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Electron Microscopic Observations on the Effect of Human Serum, Anti-Trypanosome Mouse-Serum and Acriflavine on *Trypanosoma gambiense* in Mice

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Trypanosoma gambiense is an agent protozoa of African sleeping sickness in man. The parasites maintained in laboratory mice by serial passages do not cause pathogenic effect on man (Fairbairn, 1956). Laveran (1902) and Inoki *et al.* (1952) reported that the mice infected with trypanosomes are readily cured by treatment with human serum. Mice infected with Wellcome strain of *T. gambiense* used in the present experiment recover from experimental trypanosomiasis after injection of human serum (Inoki *et al.*, 1958). Ono and Inoki (1972) studied morphologically on the mode of action of human serum against trypanosomal infections in mice and found that under light microscope many parasites were attached to the peritoneal cells in infected mice which had received an intraperitoneal injection of human serum. Lange and Lysenko (1960) and Takayanagi *et al.* (1974) demonstrated that under light microscope immune serum stimulated the phagocytosis of trypanosomes by peritoneal cells *in vitro*. Recent study showed that in mice infected with *T. evansi* the trypanocidal effect of human serum was enhanced by addition of anti-trypanosome mouse-serum (Ono and Inoki, 1973).

The present experiments were performed to study on the ultrastructural changes occurring between trypanosomes and peritoneal cells in trypanosome infected mice after treatment with human serum, anti-trypanosome mouse-serum and acriflavine, respectively. The findings in the present study did not elucidate the mode of action of curative effect of human serum on trypanosomal infections in mice. However, electron microscopy in *T. gambiense*-infected mice treated with human serum, anti-trypanosome mouse-serum and acriflavine certainly provides some interesting data on the process of phagocytosis of trypanosomes by peritoneal cells.

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Materials and Methods

The Wellcome strain of *Trypanosoma gambiense* that has been maintained in this laboratory by serial passages through ddO mice for many years was used throughout the present experiments.

ddO mice were inoculated intraperitoneally with *T. gambiense* and 3 days later they received intraperitoneal injection of human serum, anti-trypanosome mouse-serum and acriflavine. Peritoneal fluid was taken out at intervals and subjected to electron microscopy.

Inactivated human serum was obtained from the Foundation of Research Institute for Microbial Diseases, Osaka University. The method to obtain anti-trypanosome mouse-serum from mice was as follows. When parasites reached a count of 5×10^8/ml in the blood stream 3 days after intraperitoneal inoculation, mice were treated with either 1 ml of inactivated human serum or 10 mg/kg of acriflavine. Five days later,

mice were bled from the heart by opening the thorax under chloroform anesthesia. Serum was collected by centrifugation and inactivated at 56C 30 min. Acriflavine was obtained from Eli Lilly Research Laboratories (Indianapolis, Indiana, U.S.A.).

For electron microscopic observations, peritoneal fluids containing many peritoneal cells and trypanosomes were collected and fixed at 4C for 1 hr in 0.01 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. Peritoneal cells and trypanosomes were concentrated by gentle centrifugation at 2,000 rpm for 15 min, then washed for 1 hr with phosphate buffer containing 0.25 M sucrose, and post-fixed at 4C for 1 hr with 1.5% osmium tetraoxide in isotonic buffer. After dehydration, the materials embedded in Epon 812 and sections were double stained with uranyl acetate and lead citrate.

Results

1. Peritoneal macrophage obtained from non-infected mouse.

Fig. 1 shows the peritoneal macrophage obtained from non-infected mouse demonstrating the deep indented nucleus and moderate amount of cytoplasm. A few lysosomes containing some fine granular materials which stained slightly with osmium are present in the cytoplasm.

2. Peritoneal macrophage obtained from *T. gambiense*-infected mouse.

Fig. 2 shows two peritoneal macrophages obtained from *T. gambiense*-infected mouse. There is a pronounced alteration in size of macrophage associated with an increase in cytoplasmic mass, the number of lysosome, mitochondria and other organella. These changes indicate a high degree of cellular activity in macrophages. The membranes of endoplasmic reticulum show variable width. The majority of lysosome is present near the Golgi lamellae in the juxtannuclear region. A certain part of lysosomes is continuous with the membranes of endoplasmic reticulum (arrow).

3. Peritoneal macrophage obtained 20 min (Fig. 3), 1 hr (Fig. 4) and 6 hrs (Fig. 5) after intraperitoneal injection of human serum in trypanosome-infected mouse.

Fig. 3 shows photograph of phagocytosis of parasite by macrophage. Phagocytized parasite was found within phagosome (arrow) of macrophage. A few alterations in cytoplasm of trypanosomes were observed, although subpellicular microtubles and basal body did not show degeneration. Increase in number of lysosome was seen around the phagosome.

Fig. 4 shows two macrophages. Five phagolysosomes in cytoplasm of macrophage contain a large number of electron dense bodies and lysosomes. The large vacuoles containing small amount of flocculence are found in the cytoplasm of macrophage. But it is difficult to determine if this vacuole would be a remnant of phagolysosome containing trypanosome. In the other macrophage, homogeneous ectoplasm contains flagella of trypanosomes.

Fig. 5 shows alterations of phagolysosome in macrophage. Alterations are in progress. Increase in size of phagosome occurs after the lysis of cytoplasm of macrophage by enzyme from lysosome. A large phagolysosome fused with another phagolysosome contains amorphous materials and electron dense bodies.

4. Peritoneal cells obtained 20 min (Figs. 6–9) and 6 hrs (Fig. 10) after intraperitoneal injection with anti-trypanosome mouse-serum in trypanosome-infected mouse.

Fig. 6 is a low magnification showing 4 macrophages and 1 neutrophil fused by the ectoplasm containing phagocytized trypanosomes. Two or more parasites associated with pronounced alteration of cytoplasm could be found inside phagosomes in each cell. Fig. 7 is a high magnification photograph of a part of Fig. 6. Fusion of numerous lysosomes with the phagosome containing phagocytized trypanosome caused disintegration in cytoplasm of trypanosome. The cytoplasm of trypanosome is replaced by a large number of lysosomes, vacuoles and electron dense bodies. The cytoplasmic membrane of trypanosomes (arrow 1) is hardly identified, but only the kinetoplast can be recognized. A space of variable

width was observed between obscure membrane of trypanosome (arrow 1) and phagolysosome membrane (arrow 2) in which amorphous material and flagella are present. Cytoplasm display increased number of enlarged mitochondria and dilation of granular endoplasmic reticulum.

Large numbers of eosinophil leucocyte and some mast cells are present in the peritoneal fluid. Almost all of eosinophils display phagocytic activities. Fig. 8 shows phagocytosis of trypanosome by invagination in plasma membrane of eosinophil. Two arms of ectoplasm extending around parasite are fusing to make a phagosome (arrow). A large number of lysosomes and specific granules containing an equatorial band of extremely dense crystalloid material are present near the nucleus. No remarkable alterations are observed in that parasite. A large number of parasites are present in the homologous materials devoid of organella (Fig. 9). The structure similar to nucleus is demonstrated near the area of homologous materials, but it is unknown whether that structure is nucleus or not.

It is dfficult to visualize the trypanosomes taken into macrophage 6 hrs after injection with anti-trypanosome mouse-serum (Fig. 10). However, a large structure containing flagella-like structure (arrow) and membraneous component which appear to be the remnant of phagocytized trypanosomes are present within phagosome near the nucleus of macrophage.

5. Peritoneal macrophage obtained 20 min (Fig. 11) and 6 hr (Fig. 12) after intraperitoneal injection with acriflavine.

Fig. 11 shows phagocytosis of parasite by macrophage. In the engulfed trypanosome within phagosome, lysosome appears to be entering into the cytoplasm of trypanosome at a few parts of disintegrating plasm membrane (arrow). Therefore, disintegrated cytoplasm of trypanosome is completely filled with a large number of lysosomes and electron dense amorphous materials. Outside the macrophage, disintegrated trypanosomes showing a presence of flagella may be caused by direct action of acriflavine. Fig. 12 shows alteration in cytoplasm of macrophage after phagocytosis of parasite by macrophage. Phagosome in the cytoplasm of macrophage contains myelin-like substances laminated with membraneous structure. Amorphous materials are observed between the myelin-like substance and membrane of phagosome. Some structures similar to myelin are distributed around the nucleus of macrophage.

Discussion

Electron microscopic observations on the phagocytosis of bacteria and fungi by macrophage were made by many workers (North and Mackaness, 1963; Dumont and Sheldon, 1965; Dumont, 1972), but no similar report has ever been available in protozoa. The result of electron microscopy in the present experiment certainly provides an interesting information on the process of phagocytosis of trypanosomes by peritoneal cells.

Characteristics of ultrastructural changes occurring in peritoneal cells and parasites associated with inoculation of human serum, anti-trypanosome mouse-serum and acriflavine are the following. In infected mice treated with human serum, alteration in peritoneal macrophage is closely associated with the marked increase of lysosomes. Whereas, macrophage in mice treated with acriflavine displays a slight increase of lysosome and slight alterations around the phagosome. In infected mice treated with anti-trypanosome mouse-serum, engulfment of parasites by eosinophils and often by macrophage is a predominant finding. The fusion of macrophages by the intermediation of ectoplasm engulfing parasites was observed 20 min after injection with anti-trypanosome mouse-serum.

The peritoneal macrophage obtained from *T. gambiense*-infected mice indicate morphologically a high degree of cellular activity, but we could not demonstrate the phagocytosis of parasites by macrophage. However, the injection of human serum, anti-trypanosome mouse-serum and acriflavine into *T. gambi-*

ense-infected mice resulted in phagocytosis by macrophages.

The increased amount of lysosome to digest the phagocytized parasites, as seen in phagocytosis of bacteria and fungi by macrophage, was observed around parasite in macrophage. But Sanabria (1968) observed increase of lysosome while *T. cruzi* grows actively in the cytoplasm of macrophage. Threfore, release of enzyme from lysosome and activation are necessary for the process of phagocytosis.

The rate of appearance of eosinophils in the peritoneal fluid of non-infected mice is less than 1 % (Carr, 1967). Phagocytic ability of eosinophils is usually less than that of neutrophils or macrophage. However, a remarkable finding in this experiment is phagocytosis by a large number of eosinophils after the injection with anti-trypanosome mouse-serum. Phagocytosis by eosinophils appears to be same sequence of phagocytic process by macrophage. Eosinophils are attracted by substrates such as antigen-antibody complexes and histamine (Litt, 1961; Archer *et al.*, 1962; Archer, 1965). Weitz (1960) and Takayanagi *et al.* (1970) demonstrated that the exoantigen released from trypanosome was present in the blood of trypanosome-infected mice. Therefore, it is likely that antigen-antibody complex is formed in the intraperitoneal fluid of *T. gambiense*-infected mice immediately after the injection with anti-trypanosome mouse-serum. Also, mast cells that contain histamine are present in trypanosome-infected mice injected with anti-trypanosome mouse-serum.

Trypanocidal action of human serum was not evident *in vitro* and was not observed within 10 hrs after injection of human serum into mice (Goebel, 1907; Inoki *et al.*, 1958). Therefore, curative effect of human serum in *T. gambiense*-infected mice seemed to be attributed to indirect action of human serum against trypanosomes. The result obtained in the present experiment was not enough to show the mechanism of curative effect of human serum against the trypanosomal infections in mice, but alteration in peritoneal macrophage in the infected mice given human serum was characterized by marked increase of lysosomes and digestion in the early stage in the cytoplasm containing phagocytized parasite. It is probable that the engulfment of trypanosomes by macrophage is first manifestation of effect of human serum of trypanosomes.

Further experiments are necessary to clear what reactions have taken place after phagocytosis in trypanosome-infected mice injected with human serum.

Summary

Electron microscopic observations were made to study on the interaction between trypanosome and peritoneal cells in trypanosome-infected mice after treatment with human serum, anti-trypanosome mouse-serum and acriflavine, respectively.

In the infected mice given human serum, alteration in peritoneal macrophage was characterized by marked increase of lysosomes and digestion in the early stage in the cytoplasm containing phagocytized parasite. The engulfment of parasites by either macrophage or eosinophil was observed in infected mice receiving the anti-trypanosome mouse-serum. In macrophage from the mice treated with acriflavine, there was a few increases of lysosomes and minute alterations around the phagosome.

The result obtained here was not enough to show the mechanism of curative effect of human serum in trypanosome infected-mice, but electron microscopic observations were possible to provide the interesting finding on phagocytosis of trypanosomes by peritoneal cells. Moreover, the present experiment shows that the engulfment of trypanosomes by macrophage is first manifestation of effect of human serum on trypanosomes.

Refferences

1) Archer, R. K., Feldberg, W. and Kovacs, B. A. (1962): Antihistamine activity in extracts of horse eosinophils. Brit. J.

Pharmacol. Chemother., 18, 101–108.
2) Archer, R. K. (1965) : On the functions of eosinophils in the antigen-antibody reaction. Brit. J. Haematol., 11, 123–129.
3) Carr, I. (1967) : The fine structure of the cells of the mouse peritoneum. Zeit. Zellforsch. Mikroskop. Anat., 80, 534–555.
4) Dumont, A. and Sheldon, H. (1965) : Changes in the fine structure of macrophages in experimentally produced tuberculous granulomas in hamster. Laboratory Investigat., 14, 2034–2055.
5) Dumont, A. (1972) : Ultrastructural aspects of phagocytosis of facultative intracellular parasites by hamster peritoneal macrophages. J. Reticuloendothelial. Soc., 11, 469–491.
6) Fairbairn, H. (1956) : The infectivity to man of syringe passaged strains of *Trypanosoma rhodesiense* and *T. gambiense*. Ann. Trop. Med. Parasit., 50, 167–171.
7) Goebel, O. (1907) : Pouvoir preventif et pouvoir curatif du sérum humain dans l'infection due au trypanosome du nagana. Ann. de l'Inst. Pasteur, 21. 882–910.
8) Inoki, S., Kitaura, T., Nakabashi, T. and Kurogochi, H. (1952) : Studies on the immunological variations in *Trypanosoma gambiense*. I. A new variation system and a new experimental method. Med. J. Osaka Univ. 3, 357–371.
9) Inoki, S., Fukukita, S. and Matsushiro, A. (1958) : Studies on the therapeutic mechanism of normal human plasma to trypanosomal infection in mice. Jap. J. Parasit., 7, 102. (in Japanese)
10) Lange, D. E. and Lysenko, M. G. (1960) : *In vitro* phagocytosis of *Trypanosoma lewisi* by rat exudative cells. Exp. Parasitol., 10, 39–42.
11) Laveran, A. (1902) : De l'action de sérum humain sur le trypanosome du nagana (*T. brucei*). Compt. rend. Acad. Sci., 134, 735–739.
12) Litt, M. (1961) : Studies in experimental eosinophilia. III. The induction of peritoneal eosinophilia by the passive transfer of serum antibody. J. Immunol., 87, 522–529.
13) North, R. J. and Mackaness, G. B. (1963) : Electron microscopical observations on the peritoneal macrophages of normal mice and mice immunized with *Listeria monocytogenes*. I. Structure of normal macrophages and the early cytoplasmic response to the presence of ingested bacteria. Brit. J. Exp. Path., 44, 601–607.
14) Ono, T. and Inoki, S. (1972) : Studies on the phagocytosis of *Trypanosoma gambiense* by mouse ascites cell. Jap. J. Parasitol., 21 (Suppl.) 41. (in Japanese)
15) Ono, T. and Inoki, S. (1973) : Effect of human serum on *Trypanosoma evansi* infections in mice. Jap. J. Parasitol., 23, 53. (in Japanese)
16) Sanabria, A. (1968) : Ultrastructure of *Trypanosoma cruzi* in mouse brain. Exp. Parasitol., 23, 379–391.
17) Takayanagi, T., Kambara, H., Inoki, S. and Yoshikawa, K. (1970) : Immunological studies on trypanosomes, with special reference to the specificities of the antigens. Jap. J. Parasitol., 19, 260–264. (in Japanese)
18) Takayanagi, T., Nakatake, Y. and Enriquez, G. L. (1974) : *Trypanosoma gambiense:* Phagocytosis *in vitro*. Exp. Parasitol., 36, 106–116.
19) Weitz, B. (1960) : The properties of some antigens of *Trypanosoma brucei*. J. Gen. Microbiol., 23, 589–600.

マウス感染 *Trypanosoma gambiense* に対する人血清，抗トリパノソーママウス血清及びアクリフラビンの効果に関する電子顕微鏡的観察

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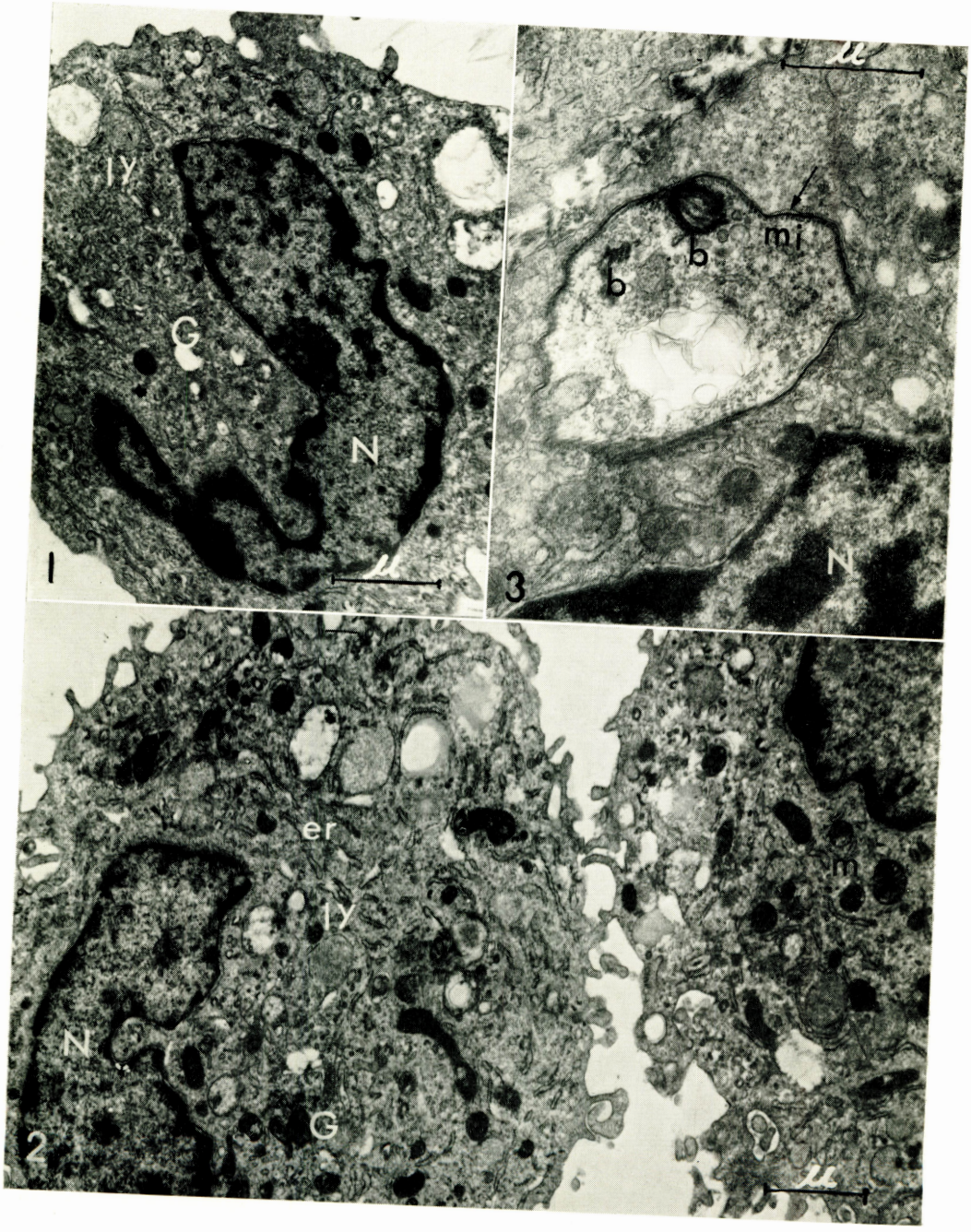
アフリカ睡眠病の病原体である *Trypanosoma gambiense* はマウスに継代を続けると人に対する病原性を失い，人血清で原虫感染マウスが治癒するようになる．人血清の原虫に対する効果はおそらく間接的なものと思われるが，その作用機序は未だ明らかでない．先に小野・猪木（1973）は *T. evansi* 感染マウスに対する人血清の治癒効果が抗トリパノソーママウス血清の注射によつて増強されることを見出した．そこで *T. gambiense* 感染マウスに人血清，抗 *T. gambiense* マウス血清（以下，抗血清）及びアクリフラビンをそれぞれ注射し，原虫と腹水細胞との間にみられる変化を電顕によつて調べた．その結果，人血清を注射した感染マウスの大食細胞では phagocyte した原虫を含む phagosome のまわりで lysosome の強い増加がみられ，大食細胞の細胞質の融解もかなり早い時期にみられた．抗血清を注射したマウスでは好酸球と大食細胞に phagocytosis がみられ，また phagocyte した原虫を含む大食細胞の ectoplasm を仲立ちとした細胞融合がみられた．アクリフラビンを注射した感染マウスでも大食細胞による phagocytosis があったが，大食細胞の細胞質における変化は比較的僅かであり，lysosome の増加も少なかつた．このように人血清の注射後だけではなく，感染マウスに治療効果をもたない抗血清，マウス体内で直接，原虫を殺すと思われるアクリフラビンをそれぞれ注射した後でも腹水細胞による phagocytosis がみられたが，原虫感染マウスに対する人血清の効果の最初の現われは大食細胞によるトリパノソーマの phagocytosis であると思われる．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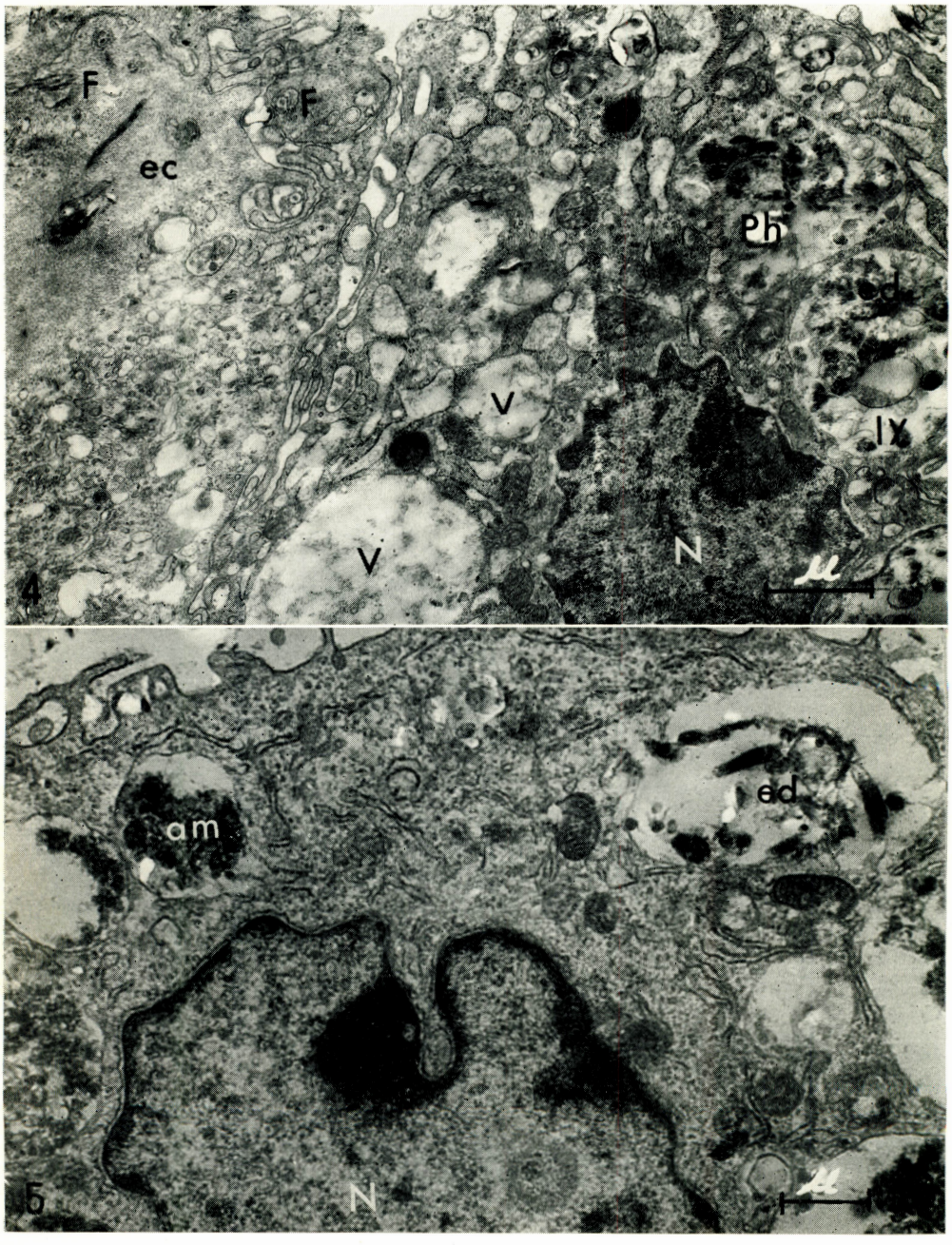
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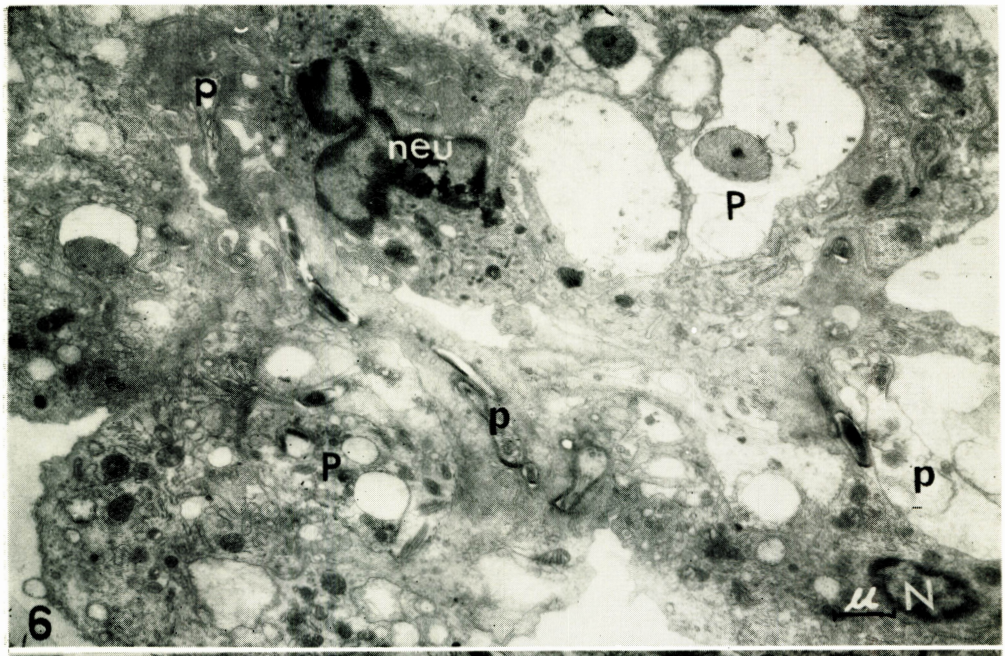
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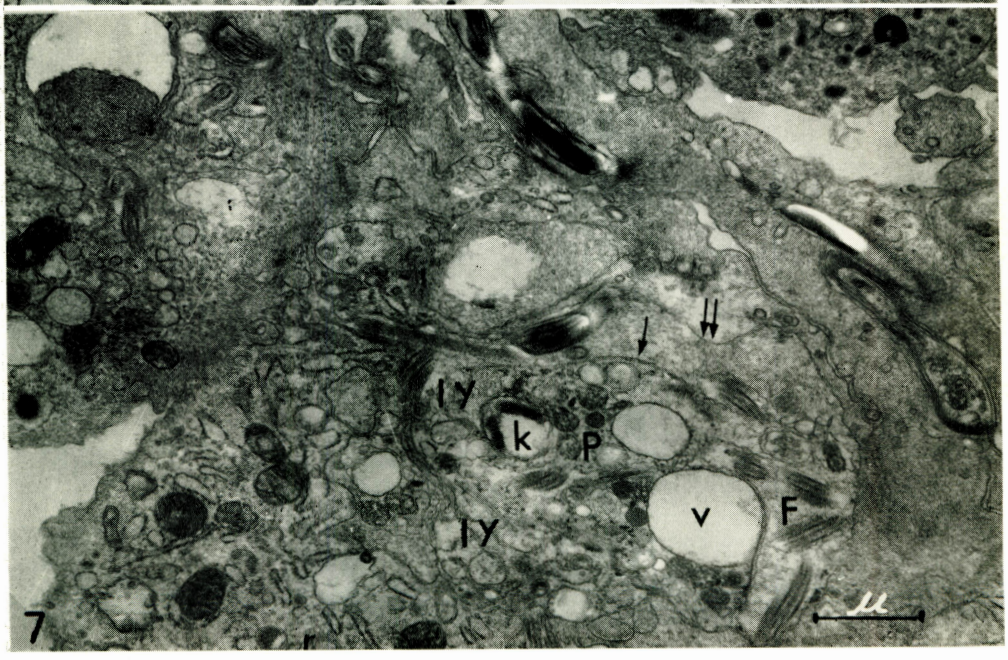
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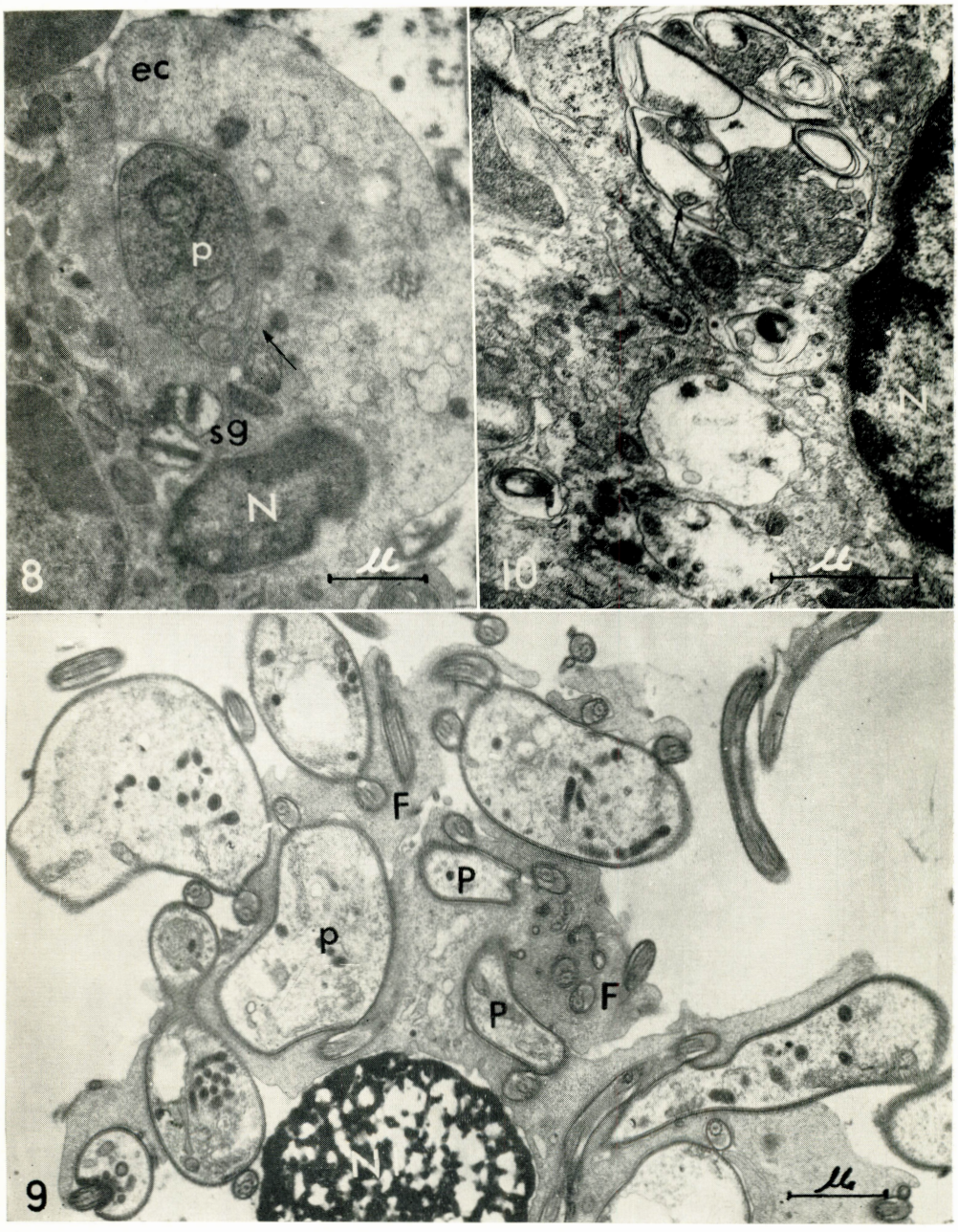
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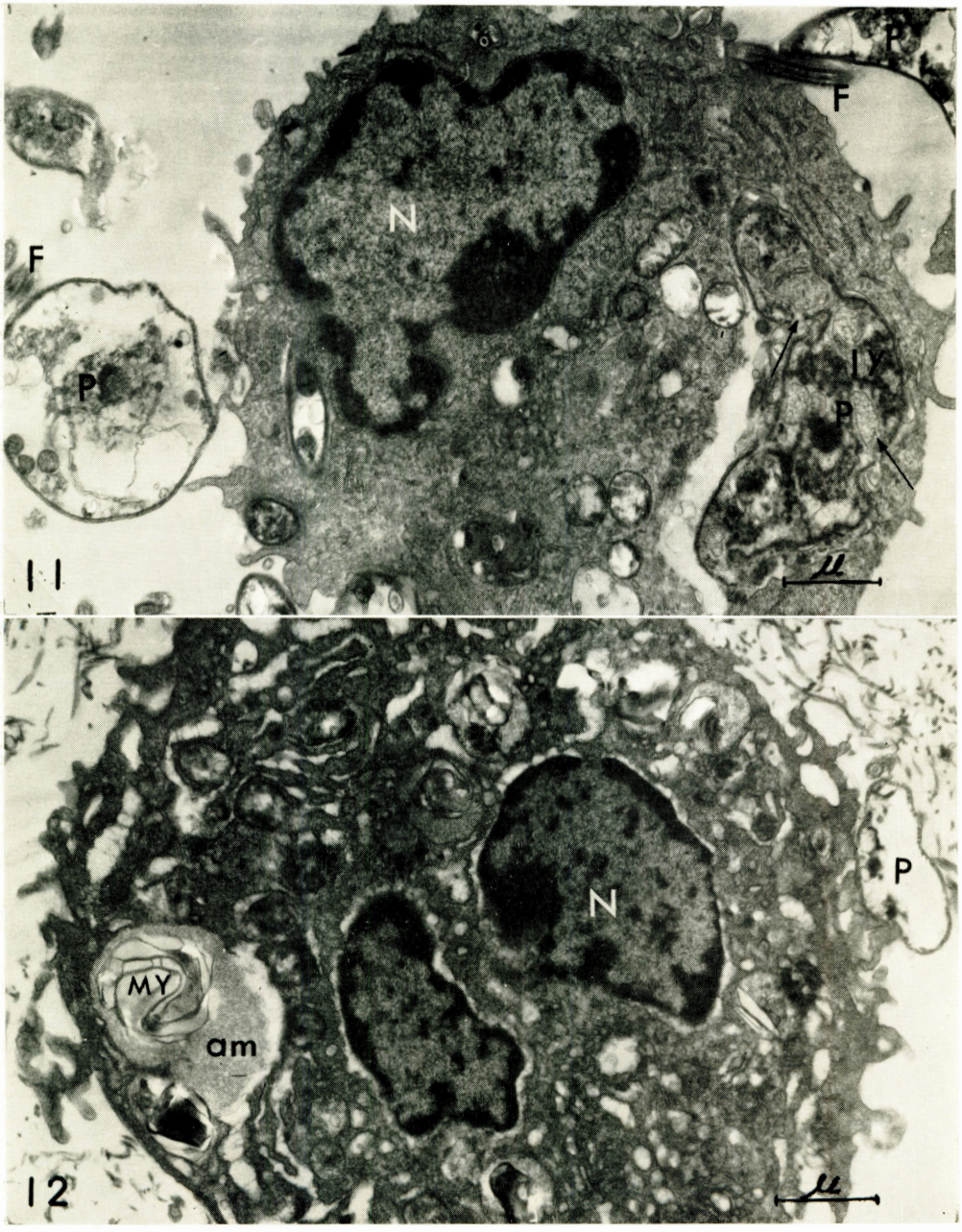
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Explanation of Photographs

Fig. 1 The peritoneal macrophage obtained from noninfected mouse.

Fig. 2 The peritoneal macrophages obtained from *T. gambiense* infected, untreated mouse. Arrow shows continuity of lysosome with the membranes of endoplasmic reticulum.

Figs. 3–5 The peritoneal macrophage obtained after intraperitoneal injection of human serum in trypanosome infected mouse.

Fig. 3 Photograph of phagocytosis of parasite by macrophage 20 min after injection. Arrow shows a phagosome of macrophage.

Fig. 4 One hr after injection.

Fig. 5 Six hrs after injection.

Figs. 6–10 The peritoneal cells obtained after intraperitoneal injection with anti-trypanosome mouse-serum in trypanosome-infected mouse.

Fig. 6 Low magnification photograph showing the fusion of 4 macrophages and 1 neutrophil obtained 20 min after injection.

Figs. 7 High magnificaiton photograph of Fig. 6. Arrow 1 and 2 show obscure membrane of trypanosome and phagolysosome membrane, respectively.

Fig. 8 Photograph of phagocytosis of trypanosome by eosinophil obtained 20 min after injection. Arrow shows a phagosome.

Fig. 9 Photograph obtained 20 min after injection showing the presence of trypanosomes in the homologous materials devoid of organella.

Fig. 10 Six hrs after injection. Arrow shows a flagellum-like structure.

Figs. 11, 12 The peritoneal macrophage obtained after intraperitoneal inoculation of acriflavine.

Fig. 11 Twenty min after inoculation. Arrow shows the invasion of lysosome into the cytoplasm of trypanosome.

Fig. 12 Six hrs after inoculation.

Abbreviations

am, amorphous material. b, basal body. ec, ectoplasm. ed, eletron dense body. er, endoplasmic reticulum. F, flagellum. G, Golgi lamellae. K, kinetoplast. ly, lysosome. m, mitochondria. mi, microtubules. MY, Myelin-like substance. N, nucleus. neu, neutrophil. Nl, nucleus-like body. p, parasite. ph, phagolysosome. sg, specific granule. V, vacuole.