[Jap. J. Parasit, Vol. 26, No. 2, 75-85, 1977]

Ultrastructural Study on the Mechanism of Entry into Host Cells in *Toxoplasma gondii*

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(Received for publication; February 14, 1977)

Introduction

The entry mechanism of *Toxoplasma gondii* into various host cells has long been debated. Using a phase contrast moving pictures, several investigators (Bommer *et al.*, 1969; Lund *et al.*, 1961) suggested an active penetration in non-phagocytic cells. The active penetration was later confirmed electronmicroscopically using mouse peritoneal macrophage (Zaman and Colley, 1972). On the other hand, the entry of *Toxoplasma* by phagocytosis was also demonstrated electronmicroscopically (Jones *et al.*, 1972), and its relation with lysosome was discussed (Jones and Hirsch, 1972). Thus the detailed mechanism of the entry of *Toxoplasma* into host cells is not definitely concluded yet.

During the course of investigation on the obligate intracellular parasitism of this parasite, the present author recognized importance of the membrane of the parasitophorous vacuole in understanding the parasitism clearly. Formation of the vacuole undoubtedly relates with the entry process of *Toxoplasma*. Unfortunately, however, it is still impossible to isolate and analyze chemically the vacuolar membrane yet. This prompted us to initiate the characterization of the parasitophorous vacuole with making clear electronmicroscopically the entry mechanism of *Toxoplasma* as well as the process of the formation of the vacuole. The present communication deals with the ultrastructural demonstration of the entry of *Toxoplasma* into mouse peritoneal cells *in vivo*.

Materials and methods

RH strain was used throughout the present experiment. *Toxoplasma* tachyzoites were collected by washing the peritoneal cavity of mouse infected with this strain with sterilized buffered saline, and were inoculated into adult male ICR mice intraperitoneally. After 30, 60 and 120 minutes, approximately 5 ml of cooled and buffered 2.5% glutaraldehyde in 0.1 M potassium phosphate, pH 7.2 was injected into the peritoneal cavities of alive or killed mice by cervical dislocation, and *Toxoplasma* and peritoneal cells were fixed *in situ*. The cells and the parasites were collected by aspiration and centrifuged. After the supernatant fluid was discarded, glutaraldehyde was added to the pelet, and the cells and the parasites were further fixed for 60 minutes at 4 C. Postfixation was done with 1% osmium tetraoxide in 0.1 M potassium phosphate, pH 7.2 for 60 minutes at 4°C. Then the specimen was dehydrated in ethanol and embedded in Epon. Thin sections were made with Porter-Blum MT 2-B ultramicrotome using a diamond knife, and were observed with Hitachi HU-12 AS electron microscope following staining with uranyl acetate and lead nitrate.

Electronmicroscopical demonstration of polysaccharide on the cellular surface of an exozoite, which represents an extracellular parasite, was performed by the procedure of Behnke (1968). The reason why the term "exozoite" was employed is it now appeares reasonable to discriminate it from endozoite which refers to "rapidly multiplying forms reproducing by endodyogeny"

(Jacobs, 1973), since it is accepted that the parasite outside the host cell "exozoite" does not multiply.

Results

Figures I and II illustrate a peritoneal macrophage which several exozoites are entering. No apparent active penetration which disrupts the plasma membrane of the host cell was observed. Although exozoites push and invaginate the host cell membrane to various extent, it appears undisrupted. The tiny cytoplasmic protrusions, which must be a kind of micropseudopod, extend from the host cell, as if they react to pressing and invaginating of the host cell membrane by exozoites. These findings suggest that *Toxoplasma* does not penetrate the plasma membrane of macrophage (Here "penetrate" means entry in which host cell membrane is disrupted by parasite).

Figures III and IV substantiate this proposition. These figures illustrate exozoites pressing and invaginating the host cell membrane. Although the anterior end of the exozites clearly attaches the host cell membrane, it is undoubtedly undisrupted. Micropseudopods extend as observed in Figures I and II. It now appears clear *Toxoplasma* does not disrupt the host cell membrane even when it enters the host cell from its anterior end.

The observation that the plasma membrane of the host cell is pushed and invaginated but not disrupted by exozoite during its entry is further confirmed by Figures V-VII which exhibit highly magnified pictures of exozoite entering the host cells. It is apparent that the exozoites push the host cell membrane but do not disrupt it, and that micropseudopods extend and surround the exozoites. Moreover, these figures suggest that *Toxoplasma* is able to push and invaginate the host cell membrane even when the site other than the anterior end of the parasite attaches the host cell (Figure VI). This ability might be ascribed to operation of submembranous microtubles.

Although Figures I-VII obviously suggest that the plasma membrane of the host cell is not disrupted during entry of the exozoite, the mode of entry is probably not regarded as typical and active phagocytosis. It certainly can be involved in phagocytosis in a broad sense, but the obervations that the parasite pushes and invaginates the host cell membrane suggest that the mode of entry shown in Figures I-VII may be termed "Engulfing".

In contrast, an active phagocytosis of exozoites by an eosinophilic leucocyte and a peritoneal macrophage is illustrated in Figure VIII and IX respectively. It is obvious that extension of micropseudopods is more prominent than in "Engulfing" shown above, and that the exozoites do not push the host cell membrane at all. Accordingly this mode of entry may be termed "Active phagocytosis" in order to discriminate this from "Engulfing".

In "Engulfing" as well as in "Active phagocytosis", it can be observed that the plasma membrane of the host cell does not contact directly with that of the exozoite during its entry. There always is some kind of fuzzy coat on the surface of the exozoite. This coat probably surrounds the whole body of exozoite and is present on the cellular surface of the parasite which has just finished entry into the host cell. Highly magnified picture of the cellular surface of exozoite is shown in Figure X. A fuzzy coat is obviously present on the cellular surface. Interestingly, however, this coat is absolutely removed from the parasite in the parasitophorous vacuole (Figure XI). Although the origin and the physiological role of this coat is not known, it may have a function in host cell recognition of *Toxoplasma*. Otherwise, the removal of the coat from the parasite may stand for some kind of intracellular differentiation of *Toxoplasma*. Concerning the chemical characteristics, this kind of coat has been considered to be a glycoprotein. Figure XII clearly shows that the fuzzy coat of exozoite also contains polysaccharide possibly a glycoprotein. Deposit of ruthenium red is ap-

parent. Interestingly, not on the cellular surface but on the outer layer of the plasma membrane of exozoite is the deposit of ruthenium red.

Discussion

The present study electronmicroscopically classified the modes of entry of *Toxoplasma* into two groups, i.e., "Engulfing" and "Active phagocytosis", on the basis of presence of pressing of the host cell by exozoite and extent of micropseudopodial extension. "Active phagocytosis" may correspond to active em-membranosis (Ogawa 1965). In contrast, "Engulfing" may resemble the mechanism of entry of malaria parasite (Aikawa and Sterling, 1974). In both modes of entry, it is now apparent that the host cell membrane is not disrupted during entry of exozoite, even when the anterior end attaches the host cell. However, Zaman and Colley (1972) delineated quite opposite findings using the similar system to that of the present study. They proposed an active penetration as the mode of entry of *Toxoplasma* into mouse peritoneal macrophage. Although the accurate reason of this discrepancy is still not known, difference of detailed aspects of the experimental procedure such as the method of fixation may be one of the causes. Supposedly, the present procedure of fixation may be better suited for the study on the entry mechanism, since the parasite and the peritoneal cells are fixed *in situ*. In contrast to Zaman and Colley (1977), Hirsch *et al.* (1972) insisted the mechanism of entry was phagocytosis in tissue-cultured macrophage, Hela cell and fibroblast. They did not observe any other mode of entry into these cells. It is supposed, however, their technique to enhance entry of the parasite may affect its entering process. They cooled and centrifuged tissue culture cylinder to promote the parasite to attach the host cell, and activated the parasite and the host cell by warming the cylinder. It is still obscure, however, whether both *Toxoplasma* and the host cell are activated to the same extent by this technique. It may be possible that only the host cell is activated and initiates to phagocytose the parasite. As far as judged from the pictures in their report (Jones *et al.*, 1972), phagocytosis that they referred to is quite similar to "Active phagocytosis" of the present study. Works now being done in our laboratory utilizing the Beige mice, however, also confirm that *Toxoplasma* at least actively pushes the host cell membrane during its entry into mouse peritoneal macrophage.

Through their studies, Jones *et al.* (1972) and Jones and Hirsch (1972) suggested that the organellas located at the anterior end of the parasite such as rhoptry and microneme secrete a substance to induce an effective phagocytosis. The present study also substantiates at least partially their proposition, since an eosinophilic leucocyte phagocytosed exozoites, although it has not been normally considered phagocytic (Bloom and Fawcett, 1975). But the observation that exozoite enters macrophage by "Engulfing" very often makes the role of these organellas rather complicated. Even when the anterior end of the parasite attaches the host cell (Figures III and IV), extension of micropseudopods is not so prominent. Presence of monovalent cations like K^+ and Cl^- at rhoptry, which was revealed by X-ray spectroscopy (Takeuchi and Fujiwara, unpublished observation), also suggest some other important role of these organellas.

The importance of the membrane of the parasitophorus vacuole in understanding the parasitism of this parasite is quite obvious. If the vacuole is actually a phagosome as insisted by Jones *et al.* (1972) as well as by the present study in part, the intracellular differentiation of the parasite should deeply relate with host lysosome. Accordingly, the mechanism of the parasitophorous vacuole to escape fusion with host lysosome is of primary importance in understanding the parasitism of this parasite. However, it is not accurately known if the vacuole formed by the parasits which has entered the host cell by "Engulfing" is a typical phagosome.

The relation of the parasitophorous vacuole with lysosome should be investigated in more details.

Concerning the surface coat, several instances have been available with parasitic protozoans, particularly with African trypanosomes (Vickerman, 1974). Relation of the surface coat with immunological events has been discussed with these parasites. Interestingly, *Plasmodium knowlesi* has been found to have a surface coat which also has something to do with immunological events and is pushed back during entry into red blood cell (Miller *et al.*, 1975). The present study also demonstrated the removal of the coat after the exozoite entered the host cell (Figure XI). However, the detailed analysis on the origin and the physiological significance of this coat awaits further investigations.

Summary

In order to characterize the parasitophorous vacuole, which has an important role in intracellular differentiation of *Toxoplasma gondii*, an electronmicroscopical analysis on the entry mechanism of this parasite was attemped, since formation of the vacuole certainly relates with the mode of entry.

The repeated experiments revealed that *Toxoplasma* was sometimes phagocytosed by a peritoneal macrophage as well as by an eosinophilic leucocyte *in vivo*. However, in most cases, the parasite certainly pushes and invaginates the plasma membrane of the host cell, and micropseudopods extend and surround the parasite. But extension of micropseudopods is not prominent compared with active phagocytosis. The plasma membrane of the host cell is not disrupted in spite of deep invagination. This mode of entry was termed "Engulfing".

A kind of fuzzy coat was found to be present on the cellular surface of the parasite outside the host cell (Exozoite). This coat was stained with ruthenium red. In contrast to exozoite, the coat was absolutely removed from the parasite in the parasitophorous vacuole.

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トキソプラスマにおける宿主細胞内侵入機構に関する電子顕微鏡的研究

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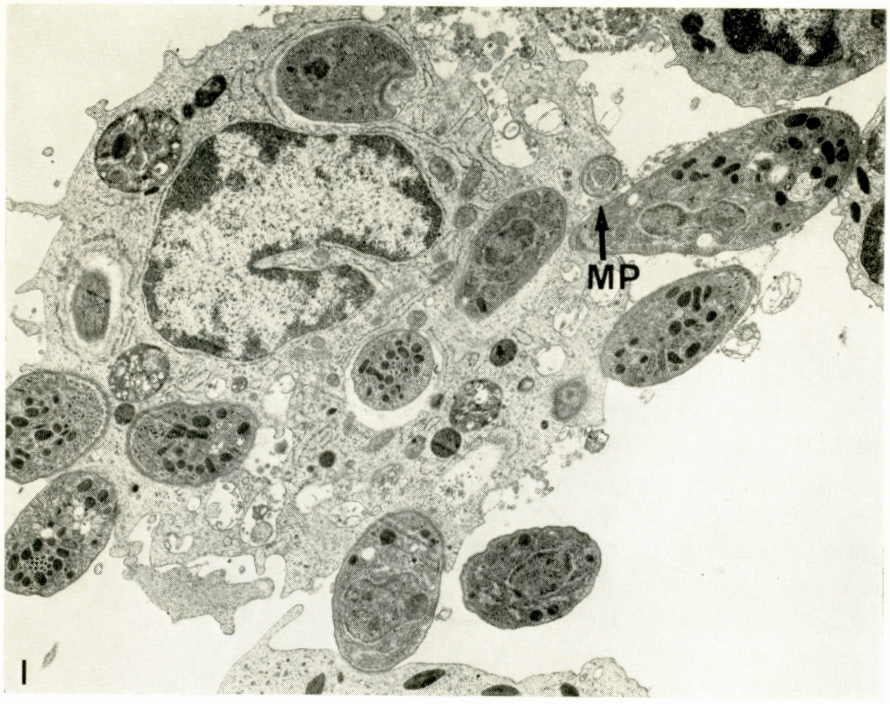
トキソプラスマ（Toxoplasma gondii）の偏性細胞内寄生のメカニズムについて一連の研究を行つてきたが，いわゆる parasitophorous vacuole の性質の解明が必要となり，おそらく vacuole の形成はトキソプラスマの細胞内侵入機構と密接に関連していると考えられることから，今回はトキソプラスマのマウス腹腔細胞に対する侵入機構を電顕的に研究した．

観察された宿主細胞は大部分腹腔マクロファージであるが，時に好酸球性白血球も見られた．侵入機構としては active な phagocytosis も見られたが，いわゆる典型的な phagocytosis とは考えられない像も見られた．すなわち，トキソプラスマ自身が宿主細胞膜を内側に押して invaginate させ，これに反応するような形で宿主細胞から現れた micropseudopod が伸長する（engulfing）といつた像が得られた．トキソプラスマはかなり深くまで宿主細胞膜を内側に押しこむことができるようである．active phagocytosis の場合はこのような像は見られなかつた．しかし何れの場合も従来いわれていた宿主細胞膜が虫体侵入時に破壊される像は認められなかつた．

更に宿主細胞外にいるトキソプラスマではその表面にルテニウムレッド陽性の coat があり，これが parasitophorous vacuole 内の虫体では失なわれていることも判つた．しかしこの coat の起源，および機能は明らかとなつていない．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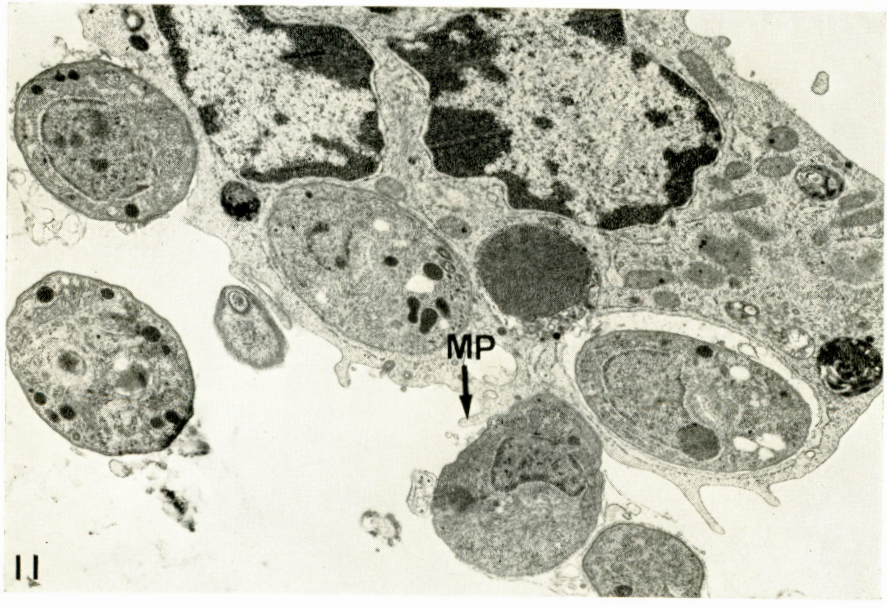
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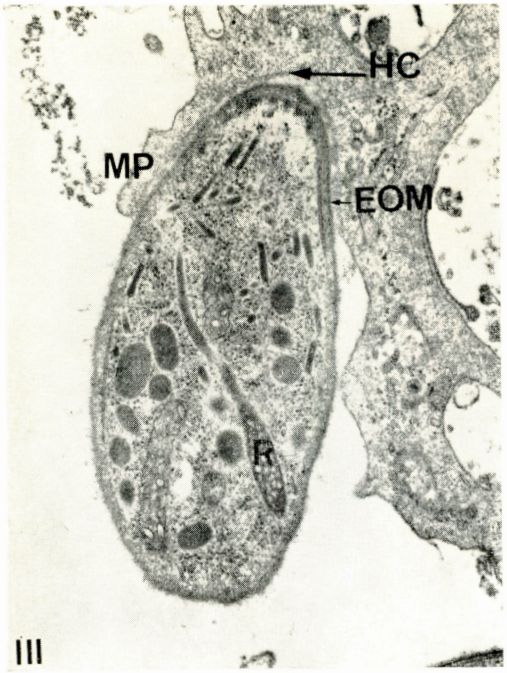
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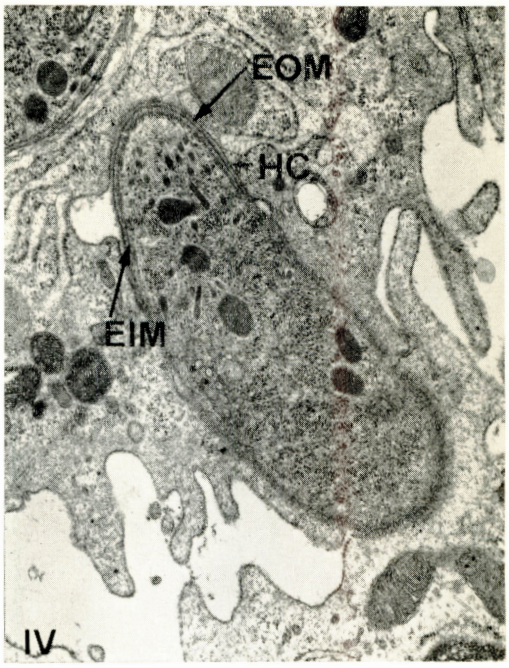
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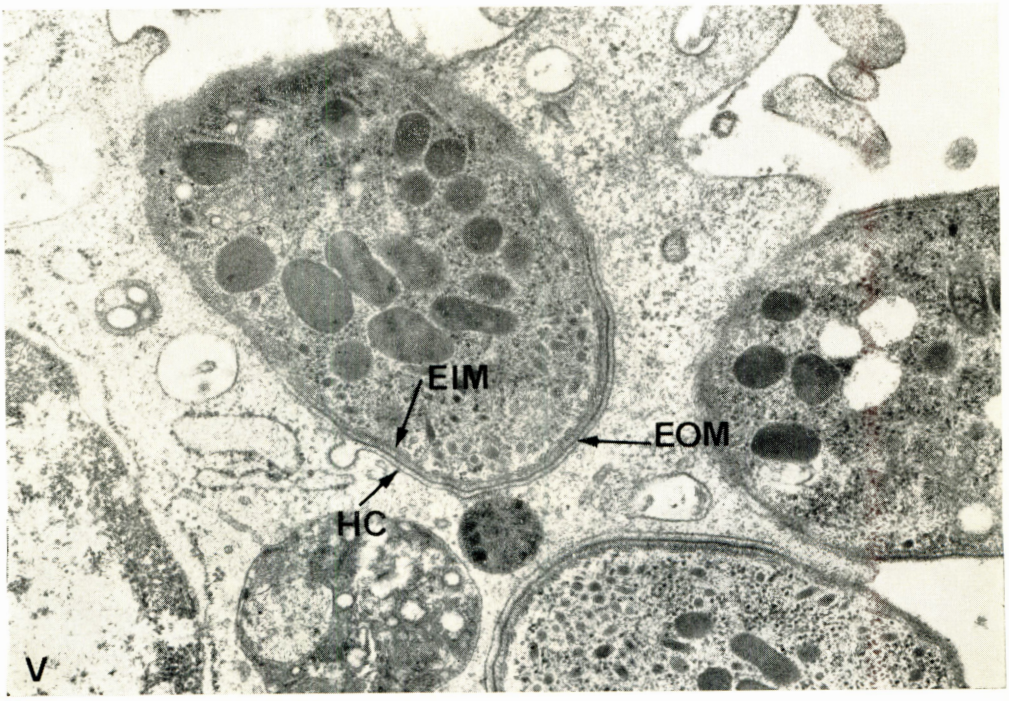
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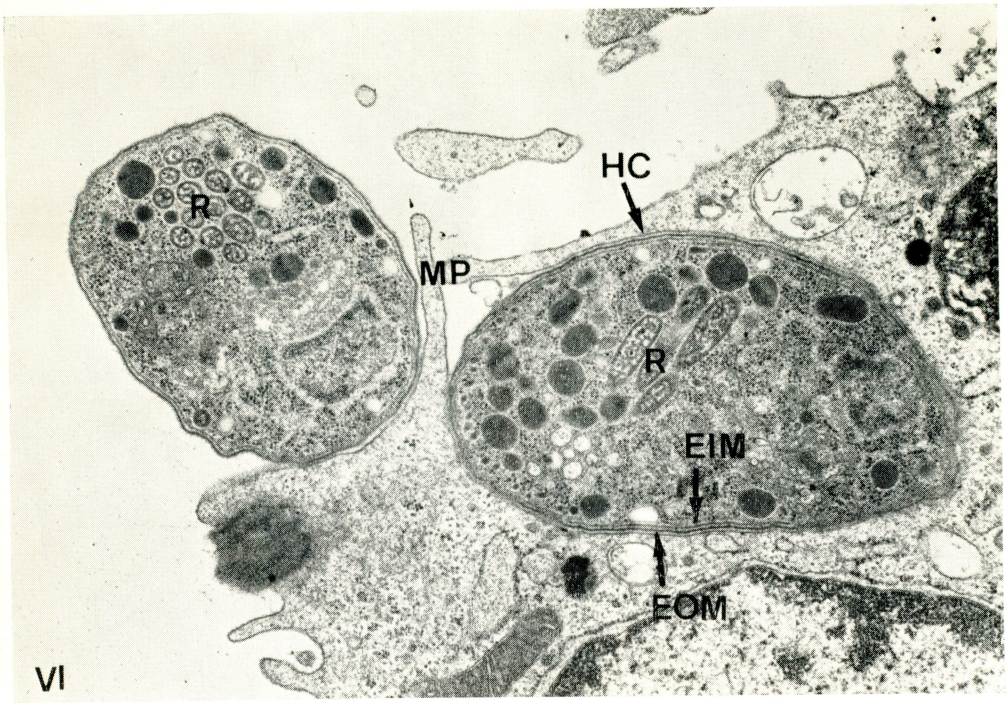
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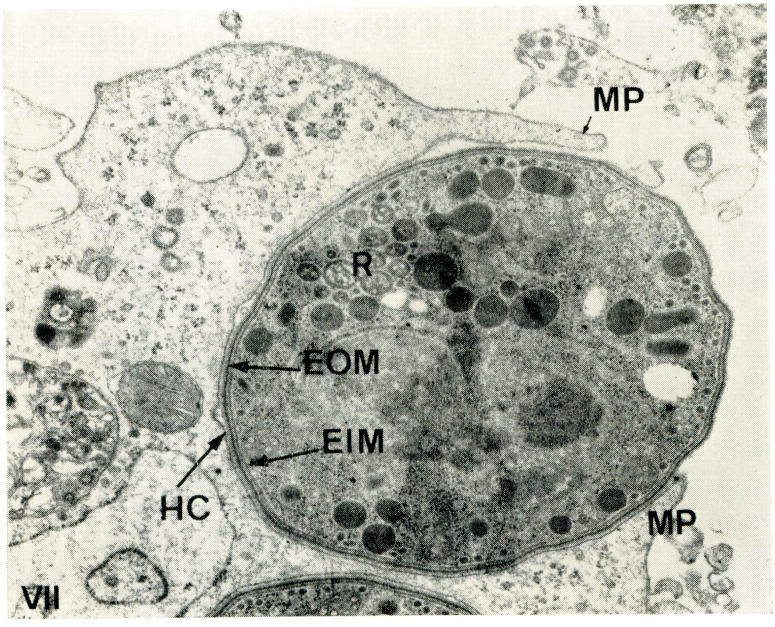
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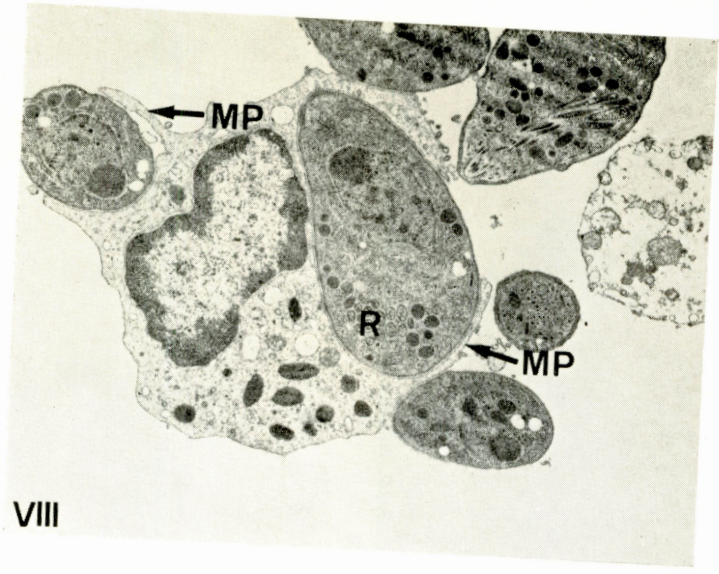
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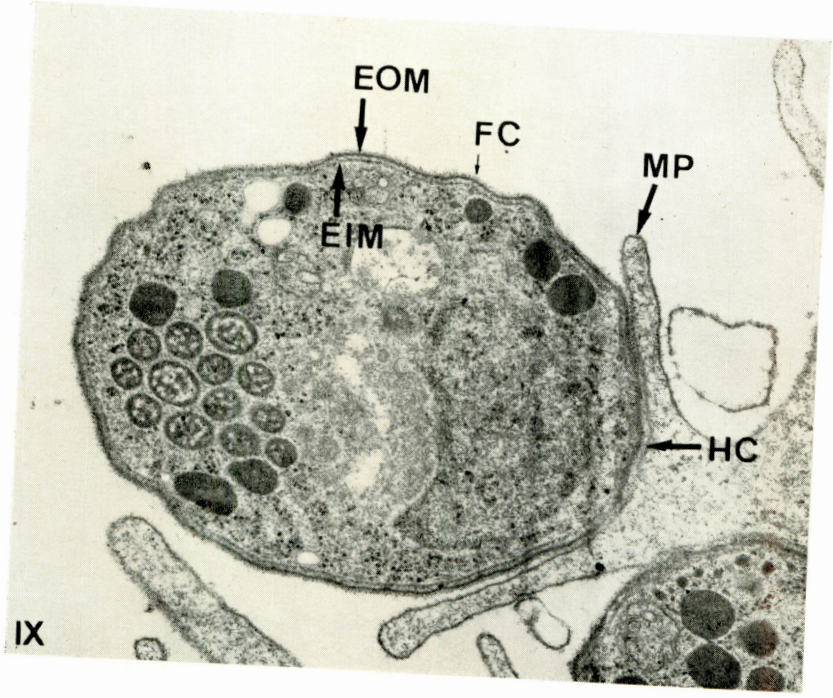
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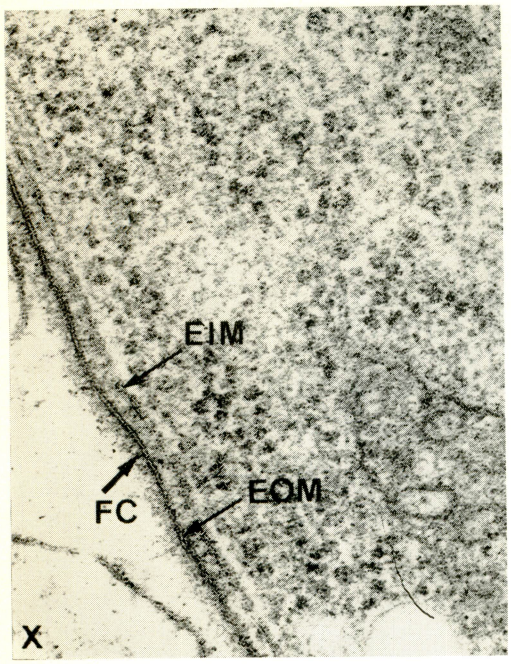
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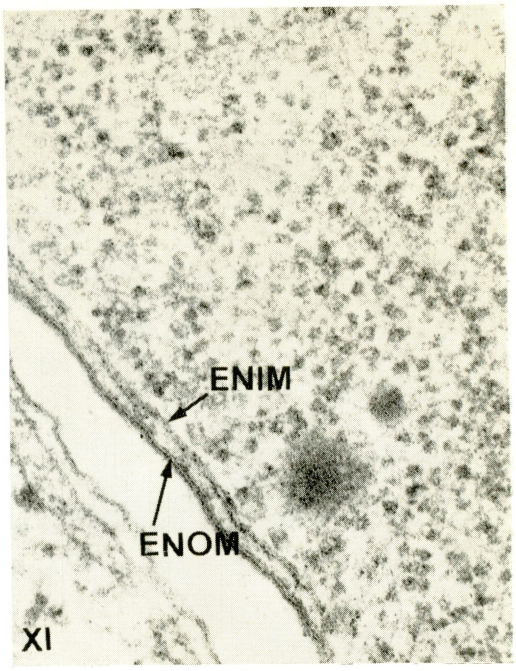
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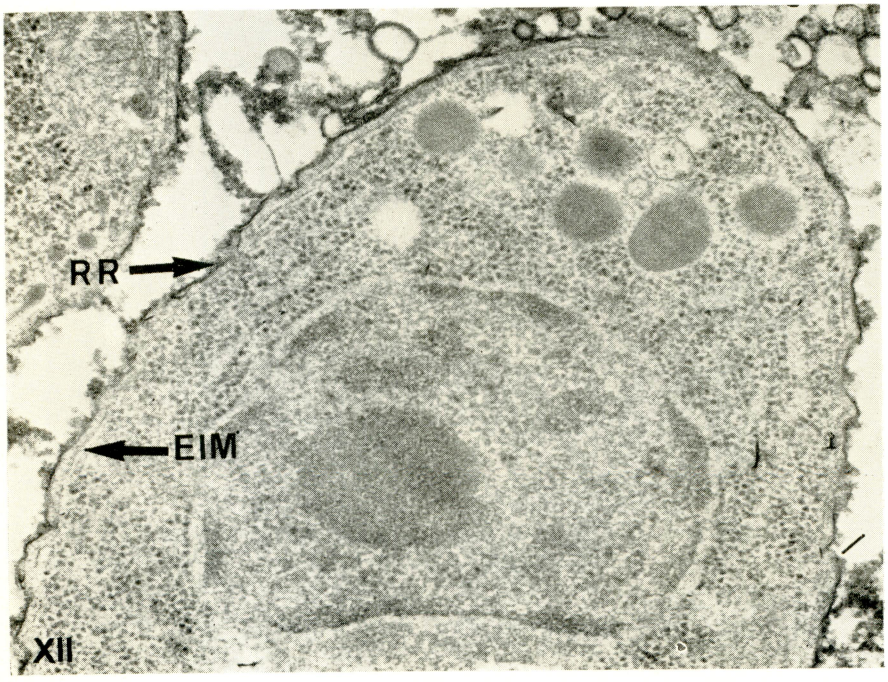
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Explanation of figures

Figure I. A low magnified picture of a peritoneal macrophage which several exozoites are entering simultaneously. These exozoites push and invaginate the plasma membrane of the macrophage. No apparent disruption of the plasma membrane is observed. (× 9,000)

Figure II. A low magnified picture of a peritoneal macrophage. Two exozoites are pressing and invaginating the host cell membrane. (× 11,000)

Figure III. An exozoite pressing the host cell membrane. A tiny micropseudopod extends from the cell. As judged from localization of rhoptry and microneme, the anterior end of the exozoite attaches the host cell. But the host cell membrane appears undisrupted. (× 23,000)

Figure IV. A similar finding to Figure III. The anterior end of the exozoite attaches the host cell. The plasma membrane of the host cell remains undisrupted. Microneme is clearly seen. (× 22,000)

Figure V. Three exozoites are pressing and invaginating the host cell membrane. Note that the host cell membrane is undisrupted, and the space between the host cell and the exozites is filled with the fuzzy coat of the exozoite. (× 36,000)

Figure VI. An exozoite has almost entered the host cell in the same manner as in Figure V. Tiny micropseudopods are seen. Another exozoite may be phagocytosed from its posterior end. (× 24,000)

Figure VII. An exozoite pressing the host cell. Note the similar findings as in Figures V and VI. (× 25,000)

Figure VIII. An eosinophilic leucocyte which is phagocytosing two exozoites simultaneously. Note prominent micropseudopods extending from the host cell. (× 10,000)

Figure IX. An exozoite which is phagocytosed by macrophage. Note that the exozoite does not push and invaginate the host cell membrane. (× 36,000)

Figure X. A high magnified picture of the plasma membrane of an exozoite. An apparent fuzzy coat is present on the cellular surface. (× 120,000)

Figure XI. A high magnified picture of the plasma membrane of the parasite in the parasitophorous vacuole. Note that the fuzzy coat is lost from the surface. (× 120,000)

Figure XII. A picture showing deposit of ruthenium red on the cellular surface as well as on the outer layer of the plasma membrane of an exozoite.

Abbreviations used

M: microneme, R: rhoptry, HC: host cell membrane, EOM: outer cell membrane of exozoite, EIM: inner cell membrane of exozoite, MP: micropseudopodium, FC: fuzzy coat on the cellular surface of exozoite, ENOM: outer cell membrane of endozoite, EMIM: inner cell membrane of endozoite, RR: deposit of ruthenium red