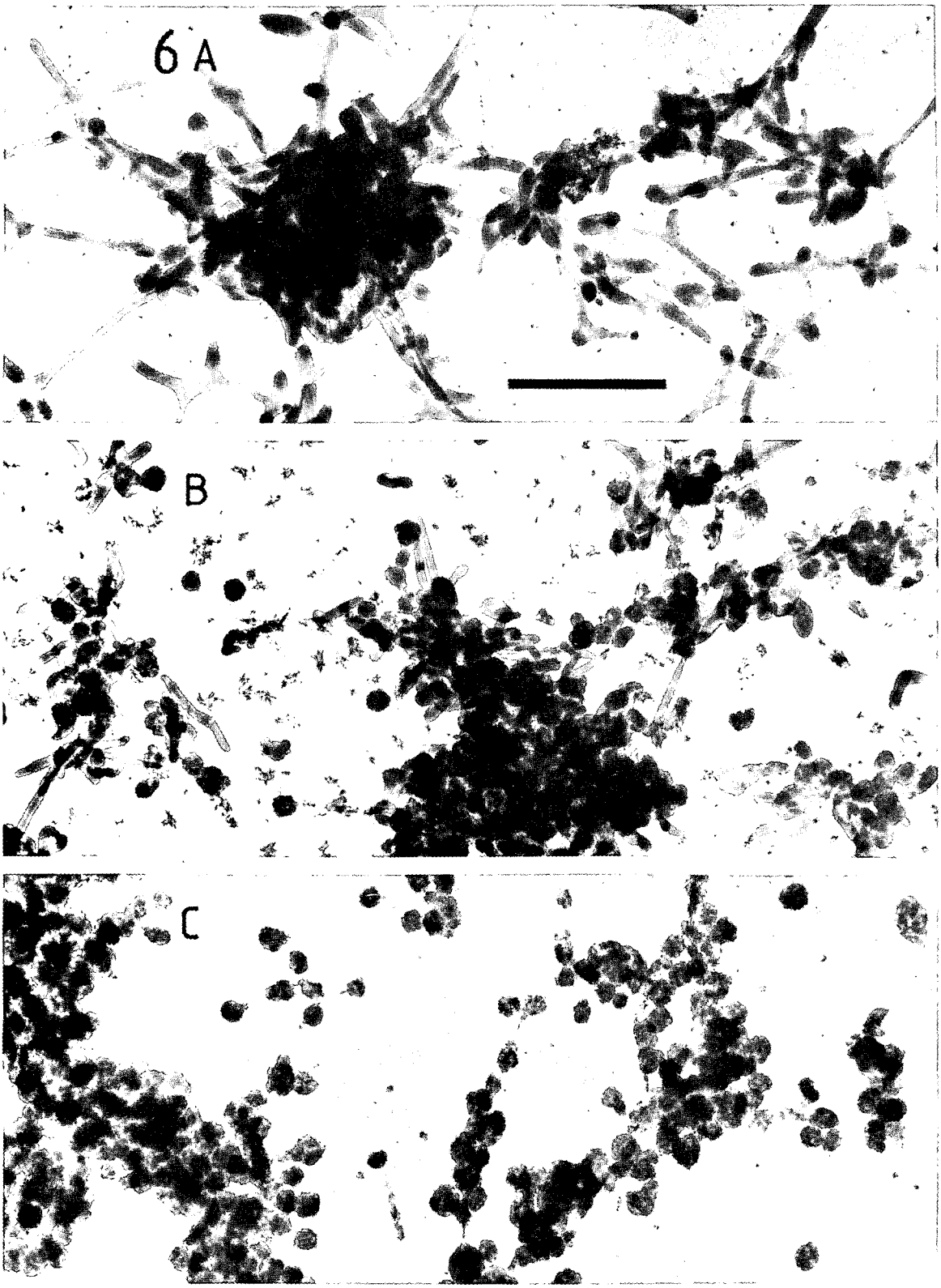
6 A
B
C