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Intracellular Digestion and Endosymbiosis in *Amoeba proteus*

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아메바에 있어서 공생과 세포내소화에 관한 연구

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적　요

Chloramphenicol 용액으로 배양한 amebae의 endosymbiotes를 전현적 세포화학법으로 연구하였다. 대조군에 있어서 acid phosphatase 활성은 오로지 최근에 섭취한 Tetrahymena 주위의 식포내에만 관찰되었으나 chloramphenicol로 처리한 실험군에서는 항균제로 변성된 endosymbiotes를 함유한 vacuoles내에도 다량의 acid phosphatase 활성이 관찰되었다. 이는 endosymbiotes가 chloramphenicol에 의한 정균작용으로 인하여 숙주의 세포내소화작용을 저지하는 힘을 소실한데 기인한다고 사료된다.

INTRODUCTION

A strain of *Amoeba proteus*, xD amebae, is associated with two kinds of endosymbiotes, DNA containing bodies and x bacteria. Initially the x bacteria were harmful parasites (Jeon and Lorch, 1967), but they are now required for the survival of hosts (Jeon, 1976). Electron microscopic investigation revealed that the bacterial symbiotes are located in vacuoles dispersed throughout the host cytoplasm. In their apparent immunity to digestion by the host cells, the bacteria and their surrounding peribacterial vacuoles resemble organelles.

Chloramphenicol selectively inhibits protein synthesis in prokaryotes, and brings about diverse morphologic and biochemical changes when added to cultures of various prokaryotic cells (Malik, 1972). Some of the short-term changes in bacteria include disappearance of ribosomes, aggregation of nuclear material toward the center (Morgan et al., 1967). Bacteriostasis by chloramphe-

nicol has served to free some flagellates of their intracellular bacterial symbiotes (Chang, 1974, 1975). Thus a single treatment of symbiotic bacteria with chloramphenicol at 0.1~0.8 mg/ml renders the flagellates symbiote free, and they can grow in the presence of chloramphenicol. If chloramphenicol could eliminate the symbiotic bacteria, study of the mechanism for the host's dependence on the symbiotes would be facilitated. The present study was initiated in order to clarify some of the events in host cell digestion relevant to an eventual understanding of the specificity and stability of the endocellular symbiotic relationship to chloramphenicol.

MATERIALS AND METHODS

Amebae from the xD strain of *Amoeba proteus* was cultivated in a modified Chalkley's medium (Jeon and Jeon, 1975), with Tetrahymena as food. To study the effect of chloramphenicol (Sigma Chemical Co.) on the endosymbiotes, ~1000 healthy xD amebae were grown in Falcon plastic dishes each containing 5 ml culture solution and chloramphenicol at different concentrations. Culture media were changed daily and newly harvested Tetrahmena were added every other day. Control groups were kept in Chalkley's solution without the antibiotic. Amebae and their symbiotes were examined at intervals during chloramphenicol treatment.

For electron microscopy amebae were first fixed in Karnovsky's (karnovsky, 1965) phosphate-buffered fixative for 1 hr at 4°C, washed overnight in 0.1 M phosphate buffer in the cold, and postfixed in 1% (W/V) OsO_4 at room temperature.

For the acid phosphatase reaction, prior to postfixation they were incubated in solution containing 0.3 percent sodium β-glycerophosphate as the substrate with 0.05 M acetate buffer at pH 5.0 for 1 hr at 38°C.

The fixed and incubated amebae were then dehydrated throughout a graded series of ethanols, clearing with toluene and embedded in Epon. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and examined in a JEM electron microscope.

RESULTS

xD strain of *Amoeba proteus* included two kind of endosymbiotes. The first kind of endosmbiotes is DNA containing bodies (Fig. 1). They had granular cytoplasm with fibrils and were individually enclosed in vesicles. The vesicle consists of two unit membranes which are separated by a space of amorphous

substances. The outer membrane is derived probably from the host cell. Second kind of endosymbiotes, x bacteria (Fig. 2), had also granular cytoplasm with fibrils but they were contained from several to hundreds in a single vesicle which consisted by unit membrane. Between the individual symbiote is separated by a space of amorphous substances which are often contain fractionated fibrillar materials. These fibrillar substances are seen scarcely in a young vesicle but appeared as it becomes older. The matrix of the endosymbiote is composed of dense ribosome-like particles and areas of low density containing fine fibrils. The particles are of the same size as ribosomes in bacteria and the fibrils have the characteristics of bacterial DNA. In the newly formed symbiote, the matrix is composed of granules and fibrils in a less compact arrangement. However, the matrix of the symbiote increases their electron opacity, perhaps due to the increases in their granules and fibrils density as they become older. On the other hand, they seemed like to be transformed into the fibrillar matrix which was fractionated with the unit membrane.

The symbiotes were enclosed in membranous vesicles of varying sizes, each bacterium measuring $\sim 0.5 \times 2\,\mu m$. When bacteria were few in numbers, the vesicles contained only the bacteria, but when numerous, the interbacterial space was filled with irregular membrane-bound vesicles that contained fibrous materials of unknown nature. The ultrastructure of the individual symbiote was like that of *E. coli* (Morgan et al., 1967) and symbiotic bacteria present in other cells, with dense cytoplasm, electron-lucent nuclear zone, and limiting plasma membrane. In some cases, the fuzzy material attached to the outside of the plasma membrane of the bacteria.

In initial studies, the growth rate of amebae grown in 0.1 mg/ml chloramphenicol was reduced by 25%. However, chloramphenicol at this concentration had little effect on number of symbiotes per amebae during a 3 week period of cultiv ation. In media containing 2.4 mg/ml chloramphenicol, Tetrahymena, the food organism, was lysed within 30 min and amebae could not be adequately fed. Therefore, the author used chloramphenicol at 0.5, 0.8 and 1.6 mg/ml concentrations. The mean generation time of amebae in chloramphenicol was 6 days as compared to 2.5 days for the control cells during the 2 week period.

During the first few days of chloramphenicol treatment, there were no detectable ultrastructural changes in the symbiotes. After a week in chloramphenicol, however, a change was detected in the contents of symbiotes' vesicles; the number of intact bacteria decreased. Some of the vesicles containing fibrous material were about the size of the symbiotes, and it appeared possible that the symbiotes changed into the fibrous material but no intermediate forms were found. The numbers of bacterial vesicles and individual symbiotes were reduced

to 40—80% in cultures a week after the chloramphenicol treatment. Many of the bacterial showed the irregulary ill-formed shapes and transformed so often into the fibrillar structures that the amorphous ground substance of the treated vesicles were occupied almost with the fractionated fibrillar elements. Electron microscopic cytochemical investigation indicated that xD amebae do not normally digest their symbiotes. In the control section, acid phosphatase reaction product is visible only in food vacuoles containing recently ingested Tetrahymena and is absent in peribacterial vacuoles (Fig. 3).

After a week of chloramphenicol treatment, copius amounts of acid phosphatase reaction product are clearly visible between and around symbiotes in many electron micrographs of the preparations.

After 2 weeks of chloramphenicol treatment, expansion of the nuclear zone was seen in some symbiotes, in other signs of overall deformation besides nuclear expansion were observed and the amount of acid phosphatase reaction product was greatly increased in chloramphenicol-treated symbiote (Fig. 4). There was noticeable deformation and degeneration and the percentage of normal symbiotes decreased sharply during the first 3 weeks of treatment. The extent of morphologic changes in symbiotes appeared to be dose-dependant during the first 2 weeks of chloramphenicol treatment, with larger proportions of symbiotes being deformed and malformed symbiotes were often segmented into the electron dense fragments, while fibrous ground substance and fragmented dense bodies in the vesicles were gradually dissolved and disappeared in the cytoplasm perhaps depending on the intracellular digestion and autolysis of the symbiotic bacteria (Figs. 5, 6). After 4 weeks of chloramphenicol treatment, amebae stopped multiplying and became unhealthy and many lysed. Therefore the effect of chloramphenicol was followed only for 4 weeks with large groups of amebae.

DISCUSSION

The symbiotes of flagellates are rendered easily symbiote-free by a lower concentration of chloramphenicol (Chang, 1975). However, it could not eliminate the symbiotic bacteria from the amebae without killing the host cells, although treatments with chloramphenicol reduced the number of endosymbiotes per ameba. Thus the response of ameba symbiotes to chloramphenicol differed from that of the symbiotes of flagellates. When returned to normal medium after chloramphenicol treatments, most remaining amebae lysed within a week. This indicates that the amebae cannot live without their symbiotes. The reasons for the ameba's dependence on the symbiotes is not known, but they supply an

essential growth factor which is not provided by the ameba's main food, Tetrahymena. Amebae normally required the presence of bacteria growing in the culture medium in addition to Tetrahymena (Griffin, 1973). It may be that the symbiotic xD amebae obtain such an essential factor from their symbiotes and that during the establishment of symbiosis they have lost the ability to aquire the factor from bacteria living in the medium (Jeon, 1976).

The effect of chloramphenicol on gross and fine structure of symbiotic bacteria resembles that found for other free-living or symbiotic bacteria (Hahn et al, 1957; Chang, 1974, 1975), This might be due either to the indirect effect of inhibited synthesis of enzyme proteins, as suggested by Anraku and Landman (1968) for *B. subtilis*, or to a direct action on the system synthesizing membrane components, as suggested by Stow et al. (1971). Chloramphenicol, well-known for its inhibitory action on DNA and protein synthesis has been used extensively indifferent biological systems (Taylor, 1959). In the study of the ultrastructural changes in ameba hybrids, Jeon (1973) found that x bacteria and mitochondria became abnormal first among the cell organelles, whereas Golgi complexes had been known to degenerate first in enucleated amebae (Flickinger, 1968, 1973). Thus it appeared that the effect of enucleation was different from those of the presence of a heterologous nucleus and of chloramphenicol on the cytoplasmic organelles, and that organelles differed in their responses. It is evident from the results that the nucleic acid-containing components, such as x bacteria and mitochondria, degenerate earlier than Golgi complexes in the lethal hybrids and chloramphenicol treated amebae, whereas the latter are affected more severely in enucleated cells. Structural alterations occurring in the cytoplasm of chloramphenicol treated amebae are not the same as those found in enucleated amebae. Thus, in the presence of chloramphenicol, bacterial symbiotes degenerated sooner than Golgi complexes. On the basis of known nucleocytoplasmic relations, it seems reasonable to conclude that the different responses of the two groups of cytoplasmic organelles are due to the presence or absence of nucleic acids in these organelles and hence their interaction with the nuclear genome.

The physiologic substrate and function of acid phosphatase have been clearly defined. Cell fractionation studies (De Duve et al., 1955) later followed by ultrastructural cytochemical studies (Essner and Novikoff, 1961; Novikoff, 1963; Gordon et al., 1965) have given rise to the concept of the lysosome as a membrane-bound structure containing several hydrolytic enzymes including acid phosphatase. These findings were confirmed by many other investigators (Bowers and Korn, 1973; Barham and Berlin, 1974; Barham, et al., 1976) and subsequently several other hydrolytic enzymes have been identified to be associated with cell functions

like phagocytosis, pinocytosis, intracellular digestion or secretioin. Electron microscopic investigations of intracellular digestion have been undertaken in ciliates (Karakashian et al., 1968). This results indicated that the digestive process in *Paramecium bursaria*, like that of other ciliates, involves the release of digestive enzymes into food vacuoles following an appropriate stimulus. In *P. bursaria*, the enzyme-releasing stimulus is not simply the presence of nutritive material within a phagosome, since neither soluble nor particulate nutrients were present when enzymes were released into phagosomes containing either of several non-nutritive particles. Perhaps the mere formation of a phagosome triggers an eventual discharge of digestive enzymes into it (Karakashian and Karakashian, 1973).

Whatever the signal for the conversion of a phagosome into a digestive vacuole, it is clear that symbiotic bacteria, if present in the vacuole, actively interfere with the transformation. Possibly this interference is effect by their secretion of an inhibitor of the digestive enzymes, or the bacteria may induce a change in the phagosome membrane so that it can no longer fuse with enzyme-containing lysosomes. The inhibitor hypothesis implies that there would be a continual production of an except of an agent or agents which block the activity of any digestive enzymes discharged into the peribacterial vacuoles. However, bacteriostasis by chloramphenicol has served to lost their apparent interference to intracellular digestion by the host cell. Thus the treatment of symbiotic bacteria with chloramphenicol rendered some of the symbiotic bacteria degeneration and digestion. It is thought that lysosomes play a role in the phagocytosis of degenerating symbiotic bacteria and possibly phosphatase enzymes have been implicated in intracellular digestion of them.

SUMMARY

Electron microscopic cytochemical methods reveal that acid phosphatase activity appears exclusively in vacuoles containing recently ingested Tetrahymena as a food and not in the vacuoles surrounding established symbiotes. However, copius amounts of acid phosphatase reaction product are visible between and around some of the degenerating symbiotes in the amebae after treatment of chloramphenicol. It is thought that bacteriostasis by chloramphenicol has served to lost the symbiotic interference to intracellular digestion by the ameba and possibly phosphatase enzymes have been implicated in phagocytosis and intracellular digestion of the symbiotic bacteria.

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Fig. 1. Electronmicrograph of DNA containing bodies, one kind of endosymbiotes, of *A. proteus*. Vacuole with 4 symbiotes. Note the electron-dense cytoplasm and electron-lucent nuclear zones in the symbiotes. ×46,000.

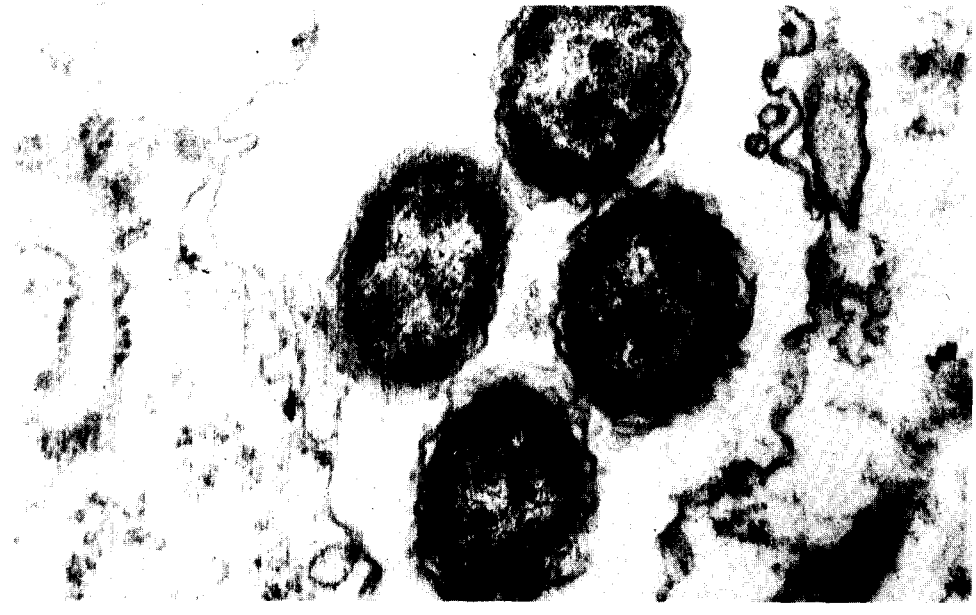
Fig. 2. Electronmicrograph of bacterial endosymbiotes of *A. proteus*. Large vacuole with irregular membrane-bound vesicles containing fibrous materials which fill the interbacterial space. ×35,000.

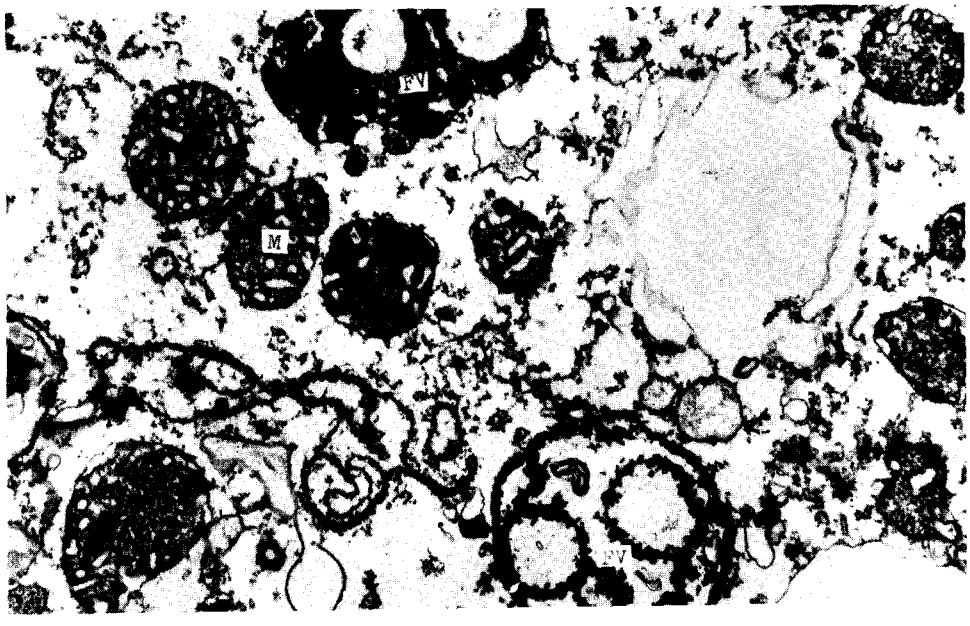
Fig. 3. Acid phosphatase reaction product can be seen in food vacuole(FV) containing Tetrahymena and in nearby many mitochondria (M) which are lack of enzyme activity. ×35,000.

Fig. 4. Electronmicrograph of bacterial endosymbiotes of *A. proteus*. grown with chloramphenicol. Vacuole containing bacterial endosymbiotes undergoing digestion (DB) and large amounts of acid phosphatase reaction product can be seen between and around endosymbiotes. ×40,000.

Fig. 5. Electronmicrograph of bacterial endosymbiotes of *A. proteus*, grown with chloramphenicol. Acid phosphatase reaction product can be seen in vacuoles containing degenerating bacteria (DB) undergoing phagocytosis and lysis. ×35,000.

Fig. 6. Electronmicrograph of bacterial endosymbiotes of *A. proteus*, grown with chloramphenicol. Large amounts of acid phosphatase reaction product can be seen in a vacuole containing degeneration bacteria (DB) and in nearby other vacuole containing DNA bodies (D), one kind of symbiotes. Note that the latter was lack enzyme activity in perisymbiotic vacuole (PV). ×35,000.

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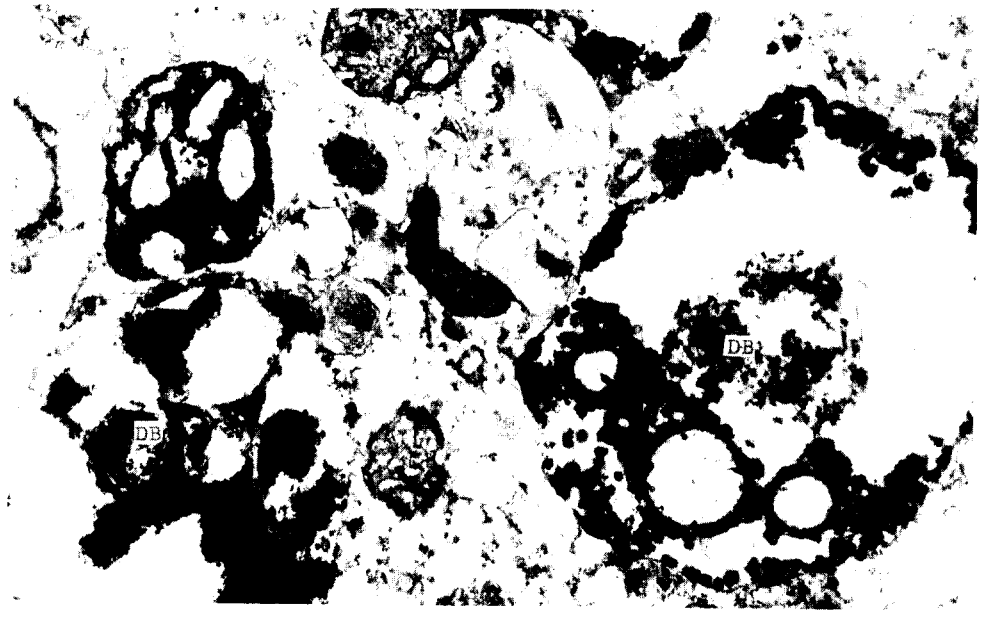
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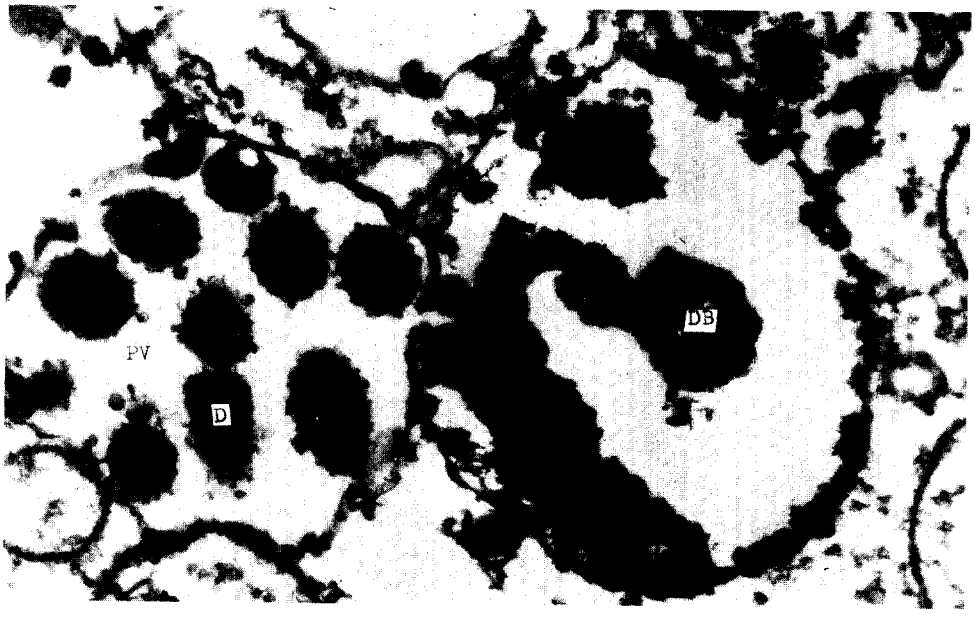
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